



The cuticle micromorphology of extant and fossil plants as indicator of environmental conditions : A pioneer study on the influence of volcanic gases on the cuticle structure in extant plants

Antonello Bartiromo

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**The cuticle micromorphology of extant and fossil plants as indicator of
environmental conditions.**

**A pioneer study on the influence of volcanic gases on the cuticle structure in
extant plants**

Bartirromo Antonello

2011

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Antonello Bartiromo

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*Il nostro destino è inestricabilmente legato a quello di tutte le
specie e di tutti gli ecosistemi della Terra.*
Niles Eldredge, 2000

Alla mia Terra, che non merita tutto questo ...

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RIASSUNTO

Lo strato acellulare che ricopre le parti aeree delle piante vascolari superiori è chiamato cuticola. Quest'ultima funge da barriera protettiva ed è un efficace rilevatore d'inquinamento ambientale. Lo studio delle cuticole vegetali e in particolare degli apparati stomatici delle conifere, è estesamente utilizzato quale strumento di analisi ai fini della comprensione delle caratteristiche ecologiche e paleoecologiche. E' interessante notare che, sebbene siano numerosi gli studi inerenti le cuticole delle piante, poco o niente è stato fatto relativamente agli effetti prodotti sulle cuticole delle piante da parte dei gas vulcanici. La Campania, con la presenza di numerose località caratterizzate da emissioni di gas di origine vulcanica (Pisciarelli, Solfatara, complesso del Somma-Vesuvio, ecc.), consente di effettuare studi di questo tipo.

L'obiettivo di questa ricerca è quello di contribuire a individuare le potenzialità delle conifere e delle angiosperme (attuali e fossili) quali indicatori ecologici utili per la comprensione delle variazioni dei parametri ambientali. A tale scopo sono state compiute osservazioni macroscopiche e microscopiche di piante vascolari in relazione all'influenza di fattori ambientali quali: aerosol vulcanici, intensità luminosa, disponibilità di acqua e salinità. Nel corso della ricerca sono state campionate numerose aree e sono state utilizzate apparecchiature quali: microscopio ottico, SEM, TEM e EDS. La statistica è stata utilizzata per l'analisi delle caratteristiche micromorfologiche.

Le osservazioni condotte su piante attuali hanno consentito di studiare, per la prima volta, gli effetti dei gas vulcanici sull'ultrastruttura delle cuticole della conifera *Pinus halepensis* [pino d'Aleppo; siti di raccolta: Pisciarelli (presenza di gas vulcanici) e Cigliano (assenza gas vulcanici)] e dell'angiosperma *Erica arborea* [siti di raccolta: Solfatara di Pozzuoli e Pisciarelli (presenza di gas vulcanici per entrambi) e Cigliano (assenza gas vulcanici)].

Le osservazioni al TEM effettuate su cuticole di *P. halepensis*, influenzate e non da gas vulcanici, hanno evidenziato che lo spessore totale di CM (cuticola) + CW (parete primaria) non subisce sostanziali variazioni di spessore. In particolare, la cuticola degli aghi influenzati da gas vulcanici mostra (a forti ingrandimenti: TEM) un aumento dei depositi di ossalato di calcio e un riarrangiamento delle fibrille che si dispongono parallelamente alla superficie. Le osservazioni condotte al SEM e al TEM su aghi di *P. halepensis* attuali hanno permesso altresì di realizzare una chiave dicotomica che consente di identificare possibili alterazioni (dovute alla presenza di gas potenzialmente tossici come quelli vulcanici) anche in cuticole di pino sub-fossili o fossili.

Le osservazioni condotte su *E. arborea*, hanno permesso di constatare che gli spessori totali delle cuticole, “fumigated and not fumigated”, sono diversi. Comunque, in presenza di gas vulcanici lo strato esterno A2 subisce un sensibile incremento di spessore. Quest’ultimo aumenta quando la concentrazione di CO₂ in atmosfera è elevata, mentre non subisce sostanziali variazioni quando la quantità di CO₂ al suolo varia drasticamente. Ciò conferma che la cuticola è il mediatore principale negli scambi gassosi tra ambiente interno ed esterno.

Per entrambe le specie attuali studiate non è stata riscontrata presenza di zolfo nella cuticola, nella parete cellulare o nel citoplasma. Ciò conferma l’ipotesi che gli scambi gassosi avvengono essenzialmente attraverso gli apparati stomatici e che lo zolfo in eccesso è metabolizzato nelle foglie.

Relativamente ai macroresti vegetali fossili, sono state studiate le cuticole rinvenute nei Fossil-Lagerstätten cretaci di Cusano Mutri (Aptiano superiore) e Pietraroja (Albiano inferiore). Il primo sito fossilifero ha consentito di: 1) identificare svariati taxa riconducibili alle conifere; 2) descrivere una nuova specie di conifera caratterizzata dalla presenza di caratteri xeromorfi: *Frenelopsis cusanensis* Bartiromo et al.; 3) rinvenire, per la prima volta al di fuori dei confini spagnoli, un’angiosperma ancestrale: *Montsechia vidalii*. Lo studio tassonomico condotto sulle cuticole cretache di Cusano Mutri e Pietraroja ha permesso di descrivere entità tipiche della Provincia Euro-Siniana. Lo studio sedimentologico e sistematico denota un clima tropicale-subtropico piuttosto arido. È interessante notare come per il sito di Cusano Mutri, il rinvenimento di abbondanti “fusain” sulle superfici di strato sia la prova che incendi naturali, frequentemente innescati da fulmini, interessavano le terre emerse.

Questo studio (almeno per quanto riguarda le piante attuali) può essere considerato pionieristico proprio perché per la prima volta sono state studiate le variazioni dell’ultrastruttura della cuticola in presenza di gas vulcanici.

RÉSUMÉ

La couche qui recouvre les parties aériennes des plantes vasculaires supérieures est appelée cuticule. Cette dernière agit comme une barrière protectrice et est un détecteur efficace de la pollution de l'environnement. L'étude de la cuticule des plantes, en particulier des appareils stomatiques des conifères, est largement utilisée comme un outil d'analyse pour comprendre les caractéristiques écologiques et paléoécologiques. Il est intéressant de noter que, bien que les études sur la cuticule des plantes soient nombreuses, peu ou rien n'a été réalisé sur les effets sur la cuticule des plantes par les gaz volcaniques. La Campanie, avec ses nombreux endroits caractérisés par des émissions de gaz d'origine volcanique (Pisciarelli, Solfatara, complexe du Somma-Vésuve), permet d'effectuer ce type d'études.

L'objectif de cette recherche est donc de contribuer à individualiser les potentialités des conifères et angiospermes (actuelles et fossiles) comme indicateurs écologiques dans la reconnaissance des variations de paramètres environnementaux. Pour cela, des observations macroscopiques et microscopiques de plantes vasculaires ont été effectuées par rapport à l'influence des facteurs environnementaux tels les aérosols volcaniques, l'intensité de la lumière, disponibilité d'eau et la salinité. Au cours de la recherche un certain nombre de localités ont été échantillonnées et on a utilisé des équipements comme le microscope optique, le MEB, le MET et l'EDS. La statistique a été largement mise à contribution, avec l'intervalle de confiance portant sur 30 mesures.

Les observations effectuées sur les plantes actuelles ont permis d'étudier, pour la première fois, les effets des gaz volcaniques sur l'ultrastructure des cuticules du conifère *Pinus halepensis* [le pin d'Alep, sites de récolte: Pisciarelli (fumigé) et Cigliano (non fumigé)] et de l'angiosperme *Erica arborea* [la bruyère arborescente, sites de récolte: Solfatara et Pisciarelli (fumigé) et Cigliano (non fumigé)].

Les observations conduites au TEM sur les cuticules de *P. halepensis*, influencé et non influencé par les gaz volcaniques, ont montré que l'épaisseur totale de la CM (cuticule) + CW (paroi pectocellulosique) ne subit pas de variations significatives d'épaisseur. En particulier, la cuticule des aiguilles influencée par les gaz volcaniques montre (à fort grossissement TEM) une accumulation d'oxalate de calcium ainsi qu'un réarrangement des fibrilles disposées parallèlement à la surface. Les observations SEM et TEM sur des aiguilles de *P. halepensis* actuelles ont permis également de réaliser une clé dichotomique permettant d'identifier les altérations possibles (dus à la présence de gaz potentiellement toxiques comme les gaz volcaniques) des cuticules de pins sub-fossiles ou fossiles.

Les observations conduites sur *E. arborea* ont permis de constater que les épaisseurs totales des cuticules, influencées ou non par les gaz, sont significativement différentes. En présence de gaz volcaniques la couche externe A2 subit un sensible accroissement d'épaisseur. Cette dernière augmente quand la concentration en CO₂ en atmosphère est élevée, alors qu'elle ne subit pas de variations substantielles quand la quantité de CO₂ au sol varie de manière drastique. Ceci démontre que la cuticule est le médiateur principal dans les échanges entre l'environnement interne et externe.

Grâce à des analyses EDS, pour les deux espèces actuelles étudiées il n'a pas été trouvé de présence de soufre dans la cuticule, dans la paroi cellulaire ou dans le cytoplasme. Ceci confirme que la cuticule est le principal médiateur des échanges gazeux entre l'environnement interne et externe.

Par rapport aux macro-restes végétaux fossiles, les cuticules du Fossil-Lagerstätten du Crétacé de Cusano Mutri (Aptien sup.) et de Pietraroja (Albien inf.) ont été étudiées. Le premier site fossilifère a permis 1) d'identifier plusieurs taxa appartenant aux conifères; 2) de décrire une nouvelle espèce de conifère caractérisée par la présence de caractères xéromorphiques: *Frenelopsis cusanensis* Bartiromo et al.; 3) de trouver, pour la première fois à l'extérieur de l'Espagne, une angiosperme ancestrale: *Montsechia vidalii*. L'étude taxonomique conduite sur des cuticules du Crétacé de Cusano Mutri et Pietraroja a permis de décrire entités typiques de la Province Euro-Sinienne. L'étude sédimentologique et systématique montre un climat tropical-subtropical plutôt sec. Il est intéressant de noter, comme pour le site de Cusano Mutri, la présence des abondants fusains sur les surfaces des couches rocheuses, montrant les incendies naturels fréquemment amorcés par des éclairs intéressant les terres émergées.

Cette étude (au moins en ce qui concerne les plantes actuelles) peut être considérée comme pionnière car, pour la première fois, a été étudié les variations de l'ultrastructure de la cuticule en présence de gaz volcaniques.

ABSTRACT

The leaves of many tracheophytes are covered with a cuticle, an extracellular membrane covering aerial organs of plants. The gas exchanges between the plant and the surrounding atmosphere are mediated by the cuticle; its acts as the main barrier to air pollutants. The study of the plant cuticle, in particular the stomatal apparatuses of conifer, is largely used as a tool analysis revealing ecological and paleoecological features. It is worth noting that little is known about the long-term response of micromorphology of natural vegetation to volcanic toxic gases. Fortunately, Campania Region with its numerous volcanic localities (Pisciarelli, Solfatara, complexe du Somma-Vésuve) represents a natural laboratory allowing experiments involving plant-volcano interactions.

The object of this research is to study the conifer and angiosperms potentialities (extant and fossil) as ecological indicators useful in the identification of the environmental parameters variations. That is why, macroscopical and microscopical observations in vascular plants in relation to various environmental factors (volcanic gases, light intensity, water availability and salinity), have been analysed. A number of localities have been sampled and SEM, TEM and EDS equipments have been used together with statistic.

Observations made on extant plants allowed for the first time, the study of the effects of volcanic gases on the cuticle ultrastructure of *Pinus halepensis* [Aleppo pine; Pisciarelli (fumigated) and Cigliano (not fumigated) localities] and *Erica arborea* [tree heather; Solfatara, Pisciarelli (fumigated) and Cigliano (not fumigated) localities].

TEM observations on *P. halepensis* cuticles fumigated or not by volcanic gases revealed insignificant thickness variations of the cell wall plus cuticle among current- and first-year-old needles of both fumigated and not fumigated trees. In particular, the needle cuticles experiencing chronic fumigation display (TEM) a calcium oxalate accumulation. Moreover, in respect to the cell surface, fibrils are parallel disposed. SEM and TEM observations allowed an identification key enabling distinction between not fumigated and fumigated material with 9 characters, providing a good tool detecting the influence of volcanism for extant and fossil plants.

In specimens of *E. arborea* fumigated or not by volcanic gases, the total thickness of cuticles varies significantly. In plant experiencing chronic fumigation the A2 layer records an increase of its thickness. Within three localities, a good correlation between the atmospheric CO₂ concentration and the thickness variation of A2 layer has been found.

This fact confirms that the cuticle is the main mediator between the plant and the atmosphere.

As for fossil plants, the cuticles of Cretaceous Fossil-Lagerstätten of Cusano Mutri (Late Aptian) and Pietraroja (Lower Albian) have been studied. In the former: 1) numerous taxa belonging to conifers have been identified; 2) the new species *Frenelopsis cusanensis* Bartiromo et al. bearing xeromorphic features has been described; 3) the occurrence of *Montsechia vidalii* is recorded for the first time outside of Spain. Taxonomical studies carried out on Cretaceous cuticles from Cusano Mutri and Pietraroja allowed the description of typical Euro-Sinian fossil plants. Sedimentological and taxonomical studies suggest semi-arid or arid conditions in a subtropical or tropical climate. It is worth noting as for Cusano Mutri locality, evidence of wildfire (fusain) suggests a periodic combination of arid periods, high temperatures and lightning strikes.

This study (at least for extant plants) can be considered pioneering, because, for the first time, the relationships between cuticle ultrastructure variations and volcanic gases have been studied.

Parole chiave:

Campi Flegrei, gas vulcanici, *Pinus halepensis*, *Erica arborea*, epidermide, ultrastruttura della cuticola, idrogeno solforato (H₂S), piante fossili, Cretacico inferiore, Sud Italia, Cusano Mutri, Pietraroja, Cheirolepidiaceae, *Frenelopsis cusanensis* sp. nov., paleoecologia, Provincia Euro-Siniana.

Mots clés:

Campi Flegrei, gaz volcaniques, *Pinus halepensis*, *Erica arborea*, épiderme, ultrastructure de la cuticule, hydrogéné sulfuré (H₂S), plant fossiles, Crétacée inférieur, Italie du Sud, Cusano Mutri, Pietraroja, Cheirolepidiaceae, *Frenelopsis cusanensis* sp. nov., paléoécologie, Province Euro-Sinien.

Keywords:

Campi Flegrei, volcanic gases, *Pinus halepensis*, *Erica arborea*, epidermis, cuticle ultrastructure, hydrogen sulphide (H₂S), fossil plants, Early Cretaceous, Southern Italy, Cusano Mutri, Pietraroja, Cheirolepidiaceae, *Frenelopsis cusanensis* sp. nov., Palaeoecology, Euro-Sinian Province.

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GENERAL INTRODUCTION

The leaves, fruits, and primary stems of higher plants are covered by a cuticular membrane (CM), or cuticle, that occupies approximately the outer 0.1-10 μm of the aerial plant surface (Stark and Tian, 2006). On leaves the cuticle is present in both adaxial and abaxial surfaces. The CM occupies the outer surfaces of epidermal cell walls and is impregnated with an extracellular matrix (Domínguez et al., 2011). The cuticle has often been called the “skin” of the primary parts of higher plants and has a very long history on the palaeobiological timescale (Riederer, 2006). With exception of fossil pollen and spores, cuticles represent the most widespread unaltered fossil plant remains and are known from the Devonian to the recent (Taylor et al., 1989). For this reason paleobotanists always played an important role in the study of plant cuticles (Kerp, 1990). The earliest references to fossil cuticles are by Göppert (1841–1846), while Brongniart (1834) gave the name “cuticula” to a superficial membrane isolated from the cabbage leaf epidermis by retting in water. Later, Norris and Bukovac (1968) defined the limits of the cuticle as “all of the layers that can be separated from the underlying cellulose cell wall”.

Cuticular membrane is a translucent film of polymeric lipids and soluble waxes (Jeffree, 2006). The cuticle is usually

thicker above the anticlinal epidermal cell walls (CW), often forming pegs or spandrels by penetrating deeply between the anticlinal walls of adjacent epidermal cells (Jeffree, 2006). The CM bears an imprint (a “ghost”) of the epidermal cell pattern of the plant organs on which it was formed (Fig. 1), which may survive

as the only remaining fossil evidence of multicellular structure of the earliest land plants (Edwards et al., 1996).

In most plants the CM is not structurally or chemically homogeneous but composed of a number of layers, each of which is defined by its position and chemical constitution (Holloway, 1982). As Taylor et al. (1989) claimed, the cuticle consists of various layers which can be delimited based on the substances (e.g. waxes) embedded within the polymeric matrix and various structural features (e.g. lamellae and fibrillae). Generally

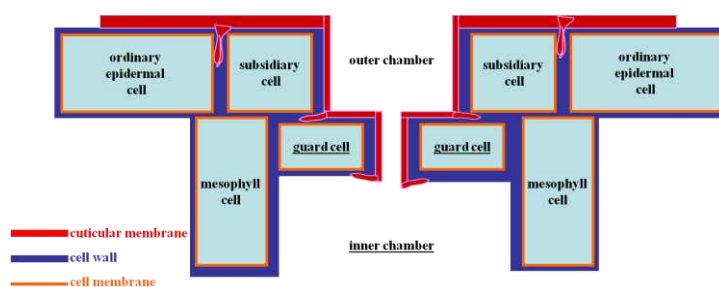


Fig. 1. Scheme showing the location of cuticle (= cuticular membrane in red) within the epidermis and in a stomatal apparatus context.

speaking, TEM revealed that the cuticle of the cells of the upper epidermis is made of an outer stratum named cuticle proper (CP) and an inner one called cuticular layer (CL). According to the international terminology, the former (CP) can be constituted by an A1 (lamellate) or/and an A2 (uniformly electron dense with areas of lacunae) layers; the latter (CL) is constituted by a reticulate (B = B1) layer (see: Holloway, 1982; Archangelsky, 1991; Jeffree, 2006), in some rare cases an innermost B2 granular layer is present.

The outer surface of cuticle can be coated with epicuticular waxes which confer water repellency (Adam, 1963). Cuticular wax plays pivotal physiological and ecological roles in the interactions between plants and their abiotic and biotic environments, respectively (Jetter et al., 2006).

The cuticle performs numerous functions such as: transpiration control; control of loss and uptake of polar solutes; control of the exchange of gases and vapours; interface for biotic interactions and so on. However, the primary function of the cuticle is a permeability barrier against water vapour loss from tissues (Schreiber et al., 1996). For these and other reasons (see: Huttunen, 1984; Jeffree, 1986; Archangelsky et al., 1995; Garrec, 1996) the cuticle can be considered as an “external skeleton” as it represents the interface between the plant and the atmosphere (McElwain and Chaloner, 1996). As cuticle forms the interface between plants and atmospheric environment, it is the first point of contact between plants and air pollutants and it presents an effective barrier to pollutant entry.

During recent years considerable progress has been made for investigating this more external part of plants and its relations with external environment (Holloway, 1982; Hill and Dilcher, 1990; McElwain and Chaloner, 1996; Newrath, 2006; Jeffree, 2006; Shepherd and Griffiths, 2006). However, few studies have been carried out in the study of plant-volcanic gases interaction.

This doctoral thesis represents a contribution to the study of extant and fossil plant cuticles by means of optical and electronic (SEM and TEM) microscopy as well as EDS analyses. In particular, this research is pioneer in the study of ultrasctructure of plant cuticle submitted to the influence of volcanic gases representing the first part of this thesis. Similar researches are highly advisable in the Campania Region (Ischia island, Phlegrean Fields, Roccamonfina and Somma–Vesuvio complex) where volcanic and not volcanic gas emissions, give great opportunities in the analysis of plant–volcano interaction. However, Campania Region represents also a “virgin reservoir” for the study of Cretaceous plants that exactly make the object of the second part of this thesis (Fig. 2).

Organisms of the past, in the same ways as those of the present, became adapted to their environments. The distributions of plant and animal species, as well as community characteristics, are strongly influenced by climate (Wing and Greenwood, 1993). As a matter of fact, thick cuticles and sunken stomata of fossil leaves also suggest lack of available water (Stewart and Rothwell, 1999).

Therefore, the “ability” of cuticle to register the environmental conditions is herein used as “trait d’union” between the first (extant plants) and second (fossil plants) part of this research as detector of environmental conditions.

For extant plants, a representative of conifers (*Pinus halepensis* Mill.) and angiosperms (*Erica arborea* L.) long-term fumigated by toxic volcanic gases have been studied. In Pisciarelli area *P. halepensis* is the only conifer growing near the vent. In Pisciarelli and Solfatara localities, *E. arborea* represents the only xeromorphic species capable to grow in the volcanic plume.

The present study, carried out over three years, rises from the collaboration between the Université Claude Bernard Lyon–1 and the Università degli Studi di Napoli Federico II and has as main goals the following:

- 1) analyze microscopical (essentially) and macroscopically features of the extant species *Pinus halepensis* Mill. and *Erica arborea* L. submitted to the influence of volcanic gases;
- 2) use experiences and observations made in the first part of the project to carry out in-depth studies of Cretaceous plants from Campania Region as to improve the knowledge of these “virgin” fossil site.

For every chapter a dedicated introduction and a “material and methods” section are proposed, because different techniques have been used for extant and fossil plant cuticles analyzed and for every site studied.

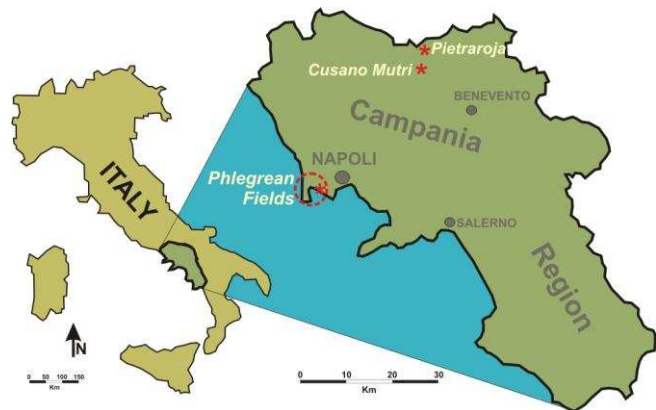


Fig. 2. Showing the location of the studied sites in Campania Region (the red asterisks). The dotted circle indicates the area occupied by the volcanic area of Phlegrean Fields.

CHAPTER I

Influence of volcanic gases on the epidermis of *Pinus halepensis* Mill. in Campi Flegrei, Southern Italy: A possible tool detecting volcanism in present and past floras

1.1. Introduction

Over the geological history of the planet, among chronic environmental stress factors advocated as killing agents (Visscher et al., 2004), changes in atmospheric chemistry had world-wide dramatic effects on plant life in land (e.g. Visscher et al., 1996; Meyer and Kump, 2008). For instance, among chemical contaminants that could have disrupted end-Permian biota, volcanogenic SO₂ (Visscher et al., 2004) and biological H₂S (euxinia mechanism: Kump et al., 2005; Berner and Ward, 2006) gases are favoured to explain extinction. In particular, volcanism subsequently played a role in both maintaining and perturbing the atmosphere chemistry and physics, with important implications in terms of the evolution of life (Mather, 2008). The development of large igneous province (LIP) and continental flood basalt province (CFBP) (Courtillet and Renne, 2003; Jerram et al., 2005; Keller, 2008; Bryan et al., 2010) commonly coincides with mass extinction events (Wignall, 2001, 2005; Rampino, 2010; Whiteside et al., 2010) and results in the release of significant volumes of gases, such as CO₂, H₂S and SO₂ into the atmosphere (Beerling and Berner, 2002; Berner and Beerling, 2007; Hori et al., 2007). It is widely recognized that volcanic sulfur dioxide (SO₂) and hydrogen sulfide (H₂S) emissions are significant sources of sulfur release to the atmosphere (Bates et al., 1992; Berner and Berner, 1996).

Gases emitted by volcanoes represent both a factor inhibiting vegetation development (Whittaker et al., 1989) and could have been responsible of the decline of vegetation during periods of global-scale volcanism (Bond et al., 2010; Visscher et al., 2004; Whiteside et al., 2010; McElwain and Punyasena, 2007). In particular, H₂S is often thought to be a phytotoxin, being harmful to the growth and development of plants (Lisjak et al., 2010) especially when the quantities are higher than plant necessity (Thompson and Kats, 1978; Lorenzini and Nali, 2005). Moreover, atmospheric pollutants produced by volcanic activity and OAEs, such as SO₂ and H₂S, are said to be absorbed via the cuticle as well as the stomata (Haworth and McElwain, 2008).

Plants exposed to poisonous volcanic gases may show signs of diseases to total defoliation and death (e.g. Dickson, 1965; Clarkson and Clarkson, 1994; Delmelle et al., 2002). However, plant damages are related to both gas concentration (Delmelle, 2003) and its persistence (Grattan et al., 1998) in the atmosphere. Under severe pollution conditions, the direct phytotoxic effects of gaseous pollutants as well as long-term effects of acid washout (Grattan and Pyatt, 1994) can even be considered as potential environmental mutagens disturbing plant growth and community structure (Visscher et al., 1996). As a matter of fact, as Visscher et al. (2004) pointed out, variation in structure and composition of leaf cuticles is a potential source of botanical evidence on mutational effects of environmental stress factors.

Therefore, leaves in natural environments are subjected to a range of physical processes which may damage their surfaces, leading to alterations in the structure and integrity of the cuticle, and consequently changes in the physical properties of the leaf surfaces (van Gardingen et al., 1991).

To this end, numerous articles have been published in relation to the effects and interactions of the volcanic activity products (e.g. tephra or ash fall) on both fossil (e.g. Kovar-Eder et al., 2001; García Massini and Jacobs, 2011) and extant plants (Winner and Mooney, 1980b; Cook et al., 1981; Seymour et al., 1983; Dale et al., 2005). Moreover, in extant plants the concentration of chemical elements in the leaves (Notcutt and Davies, 1989; Martin et al., 2009a and b) and the analysis of the log (Baillie and Munro, 1988; Battipaglia et al., 2007) together with field studies led to significant advances in understanding the composition and dispersion of volcanic emissions at source (e.g. Kempter et al., 1996; Delmelle et al., 2002), including major “gas species” (Costa et al., 2005; Chiodini, 2008; Chiodini et al., 2010a).

Leaves of plants act as passive and active collectors for natural (e.g. Martin et al., 2009a) and anthropogenic (e.g. Bačić et al., 1999) airborne pollutants (e.g. gas, aerosols and dusts) and are more sensitive to air quality than other plant organs (e.g. roots) (Landolt et al., 1989; Casseles, 1998; Kabata-Pendias, 2001); the gas exchanges between the plant and the surrounding atmosphere are mediated by the cuticle; this non-living (Riederer, 2006) thin (<0.1-10µm thick in extant plants) and heterogeneous membrane (van Gardingen et al., 1991) covers the epidermis of the aerial part of many tracheophytes (Guignard et al., 2004) and consists of a polymer matrix (cutin), polysaccharides and associated solvent-soluble lipids which are synthesised by the epidermal cells and deposited on their outer wall (Kirkwood, 1999; Riederer and Schreiber, 2001). The outer

surface of the cuticle is coated with epicuticular waxes, a general term (Jeffree, 2006) designating very long chain hydrocarbons found embedded within the cuticle and also in the crystalline epicuticular wax layer (Bird and Gray, 2003). The main function ascribed to waxes is to limit the diffusional flow of water and solutes across the cuticle (Heredia and Dominguez, 2009), providing protection for the leaf cells (Turunen and Huttunen, 1990) and acting as the main barrier to air pollutants (e.g. Jeffree, 1986). The composition and amount of waxes in the cuticle have been shown to vary depending to environmental conditions of the plant (Baker, 1982; Bird and Gray, 2003) and according to many authors air pollution seems to increase the rate of wax tubules degradation (e.g. Huttunen and Laine, 1983; Riding and Percy, 1985; Berg, 1987; Turunen and Huttunen, 1990, 1991, Huttunen, 1994). In particular, wax load and structure can be used as an indicator of pollution level (Hansell and Oppenheimer, 2006; Holroyd et al., 2002). The epicuticular wax of pine needles undergoes an ageing procedure during the needle lifetime (Turunen and Huttunen, 1996; Bačić et al., 1999) and is disturbed by polluted air (Huttunen, 1994). The literature is replete with references to structural changes in epicuticular waxes following exposure to air pollutants (see Turunen and Huttunen, 1990), and as a matter of fact, the erosion of epicuticular waxes is a relevant factor of the multiple forest decline syndrome (Turunen and Huttunen, 1990).

Few paleobotanical works have been achieved on cuticular characters related to volcanic stress. Archangelsky et al. (1995) and Villar de Seoane (2001) studied Early Cretaceous plants from Patagonia (recovered in Baqueró and Springhill Formations, respectively) demonstrating that the volcanic ash fall played an important role in the formation of xeromorphic structures. As Haworth and McElwain (2008) claimed, the effect of toxic atmospheric gases and volcanic dust would explain xeromorphic features of *Pseudofrenelopsis parceramosa* (Fontaine) Watson from the Early Cretaceous of England. Moreover, the relationship between ultrastructural characteristics of cuticle and the environment is still poorly understood for extinct as well as extant plants (Guignard et al., 2001) and cuticular ultrastructure data are not numerous for fossil conifers (e.g. Guignard et al., 1998; Villar de Seoane, 1998; Yang et al., 2009) and seem to be still lacking for some species belonging to the genus *Pinus* (Jeffree, 2006).

However, to date, no studies have been carried out relatively to the response of the ultrastructural features of plant cuticle exposed to the persistent volcanic gases. Conifers are well suited for studies of pollutant levels because they are evergreen and often have long-lived foliage. Usually the needles have a life cycle of several years (Hellström, 2003).

Therefore, the protective role of the epicuticular waxes is particularly important for conifers that have to ensure their investment in leaf tissue for several years (Chabot and Chabot, 1977). In the volcanic area of Pisciarelli (Campi Flegrei, Southern Italy) the gymnosperm *Pinus halepensis* Mill. (Aleppo pine) is the only conifer growing adjacent to the fumaroles, and much of the surrounding vegetation (under study) displays indications of damage caused by toxic gases. *P. halepensis* is the most abundant pine species in the western Mediterranean Basin, where it occupies 2.5 million ha (Quézel, 2000) and it is considered as an opportunistic species (Nathan and Ne'eman, 2000) which is able to regenerate either in the absence or as a result of fire. In addition, *P. halepensis* has an elevated resistance to drought (Boddi et al., 2002), so much so that Emberger (1930) identifies it as being semiarid, and Oppenheimer (1968) considers it as the most arid-tolerant of all the *Pinus* species. As a matter of fact, the present study aims to assess the cuticular response of this conifer at a prolonged exposition to the volcanic gases using both SEM and TEM approaches. Moreover, to our knowledge, this is the first study that tests the cuticle ultrastructure behaviour during two subsequent years (current- and first-year-old needles) in response to the fumigation of volcanic gases containing H_2S .

In particular, this research aimed to investigate: 1) response of plants to volcanic gases through different aspects: epicuticular and epistomatal waxes and ultrastructural features of the cuticle; 2) potential implications of the conifer cuticle response across environmental stress periods during the geological past; 3) a new method detecting the influence of volcanism for extant and fossil plants.

1.2. Material and methods

The material was collected from two localities in the Phlegrean Fields (Campi Flegrei, Campania Region), an active caldera which spans the last 50000 years (Scandone et al., 2010), characterized by significant recent ground deformation (Morhange et al., 2006) and considered as one of the most dangerous volcanic areas in the world (Chiodini et al., 2010a). In particular, pine needles were recovered from the famous fumaroles field in Pisciarelli locality (40°49'48.88''N, 14°08'46.95''E) about 1 km SE of the Solfatara volcano, both characterised by volcanic gas emissions (Fig. 1A,B). Control sample of needles were collected from a volcanic quiescent area (Cigliano: 40°50'46.46''N, 14°07'36.31''E) about 2.5 km from Pisciarelli and characterised by the absence of volcanic gas emissions and the presence of clean air. Both localities are characterised by the same

soil features (Di Gennaro and Terribile, 1999; Di Gennaro, 2002) and sun exposition and are far away from traffic and industries.

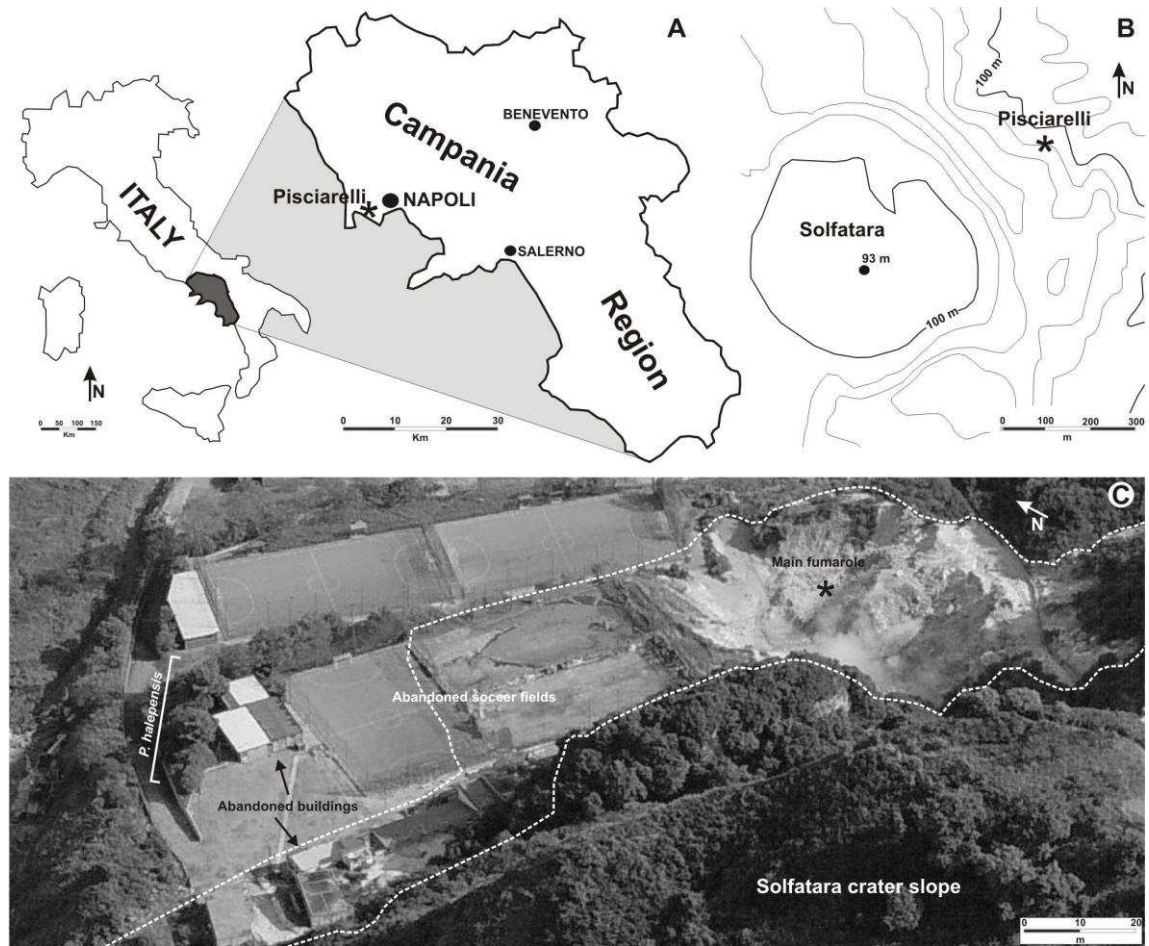


Fig. 1. (A) Location of the Pisciarelli area in the Campania Region. (B) Sketch map showing the location of Solfatara crater and Pisciarelli localities. (C) Close up view of Pisciarelli area with the main fumarole. Dotted line indicates the area of diffuse degassing.

The temperature reaches about 97°C at the Pisciarelli fumaroles (Chiodini et al., 2010a) and the analysis of gaseous compositions (Caliro et al., 2007) revealed that the main component of the fumaroles is H₂O followed by CO₂, H₂S, N₂, H₂, CH₄, He, Ar, and CO. The absence of acidic gases (SO₂, HCl, and HF) can be also noted (Chiodini et al., 2010a). Natural high atmospheric concentration of sulphur gas may occur locally in areas with volcanic and geothermic activity (De Kok et al., 2007). As a matter of fact, at Pisciarelli volcanic vent, the high H₂S air concentration (equal to ca 600 µm/mol) at source (Chiodini et al., 2010a) is also testified by both typical smell (the odour threshold is >0.02 µm l⁻¹: De Kok et al., 2007) of rotten eggs in the surrounding air and by indirect corrosive action of this gas -well visible on the iron objects- which caused people to abandon some buildings in the area (Fig. 1C). Nevertheless, this extreme environment is inhabited by very few

angiosperm species providing xeromorphic features (e.g. *Erica arborea*) and the boiling water of fumaroles retains the cyanidiallean alga *Galdieria phlegrea* (Pinto et al., 2007).

Distal volcanic impacts have shown that plants are generally less sensitive to eruptions outside the growing season (Zobel and Antos, 1997; Hotes et al., 2004) and, as Payne and Blackford (2008) pointed out, in winter, plants are senescent and higher rainfall may serve to remove rapidly volcanic pollutants. In case of *Pinus halepensis*, an evergreen plant permanently fumigated by volcanic gases, retaining leaves for over a year, these “ground noises” do not exist.

The trees present diffuse damages along the North sides of the crown, while needles show symptoms consisting in leaf-tip non-specific discoloration which gradually increasing shootward (terminology from Baskin et al., 2010). Current- and first-year-old needles were collected from branches at heights over 1.5 m from three trees (15-20 years old) at each site (Pisciarelli and Cigliano). Needles were carefully handled to avoid damaging the epicuticular waxes. Following Reed’s remarks (1982) and also Crang and Klomparens’ ones (1988) about possible changes in epicuticular wax structures occurring during sample preparation, in order to limit any chemical or physical damages, especially for preserving and dehydrating samples for wax morphology studies (e.g. Turunen and Huttunen, 1991; Tuomisto and Neuvonen, 1993), needles were air-dried for 1-week at mild room temperatures. Among several hundreds of pine needles collected at each site (fumigated and not fumigated), 60 were selected for scanning electron microscope (SEM), then 16 were carefully selected for transmission electron microscope (TEM). Stomata were observed on 15 current- and first-year-old needles for each site. Analysis was performed within two weeks from sampling. Taxonomical identification of García Álvarez et al. (2009) approach has been used. Light microscope observations were made using a Leitz microscope.

In order to quantify the quality of epicuticular and epistomatal waxes, SEM observations were carried out. Untreated needle sections of approximately 5 mm in length - obtained from the middle of each needle- were mounted on stubs using double-sided adhesive tape; both abaxial and adaxial surfaces have been studied. Part of specimens were sputter-coated with gold using an AGAR Auto Sputter Coater, while the specimens for energy diffractive x-ray (EDX) analyses were coated with carbon in a Emitech K450, observed and photographed with JEOL JSM-5310 SEM adapted with an Energy Diffractive X-ray Oxford Inca X-act at the CISAG (Centro Interdipartimentale di servizi per analisi Geomineralogiche) in the Dipartimento di Scienze della Terra, Università degli

Studi di Napoli “Federico II”. The operative conditions were as follows: 25-30 KV accelerating voltage, 100 μ A emission current, 15 μ m spot size, 20 mm microscope work distance and 1 min spectra collection time. To quantify the wax change in stomatal chamber, the Nicolotti et al. (2005) needle damage classes have been used as a criterion for the level of crystalline wax degradation.

Samples for TEM were dropped in paraformaldehyde solution mixed in a phosphate-sodium buffer for 3 weeks using Lugardon's technique (1971), washed and postfixed in a 1% osmium tetroxide solution mixed in a phosphate-sodium buffer for 24 hours. Dehydrated in graded ethanol series during 48 h, the samples were dropped in propylene oxide with an increasing percentage of Epon resin for 24 h. Transferred into pure Epon resin during 24 hours, they were embedded in fresh Epon resin using flat moulds. The preparations were subsequently treated for polymerization at 56 °C for 3 days. Ultrathin (60-70 nm) sections were sectioned with a diamond knife, using a Reichert Ultracut microtome. Ultrathin sections were placed on uncoated 300 Mesh copper grids and stained manually both with a methanol solution of 7% uranyl acetate for 15 min and an aqueous lead-citrate solution for 20 min, then observed and photographed with a Philips CM 120 TEM at 80 kV, in the Centre de technologie des microstructures (CT μ) of Lyon-1 University, Villeurbanne, France. Totally 16 pieces of material were embedded in Epon resin blocks. 90 uncoated mesh copper grids were prepared (80 as transversal sections, i.e. perpendicular to the leaf length; 10 as longitudinal sections, i.e. parallel to the leaf length). In order to detect the presence of sulphur in the cuticle, 40 measurements (i.e. 10 measures for each current- and first-year-old needles, fumigated and not fumigated by volcanic gases) with EDS microanalysis were carried out on different parts of the cuticle and also on the cytoplasm remnants of the epidermal cells. The sulphur analysis was performed on 25 coated 300 Mesh grids with transmission electron microscope JEOL 1200EX coupled to a microanalysis system EDS Si(Li) 30mm² NORAN VOYAGER III with an acceleration voltage = 80 kV and a spectres acquisition time = 60 s.

All the quantitative TEM measurements were made with tools in the ImageJ program (Abramoff et al. 2004). The terminology of Holloway (1982) and Archangelsky (1991) was used for the ultrastructural analysis. All specimens and SEM stubs are housed in the Dipartimento di Scienze della Terra, Largo San Marcellino, 10, Napoli, Italy. The resin blocks and TEM negatives are stored in the Lyon-1 University, Villeurbanne, France.

1.3. Results

1.3.1. Sulphur measures

Energy diffractive X-ray analysis with the SEM, carried out on needles undergone chronic fumigation, showed two different graphics displaying typical volcanic products coming from Pisciarelli area (Valentino and Stanzione, 2004): the leaves were covered with thin volcanic dust coming from the main fumarole of Pisciarelli (Fig. 2A); they also displayed numerous CaSO_4 crystals (Fig. 2B).

EDS microanalyses, within fumigated and not fumigated needles, concerned 4 sets of 10 measurements on outer, middle and inner part of cuticle + cytoplasm remnants of the epidermal cells. The elements are largely homogeneous (Fig. 3A-D) and the absence of sulphur is clear in all analysed parts. Extraneous elements detected by means of TEM X-ray analyses are Cu, Os and Cl derived from: the TEM-grids, chemical treatments and polyvinyl formal (FORMVAR, the most widely film for TEM grids) respectively. However, TEM electron energy-loss spectroscopy has proved that the cation calcium can be found in cuticle and cytoplasm of epidermal cells, especially in correspondence of low electron-density areas related to calcium-oxalate (CaC_2O_4) deposit.

1.3.2. Scanning electron microscopy observations

Stomatal apparatuses together with epicuticular and epistomatal waxes of fumigated and not fumigated needles have been observed (Plate I).

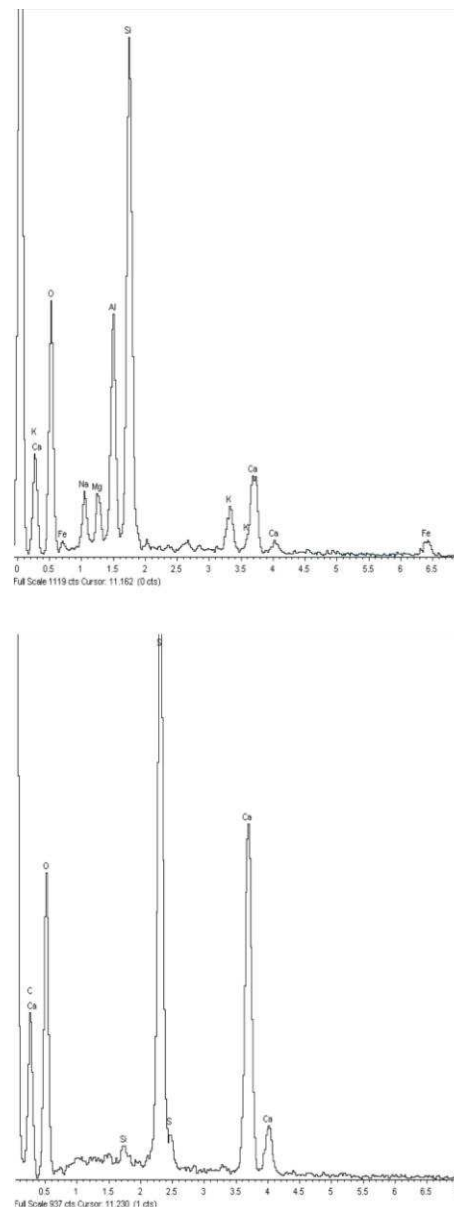


Fig. 2. Two different qualitative SEM X-ray diffractograms. A) Example of volcanic dust spectrum. B) Example of CaSO_4 crystals spectrum. Current-year-old needle. Abaxial side. Pisciarelli.

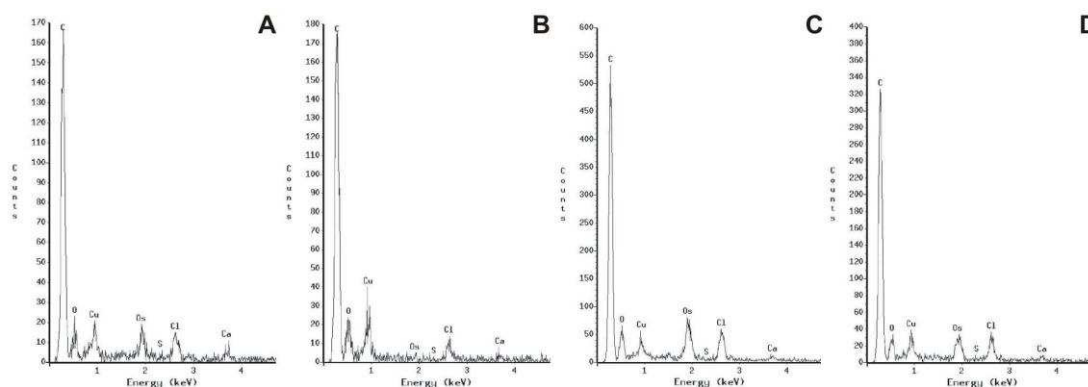


Fig. 3. Cuticle qualitative TEM X-ray diffractograms made on 70 nanometers sections, in three parts of the cuticle (outer, middle and inner parts) plus cell cytoplasm remnants, showing four examples among the elements content of: not fumigated, current- (A) and first-year-old (B) needles; fumigated, current (C) and first-year-old (D) needles.

Current- and first-year-old needles which did not experience chronic fumigation, retain quite well-preserved wax tubes in both abaxial and adaxial needle sides (Plate 1, Figs. 3,4). The abaxial side of not fumigated current- and first-year-old needles displays the borders of the epidermal cells making folds between them (Plate I, Fig. 1).

The abaxial sides, and to a lesser degree the adaxial ones, of current- and first-year-old needles experiencing chronic fumigation, have tubular wax crystals which are converted into scale-like crystalloid formations on epicuticular surface (Plate I, Fig. 2) owing to the wide wax fusion phenomena. Rare crystalloid structures have been preserved underneath the epicuticular crusts (Plate I, Fig. 7, arrows). Epistomatal chambers show eroded and fused crystalloids wax, clearly visible in transverse sections (Plate I, Figs. 7,8) as a top sheet distinct to waxes embedded in the cuticle. Crusts were not usually seen in samples from Cigliano (not fumigated), where, apart from some scattered particles and granules the surface was quite smooth, even relatively clean (Plate I, Fig. 1). Crusts appear on most of the samples from Pisciarelli and are clearly visible in the area which surrounds stomatal apparatuses.

The abaxial side of the needles fumigated by volcanic gases presents the most part of stomatal apparatuses affected by crystalloids wax erosion and fusion phenomena in both epistomatal chamber and cuticle surface (Plate I, Figs. 5,7,8). On the other hand, the adaxial surface is less damaged and crystal tubules, even if eroded, can be seen (Plate I, Fig. 9). For not fumigated needles epicuticular waxes are more or less well-preserved in both epistomatal chamber and cuticle surface (Plate I, Figs. 1,3,4,6).

The majority of the stomatal apparatuses in abaxial side of current- and first-year-old needles from Pisciarelli (= fumigated) have fused tubules inside the epistomatal chambers

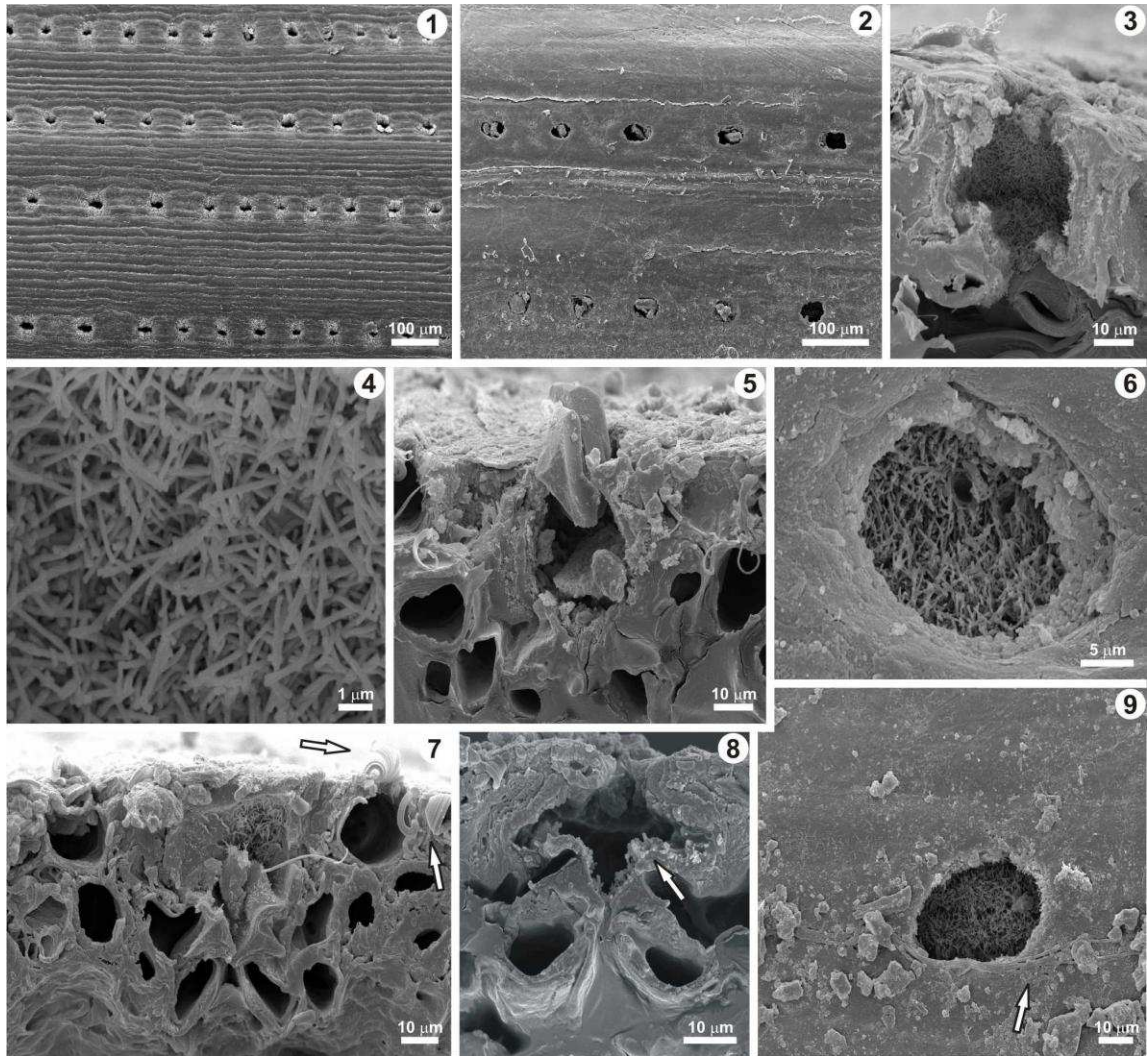


Plate I. *Pinus halepensis*, SEM of abaxial and adaxial cuticles, for to both trees experiencing and not chronic fumigation by volcanic gases. Stomatal class refers to Nicolotti et al. (2005) classification in which: class 0 = stomatal structure displaying no sign of alteration, class 1 = for slight sign of alteration, class 2 = for moderate sign of alteration, class 3 = for severe alterations.

1. Abaxial side of not fumigated current-year-old needle showing crystalline wax structure around epistomatal rim and along the contact zones of epidermal cells. Photo MC3lon1. 2. Abaxial side of fumigated current-year-old needle showing heavily melted epicuticular waxes partially occluding epistomatal chambers. Numerous volcanic dust particles obstruct stomatal apparatuses. Photo PP1.2long4. 3. Transversal section of not fumigated abaxial side of current-year-old needle showing a stomatal apparatus with well-preserved crystalline wax in epistomatal chamber (Class 0). Photo MC3s.t.1.1. 4. Detail of Fig. 3, showing completely uninfluenced wax crystal in stomatal antechamber (Class 0). Photo MC3s.t.1.2. 5. Transversal section of fumigated abaxial side of current-year-old needle showing a stomatal apparatus displaying severe alterations with the formation of wax granules and a volcanic dust particle that totally obstruct the epistomatal chamber; the network of microtubules is almost all melted (Class 3). Photo PP1.2s.t.3. 6. Abaxial side of not fumigated first-year-old needle showing slightly influenced wax crystals in epistomatal chamber (Class 1). Photo MC2.3long4. 7. Transversal section of adaxial side of fumigated current-year-old needle showing a stomatal apparatus with its epistomatal chamber almost filled with a wax clump, except a small area in the right part in which the wax tubes are visible (Class 2). The arrows show crystalloids structures underneath the epicuticular crusts. Photo PP2.1s.t.2. 8. Transversal section of fumigated first-year-old adaxial side of needle showing a rather flattened and deformed stomatal apparatus lacking in wax tubes in the epistomatal chamber, except for the base (arrow) in which the wax is heavily fused (Class 2-3). Photo PP2.2s.t.2. 9. Outer view of fumigated adaxial side of current-year-old needle showing stomatal aperture with more or less dense tuft of tubular wax structures in the upper part of epistomatal chamber. Mycelia fungi are also visible (arrow) (Class 1). Photo PP1.1long5.

forming a flat and solid wax plug or amorphous crusts above the pore which was completely or partially occluding the stomata in most cases (Plate I, Fig. 7). Instead, current- and first-year-old needles that do not experience chronic fumigation, regardless of the age of the needles sampled, present most of their stomatal apertures not occluded, thereby remaining almost completely open, apart from some minor fusing tubules (Plate I, Fig. 3). On the abaxial side of current- and first-year-old needles experiencing chronic fumigation, crystalloid wax tubules rarely persisted and they occupy limited portion only in epistomatal chambers (Plate I, Fig. 7 top right). It was noticed that in current-year-old needles from Pisciarelli the wax degradation was beginning very early and it increases.

Frequently, in abaxial side of some Pisciarelli stomatal apparatuses, amorphous wax and/or particles of other material -30-40 μm width- filled the stomatal aperture, therefore blocking the direct view into the chamber (Plate I, Figs. 5,7).

In abaxial side of not fumigated needles, the rim of stomatal apparatuses retains crystalloid wax, whereas in fumigated needles the wax tubes are absent (Plate I, Figs. 1,2).

Frequently, the abaxial side of needles experiencing chronic fumigation presents deformed stomatal apparatuses so losing the typical funnel-like cavity (Plate I, Fig. 8). Stomatal damages are common and may include collapses (Plate I, Fig. 8), depression, degradation of guard cells (Plate I, Fig. 2), and occlusion with wax clumps (Plate I, Figs. 2,7). Decay of the epistomatal chambers and empty cavities in stomatal chambers can also be noted (Plate I, Figs. 5,8).

On both abaxial and adaxial surfaces of fumigated needles, a great amount of dust occurred (Plate I, Figs. 2,9), while on the needles not fumigated by volcanic gases non-reactive dusts only mechanically disturb the wax structures (Plate I, Fig. 1). Moreover, most of Pisciarelli needles are affected by fungal infection (Plate I, Fig. 9).

1.3.3. Transmission electron microscopy observations

The ultrastructure (cuticle and cell wall) of abaxial epidermal cells from fully grown needles (current- and first-year-old needles) of both localities have been studied in details. Cuticular membranes (CM) of all ordinary epidermal cell are composed of a fibrillar layer B (CL) in which an outer, middle and inner zones can be distinguished (Plate II). The cuticle proper (CP = A) is absent. Differences between the cuticular structures are given below. All the data given below are the means based on 30 measurements, the percentages of each component of the cuticle and of cell wall are also given (Table I).

In the current-year-old not fumigated needles (Plate II, Figs. 1-5) the total thickness of B1 + cell wall (CW) is 3.80 μm (Plate II, Figs. 1,2). 58.7% is composed of the cuticular membrane, which may be further divided into three fibrillous zones: B1 outer (14.6%; 0.56 μm), B1 middle (19%; 0.72 μm) and B1 inner (25.1%; 0.95 μm). In the first-year-old needles (Plate II, Figs. 6-11) the total thickness of CM + CW is 4.09 μm (Plate II, Figs. 6-7). 46.9% is composed of the cuticular membrane, which may be further divided into an outer fibrillous zone B1 (13.4%; 0.55 μm), and middle zone (33.5%; 1.37 μm). The B1 inner zone is lacking in this case.

In the current-year-old fumigated needles (Plate II, Figs. 12-16) the total thickness of CM + CW is 4.14 μm (Plate II, Figs. 12,13). 74.4% of the CM + CW is composed of the cuticular membrane, which may be further divided into three fibrillate zones: B1 outer (17.1%; 0.71 μm), B1 middle (33.4%; 1.38 μm) and B1 inner (23.9%; 0.99 μm).

Table 1. Statistical values, made with 30 measurements for cuticular membrane (CM) and cell wall (CW) of the epidermal cells. Note: the cuticular membrane CM is made up with cuticular layer CL (= B1 outer, middle and inner layers). All the measurements are in μm . min-max = minimum and maximum values observed; % = percentage of each detailed part of the cuticle and cell wall; st-d = standard deviation; var = variance.

not fumigated (current year)						not fumigated (first year)				
	mean	min-max	%	st-d	var	mean	min-max	%	st-d	var
Total CM + CW	3.80	3.03-5.01	100	0.53	0.28	4.09	2.64-7.58	100	1.47	2.17
CM = B1	2.23	1.48-2.79	58.7	0.35	0.12	1.92	1.17-2.88	46.9	0.49	0.24
B1 outer	0.56	0.29-0.69	14.6	0.09	0.01	0.55	0.34-1.14	13.4	0.21	0.04
B1 middle	0.72	0.39-1.15	19.0	0.18	0.03	1.37	0.52-2.17	33.5	0.46	0.21
B1 inner	0.95	0.39-1.60	25.1	0.34	0.12			0		
CW	1.57	0.87-2.32	41.3	0.37	0.14	2.17	0.84-5.02	53.1	1.12	1.26
fumigated (current year)						fumigated (first year)				
Total CM + CW	4.14	3.40-5.86	100	0.70	0.49	3.92	3.25-5.52	100	0.50	0.25
CM = B1	3.08	2.27-4.29	74.4	0.56	0.31	2.30	1.93-3.46	58.7	0.48	0.23
B1 outer	0.71	0.37-1.27	17.1	0.28	0.08	0.38	0.22-0.80	9.7	0.16	0.03
B1 middle	1.38	0.94-1.94	33.4	0.24	0.06	0.86	0.44-1.60	21.9	0.29	0.08
B1 inner	0.99	0.36-1.53	23.9	0.28	0.08	1.06	0.40-1.61	27.1	0.27	0.07
CW	1.06	0.66-2.40	25.6	0.37	0.14	1.62	0.22-2.15	41.3	0.52	0.27

In the first-year-old needles (Plate II, Figs. 17-21) the total thickness of CM + CW is 3.92 μm thick (Plate II, Figs. 17,18). 58.7% of the CM + CW is composed of the cuticular membrane, which may be further divided into the fibrillous zones: B1 outer (9.7%; 0.38 μm), B1 middle (21.9%; 0.86 μm) and B1 inner (27.1%; 1.06 μm).

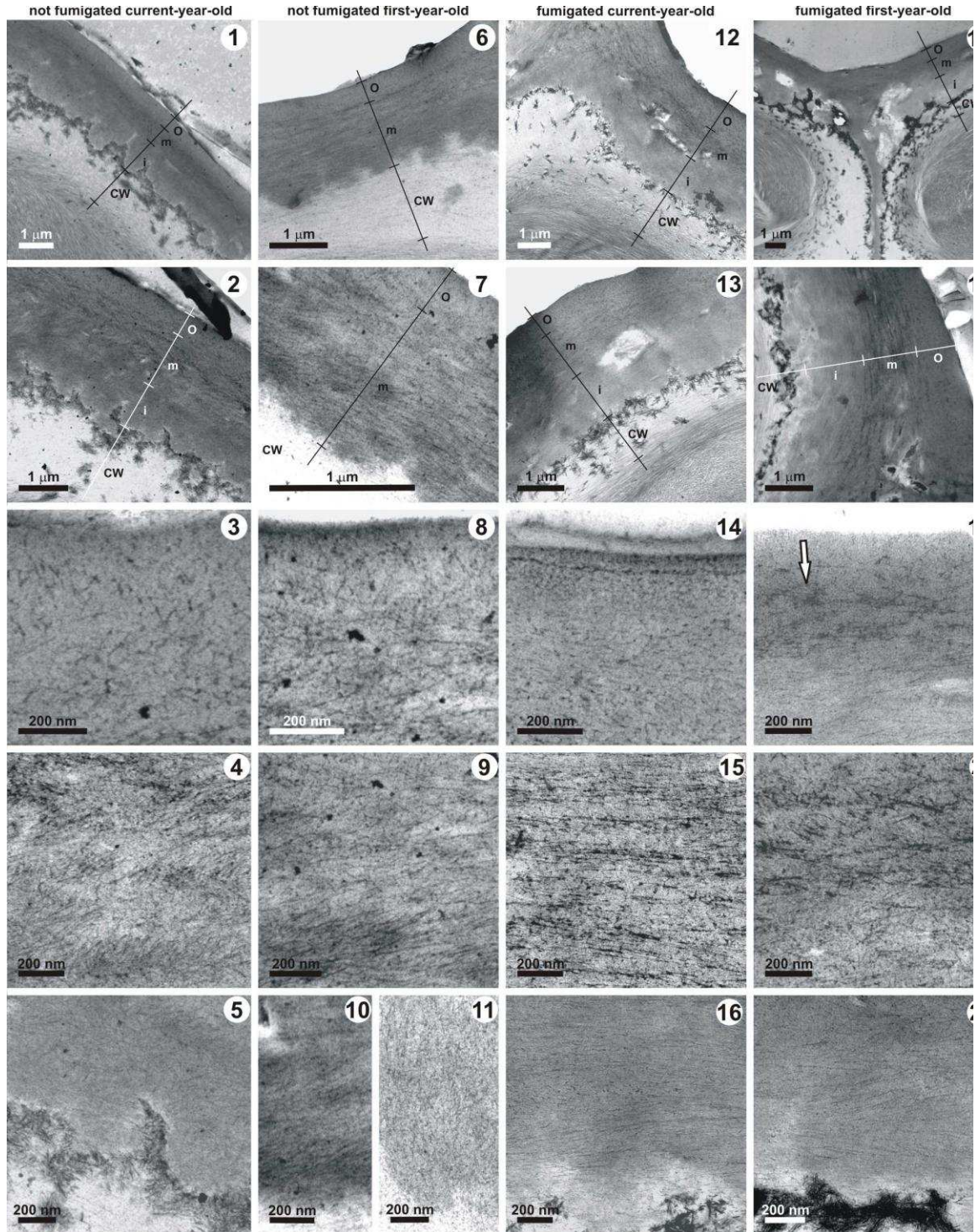


Plate II. *Pinus halepensis*, TEM of fumigated and not fumigated needles. All photographs were taken from ordinary epidermal cells in transversal sections, except a longitudinal section of photograph 11. The cuticle is made up with B1 fibrillous layer (= CL, cuticular layer) divided in three zones: o = outer zone, m = middle zone and i = inner zone.

1-5. Not fumigated current-year-old cuticle. 1. General view of cuticle and cell wall. Photo GGAB0018. 2. Magnification of cuticle and cell wall showing also the three B1 zones. Photo GGAB0007. 3. Detail of B1 outer zone showing slightly dense fibrils more or less random orientated. The upper part of middle zone just appears in the bottom of the photo, more densely stained. Photo GGAB0013. 4. Detail of B1 middle zone of cuticle showing more dense and crowded fibrils randomly orientated and in variable densities. Photo GGAB0012. 5. Detail of B1 inner zone showing less dense and the random disposition of fibrils. Note the lower part of middle zone just at the top right of the photo. Photo GGAB0011.

6-11. Not fumigated first-year-old cuticle. 6. General view of cuticle and cell wall. Photo GGAB0012. 7. Magnification of cuticle and cell wall: the lack of B1 inner zone can be noted. Photo GGAB0006. 8. B1 outer less dense zone showing a prevalent random orientation of fibrils. Photo GGAB0008. 9. B1 middle zone more dense in parallel fibrils (especially visible downwards) and randomly orientated (upwards). Photo GGAB0008. 10. B1 base part of middle zone showing fibrils oblique or parallel to the cell wall. Photo GGAB0006. 11. B1 other middle zone of cuticle showing fibrils perpendicularly orientated, the upper part of cell wall being at the bottom of the photo. Photo GGAB0009. 12-16. Fumigated current-year-old cuticle. 12. General view of cuticle and cell wall with numerous calcium oxalate crystal deposits between middle and inner zones of the cuticle. Photo GGAB0017. 13. Magnification of cuticle and cell wall showing the three B1 zones and a big amount of calcium oxalate crystals deposit between the B1 middle and B1 inner zones. Photo GGAB0028. 14. B1 outer zone of cuticle showing a prevalent parallel orientation of fibrils and quite numerous granules more or less aligned tending to simulate the A1 polylamellate layer of the cuticle proper A. Photo GGAB0042. 15. B1 middle zone more dense in fibrils showing fibrils orientated parallel. Photo GGAB0019. 16. B1 inner zone of cuticle less dense showing a parallel disposition of fibrils, above the upper part of cell wall at the bottom of the photo. Photo GGAB0020. 17-21. Fumigated first-year-old cuticle. 17. General view of cuticle and cell wall with numerous and extensive calcium oxalate crystal deposits, especially at the anticlinal wall location between the two cells. Photo GGAB0040. 18. Magnification of cuticle and cell wall showing the three B1 zones and calcium oxalate crystal deposits. Photo GGAB0016. 19. B1 outer zone of cuticle showing the parallel orientation of fibrils tending to form dense clusters (arrow) and quite numerous granules at the top part tending to simulate a cuticle proper "A". Photo GGAB0027. 20. B1 middle zone with more dens and thicker fibrils showing fibrils more or less parallel orientated tending to form clusters. Photo GGAB0010. 21. B1 inner zone showing the parallel disposition of fibrils, above the upper part of cell wall at the bottom of the photo. Photo GGAB0044. CW = cell wall, o = outer, m = middle, i = inner. All the microphotographs are presented with the same orientation, the top of each print corresponding to the upper side of the cuticle, the bottom corresponding to the lower side of the cuticle.

For both fumigated and not fumigated needles a decrease of CM thickness between current- and first-year-old needles, has been observed.

A tendency to a reduction in the fibrillar structure followed by an increase of the granular component has been noted between not fumigated and fumigated needles. This feature is visible for all B1 zones but especially for B1 middle zone (Plate II, Figs. 4,9,15,20). Moreover, it is worth noting the disposition of fibrils: they are more or less chaotic in the three B1 zones of not fumigated needles, while they are much more parallel to the cell surface in the B1 outer (Plate II, Figs. 14,19) and inner (Plate II, Figs. 16,21) zones of influenced material. The cuticle of current- and first-year-old symptomatic needles, in respect to control needles, exhibits a much higher number of more or less rounded shaped areas of low electron-density; these areas represent calcium oxalate deposits, usually located between the middle and inner B1 zones (Plate II, Figs. 12,13,17,18).

1.4. Discussion

The present study based on 30 measurements is the most precise statistic analysis so far done for extant plant cuticles. Similar statistic analyses were provided for fossil plants as *Pachypteris gradinarui* from the Early Jurassic of Romania (Guignard et al., 2004) and the

cheirolepidiacean *Pseudofrenelopsis dalatziensis* from the Cretaceous of China (Yang et al., 2009). The relation among the different zones [cuticular membrane (CM) (B1 outer, middle and inner) + cell wall (CW)] can be traced based on the confidence interval value ($CI = \bar{X} \pm \sqrt{\frac{\text{var}}{n}} \times 1.96$, giving 95% α risk) among 6 characters (Fig. 4). It allows to estimate the significance of the differences between the four types of studied material. The results may be also summarised in the schemes representing different layers of the epidermal cells cuticle among the four types of studied material (Fig. 5).

Moreover, this multidisciplinary approach also allows to evaluate different changes, in relation to the degree of epicuticular and epistomatal waxes degradation, between CM and CW experiencing different environmental conditions and in the quantification of Ca-oxalate crystals deposits and stomatal damage (Table 2). It is possible to trace these changes not only between the two types of environment (not fumigated and fumigated) but also during one year of needle growth. Exposition to volcanic environment characterized by gas emissions, causes an acceleration in needles ageing, and through the analysis of the degree of needles damage, one can clearly distinguish between fumigated and not fumigated needles (Table 3). Comparing not fumigated to fumigated needles of the same age, in the latter symptoms amplify (Table 2): an increase in epicuticular waxes fusion, a degradation in epistomatal waxes and an increase in Ca-oxalate deposits can be observed. All these syntheses allow to discuss several diverse environmental and fossil aspects.

1.4.1. Environmental response of *Pinus halepensis* to volcanism

1.4.1.1. Epicuticular and epistomatal wax

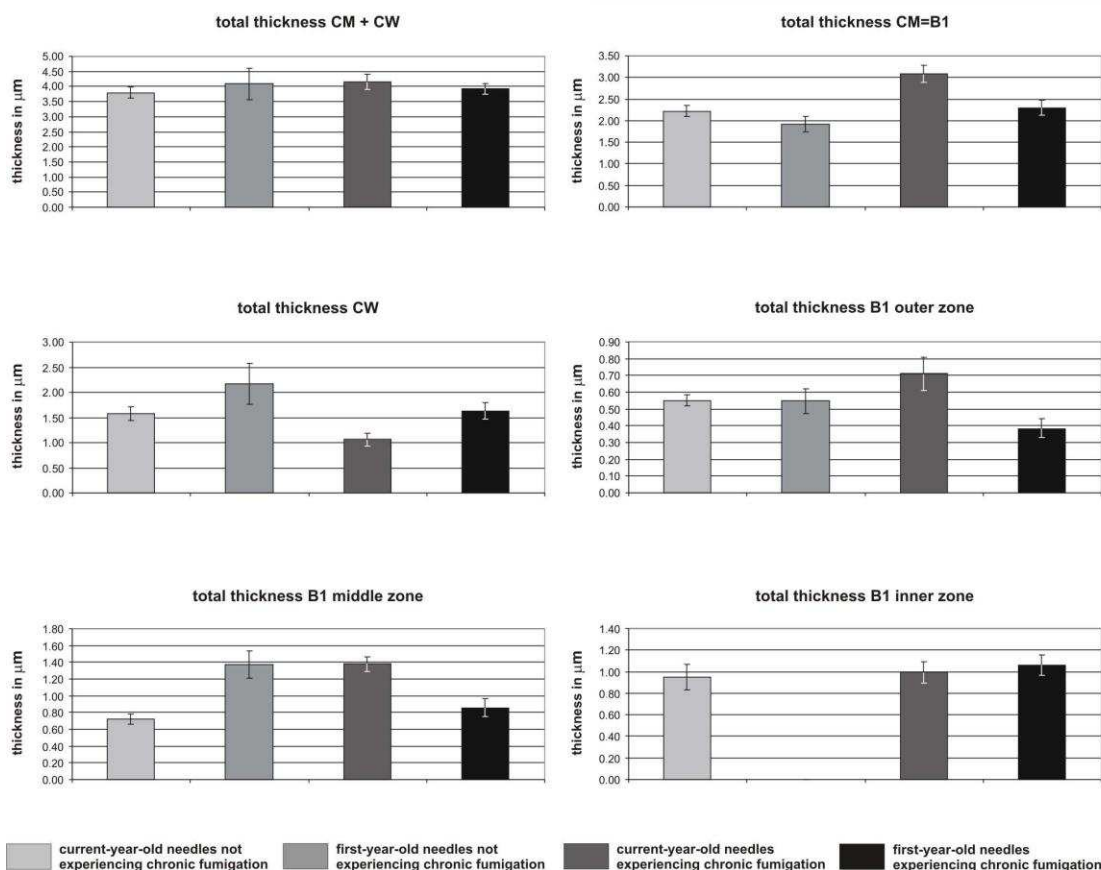
Stomatal aperture

In conifers growing wild, like *Pinus*, the thin, fibrillar and netted epicuticular and epistomatal waxes are well-developed especially in (with dense tufts of tubular wax structures) and around stomatal entrance (Hanover and Reicosky, 1971; Yoshie and Sakai, 1985; Huttunen, 1994). The stomatal wax tubes forms in young leaves above the guard cells, filling the stomatal pits and making appear stomata as white disks under a microscope (Jeffree et al., 1971; Feild et al., 1998). In conifers, the genus *Pinus* is characterized by a type D Florin ring (Yoshie and Sakai, 1985) and, in particular, according to the distributional pattern of epicuticular wax, *P. halepensis* can be ascribed to

the “Type I” of the Yoshie and Sakai (1985) classification in which the wax crystals are deposited in the epistomatal chambers and in the stomatal bands.

Usually the leaves from Pisciarelli display an overall wax degradation both on epicuticular surface and in the epistomatal chamber (Plate I, Figs. 2,5). Permanent emissions of volcanic toxic gases affect needles already during the initial growing season. Wax tubes may start fusing early with a faster rate than in clean areas, and as Turunen and Huttunen (1990) claimed in their overview of many conifers, it usually starts during the second year of needles growth. Probably, the waxes erosion is indirectly due to the H₂S [that can originate H₂SO₄ (Delmelle, 2003)] but, as Turunen and Huttunen (1990) point out, the specificity of the symptoms in the wax structures to different air pollutants is limited. In the present case of Aleppo pine the crystalloid waxes not only of first-year-old but also of current-year-old needles of not fumigated stomatal antechambers show some typical signs of ageing erosion (e.g. Turunen and Huttunen, 1996). However, comparing the two sets of data (fumigated and not fumigated trees) it is obvious that the degree of

Fig. 4. Mean and confidence interval CI ($= \bar{X} \pm \sqrt{\frac{\text{var}}{n}} \times 1.96$, giving 95% α risk) for each component of the cuticle and of cell wall (CW). Note: the values represent the mean \pm CI. The cuticular membrane (CM) is made up of cuticular layer CL (= B = B1 divided in outer, middle and inner zones). The B1 inner zone is lacking in not fumigated first-year-old needles. All the measurements are in μm .



occlusion of suprastomatal chambers (with wax crusts) increased in trees growing in the polluted site in respect to the control site used in this study (Table 2). In normal conditions, large amounts of wax occlude the epistomatal chambers and the Florin rings are characterised by large opening size. Instead, in the needles under the influence of volcanic gases of Pisciarelli, the reduction of wax tubes in epistomatal chambers is associated to a reduction of the stomatal aperture. Numerous stomatal apparatuses even display collapse and decay of the epistomatal chambers (Plate 1, Fig. 8). On the abaxial side of Pisciarelli needles, amorphous wax filling epistomatal chambers, as well as the rims, could probably disturb normal gas exchange and, as Holroyd et al. (2002) claimed in their study on *Arabidopsis* mutant plants, alterations in wax composition could also affect stomatal development.

Volcanic toxic compounds and wax alterations

After water and carbon, sulphur is the major constituent of the fumes emitted by volcanoes (Le Guern et al., 1988) and as a matter of fact, among Pisciarelli fumarolic effluents a relatively high quantity of hydrogen sulphide (H_2S) is released (Chiodini et al., 2010a). Brown (1982), Lorenzini and Nali (2005) and also Haworth et al. (2010) claimed that the H_2S oxidizes rapidly in the atmosphere to form SO_2 ; this last rapidly converts into H_2SO_4 (Visscher et al., 2004) considered as an oxidation product of SO_2 or H_2S (Mather et al., 2003). In extant plants SO_2 concentrations between 0.1 and 1.0 ppm can cause rapid changes in stomatal conductance for a wide range of plant species. Changes in stomatal conductance during period of SO_2 exposure are apparently associated with regulating the extent to which foliar injury develops (Winner and Mooney, 1980b). Winner and Mooney (1980a), studying the fumigation with SO_2 of two Californian shrub chaparral species (*Diplacus aurantiacus* and *Heteromeles arbutifolia*, angiosperms), observed a decline in both photosynthesis and transpiration during the fumigation periods and a tendency to close during fumigation for guard cells. They also state that the response of guard cells during periods of SO_2 exposure may be one of the factors contributing to variations in SO_2 resistance between plants. According to the same authors, owing to the effects on plant community structure and plant metabolism, the SO_2 may be considered as a chronic environmental stress for vegetation. Actually, the impact of these sulphurous gases on plants is much more complex, since they may act as toxin or plant nutrient upon foliar deposition (De Kok et al., 2007). SO_2 and H_2S affect leaves causing also dwarfism phenomena (Wood and Boorman, 1977). More precisely for conifers, in their study on

Picea abies (Norway spruce) needles with a treatment simulated acid deposition, Raddi et al. (1994) showed that H_2SO_4 altered significantly both the epicuticular wax and fibrillar wax structure in the epistomatal chamber. Rinallo et al. (1986) observed that aqueous sulphuric acid of pH 3.5 had greater effects on epistomatal waxes of silver fir (*Abies alba* Mill.) and Norway spruce needles than aqueous nitric acid of the same concentration.

The present study of *Pinus halepensis* also contributes to the analysis of sulphur influence since, on control needles (Cigliano locality) no CaSO_4 crystals were found on needle surfaces. Conversely, in fumigated needles from Pisciarelli, the H_2SO_4 action is testified by the occurrence of CaSO_4 crystals on needle surface, the result of a reaction between the calcium leached out of the needles as observed by Turunen et al. (1994) on *Pinus sylvestris* and *Picea abies* needles, subjected to acid rain treatment, and the sulphur deriving from both H_2SO_4 and/or from tephra (Smith et al., 1983) deposited on needle surfaces.

As it was already shown that removal of waxes from cuticles results in an increase of gas and water permeabilities of up to three orders of magnitude (Lendzian and Kerstiens, 1991), the formation of wax crusts on needles epidermis of the present Aleppo pine could act as a protecting (closing) device against further H_2S , SO_2 and H_2SO_4 attacks increasing the boundary layer resistance. This was already noticed by Kayode and Otoide (2007) in matured leaves of an angiosperm *Newbouldia laevis*. As Haworth and McElwain (2008) claim, in extant plants resistance to fumigation with toxic gases is often associated with an increase in the boundary layer resistance. The angiosperm *Metrosideros polymorpha* growing in the Kilauea crater region (Island of Hawaii) is resistant to persistent fumigation with toxic volcanic gases (SO_2 , H_2S , HCl , HF) while other species (e.g. *Dodonaea eriocarpa*, an angiosperm) growing in the same region displays a noticeable damage (Winner and Mooney, 1980b). *M. polymorpha* from the Kilauea crater region displays heavily trichomous abaxial surfaces, while those in neighbouring regions, not experiencing chronic fumigation, possess non-trichomous cuticles (Haworth, 2006). This pattern suggests (Haworth, 2006) that the development of trichomes in the Kilauea Crater region is an adaptation to increase boundary layer resistance, and consequently reduces entry of toxic gases into the leaf. With present observations and measurements for *Pinus halepensis* a sulphur reaction seems to exist too.

The almost total fusion of wax crystals in needles from Pisciarelli volcanic area is not comparable to the mechanical injury under windy condition where leaves scrape against each other. In her SEM study on *Acer pseudoplatanus* leaves under the effect of wind,

Wilson (1984) showed different microscopic damages like dark and light brown lesions, holes and deformations of the leaf. The wax degradation in specimens from Pisciarelli presents different features in respect to the impact of wind blown particles. van Gardingen et al. (1991) studied *Picea sitchensis* and *Pinus sylvestris* in a wind tunnel showing different diseases in respect to the present wax degradation in *P. halepensis*. In particular, in *P. sylvestris* the authors noticed that the most damage was on the cells surrounding the stomatal antechamber or on needle ridges. Moreover, needles damage cannot be related to the ozone exposition because the gaseous nature of these molecules should have affected the overall needles. The structural injuries observed in the stomatal apparatuses are not correlated to the surfactant action. Usually this kind of damages are observed in plants growing 100 m far from the sea (because Pisciarelli is located far from sea), near the mouth of a river or stream, near the outlet of sewage canals and in any other coastal areas where the surface currents carry a surfactant load (Busotti et al., 1995; Nicolotti et al., 2005). Moreover, spraying with NaCl solutions can be excluded since it does not induce the occlusion of stomatal openings (Krause, 1982).

Other sulphur considerations

Although it is not directly the aim of this study, some EDS measurements allow to discuss about sulphur input. Cuticle and stomata are commonly involved in sulphur and other molecules absorption (Haworth and McElwain 2008; Haworth et al., 2010; Kivimäenpää et al., 2010). In their article about various ecological and cuticular micromorphology aspects, Haworth and McElwain (2008) summarised data of various authors on different taxa, sulphur (combined with oxygen or hydrogen) is said to be “absorbed via the cuticle as well as the stomata”. In the present study of *Pinus halepensis*, 40 analyses of ultrathin sections (70 nm) of both fumigated and not fumigated needles (Fig. 3A-D) have demonstrated the absence of sulphur within the overall cuticle thickness of epidermal cells and cytoplasm. Wide epicuticular and epistomatal wax fusion phenomena can be also observed. The lacking of sulphur in the structure of the cuticle suggests that the uptake of volcanic toxic gases takes place via stomatal apparatuses. As Le Guern et al. (1988) point out, the sulphur is metabolized because it is an essential element for plants, and it participates in physiological reactions and is eliminated accordingly. Haworth and McElwain (2008) point out that cuticles of evergreens tend to have lower permeability than those of deciduous species, possibly reflecting adaptation of species with long-lived foliage to conserve water during periods of reduced water availability (Kirsch et

al., 1997; Gratani and Bombelli, 2000). With the present study we may think that epicuticular and epistomatal wax degradation in *P. halepensis* needles experiencing chronic fumigation with volcanic gases from Pisciarelli could be related to the H_2S and/or H_2SO_4 action and inputs.

Wettability of leaf surface

Wettability of plant surfaces is related to the composition, structure and cristallinity of epicuticular waxes (Heredia and Dominguez, 2009). Airborne pollutants accelerate erosion of cuticular waxes of conifers (Huttunen, 1994; Riederer, 2006; Shepherd and Griffiths, 2006) and thus may be responsible for forest decline in Europe and elsewhere (Riederer, 2006). Erosion of the waxes can change needle wettability (Cape, 1983) and rain retention (Turunen and Huttunen, 1990).

As seen above, needles from Pisciarelli experiencing chronic fumigation, is lacking of well-preserved epicuticular wax crystalloids, therefore, the Lotus-effect (an anti-adhesive property against particulate contamination; see Barthlott and Neinhuis, 1997; Neinhuis and Barthlott, 1997) compromises the removal of contaminant particles.

An added point concerns dust particles. As Ots et al. (2010) claimed, tree leaves, such as conifer needles, can efficiently accumulate dusts, which are an important stress factor. Moreover, Cape et al. (1989) found that leaf wettability of the conifers *Picea abies* and *Pinus sylvestris* rose with increasing amount of dust on leaf surfaces. As a matter of fact, in the present study, although it is not directly the aim of this study, the great amount of dust covering needle surfaces (Plate I, Figs. 2,5,9) emitted by principal fumarole of Pisciarelli and derived from volcanic airborne (Delmelle, 2003) together with degradation of wax could determine an increase of wettability. As it is certainly affecting plant biology it could be one of the multiple reasons of the rarity of plant taxa in this extreme environment.

Additional observation is that a heavy fungal infection affects almost all Pisciarelli needles (Plate 1, Fig. 9). This is probably related to several factors, making unfavourable abiotic conditions in these trees, and becoming then more susceptible to pathogens as noticed by Sieber (2007) for conifers. For instance, the particular topographic leaf surface of *P. halepensis* together with the presence of hydrophobic epicuticular waxes -that are frequently crystals-, may have considerable influence on all fungal spore adhesion and retention (see Carver and Gurr, 2006). Moreover, the acidified aqueous environment on needle surface (H_2SO_4) could favour fungus proliferation as it was shown in *Phyllosticta ampellicida* pycnidiospores which adhere firmly to hydrophobic artificial substrata in an

acidified aqueous environment (Kuo and Hoch, 1996). Another factor is that water collecting on needle surfaces is essential for all fungal infections (Huttunen, 1984) and thus related to wettability. In their study of *Picea pungens* needles, Patrie and Berg (1994) noticed that the epicuticular wax progressively deteriorated as fungus proliferated, so for the present *Pinus halepensis* the degradation of epicuticular waxes and the consequent increase in wettability could be also responsible of fungus proliferation.

Microenvironment

According to Nicolotti et al. (2005) stomatal damage classes, the abaxial side of the current- and first-year-old fumigated needles can be placed in the Classes 2 and 3, namely a “stomatal structure displaying moderate sign of alteration” and “severe alteration”, respectively (Plate 1 and Table 2). On the other hand, the adaxial side of needles can be ascribed to the Classes 0 and 1, namely a “stomatal structure displaying no sign of alteration” and “slight sign of alteration”, respectively (Table 2). So, for one single needle, the difference in adaxial and abaxial stomatal Class attribution seems to be related to their disposition on the two sides of needles. As a matter of fact, *P. halepensis* retains leaves spreading by fascicular sheath which are coupled in pairs, therefore the adaxial side of a needle is more protected than the abaxial one, demonstrating a very differentiated microenvironmental influence of the epidermis of the leaves.

Another point is that the needles of *P. halepensis* experiencing chronic fumigation (Pisciarelli) show another kind of sensibility to environment, as the degree of epicuticular wax tubule fusion decreases on the adaxial side (more protected) of needles, but increases towards the needle-tip (less protected). This fact leans towards a H_2SO_4 action, probably also in combination with H_2S or SO_2 gases that would act less on the adaxial side of needles. The present study adds another very sensible microenvironmental example to those in plants; in conifers it seems to be a general case as Thompson and Kats (1978) treated a number of plants (five angiosperms and two conifers) with continuous fumigation of H_2S founding that Douglas fir (with free-leaves) was much more sensitive than Ponderosa pine (which has leaves in bundle of threes, occasionally in two's) to foliar injury.

1.4.1.2. Cuticular membrane (CM) + cell wall (CW)

Pinus halepensis cuticle type

The effect of volcanic gases on the epidermis in conifers has not been previously studied by TEM, and no detailed ultrastructural studies have been made so far on the cuticle of *Pinus halepensis*. As Guignard et al. (2001) point out, according to the Achangel'sky et al. (1986) terminology used commonly for both fossil and extant plants, within cuticle thickness it is possible to distinguish a cuticle proper (termed A, often lamellate in its outermost part and granulous below) and cuticular layer (termed B, mostly fibrillous). The cuticle proper is absent in the studied species. Since the cuticular material of fumigated and not fumigated plants by volcanic gases is predominantly made up of fibrils, *P. halepensis* cuticle can be attributed to cuticular layer B and divided into three zones, respectively named in the present study B1 outer, middle and inner. Although unfortunately to our knowledge previous statistical detailed studies of the cuticle do not exist, it has to be noticed a certain homogeneity in the cuticle structure among species of the genus *Pinus*, as the studied *P. halepensis* cuticle belongs to the type 4 ("All regions *reticulate*") of Holloway's (1982) classification in which he included other species of *Pinus* (*P. sylvestris* and *P. nigra*), as well as other conifers (*Picea sitchensis* and *Abies balsamea*). Besides of the identification to one of Holloway's general types, the present material seems to be a good example to conduct further statistical ultrastructural studies of the cuticle among *Pinus* species in order to verify the Holloway's (1982) remark: "Because the CM (= cuticular membrane) of plants is so heterogeneous in structure it is dangerous to oversimplify and generalize about its morphology and construction - there is no typical plant cuticular membrane. Consequently, each species must be considered individually and it should not be assumed that any structural features which may be observed are of universal occurrence.". However, taking into account a TEM photo of *Picea sitchensis* type 4 cuticle (Holloway, 1982, fig. 16), it seems that there exist clear differences with *Pinus halepensis*, although in this latter genus the cuticle proper A is lacking, but only B1 layer is present.

CM + CW thickness

Plants of the same species experiencing different environments have a response at histological level. In the studied material, the total thickness of CM + CW is roughly constant with an average thickness of 3.99 μm (3.80-4.14 μm , see Table 1) among the four

types of studied cuticles. However, susceptible variations in the proportion of different zones between needles experiencing- or- not chronic fumigation can be noted (Table 1). It is worth noting that in both fumigated and not fumigated needles the cell wall thickness

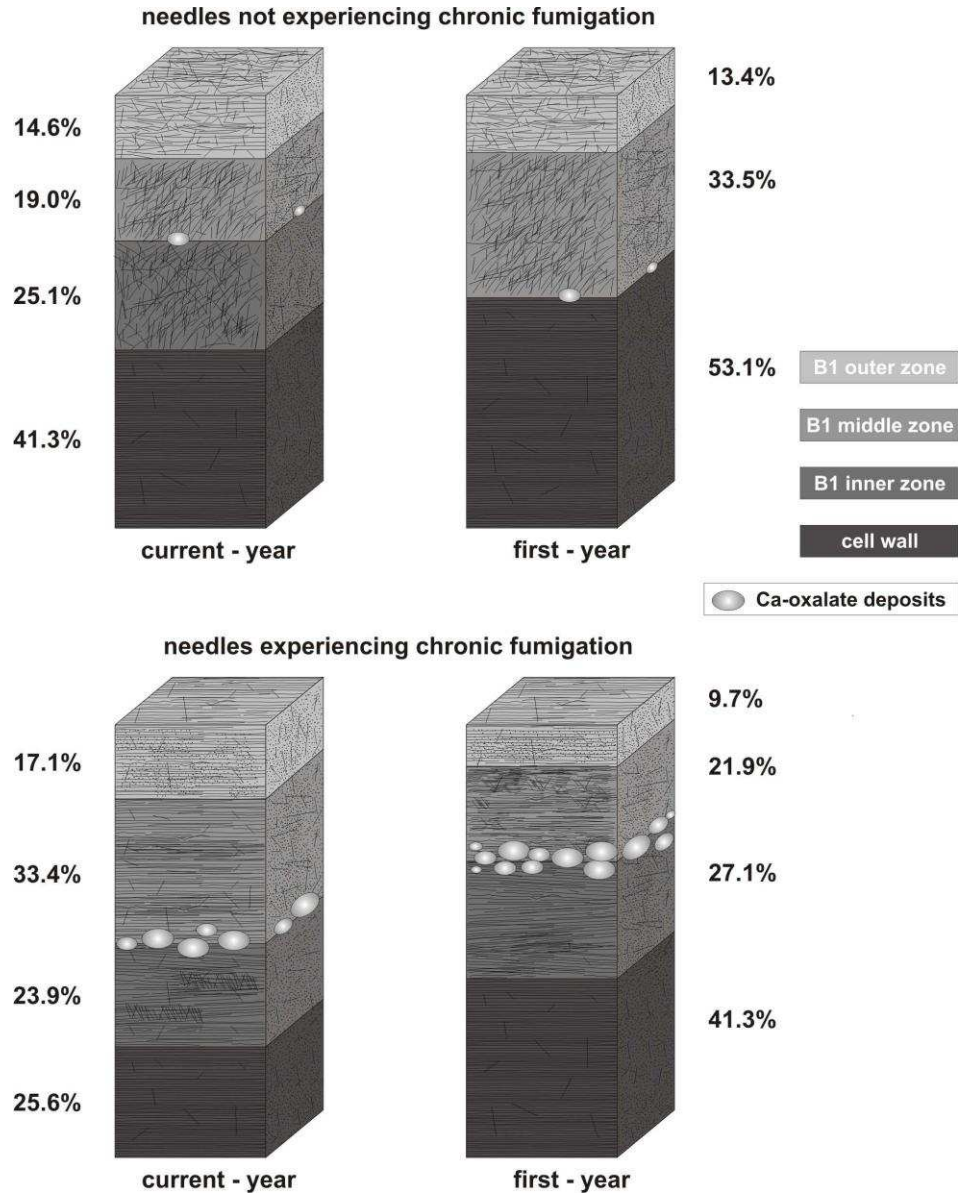


Fig. 5. Three-dimensional reconstruction of both cuticle and cell wall (CW) for needles experiencing- or- not chronic fumigation. Note: the cuticular membrane CM is made up with cuticular layer CL (= B = B1 divided in outer, middle and inner layers).

increases with needles ageing. In Pisciarelli area, *P. halepensis* needles respond to volcanic gases by producing a thicker cuticle membrane (CM = B1) than those that are not fumigated. However, the cuticular membrane (CM) is always greater than cell wall (CW) except for first-year-old needles from Cigliano, not fumigated (see Table 1). This would suggest that higher energy compounds are allocated to protection mechanisms in the

cuticular membrane, demonstrating once again that cuticle protects basic leaf functions. In detail, the lacking of B1 inner zone in not fumigated first-year-old needles (Table 1; Plate II, Fig. 7; Figs. 4,5) can be explained by an increase in fibril amount in the inner zone (usually characterized by a low density of fibrils), thus making this layer indistinguishable from the middle zone (usually with a high density of fibrils).

CM + CW development

This multidisciplinary study clearly shows the sensitivity of *P. halepensis* to an extreme environment, during needle ontogeny and therefore the CM and CW formation (Plate II). For both fumigated and not fumigated current-year-old needles, the total thickness of cuticle (CM) + cell wall (CW) is statistically comparable (Table 1, Fig. 4), but the percentage of each layer is different according to the age and exposition to gases (Fig. 5), thus showing a diverse equilibrium among zones of the same needle as response to growth and environmental factors. The needles seem to react early in ontogeny to volcanic environment because the thickness of B1 or CW already differs statistically between not fumigated and fumigated current-year-old needles (15.7%). After one year of growth, these differences are still maintained, although they slightly decreased (11.8% of reduction). To sum up, the variations of the three zones of the cuticle between fumigated and not fumigated needles are statistically significant (Fig. 5) and this fact mainly concerns B1 outer and middle zones, while B1 inner zone does not change considerably.

CM and CW ageing

If the response of the plant starts early in the growth of cuticle membrane (CM) + cell wall (CW), after one year *Pinus halepensis* responds more actively to volcanic environment than to no-volcanic one (Table 1). Although the CM + CW thickness in current- and first-year-old needles does not record significant variations statistically, an equilibrium between CM and CW (i.e. a decrease of CM and an increase of CW) exists. The rate of reduction is more pronounced for fumigated needles (15.7%) than for those that are not fumigated (11.8%) inferring that the volcanic inputs have more important effects on the outermost part of the cuticle. Comparing current- and first-year-old needles, it was observed that the B1 outer and middle zones of the cuticle decrease (17.1% versus 9.7% with a difference of 7.4% and a reduction of 57%; 33.4% versus 21.9% with a difference of 11.5% and a reduction of 66% respectively, see Fig. 5), while the thickness of B1 inner zone does not vary significantly.

In current- and first-year-old needles experiencing chronic fumigation, the parallel orientation of fibrils in respect to the cuticle surface in B1 outer and inner zones could be interpreted as a further protection device which adds to wax fusion discussed above. The amount of parallel fibrils of *Pinus halepensis* from Pisciarelli is mainly located in B1 outer and inner zones. The sum of the two latter zones (41% in current-year-old needles versus 36.8% in those of first-year) is higher than B1 middle zone that mainly contains random arranged fibrils (33.4% in current-year-old needles versus 21.9% in those of first-year). The parallel arrangement of fibrils could assume a role –analogous– like the outermost polylamellae (layer A1) which are interpreted as the main barrier to the diffusion in *Hedera helix* L. (Viougeas et al., 1995), and the main cuticular permeability barrier in *Agave americana* (Jeffree, 2006).

Calcium-oxalate deposits

In fumigated and not fumigated needles areas of very low electron-density which occur between B1 middle and inner zones of the cuticle, represent calcium oxalate (CaC_2O_4) crystals deposits. Additionally to morphological observations, Ca is clearly chemically identified with TEM X-ray analyses (Fig. 3A-D) in these areas. This is already known in living conifers which precipitate calcium in the apoplast as oxalate (Fink, 1991b). This latter author observed, in *Picea abies* needles, tiny Ca oxalate crystals in between epidermal cell wall and proper cuticle as well as numerous small crystals within epidermal cells walls, especially in the cuticular layer. Boddi et al. (2002, Fig. 10), thanks to TEM observations, reported calcium oxalate-like crystals in the cuticle of *Pinus halepensis*. Kivimäenpää et al. (2010, Fig. 5), by TEM analysis, claim that calcium oxalate crystals are typical of *P. halepensis* needle epidermis. Soda et al. (2000, Fig. 6C,D), studying impacts of urban levels of ozone on *P. halepensis* foliage with TEM, remarked symptomatic needles exhibit a great accumulation of calcium oxalate-like crystals in epidermal tissue. Although the distribution of calcium oxalate in needles is not completely understood (Fink, 1991a and b), abnormal distribution pattern could be possibly associated with pollution effects such as acid precipitation (pH 2-5) and ozone fumigations (Fink, 1991a). Our research demonstrates an increasing number of calcium oxalate deposits occurring in current- and first-year-old needles recovered from volcanic area of Pisciarelli, (table 2, Plate 2 and Fig. 5).

1.4.2. Potential application for extant and fossil material

Environmental responses of fossil and extant plants, based on ultrastructural cuticle studies, are deduced till now from three gymnosperms (latu sensu): *Komlopteris nordenskiöldii* (Nathorst) Barbacka, a Jurassic pteridosperm, was used by Guignard et al. (2001) to obtain numerical data by means of 10 measurements. In *Ginkgo yimaensis* Zhou and Zhang (the oldest *Ginkgo* in China), a Jurassic ginkgoalean species, and in the extant *Ginkgo biloba* L., statistics were made on 20 measurements (Guignard and Zhou, 2005). Finally in the present study statistics are based on 30 measurements for extant *Pinus halepensis* Mill. (Aleppo pine, extant Pinaceae). Although data are not yet enough to have a general view of the cuticle ultrastructure of gymnosperms, five main comments can be done.

Table 2. Differences between needles experiencing- or- not volcanic fumigation. Characters - = no changes, + = small changes, ++ = medium changes, +++ = great changes; CM = cuticular membrane, CW = cell wall; o = outer zone, m = middle zone, i = inner zone of B1 layer.

		statistically significant changes in thickness						sulphur presence	epicuticular wax	epistomatal wax	Ca-oxalate crystals	stomatal damage
		total CM + CW	cuticle B1				CW					
			total B1 = o + m + i	B1 outer	B1 middle	B1 inner						
changes -or-not between same environment needles	not fumigated current-year-old vs not fumigated first-year-old	-	+	-	++	+++	+	-	+	+	-	+
	fumigated current-year-old vs fumigated first-year-old	-	++	++	++	-	++	-	++	++	++	++
changes -or-not between different environment needles	not fumigated current-year-old vs fumigated current-year-old	-	++	+	+++	-	++	-	+++	+++	+++	+++
	not fumigated first-year-old vs fumigated first-year-old	-	+	++	++	+++	+	-	+++	+++	+++	+++

Firstly, two of those four taxa show a real sensibility to environmental conditions with an ultrastructural cuticle response. Solar radiation (sunny vs. shade leaves), wind and rainfall (i.e. low or high exposure), are invoked as factors influencing cuticle ultrastructure of *K. nordenskiöldii* leaves (Guignard et al., 2001). *P. halepensis* needles from Cigliano are influenced by “standard” degree of solar radiation, wind and rainfall; those of Pisciarelli are set under the volcanism effect.

Secondly, cuticular responses seem to affect in a greater degree “simple” cuticles than “complex” ones: in *P. halepensis* and *K. nordenskiöldii* the cuticles are made of one layer, B1 and A2 respectively. In both species A1 polylamellate layer is lacking, this fact reinforces the hypothesis that A1 layer acts as a barrier (Viougeas et al., 1995; Jeffree, 2006), although without the A1 barrier the cuticle seems to be more sensible and variable. On the other hand, only slight differences were found among the cuticles of two Ginkgo species (*G. biloba* and *G. yimaensis*) as well as a poor cuticular environmental response

Table 3. Key obtained by means of both SEM and TEM observations enabling the identification of each of four types of cuticles observed in *Pinus halepensis* needles experiencing- or- not volcanic gas fumigation. Cuticular membrane (CM = B1) and cell wall (CW) values are given as mean values (measurements) and percentage of total thickness. Stomatal class refers to Nicolotti et al. (2005) classification in which: class 0 = stomatal structure displaying no sign of alteration, class 1 = for slight sign of alteration, class 2 = for moderate sign of alteration, class 3 = for severe alterations. B1 oz = B1 outer zone, B1 md = B1 middle zone.

				measurements		proportion		
not fused epicuticular wax		B1 inner zone present (0.95 µm), 25.1%	→	CW 1.57 µm B1 2.23 µm B1 mz 0.72 µm		41.3% 58.7% 19% (B1 mz/CM+CWx100)		not fumigated current-year-old cuticle needle
stomatal class 0-1	→	not fumigated cuticle needle						
Ca-oxalate rare		B1 inner zone absent	→	CW 2.17 µm B1 1.92 µm B1 mz 1.37 µm		53.1% 46.9% 33.5% (B1 mz/CM+CWx100)		not fumigated first-year-old cuticle needle
fused epicuticular wax			→	CW 1.06 µm B1 3.08 µm B1 oz 0.71 µm B1 mz 1.38 µm		25.6% 74.4% 17.1% (B1 oz/CM+CWx100) 33.4% (B1 mz/CM+CWx100)		fumigated current-year-old cuticle needle
stomatal class 2-3	→	fumigated cuticle needle						
Ca-oxalate abundant			→	CW 1.62 µm B1 2.30 µm B1 oz 0.38 µm B1 mz 0.86 µm		41.3% 58.7% 9.7% (B1 oz/CM+CWx100) 21.9% (B1 mz/CM+CWx100)		fumigated first-year-old cuticle needle

(Jurassic *G. yimaensis* is supposed to have lived in a high CO₂ “greenhouse” climate while extant male and female *G. biloba* trees were planted in different environments in Nanjing, China). The cuticle complexity of these two Ginkgo species (occurrence of A1, A2 and B1) could explain their resistance.

Thirdly, the cuticle analysis can become a taxonomical tool of a certain weight. It is a fact that the layers retain their sequence and basic structure, but their relative thickness and minor structural elements (fibrils and granules) may vary under changes of the environmental conditions. Actually the A2 granular layer made with four types of material (four types of cuticle: sun upper, sun lower, shade upper and shade lower) of *K. nordenskiöldii* and B1 fibrillous layer divided in 2-3 zones variable in thickness of *P. halepensis* can be influenced by the environment. Conversely, A1 layer consisting of A1 upper and A1 lower zones, A2 layer, and B1 layer, typically present in *G. biloba* and *G.*

yimaensis, can be considered useful for taxa identification because they do not change under different environmental conditions.

The fourth remark concerns further ultrastructural details, i.e. the progressive reduction in the fibrillar structure and the corresponding increase in granular component from current- to first-year-old needles of *Pinus halepensis* (Plate II, 14-16, 19-21) that can be interpreted as a response to enhanced environmental stresses. On this regard Villar de Seoane (2001) illustrates the alteration of the epidermal wall ultrastructure of *Otozamites patagonicus* from the Early Cretaceous Springhill Formation of Patagonia, where multilamellate and reticulate-alveolate layers are transformed to amorphous layers (Villar de Seoane, 2001 sense) and interpreted as the result of high temperature owing to persistent volcanic ash fall. Del Fuejo and Archangelsky (2002) point out that amorphous areas in *Araucaria grandifolia* Feruglio from the Lower Cretaceous of Patagonia (Argentina) may be the result of poor preservation of the cuticular membrane.

Moreover deposits of calcium oxalate crystals in *P. halepensis* could be equivalent structures to the electron lucent amorphous material of *K. nordenskiöldii* (Guignard et al., 2001, Plate II, Fig. 4), although the role of this material is unclear (Guignard et al., 2001). Similar electron lucent areas are visible in the cuticle of two fossil species (*Pseudoctenis ornata* and *Restrepophyllum chiguoides*) from the Early Cretaceous of Patagonia (Passalia et al., 2010). It is noteworthy that this vegetation grew under environmental stressful conditions for persistent volcanic activity (Archangelsky et al., 1995; Archangelsky, 2001; Del Fuejo and Archangelsky, 2002). The epidermal cells of *P. ornata* show the sublayer A2 with irregular isodiametric areas containing material of less electron density (Archangelsky et al., 1995, Pl. V, Fig. 31). Cuticle degradation is recorded in *R. chiguoides* (Passalia et al., 2010, Pl. IV, Fig. 5). To sum up, the increase of Ca-oxalate crystals could be a key character to identify environmental stress in fossil and extant plants.

The last remark reveals a possible tool to identify current- versus first-year-old *Pinus* needles in extant and fossil contexts and make inferences on gas fumigation disease (van de Schootbrugge et al., 2009 suppose SO₂ as a probable cause of plant stress during periods of global-scale volcanism). According to the present study one can distinguish not fumigated from fumigated needles (Table 3) by means of eight characters (epicuticular and epistomatal wax, Ca-oxalate, total B1, CW, B1 inner, outer and middle). Therefore the degree of wax crystals degradation in stomatal antechamber and epidermal surface, together with the amount of calcium-oxalate deposits in the cuticle, allow a first distinction between needles experiencing- or- not volcanic gas fumigation (Table 3). Furthermore

three characters (i.e. thickness of CW, B1 and B1 middle zone) allow to make a distinction between current- and first-year-old not fumigated cuticle needles; while four characters (i.e. thickness of CW, total B1, B1 outer and middle zones) enable to detect fumigated needles. In particular the presence or absence of B1 inner zone (see Figs. 4,5) can be used to distinguish not fumigated current- from first-year-old needles. For the thickness of cuticular membrane (CM) + cell wall (CW) keeps constant in *P. halepensis* needles regardless the age and environmental conditions, this character could be considered as a taxonomical one at species level.

CHAPTER II

The cuticle micromorphology of in situ *Erica arborea* L. exposed to long-term volcanic gases in Phlegrean Fields, Campania, Italy

2.1. Introduction

Within plant organs, the leaf has been the target of many studies (see Beck, 2010) and is the most sensitive organ to pollution (Sant'Anna-Santos et al., 2006). The leaves of many tracheophytes (Guignard et al., 2004) are covered by the cuticle, an extracellular membrane (Graham, 1993) covering aerial organs of plants (Riederer, 2006). The bulk of the cuticle in modern plants is composed of a solvent-insoluble matrix makes up the framework of the cuticle (Tegelaar et al., 1991). This composite matrix provides several functions at the interface level that enable plants to thrive in different habitats and withstand adverse environmental conditions (Domínguez et al., 2011). The cuticle could be considered an “external skeleton” as it represents the interface between the plant and the atmosphere (McElwain and Chaloner, 1996). For these reasons, in the last three decades numerous studies have been carried out on fossil and extant plant cuticles in order to attempt paleoenvironmental reconstructions and evaluate the gaseous atmospheric composition. This latter composition varied consistently during geological time (Berner, 1999) and major volcanism -e.g. Large Igneous Provinces formation (Courtilot and Renne, 2003)- is frequently invoked as a possible cause of mass extinctions (Rhode and Muller, 2005) by means of massive degassing (Scaillet, 2008; Sobolev et al., 2011; Wignall, 2011) of CO₂, H₂S and SO₂ into the atmosphere (Beerling and Berner, 2002; Berner and Beerling, 2007; Hori et al., 2007). The volumes of SO₂ outgassed during large basaltic eruptions are comparable in magnitude to those of CO₂ (Thordarson et al., 1996). For this reason, volcanism played an important role in past plant communities composition (Falcon-Lang and Cantrill, 2002) insomuch as, climatic fluctuations and proximal volcanic eruptions probably contributed to the formation of disturbed habitats favourable for early angiosperms (Herman, 2002).

Numerous researches spent their efforts onto the study of cuticle topography (number of stomata per unit area = stomatal density; number of stomata relative to the number of epidermal cells = stomatal index), at mesoscopic scale (see Collinson 1999), and its relationships with atmospheric CO₂ concentration ([CO₂]) in the geological past

(Woodward, 1987; Beerling et al., 1998; Royer, 2001). It has been assumed that plants' stomatal density adjust in response to changing atmospheric $p\text{CO}_2$ (Woodward, 1987). However, little is known (Winner and Mooney, 1980) about the long-term response of micromorphology of natural vegetation to volcanic toxic gases. Moreover, it is unclear if small community experiments are capable of both reconstruct and predict gas atmospheric composition because these experiments often lack the necessary range of natural disturbances that occur in the long term (Woodward, 1992).

Because of the difficulties to simulate complex environmental conditions within plant-volcano interactions, we studied the in situ angiosperm *Erica arborea* L. submitted to volcanic gases in two solfataras (sulphur-emitting vents): Solfatara di Pozzuoli and Pisciarelli. Solfataras are exciting habitats for studying wildlife adaptations to a very peculiar but extreme environment (see Paoletti et al., 2005). The populations of *E. arborea* growing around the vents have a multiple-generation and a life-time exposure to volcanic gases, resulting in long-term adaptations of plants to these naturally environments. The occurrence of vents emitting volcanic gases provides a rare opportunity to study long-term response and microevolutionary adaptation (Bettarini et al., 1995) of natural vegetation to volcanic gases.

Numerous studies have been carried out on in situ plants exposed to geogenic gases (Bettarini et al., 1995; Bettarini et al., 1998; Tognetti et al., 2000; Paoletti et al., 2005; Pfanz et al., 2007; Haworth et al., 2010) but as far as we know, there are no studies relatively to the response of the ultrastructural features of angiosperms plant cuticle exposed to the persistent volcanic gases.

Therefore, this study aims to try understand: 1) how plants respond to realistic extremes outdoor conditions; 2) what is the response of the cuticle micromorphology long-term exposed to volcanic gases, 3) in particular, whether exposure and/or resistance with volcanic gases is associated with a change in ultrastructure of cuticle; 4) potential implications of the angiosperm cuticle response across environmental stress periods during the geological past.

Investigations on volcanic toxic gases-emitting vents offer opportunity to study plant physiological response to these extreme environments. The volcanic areas in Campania Region (Roccamonfina, Phlegrean Fields and the complex of Somma-Vesuvio) are suitable for studies of this type because they are historically monitored and easily accessible representing natural laboratories allowing experiments to realistic extremes outdoor

conditions. In the present study, *E. arborea* was chosen and X-ray analyses and SEM and TEM observations were performed focusing on leaf cuticle micromorphology.

2.2. Material and methods

2.2.1. Plant material and sites description

Leaf samples were obtained from *Erica arborea* L., one of the common and widespread Mediterranean macchia shrub species belonging to the family Ericaceae including species that are efficient colonizers (Luteyn, 2002). *E. arborea* is characterized by hypostomatic leaves with revolute margins protected by trichomes (Gratani and Varone, 2004). Samples were collected from three localities in the Phlegrean Fields, an active caldera which spans the last 50000 years (Scandone et al., 2010), characterized by vertical ground movements locally called “Bradyseim” (Del Gaudio et al., 2010). This is a very dangerous volcanic area because it includes part of the city of Napoli, the town of Pozzuoli, and numerous densely inhabited villages (Chiodini et al., 2011).

Samples long-term fumigated by volcanic gases were recovered from Solfatara crater, ca. 30 m NNW from the Bocca Grande vent ($40^{\circ}49'38.91''\text{N}$, $14^{\circ}08'32.98''\text{E}$), and ca. 10 m NE from the main fumarole in Pisciarelli locality ($40^{\circ}49'48.88''\text{N}$, $14^{\circ}08'46.95''\text{E}$) about 750 m SE of the Solfatara volcano (Fig. 1A,B). These two volcanic areas, active for centuries, are historically monitored (Gunther, 1897) and make the object of numerous studies.

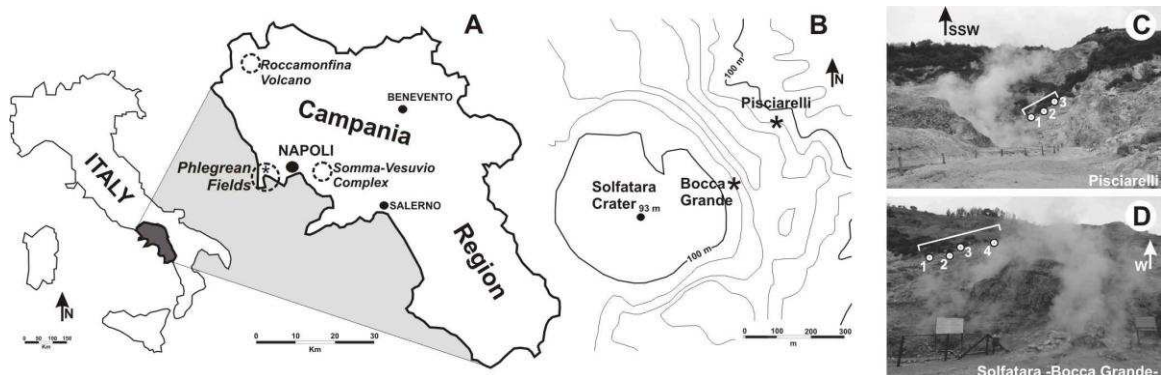


Fig. 1. (A) Location of the Phlegrean Fields and others gas-emitting volcanic areas (dotted circles) in Campania Region. (B) Sketch map showing the location of Solfatara crater and Pisciarelli locality. (C,D) Close up views of Pisciarelli and Solfatara localities with the main fumaroles. Square brackets indicate sampling areas and white circlets show the location of CO₂ measurements.

Control sample of leaves were also collected in a volcanic quiescent area (Cigliano: 40°50'46.46''N, 14°07'36.31''E) located about 2.5 km NNW from Pisciarelli, but characterized by the absence of volcanic gas emissions. The three localities share the same soil features (Di Gennaro and Terribile, 1999; Di Gennaro, 2002) and sun exposition and they are also far away from traffic and industries. The climate is typically Mediterranean, with cool, wet winters and hot, dry summers.

The samples collected around the vents have a multiple-generation resulting in long-term adaptations of plants to volcanic gases. The outskirts of these extreme environments (Pisciarelli and Solfatara) is inhabited by a Mediterranean vegetation providing xeromorphic features. *Erica arborea* (and some grasses) is the unique typical Mediterranean angiosperm shrub growing near the vents. Moreover, the boiling water of Pisciarelli and Solfatara fumaroles retains the cyanidial algae *Galdieria phlegrea* (Pinto et al., 2007) and *Cyanidium caldarium* (De Luca et al., 1979) respectively.

Distal volcanic impacts revealed that plants are generally less sensitive to eruptions outside the growing season (Zobel and Antos, 1997; Hotes et al., 2004) and, as Payne and Blackford (2008) pointed out, in winter, as plants are senescent, higher rainfall may serve to remove rapidly volcanic pollutants. For evergreen *E. arborea*, permanently fumigated by volcanic gases retaining leaves for over a year, these “ground noises” do not exist. The plants do not present significant damages along the crown, but some leaves show symptoms consisting in leaf-tip non-specific discoloration.

2.2.2. Gas vent

At Pisciarelli, the fumarolic discharge temperature from 1999 to 2005 has fluctuated around 95 °C, reaching 108 °C in October 2010, with the CO₂ flux from this pool estimated at 15 ton/d to 20 ton/d (Chiodini et al., 2011). Measurements of diffuse soil CO₂ fluxes in the Solfatara area (1.4 km²) were carried out from 1998 to 2000 and gave a daily amount of deeply derived CO₂ discharged at Solfatara of about 1500 tons (Caliro et al., 2007). The analysis of gaseous compositions (Caliro et al., 2007) revealed that the main component of the fumaroles is H₂O followed by CO₂, H₂S, N₂, H₂, CH₄, He, Ar, and CO. The absence of acidic gases (SO₂, HCl, and HF) can be also noted (Chiodini et al., 2010a). Natural high atmospheric concentration of sulphur gas may occur locally in areas with volcanic and geothermic activity (De Kok et al., 2007). As a matter of fact, at Pisciarelli and Solfatara, the high H₂S air concentration is equal to ca 600 µm/mol (at source;

Chiodini et al., 2010a) and 3.20 ppm (Carapezza et al., 1984) respectively. The great H₂S air concentration is also testified by both typical smell (the odour threshold is >0.02 µm l⁻¹: De Kok et al., 2007) of rotten eggs in the surrounding air and by indirect corrosive action of this gas that led to the destruction, around the vents and in few minutes, of any electronic device and to a relatively long-term corrosion of metalwork.

2.2.3. SEM, TEM and EDS preparations

Fumigated and not fumigated specimens were collected from branches at heights over 75 cm from the ground at each site (Pisciarelli, Solfatara and Cigliano). Leaves were carefully handled to avoid damaging the epicuticular waxes. Following Reed's (1982) and Crang and Klomparens' (1988) remarks about possible changes in epicuticular wax structures occurring during sample preparation, in order to limit any chemical or physical damages, especially for preserving and dehydrating samples for wax morphology studies, leaves were air-dried for 1-week at mild room temperature. Among several hundreds of leaves collected at each site (fumigated and not fumigated), 100 were selected for scanning electron microscope (SEM), then 60 were carefully selected for transmission electron microscope (TEM). Light microscope observations were made using a Leitz microscope.

Both untreated and frozen leaves (in liquid nitrogen at -210 °C) were cut transversally and longitudinally. Subsequently they were mounted onto the stubs using double-sided adhesive tape then coated with gold using an AGAR Auto Sputter Coater and transferred to the SEM for mesoscopic (Collinson, 1999) observations. Entire leaves were directly mounted to observe abaxial and adaxial surfaces. The Barthlott et al. (1998) classification has been used to typify epicuticular wax.

SEM observations were done in two universities: 1) with a JEOL JSM-5310 SEM adapted with an Energy Diffractive X-ray Oxford Inca X-act at the CISAG (Centro Interdipartimentale di servizi per analisi Geomineralogiche; Dipartimento di Scienze della Terra, Università degli Studi di Napoli "Federico II"). The operative conditions were as follows: 25-30 KV accelerating voltage, 100 µA emission current, 15 µm spot size, 20 mm microscope work distance and 60 s spectra collection time; 2) with a Hitachi S800 coupled to a microanalysis system EDS SiLi Samix (acceleration voltage = 15 kV and a spectres acquisition time = 60 s; in the Centre de technologie des microstructures (CTµ) of Lyon-1 University, Villeurbanne, France). The specimens for energy diffractive x-ray (EDX) analyses were then coated with carbon in an Emitech K450 at CISAG. A total of 100 EDS

analyses were carried out on both leaf sections (cuticle and cell wall with JEOL SEM) and surfaces (crystals, hairs and epidermis with SEM Hitachi) experiencing or not chronic fumigation. It is worth noting that data generated by the X-ray microanalyzer are not as accurate as other quantitative analyses, therefore they can be considered at best as semi-quantitative (Van Steveninck and Van Steveninck, 1991).

Samples for TEM were dropped in paraformaldehyde solution mixed in a phosphate-sodium buffer for 3 weeks using Lugardon's technique (1971), washed and postfixed in a 1% osmium tetroxide solution mixed in a phosphate-sodium buffer for 24 hours. Dehydrated in graded ethanol series during 48 h, the samples were dropped in propylene oxide with an increasing percentage of Epon resin for 24 h. Transferred into pure Epon resin during 24 hours, they were embedded in fresh Epon resin using flat moulds. The preparations were subsequently treated for polymerization at 56 °C for 3 days. Ultrathin (60-70 nm) sections were sectioned with a diamond knife, using a Reichert Ultracut microtome. Ultrathin sections were placed on uncoated 300 Mesh copper grids and stained manually both with a methanol solution of 7% uranyl acetate for 15 min and an aqueous lead-citrate solution for 20 min, then observed and photographed with a Philips CM 120 TEM at 80 kV, in the Centre de technologie des microstructures (CTμ) of Lyon-1 University, Villeurbanne, France. Totally 60 pieces of material were embedded in Epon resin blocks. 210 uncoated mesh copper grids were prepared (180 as transversal sections, i.e. perpendicular to the leaf length; 30 as longitudinal sections, i.e. parallel to the leaf length).

All TEM measurements were performed with tools in the ImageJ program (Abramoff et al. 2004). The terminology of Holloway (1982) and Archangelsky (1991) was used for the ultrastructural analysis. Ultrastructural measures have been taken in the middle of epidermal cells. No measures have been carried out in overlying cuticle between two adjacent epidermal cells where the cuticle is generally thicker. All specimens and SEM stubs are housed in the Dipartimento di Scienze della Terra, Largo San Marcellino, 10, Napoli, Italy. The resin blocks and TEM negatives are stored in the Lyon-1 University, Villeurbanne, France.

2.2.4. Gas concentration measurements in air and soil

CO₂ measurements in the soil and in the air surrounding the vegetation were performed with a portable gas analyser (LICOR 800). Holes were drilled into the soil until roots of

Table 1. Soil and atmospheric (atm.) CO₂ concentrations within both the rooting and crown zones of *Erica arborea* at fumaroles of Solfatara and Pisciarelli in Phlegrean Fields.

Solfatara -Bocca Grande-			
Site	Lat. and lon.	g/m ² /day CO ₂ (soil)	CO ₂ (ppm) atm.
1	40°49'38.50"N 14°08'31.71"E	676.05	1000-1300
2	40°49'38.49"N 14°08'31.92"E	442.81	500-600
3	40°49'38.51"N 14°08'32.13"E	597.87	800
4	40°49'38.35"N 14°08'32.52"E	62.93	600-700
Pisciarelli			
	Lat. and lon.	g/m ² /day CO ₂ (soil)	CO ₂ (ppm) atm.
1	40°49'45.49"N 14°08'49.00"E	64.56	1500-3000
2	40°49'45.48"N 14°08'48.92"E	57.09	1500-3000
3	40°49'45.47"N 14°08'48.86"E	48.34	1500-3000

sampled *E. arborea* shrubs; gas-probe was then inserted and values recorded. In the studied areas the vents position has not changed for long time and gases fluxes (in the soil) are rather constant (Table 1). The site topography and constant winds disperse these emissions in plumes strongly directed to the SSW (Pisciarelli) and W (Solfatara), id est. just on

sampled areas (see Fig. 1C,D). For this reason, gas measurements can be done at all times.

2.2.5. Statistical analysis

The present study is based on 30 measurements. Similar statistic analyses were provided for fossil plants (Guignard et al., 2004; Yang et al., 2009). The relation among the different layers {cuticular membrane (CM) = [(cuticle proper = CP = A2 + cuticle layer = CL = B1)] + cell wall (CW)} can be traced based on the confidence interval value ($CI = \bar{X} \pm \sqrt{\frac{\text{var}}{n}} \times 1.96$, giving 95% α risk) among 5 characters (Fig. 5). The CI value allows to estimate the significance of the differences between three types of studied material.

2.3. Results

2.3.1. Energy diffractive X-ray analysis with SEM

The cuticle surfaces of *E. arborea* leaves experiencing chronic fumigation are typically covered by dust of volcanic origin. X-ray analyses have revealed that dust is composed mainly of: crystals belonging to the Alunite Group (Fig. 2A), typical aggregates of both minerals and glasses of solfataras (Fig. 2B) and CaSO₄ crystals (Fig. 2C).

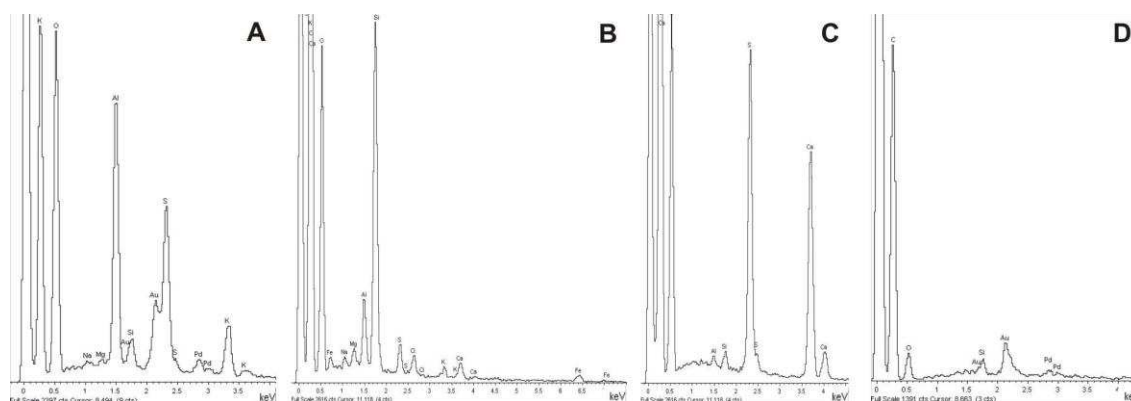


Fig. 2. Qualitative X-ray diffractograms of particles commonly recovering on fumigated leaves from Solfatara and Pisciarelli. (A) Example of crystal belonging to Alunite Group. (B) A typical aggregate of both minerals and volcanic glasses of solfataras. (C) Example of CaSO_4 spectrum. (D) Diffractogram showing silicon traces in cluster of rodlets wax from Pisciarelli.

The EDS microanalysis of cluster of rodlets wax from Pisciarelli (Fig. 3D) has revealed siliceous traces (Fig. 2D). Within fumigated and not fumigated needles, concerning 3 sets of 10 measurements on cuticle and cell wall, the elements content is largely homogeneous and the absence of sulphur can be noted. Extraneous elements [e.g. Au and Pd (see Fig. 2A, D)] derive from metallization process.

2.3.2. Scanning electron microscopy observations

The leaves of *E. arborea* experiencing chronic fumigation (Pisciarelli and Solfatara areas) have a more serrate revolute margin (Fig. 3A and B) and are covered by abundant volcanic dust (especially in the adaxial side) (Fig. 3C, D, I, K, and L). In not fumigated leaves, the adaxial side becomes the site of dust accumulation.

In outer view, both adaxial and abaxial sides of leaves epidermis experiencing chronic fumigation (especially in Pisciarelli locality) bear numerous clusters of rodlets wax 10–20 μm in length by ca. 1 μm in width (Fig. 3D).

In the the abaxial side of leaves experiencing chronic fumigation in which the margin is less revolute (Fig. 3E), a number of hairs and a high production can be noted (Fig. 3F). The typically wax rodlets are circular in section and are longitudinally aggregated (Fig. 3G).

In outer view, the abaxial side of control leaves appear intact with well-defined shape of epidermal cells and glabrous surface (Fig. 3H). Conversely, fumigated leaves show less defined pattern of epidermal cells having both wax overproduction and degradation forming crusts (Fig. 3I) especially in the adaxial side.

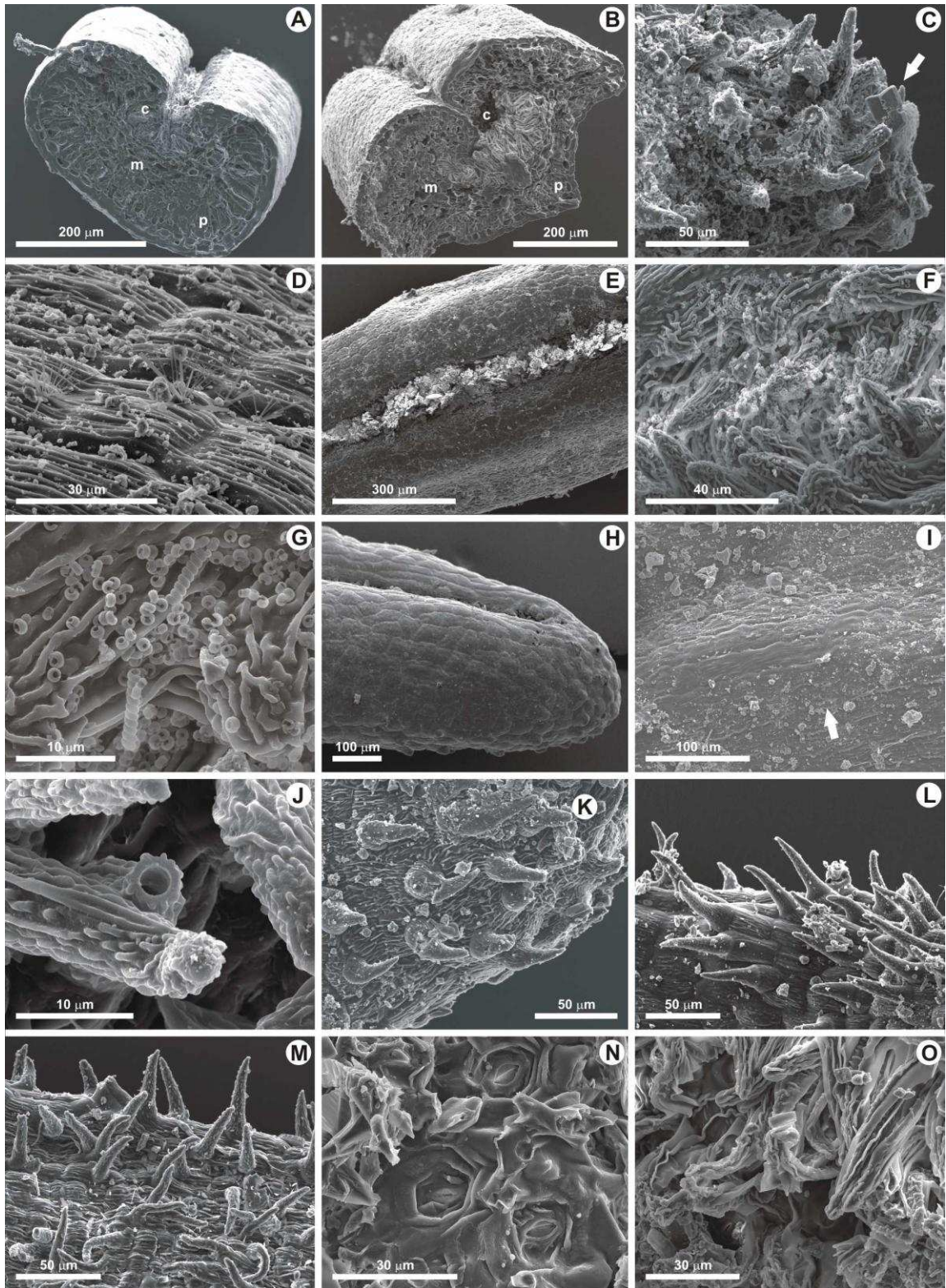


Fig. 3. *Erica arborea*, SEM abaxial and adaxial cuticles, for both shrubs experiencing or not chronic fumigation by volcanic gases.

A) Abaxial side of not fumigated (Cigliano) cryo-sectioned leaf showing not more serrate revolute margins. The palisade layer (p), the spongy mesophyll (m) and the trichome crypt (c) can be seen. Photo GGAB038. B) Abaxial side of fumigated (Pisciarelli) cryo-sectioned leaf showing a serrate revolute margin. The palisade layer (p), the spongy mesophyll (m) and the great trichome crypt (c) can be seen. Photo GGAB043.

C) Adaxial side of fumigated (Pisciarelli) leaf tip showing abundant volcanic dust and crystals (arrow) covering epidermis. Photo GGAB039. D) Adaxial side of fumigated (Pisciarelli) leaf showing clusters of rodlets wax. Photo GGAB009. E) Adaxial side of fumigated (Pisciarelli) leaf having not more serrate revolute margins showing a wax and trichomes overproduction. Photo GGAB002. F) Detail of E showing projections-bear trichomes and adhering wax. Photo GGAB014. G) Abaxial side (detail) of fumigated leaf (Pisciarelli) showing wax rodlets circular in section being longitudinally aggregated. Photo GGAB012. H) Abaxial side of not fumigated leaf (Cigliano) showing well-defined epidermal cells. Photo AB9. I) Abaxial side of fumigated (Pisciarelli) leaf replete of volcanic dust and crusts (arrow) originated from epicuticular wax fusion. Photo GGAB031. J) Adaxial side of fumigated leaf (Solfatara) showing trichomes with a marked ornamentation. In the middle of image a hollow hair is visible. Photo GGAB007. K) Adaxial side of fumigated (Pisciarelli) leaf tip showing trichomes with typical ruptures. Photo GGAB034. L) Adaxial side of fumigated (Pisciarelli) leaf showing hairs with prolonged and tough inflated bases. Photo GGAB005. M) Adaxial side of not fumigated (Cigliano) leaf showing thinner trichomes with not inflated bases. Photo GGAB026. N) Abaxial side of not fumigated (Cigliano) leaf showing three stomatal apparati. Photo GGAB006. O) Abaxial side of fumigated (Pisciarelli) leaf showing three sunken stomatal apparatuses with respect to the median level of epidermis. Photo GGAB006.

Both fumigated and not fumigated leaves have hairs distributed on the edge of adaxial side. The edge of abaxial side is devoid of hairs. In fumigated leaves the hairs display a marked ornamentation (Fig. 3J). Fumigated leaves show their apex crowded by hairs presenting typically ruptures (Fig. 3K). In fumigated leaves, along the adaxial side of petiole, most of the hairs have prolonged and tough inflated bases (Fig. 3L) compare with not fumigated one (Fig. 3M).

Leaves not experiencing chronic fumigation bear stomata less protected (Fig. 3N). Leaves from volcanic sites display more sunken stomatal apparati (Fig. 3O) in respect to the epidermal surface.

2.3.3. Transmission electron microscope observations

The ultrastructure (cuticle and cell wall) of abaxial epidermal cells from fully grown leaves of three localities have been studied in details. Cuticular membrane (CM) of all ordinary epidermal cells is composed of cuticle proper (CP = A) represented by the outermost granular A2 layer and innermost cuticular layer (CL = B) represented by the fibrillar layer B1. Differences between the cuticular structures within three localities are given below. All the data given below are the means based on 30 measurements, the percentages of each component of the cuticle and of cell wall are also given (Table 2).

In not fumigated leaves (Fig. 4A-E) the total thickness of CM (= CP + CL) + cell wall (CW) is 8.75 μm (Fig. 4A). 72.3% (4.9% of A2 and 67.4% of B1) is composed of the CM. In the fumigated leaves from Solfatara (Fig. 4F-J) the total thickness of CM + CW is 10.58 μm (Fig. 4F). 78.4% (12% of A2 and 66.4% of B1) of the CM + CW is composed of the CM. In leaves from Pisciarelli also experiencing chronic fumigation the total thickness of

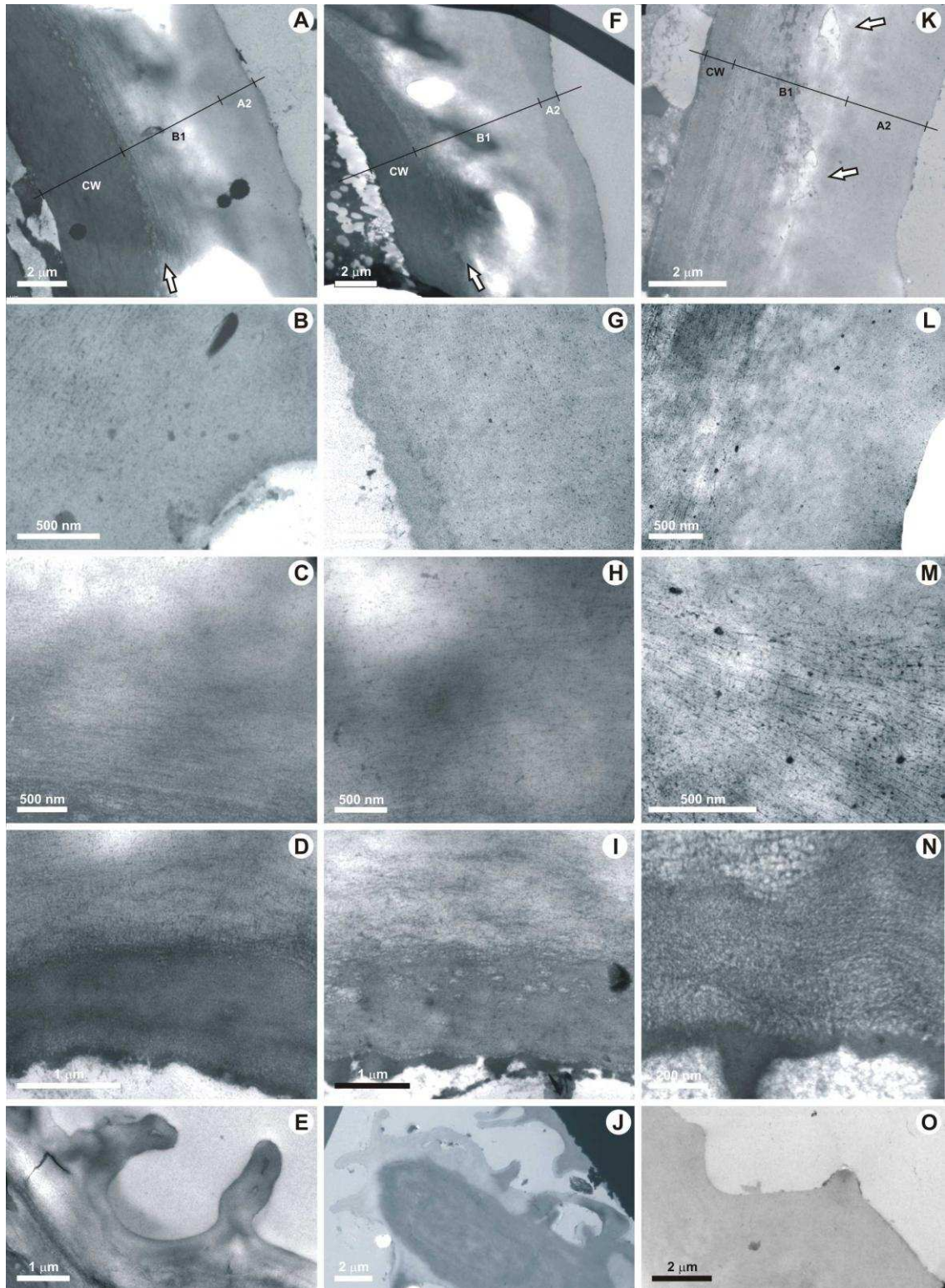


Fig. 4. *Erica arborea*, TEM of fumigated and not fumigated leaves. All photographs were taken from ordinary epidermal cells in transversal sections, except a longitudinal section of photograph N. The cuticle (CM = Cuticular membrane) is made up with A2 amorphous layer (= CP, cuticular proper) and B1 fibrillous layer (= CL, cuticular layer).

A-E. Not fumigated leaves from Cigliano.

A) General view of cuticle and cell wall. A transition zone (arrow) probably deriving by a mixture of cuticle and cell wall material can be seen. Photo GGAB0004. B) Detail of A2 outer layer clearly showing the transition zone between A2 granular and B1 fibrillate layers. Photo GGAB0021.

C) Detail of B1 layer of cuticle showing more dense and crowded fibrils (lower part) decreasing in abundance toward the A2 granular region in which electron lucent areas can be seen. Photo GGAB0005. D) Detail of cell wall showing four different stained zones as follows: a less evident densely stained zone below the cell wall; a more electron lucent zone; a dense fibrillate zone and a densely stained fibrillate zone in contact with B1 layer. Photo GGAB0009. E) Trichomes projections made of folded A2 layer entrapping small portions of fibrillar B1 layer. Photo GGAB0040. F-J. Fumigated leaves from Solfatara. F) General view of cuticle and cell wall showing a less marked transition zone (arrow). Photo GGAB0036. G) Detail of A2 outer layer clearly showing the transition zone between B1 fibrillate and A2 granular layer. The B1 layer shows some mottling caused by dark staining of certain areas. Photo GGAB0007. H) Detail of B1 layer of cuticle showing less dense fibrils, a conspicuous granular component and numerous small dark stained areas causing mottling. Photo GGAB0016. I) Detailed view of B1 layer of cuticle and cell wall showing small B1 fibrillate areas entrapped in cell wall network. Photo GGAB0015. J) Trichome showing numerous projections made of A2 layer. Photo GGAB0027. K-O. Fumigated leaves from Pisciarelli. K) General view of cuticle and cell wall. The transition zone, less marked, approach two electron lucent areas (arrows) delimited by a thin darkly stained layer. Photo GGAB0007. L) Detail of A2 outer layer and part of B1 fibrillate layer. In the thickness of A2 granular layer small dark regions can be seen. Photo GGAB0005. M) B1 layer showing more or less parallel fibrillae and densely stained mottled areas decreasing in abundance toward the A2 granular outer region. Photo GGAB0005. N) Detailed view of cell wall fibrillae in connection (arrow) with fibrillate network of cuticular layer. Photo GGAB0002. O) Detail of trichome showing dome-shaped structures made of A2 layer only. Photo GGAB0022.

CM + CW is 6.40 μm (Fig. 4K-O). 88.9% (29.4% of A2 and 59.5% of B1) of the CM + CW is composed of the CM.

The outermost A2 layer, or cuticle proper (CP), is composed of granular material. In fumigated leaves the A2 layer is thicker (1.27 μm for Solfatara specimens and 1.88 μm for Pisciarelli one) in respect to not fumigate one (0.43 μm) (Fig. 4B, G and L). In particular, the specimens from Pisciarelli have the highest value with 29.4% of the CM + CW composed of A2 layer. In leaves experiencing or not chronic fumigation, the B1 fibrillate layer is thicker than A2 and CW layers. From not fumigated to fumigated leaves a decrease of CW thickness has been observed (Cigliano: 2.42 μm ; Solfatara: 2.28; Pisciarelli: 0.71 μm). However, a tendency to a reduction in the fibrillar structure followed by an increase of the granular component has been noted between not fumigated and fumigated leaves. The cuticular layer (CL = B1) of fumigated leaves retain numerous electron lucent areas, the greatest of which are delimited by a thin darkly stained layer (Fig. 4K).

The B1 layer toward the outer surface (i.e. A2) is quite homogeneous with more or less parallel fibrillae (Fig. 4C, H and M). However, cuticles display some mottling caused by dark staining of certain areas (Fig. 4G and M). These dark regions decrease in abundance near the outer cuticle surface, but sometimes in A2 granular layer can be seen (Fig. 4B and L). In fumigated leaves the cell wall network shows less stained areas probably composed of the same material of fibrillate B1 layer (Fig. 4I). Below the CW, a densely stained zone, ca. 100-300 nm thick, can be seen especially in leaves experiencing chronic fumigation (Fig. 4D, I and N). In the abaxial side of leaves, trichomes projections (Fig. 3J), more elongated in not fumigated leaves (Fig. 4E), are made of folded A2 layer sometimes

entrapping small portions of fibrillar B1 layer (Fig. 3E). In fumigated leaves, hairs bear dome-shape structures made of A2 layer only (Fig. 3O). Some plate-like morphology of the epicuticular wax may be present as a darkly staining residue outside of the A2 granular layer (Fig. 4A, F and K).

Table 2. Statistical values, made with 30 measurements for cuticular membrane (CM) and cell wall (CW) of the epidermal cells of three sites. Note: the cuticular membrane CM is made up with cuticular proper CP (= A2) and cuticular layer CL (= B1). All measurements are in μm . min-max = minimum and maximum values observed; % = percentage of each detailed part of the cuticle and cell wall; st-d = standard deviation; var = variance.

zones measured	mean	min-max	%	st-d	var
not fumigated (Cigliano)					
total CM + CW	8.75	7.52-10	100	0.67	0.45
CW	2.42	1.23-3.76	27.7	0.76	0.58
B1	5.90	4.85-7.45	67.4	0.76	0.59
A2	0.43	0.09-0.87	4.9	0.23	0.05
total CM = B1 + A2	6.33	5.37-7.63	72.3	0.62	0.39
fumigated (Solfatara)					
total CM + CW	10.58	8.74-15.62	100	2.22	4.94
CW	2.28	0.90-4.11	21.6	1.00	1.00
B1	7.03	5.59-11.29	66.4	1.87	3.48
A2	1.27	0.46-2.37	12.0	0.59	0.35
total CM = B1 + A2	8.30	6.51-11.75	78.4	1.61	2.61
fumigated (Pisciarelli)					
total CM + CW	6.40	4.69-8.43	100	1.17	1.38
CW	0.71	0.15-1.43	11.1	0.27	0.07
B1	3.81	2.42-5.58	59.5	0.92	0.85
A2	1.88	1.24-3.61	29.4	0.62	0.39
total CM = B1 + A2	5.69	4.17-7.68	88.9	1.03	1.07

2.4. Discussion

The present study based on 30 measurements (TEM) is the most precise statistic analysis so far done for extant angiosperm cuticles. All these observations allow to discuss several diverse environmental aspects.

2.4.1. Chemical and SEM considerations

Chemical considerations - Large part of dust covering leaves experiencing chronic fumigation originates from solidification of chemical compounds of which the plume is loaded. Volcanic glasses, carried by wind, derive from neighbouring soil. EDS microanalysis have revealed the absence of sulphur within the overall cuticle thickness of epidermal cells and cytoplasm. The lacking of sulphur in the structure of the cuticle suggests that the uptake of volcanic toxic gases takes place via stomatal apparatuses. The presence of silicon in the cluster of rodlets from Pisciarelli (Fig. 2D) is not surprising as the environment in which a plants grows can affect epicuticular wax composition (see Baker, 1982).

Wax overproduction and degradation - On leaves epidermis experiencing chronic fumigation (especially in Pisciarelli locality) the presence of siliceous in clusters of rodlets (see Figs. 2D and 3D) could be the result of a “control device” avoiding the uptake of Si deriving from solidification of volcanic compounds by the plume. In fumigated plants the wax overproduction (Fig. 3E-G) could be the effect of elevate CO_2 concentration. Vanhatolo et al. (2001) found that elevated CO_2 alone and in combination with O_3

increased wax coverage on the abaxial leaf of an inland clone of pubescent birch *Betula pubescens*, but not in that of coastal clone. Among Solfatara and Pisciarelli fumarolic effluents a relatively high quantity of hydrogen sulphide (H_2S) is released (Chiodini et al., 2010; Caparezza et al., 1984). Brown (1982), Lorenzini and Nali (2005) and Haworth et al. (2010) claimed that the H_2S oxidizes rapidly in the atmosphere to form SO_2 ; this last rapidly converts into H_2SO_4 (Visscher et al., 2004) considered as an oxidation product of SO_2 or H_2S (Mather et al., 2003). Therefore, the wax degradation noticed in adaxial side of leaves experiencing volcanic gases fumigation (Fig. 3I) could be due to one of the previous listed chemical compounds.

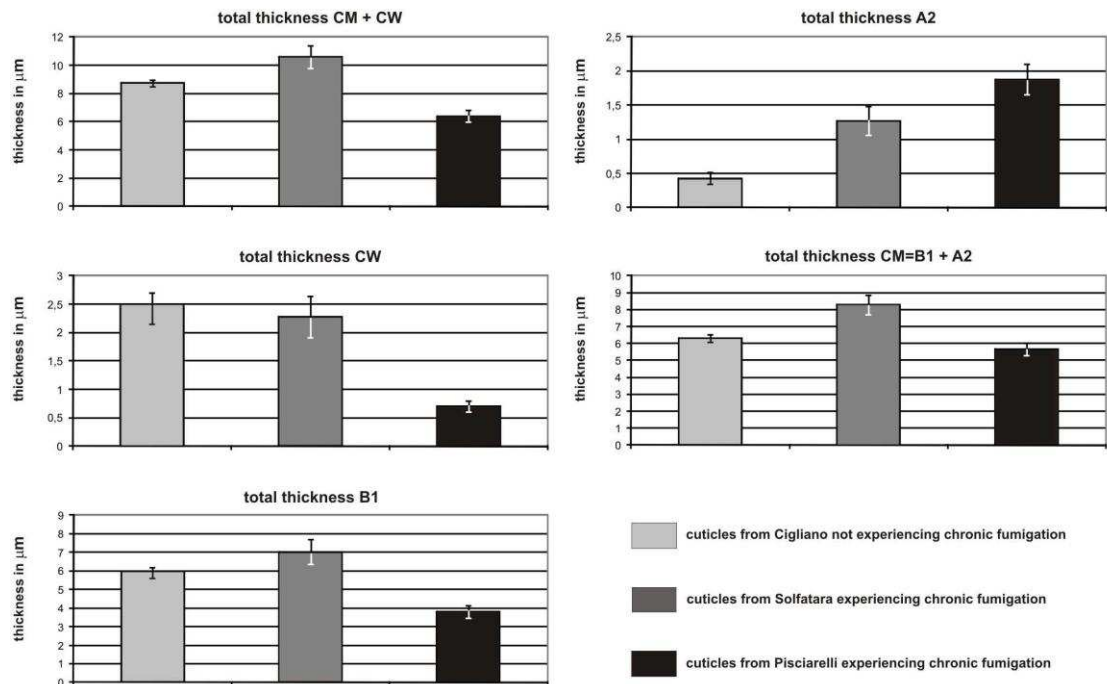
Trichomes alteration - In fumigated leaves, the high density of trichomes in the abaxial side (Fig. 3B) together with hairs having prolonged and tough inflated bases in the adaxial side of petiole (Fig. 3L) represent a barrier against toxic volcanic gases diffusion increasing boundary layer resistance (see Haworth and McElwain, 2008). A similar example can be done with the angiosperm *Metrosideros polymorpha* growing in the Kilauea crater region (Island of Hawaii) which is resistant to persistent fumigation with toxic volcanic gases (SO_2 , H_2S , HCl , HF) (Winner and Mooney, 1980b). *M. polymorpha* from the Kilauea crater region displays heavily trichomous abaxial surfaces, while those in neighbouring regions, not experiencing chronic fumigation, possess non-trichomous cuticles (Haworth, 2006). This pattern suggests (Haworth, 2006) that the development of trichomes in *M. polymorpha* from Kilauea Crater region is an adaptation to increase boundary layer resistance, and consequently reduces entry of toxic gases into the leaf.

Stomatal apparati - In fumigated leaves, the stomata are located beneath the medium level of epidermis (Fig. 3O). As stomata are the site of entrance of ca. 90% of atmospheric pollutants into the plant (Black and Unsworth, 1979), it may be also hypothesized that this feature could be a further protection device against the uptake of volcanic toxic gases. These features stress more xeromorphic features already existing in this species. As *E. arborea* is the unique typical Mediterranean angiosperm shrub able of growth close to the vents it could be attributable to a more effective stomatal control than other species with less effective stomatal control (see Haworth et al. 2011).

2.4.2. TEM considerations

The effect of volcanic gases on the epidermis in angiosperms has not been previously studied by TEM, and no detailed ultrastructural studies have been made so far on the cuticle of *E. arborea*. According to Archangelsky et al. (1986), within cuticle thickness it

Table 2. Statistical values, made with 30 measurements for cuticular membrane (CM) and cell wall (CW) of the epidermal cells of three sites. Note: the cuticular membrane CM is made up with cuticular proper CP (= A2) and cuticular layer CL (= B1). All measurements are in μm . min-max = minimum and maximum values observed; % = percentage of each detailed part of the cuticle and cell wall; st-d = standard deviation; var = variance.



is possible to distinguish a cuticle proper (termed A, often lamellate in its outermost part and granulous below) and a cuticular layer (termed B, mostly fibrillous). The cuticle of *E. arborea* appears to correspond best with Holloway's type three structure. This category (Holloway, 1982) includes cuticles with "outer region amorphous, inner region mainly reticulate".

The cuticle of *E. arborea* shows a significant response to volcanic fumigation. It reacts differently, increasing (Solfatara) or decreasing (Pisciarelli) its thickness. The CI reveals (Fig. 5) that CM + CW, CM, B1 and A2 show significant variations. Conversely, the cell wall thickness of Cigliano and Solfatara does not records significant variations.

CM and CW considerations - In fumigated leaves two types of CM + CW thickness responses to volcanic gases can be noted. In the specimens from Solfatara, as expected,

high CM (= B1 + A2) + CW (10.58 μm) and CM (8.30 μm) thickness values can be observed. Instead, the specimens from Pisciarelli both show lower CM + CW (6.40 μm) and CM (5.69 μm) values. Passing from specimens experiencing or not chronic fumigation, the CW thickness reduces of 3.4 times in Pisciarelli specimens. As Retallack (2002) claimed, vascular plants respond to high CO_2 concentration by increasing the thickness of their cuticles. However, even if the increase of cuticle thickness could be the response to high concentration of CO_2 , as Paoletti et al. (2005) claim, other gases virtually undetectable could have biological effects.

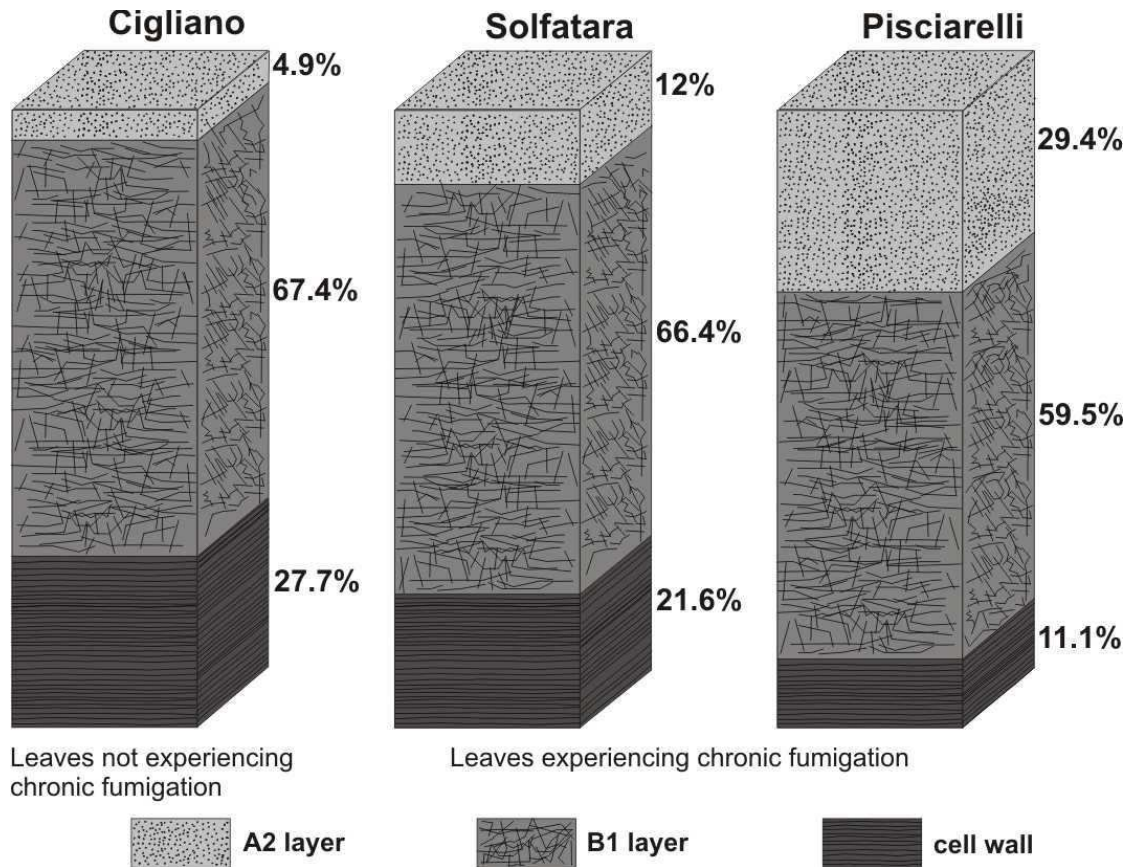


Fig. 6. Three-dimensional reconstruction of both cuticle and cell wall (CW) for leaves experiencing- or-not chronic fumigation. Note: the cuticular membrane CM is made up with cuticular proper (= A2) and cuticular layer CL (= B1).

CM (B1 + A2) considerations - Within cuticle thickness of fumigated leaves, for both statistical values (Table 2) and percentages (Fig. 6) the B1 and A2 thickness layers vary significantly. In cuticles from Solfatara, the B1 layer is 1.8 and 1.2 times greater than in Pisciarelli and Cigliano specimens respectively. Very interesting is the “behaviour” of A2 layer as in Pisciarelli specimens it is 4.4 and 1.5 times thicker in respect to Cigliano (not fumigated) and Solfatara cuticles respectively. In particular, within the cuticular

membrane, the A2 layer responds more marked in respect to B1 ones. As showed from data (Table 1 and 2; Figs. 5 and 6), a close relation between atmospheric CO₂ concentration and the A2 layer thickness seems to exist. It is worth noting that, in Solfatara and Pisciarelli localities, cuticles respond to atmospheric CO₂ concentration and not to the CO₂ soil concentration (Tables 1, 2 and Fig. 6). In specimens from Pisciarelli, the statistical reduction of the B1 layer thickness and the corresponding increasing of A2 layer thickness in respect to those of Solfatara and Cigliano (Table 1) could be the result of a carbon allocation from the inner layers to the outermost A2 layer. Therefore, the differences in layers thickness could be due to a different allocation of carbon-based secondary or structural compounds (CBSSC). According to growth-differentiation balance hypothesis (Hermes and Mattson, 1992), any environmental condition that affects photosynthesis (carbon source) and growth (carbon sink) with different intensity will affect the relative carbon pool available for allocation to carbon based compounds (Peñuelas and Estiarte, 1998). The carbon allocation could represent a further cost for plants experiencing chronic fumigation. In fumigated leaves the thickest A2 layer could be the response to toxic volcanic gases or/and to an increase of CO₂ concentration (Table 1). However, in the specimens from Solfatara and Pisciarelli, the increase in A2 layer thickness is consistent with Taylor et al. (1989) statement according to whom, amorphous remains within cuticle envelope are likely to represent the most resistant component of leaf.

CHAPTER III

An Early Cretaceous flora from Cusano Mutri, Benevento, southern Italy

3.1. Introduction

The distribution of fossil plants is one of the most reliable tools for a better understanding of terrestrial climatic conditions through geologic time (Francis and Frakes, 1993), and the addition of sedimentological context to species lists greatly refines such studies (DiMichele and Gastaldo, 2008).

This research represents a continuation of palaeobotanical studies carried out on Cretaceous plant-bearing localities in the southern Apennines (Bartiromo et al., 2006, 2008, 2009). The aim of this research is to provide supplementary data to investigations of subaerial Early Cretaceous Tethyan environments. A palaeontological survey on South Apennine Mesozoic sediments of carbonate platform facies (Campania Region) revealed in 2005 a new Early Cretaceous (Late Aptian) fossil locality near the village of Cusano Mutri (Benevento Province) (Fig. 1A). This new outcrop rivals in extent, abundance and preservation of the fossil material only the historical site of Pietraroja (early Albian) (Fig. 1A), the fossil flora of which has already been subjected to a preliminary study (Bartiromo et al., 2006, 2008). Several excavations were carried out at the site during the years 2005e2008, which provided hundreds of macro-remains among animal and plants (Bartiromo et al., 2008). This locality (Fig. 1B) displays a more complete fossil record than is usually found in the sedimentary successions of the Apenninic Carbonate Platform. The richness, taxonomic diversity and type of preservation make this outcrop worthy of consideration as a Fossil-Lagerstätte (Seilacher, 1970; Seilacher et al., 1985). Following a brief account of the invertebrate and vertebrate faunal assemblage we focus on the fossil flora to understand better the palaeoenvironment in which these plants grew. In addition, epidermal features were studied and interpreted palaeoecologically.

3.2. Geological setting

The outcrop (Fig. 1B) is located at “Peschera” (IGM Map at scale 1:25000 173 IV NO - Cerreto Sannita, long.14° 32' 08.15'', lat. 41° 17' 42. 89''), about 5 kmsouth of the village of Cusano Mutri (Campania Region, Benevento Province: Fig. 1A), on the eastern slope of

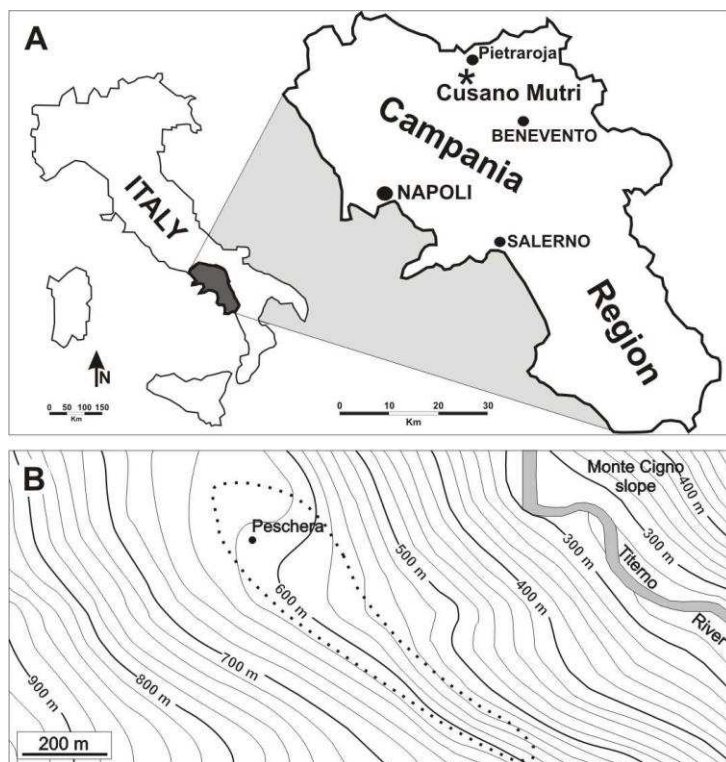


Fig. 1. A, the location of the village of Cusano Mutri in the Campania Region. B, sketch map showing the location of the fossil site. The area occupied by the fossil-bearing strata is approximately marked by the dotted line.

the mountain Monaco di Gioia, which is part of the southernmost border of the Matese Massif. From a structural point of view the southern Apennine Arc (sensu Parotto and Pratlun, 2004), stretching from the Central Apennines to Sicily, is a fold-and-thrust belt structure that originated during the late Tertiary deformation of the continental margin of the Adria Plate (Carannante et al., 2006). The Campania-Lucania Apennine is a segment of a chain of high complexity,

the most puzzling and tectonically complicated area of the whole Apennine-Maghrebian mountain range (Parotto and Pratlun, 2004). In particular, the Campanian Apennines are dominated by Mesozoic Carbonate Platform deposits and associated facies (Ciarapica and Passeri, 1998). During the Early Cretaceous the Cusano Mutri area was part of a large, shallow-water carbonate domain known as the Apenninic Platform, which developed in tropical-subtropical climatic conditions (D'Argenio, 1976; Mostardini and Merlini, 1986). The Matese Massif represents part of the platform. It comprises Upper Triassic-Cretaceous shelf limestones. Transgressive Lower Miocene limestones (Cusano Formation; Selli, 1957) overlie the Mesozoic sequence. In this area three Aptian-Albian, "Plattenkalk" intercalations bearing fossil fish and plant remains have been found (Monte Cigno, Civita di Pietraroja and the present one: Bravi, 1996; Bravi and Garassino, 1998; Bartiromo et al., 2008).

The mountain of Monaco di Gioia is a carbonate monocline intercepted by E-W trending faults and NW-SE border faults. The Cusano Mutri fossiliferous level, although of wide extent, is tectonically disturbed and poorly exposed, because it is mostly covered by vegetation and soil. Locally, very thinly stratified small outcrops are present.

3.2.1. Stratigraphy

The Cusano Mutri Plattenkalk contains scarce index microfossils, but samples collected just above and below it have revealed assemblages of chronostratigraphic value. A 50-m-thick stratigraphic succession has been sampled, including the thin-stratified Plattenkalk (Fig. 2). The succession can be subdivided in three intervals as follows:

A, Basal interval: 5 m of slightly dolomitized packstones and grainstones with *Coptocampylodon fontis* Patruilius, abundant *Salpingoporella dinarica* Radoičić, *Thaumatoporella* sp., *Aeolisaccus* sp., *Bacinella irregularis* Radoičić, miliolids, textularids, valvulinids, *Valvulineria* sp., *Spiroloculina* spp. among which is *S. cf. cretacea* Reuss, *Cuneolina* spp., *Sabaudia minuta* (Hofker), *Debarina hahounerensis* Fourcade,

Raoult and Vila, *Praechrysalidina infracretacea* Luperto-Sinni, *Volschinoides murgensis* Luperto-Sinni and Masse, *Pseudolituonella conica* Luperto-Sinni and Masse, *Nezzazatinella* sp., *Bolivinopsis* sp., *Pseudonummoloculina* sp., *Glomospira* sp., ostracods, requienid fragments, micritized gastropods and solitary corals (Fig. 3G, H, K). The rich and well differentiated microfauna and the

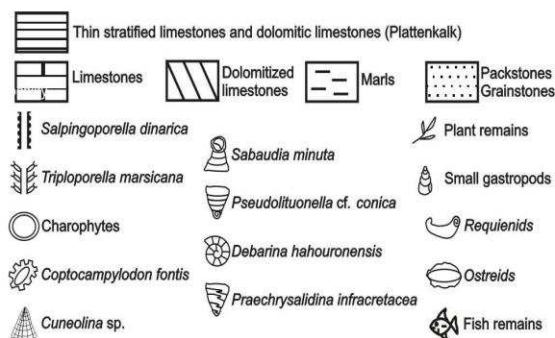
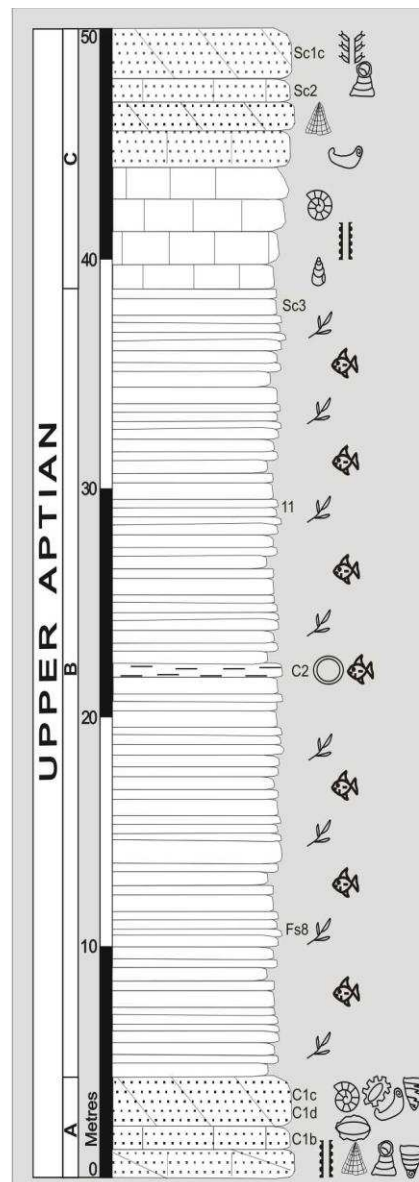


Fig. 2. Sedimentological log of the outcrop; see text for descriptions of the horizons.

sedimentological features (prevailing packstones and grainstones) may be interpreted as indicating to a moderately restricted lagoonal environment.

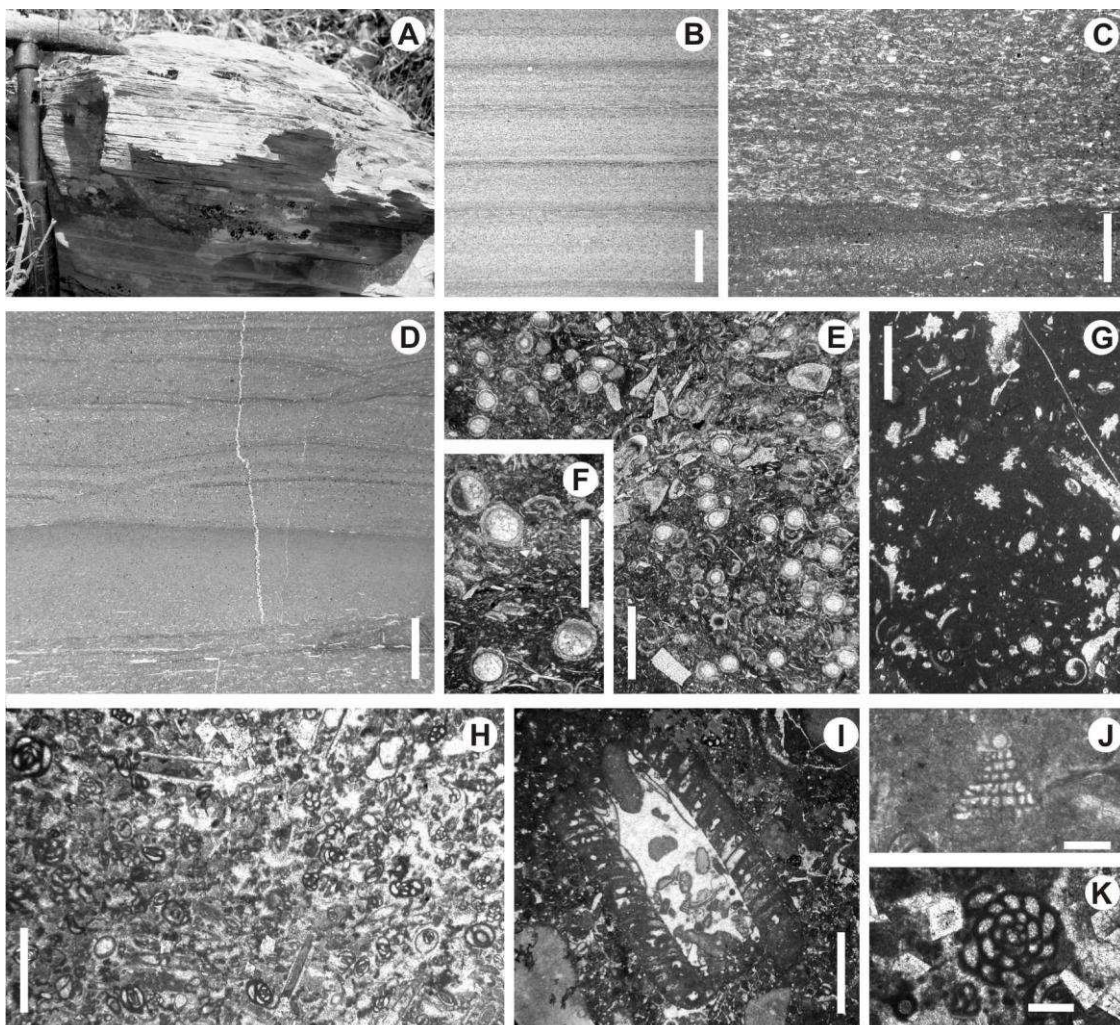


Fig. 3. Cusano Mutri, locality “La Peschera”, Late Aptian. A, typical thinly laminated strata of the “Plattenkalk facies”. B, thin section SC3: mm-thick dolo-micritic undisturbed laminae in the “Plattenkalk facies”. C, thin section 11: thinly laminated wackestone/packstones rich in ostracod tests in the “Plattenkalk facies”. D, thin section FS8: slight cross lamination (upward in the photo) and fining-upward lamination (lower part of the photo) in the “Plattenkalk facies”. E, thin section C2: marly limestones (packstones) full of charophyte gyrogonites and frustules; ganoid fish scales (*Lepidotes* sp.) are also visible. The level is located in the middle part of the plattenkalk. F, thin section C2: marly limestone rich in charophyte gyrogonites and fish scales. It is worth noting the different orientation of the geopetal infilling of some gyrogonites, indicating a mass remobilization of the material from the primary depositional site. G, thin section C1c: *Coptocampylodon fontis Patruilius* into wackestones intraclasts contained in dolomitized packstones strata. H, thin section C1d: dolomitized grainstone/packstones with *Salpingoporella dinarica* Radoičić and miliolids. I, thin section SC2: *Triploporella marsicana* Pratulon into packstones with micritized intraclasts. J, thin section SC1c: *Sabaudia minuta* (Hofker). K, thin section C1b: *Debarina hahounerensis* Fourcade, Raoult & Vila. Scale bars represent 2 mm in B-E; 1 mm in F-I; 100 μ m in J; 200 μ m in K.

B, Plattenkalk interval: 35 m of limestones, calcareous dolomites and marly-limestone beds varying in thickness from cm to dm, intercalated with sets of mm-thick, easily

exfoliated laminae (Fig. 3A). Some plattenkalk strata contain charophyte gyrogonites and frustules together with *Lepidotes* fish scales (Fig. 3E and F), the richest of these occurring in the middle of this plattenkalk interval, which consists of a 50-cm-thick marly stratum (Fig. 2). Other beds and laminae sets bear fish, crustacean and plant remains. Land, freshwater, brackish and marine fossil remains mixed together may be explained by sediment remobilization from surrounding inner landward areas in a channel context. The random orientation of the geopetal filling within the gyrogonites may support this interpretation (Fig. 3F). The typical Plattenkalk facies consists of strata and laminae of dolomicrite (Fig. 3B). Sometimes, in thin section cm-long fractures can be observed, seemingly owing to local differential compaction. At a later stage, the sediment probably underwent zone-mixing diagenesis, so that differential dolomitization developed along the fractures. Close to the fractures, dolomitization appears sometimes more marked than in the rest of the rock, and at times less marked. Finingupward structures (Fig. 3D), indicating decantation of muddy sediments brought in by tides during the normal, calm, environmental conditions, are also observed. The coarser sediment at the base of laminae often consists of ostracods (Fig. 3C) and estherid valves; the top is capped by a thin brownish layer, probably indicating the last phase of decantation of suspended organic matter. Irregular undulating laminae are also commonly observed. Some of these show cracks and strain structures, probable desiccation voids and root moulds which at present cannot be safely determined as subaerial exposure (Fig. 3D).

From an environmental point of view, the plattenkalk facies, which formed in a sheltered area of the carbonate platform, more or less close to land, strongly influenced the sedimentation and palaeontological content. Sediments deposited landward in fresh to brackish ponds were sometimes removed and washed seaward.

C, Upper interval: 10m of grainstones, packstones and packstone wackstones. The microflora is composed of *Thaumathoporella* sp., dasycladalean algae (such as *Triploporella marsicana* Pratulon and *S. dinarica* Radoičić) and *Cretaciclodus minervini* Luperto-Sinni. Foraminifers are represented by milioliids, textulariids, valvulinids and Ataxophragmidae. Most significant taxa are *S. minuta* (Hofker), *D. hahounerensis* Fourcade, Raoult and Vila, *P. infracretacea* Luperto-Sinni, *Spiroloculina* cf. *cretacea* Reuss, and *Cuneolina* spp. Ostreids, requienid fragments, small micritized and bioeroded gastropods, and thin, smooth valves of ostracods are also present (Fig. 3I and J). This last interval is broadly characterized by an increase in biodiversity, which may testify the return to marine conditions.

The stratigraphical study has also revealed the presence of numerous animal remains throughout the plattenkalk sequence. Within the invertebrates: (1) small turritellate gastropod shells (ca. 15 mm long, 5 mm wide) randomly sparse on bed surfaces (Bravi and Bartiromo, 2005); (2) small (ca. 2 mm long, 1.5 mm wide) connected valves of bivalves with concave surfaces facing upward; (3) complete and fragmented decapod crustaceans, and (4) disarticulated valves of spinicaudatan (conchostracan) branchiopods (estherids) are noted. With regards to fish: (5) disarticulated scales and complete specimens belonging to the genus *Lepidotes*; (6) some *Clupeiformes* (*Clupavus*?) not exceeding 2.6 cm in length; (7) a fragmented *Pycnodontiform* and (8) a 4-cm-long specimen of *Notagodus* were also found. Indeterminable worm traces are also common on the bedding-plane surfaces.

The upper Aptian (upper part of the “*S. dinarica* biozone”; De Castro, 1991) microfossil assemblage occurring throughout the whole succession allows us to ascribe the plattenkalk interval to this age. The Cusano Mutri plattenkalk can be considered coeval with that of Monte Cigno (Bravi and Mega, 1999), cropping out on the opposite side of the Titerno River valley (Fig. 1B). Also, the “plattenkalk 1” from the “Civita di Pietraroja” (Fig. 1A) (Bravi and Garassino, 1998) is possibly the same age as the two found previously, although further stratigraphic study is needed to confirm this.

3.3. Material and methods

The material was collected during excavations carried out by one of the authors (AB) during the period 2005-2008. The specimens are labelled with the letters CM (Cusano Mutri) followed by the identification number. Following Schopf's (1975) classification, the fossil plants described in this paper are impressions, poorly preserved cuticles and carbonaceous compressions. Well preserved cuticle fragments belong to the extinct family Cheirolepidiaceae (e.g. *Frenelopsis*), but exquisite plant impressions showing the epidermal structures are characteristic of the whole of the studied flora.

Low viscosity collodion was used to prepare casts showing fine epidermal characters. The collodion method is very useful (Walton, 1923), although Kouwenberg et al. (2007) do not recommend it because it is extremely flammable and readily forms an explosive mixture with air at relatively low temperatures. A tough film of collodion is formed from the evaporation of the solvent; this can be stripped off and examined under transmitted light or a Scanning Electron Microscope (SEM). Watson (1983) described the species *Frenelopsis silfloana* by means of casts made with low viscosity silicone rubber.

Microscopical details of some cuticles were difficult to observe after chemical treatment. For this reason, some cuticle fragments were mounted directly onto the stubs for the SEM observation. Cuticle fragments were separated from the surface of the bed by means of tweezers. They were cleaned in 37% hydrochloric acid (HCl) for 2 h. Adhering siliceous debris on the cuticle surfaces was removed by immersion in 40% hydrofluoric acid (HF) for 1 h. Finally, they were macerated in Schulze's reagent (HNO₃ and KClO₃) for 2 h; the duration of the latter depending on the degree of carbonization and preservation (Kerp, 1990). Parts of cuticle were directly mounted on glass slides for light microscope examination. Other cuticle fragments were directly mounted onto the stubs for observation under the SEM. Light microscope (LM) and Stereo-microscope (SM) observations were made using Leitz microscopes. Specimens examined under an optical microscope were photographed with a Nikon Coolpix 990 camera. The cuticles were examined using a JEOL JSM-5310 SEM at the CISAG (Centro Interdipartimentale di servizi per analisi Geomineralogiche) and a FEI-QUANTAS 200 ESEM (Figs. 6G and 7G) at the CISME (Centro Interdipartimentale di servizi di Microscopia Elettronica).

The material is housed in the Museo di Paleontologia, "Centro Museale, Centro Musei delle Scienze Naturali", Università degli Studi di Napoli Federico II (Italy).

3.4. Systematic palaeontology

Order Coniferales

Family incertae sedis

Genus Cupressinocladus Seward, 1919

Cupressinocladus sp.

Figs. 4A, 5A

Material examined. CM 21, 68

Description. Twig impressions, branched (CM 21: Figs. 4A and 5A) and unbranched. The leaves are decussately arranged. They are heavily adpressed to the axes and have a rounded apex. The free part of the leaves arises from whole basal cushion. A poor impression of a stomatal apparatus 80 µm in diameter has been obtained by means of a peel.

Discussion. The morphology of the shoots together with the arrangement of the leaves lead us to ascribe the specimens to the genus Cupressinocladus Seward, 1919. However,

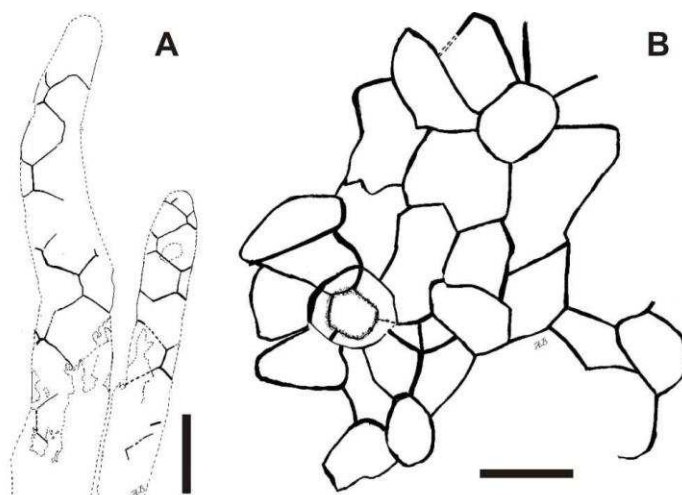


Fig. 4. Late Aptian fossil plants from Cusano Mutri: drawings of selected specimens. A, *Cupressinocladus* sp., CM 21: detail showing the leaves basal cushions. B, *Araucarites* sp., CM 53: stomatal apparatus with four subsidiary cells presenting an annular thickening on the periclinal walls. Scale bars represent 4 mm in A; 50 µm in B.

the poor preservation of the specimens and the lack of cuticle do not allow determination of the specimens at species level.

Genus *Pagiophyllum* Heer, 1881 emend. Harris, 1979

Pagiophyllum sp. 1

Fig. 5B

Material examined. CM 104

Description. Carbonaceous compression of fragmented

and unbranched vegetative shoot, 14.5 mm in length with an almost constant width of ca. 3.3 mm (Fig. 5B). The leaves are helically arranged and measure ca. 2mm in length by 1.5mm in width. They are closely adpressed to the axes forming with it an angle of ca. 20° (26° at the base and 15° in the distal part). The free part of the leaves is longer than their width. The upper surface of the leaves is difficult to elucidate. The lower surface is fairly convex with the basal part rounded; the leaf apex is rounded.

Discussion. The dimensions (leaf length is greater than its width) and disposition of the leaves lead us to ascribe the specimen to the genus *Pagiophyllum*. The specimen differs enormously from *Pagiophyllum* sp. 2 in shape and size, but the poor preservation does not allow us to determine it to species level.

Pagiophyllum sp. 2

Fig. 5C, D

Material examined. CM 19, 30

Description. Distal parts of vegetative shoots (Fig. 5C), unbranched, 18 mm in length by 5 mm in width (CM 19). The leaves are spirally arranged and falcate; the longest measures 2.9 mm in length by 1.9 mm in width (CM 19). The free part of the leaves is almost triangular in section. The upper surface of the leaves is almost flattened. The lower surface is fairly concave, keel shaped. The leaf apex curves upward and is pointed. Small

areas show epidermal cells that are polygonal in shape disposed in longitudinal rows. The anticlinal walls of the epidermal cells are frequently curved but sometimes they are straight. Periclinal walls display fine granularity. The peel technique allowed us to observe stomatal apparatuses 50 μ m in diameter, probably disposed in rows with ostioles showing a rather good preferential orientation (Fig. 5D).

Discussion. The size and disposition of the leaves leads us to ascribe the specimens to the genus *Pagiophyllum*. The specimens resemble *P. araucarinum* Pomel (Saporta) but differ by having smaller leaves. Unfortunately, the poor preservation of the specimens and the lack of the cuticle leave the specimens unassigned at species level.

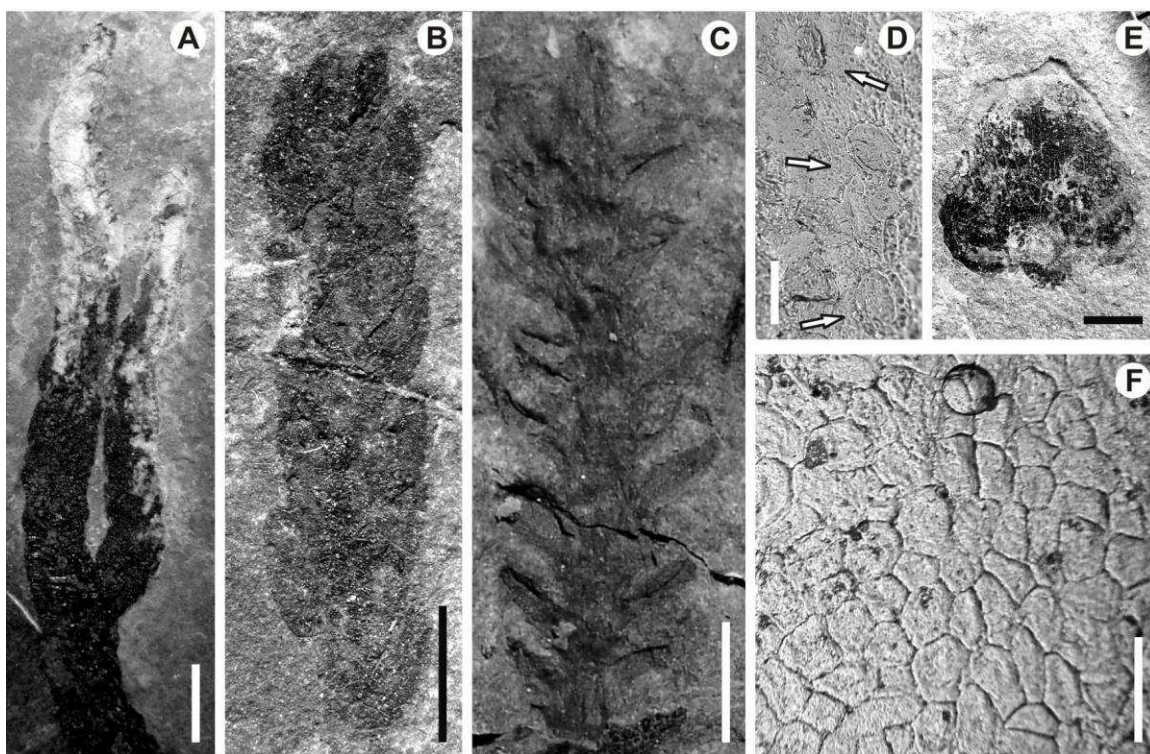


Fig. 5. Late Aptian fossil plants from Cusano Mutri. A, *Cupressinocladus* sp., CM 21: branched shoot with leaf cushions preserved in the apical part. B, *Pagiophyllum* sp. 1, CM 104: carbonaceous shoot compression with leaves spirally arranged and heavily adpressed to the axis. CeD, *Pagiophyllum* sp. 2., CM 19. C, branch fragment with large leaves in spiral arrangement. D, stomatal apparatuses disposed in rows with ostioles (arrows) showing a probable preferential orientation (collodion peel). E-F, *Araucarites* sp., CM 53. E, carbonaceous compression of a triangular woody scale. F, polygonal epidermal cells (detail of E; collodion peel). A-C, E: SM observations; D, F: LM observations. Scale bar represents 5 mm in A; 2 mm in B; 3 mm in C, E; 50 μ m in D; 100 μ m F.

Genus *Araucarites* Presl (in Sternberg, 1838)

Araucarites aff. *pedreranus*

Figs. 4B, 5E, F

Material examined. CM 53, 135

Description. Woody scales (CM 53 probably a lower surface) 10.5 mm long by 10.1 mm wide, triangular in shape with entire margin. The base of the scales is 5 mm in width. The apex is rounded (Fig. 5E). The epidermal features are visible on the distal surface of the scale (CM 53) and have been obtained by means of collodion peels. The epidermal cells are polygonal and sometimes pentagonal and or hexagonal in shape (Figs. 4B and 5F). The haplocheilic, monocyclic and incompletely amphycyclic stomatal apparatuses have a diameter of ca. 47 μ m. They are composed of four subsidiary cells, the largest of which is 28 μ m long and 12 μ m wide. The periclinal surface of subsidiary cells seems to present a coronal thickening around the stomatal rim 4 μ m thick (Fig. 4B).

Discussion. The size and shape of the scales together with the epidermal features indicate an affinity with *Araucarites pedreranus* described by Barale (1989) for the Early Cretaceous lithographic limestones of Montsech (Spain, Pedrera and Cabrera quarries). However, the scale apex is triangular in *A. pedreranus* but rounded in the specimens from Cusano Mutri. Although fragmentary, the Cusano Mutri scales share with the Spanish material the same epidermal features. Unfortunately, the poor state in the preservation of the specimens and the incompleteness of the epidermal features prevent a specific assignment.

Family Cheirolepidiaceae Takhtajan, 1963

Genus *Frenelopsis* (Schenk, 1869) emend. Watson, 1977

Type species. *Frenelopsis hoheneggeri* (Ettingshausen, 1852) Schenk, 1869

Frenelopsis cusanensis sp. nov.

Figs. 6A-J, 7A-I

Holotype. CM 100, slide number 100v1, 100v2; stub number 100s1, 100s2. Figs. 6BeH, J, 7A-I.

Paratypes. CM 3, 8, 22, 66; stub number 3s, 8s, 22s, 66s.

Type locality. Cusano Mutri village, Benevento Province, southern Italy.

Derivation of name. Refers to the village of Cusano Mutri where the species was found.

Repository. Museo di Paleontologia, "Centro Museale, Centro Musei delle Scienze Naturali", Università degli Studi di Napoli Federico II (Italy).

Stratigraphic range. Late Aptian

Diagnosis. Articulated shoots attaining at least two orders of branching in one plane. Branches arising at an angle of ca. 11° with internodes 4-10 mm long and 1.3-2.4 mm wide. Shoots bear whorls of three scale-like leaves 0.5-0.9 mm in length bordered by unicellular hairs up to 60 μm in length. Internode cuticle is ca. 7 μm thick. The stomatal apparatuses have an average diameter of 40 μm and are arranged in well-defined rows (7-10 per linear mm). Average stomatal density is 70-80 per mm^2 . The stomatal apparatuses, randomly orientated, bear four subsidiary cells (rarely five), separated into lateral and polar cells, each bearing one outer and one inner papilla. Papillae quadrangular, small, sometimes elongated, arise from epidermal cells.

Description. The material consists of impressions and compressions of branched (CM 22, 66), articulated cuticular segments (CM 8, 66, 100) and internode fragments (CM 65) 1.2 up to 11.1 cm long (Fig. 6A and B). The best preserved shoot (CM 100) as well as the holotype, measures 77 mm in length by 2.4 mm in width and is composed of part and counterpart (Fig. 6B: part). The branches are produced alternately, in one plane, and the specimens have one main axis. Branches arise at an angle of ca. 11° . The internode length varies between 4 and 10 mm; the segment width is 1.3-2.4 mm, 1.8 mm on average. At the level of every node a whorl of three squamiform leaves, laterally fused, form a leaf sheath developed along the internode length (Fig. 6C and D). At the extremity of the leaf sheath, three small teeth (Fig. 6F), related to the free leaf extremity are present. There are no suture lines along the leaf sheath. The free parts of the leaves are identical at each node: they are triangular in shape and measure 0.9-0.5 mm in length with an average of 0.7 mm. The leaves are bordered by marginal unicellular hairs up to 60 μm in length (Fig. 6D). The cuticle of the internode is 7 μm thick (Fig. 7B). In outer view, the epidermal cells bear one papilla each; close to the stomatal apparatuses the papillae measure ca. 11 μm in length and 10 μm in width extending in the direction of the stomatal apparatuses itself (Figs. 6I and 7D). Elongated epidermal papillae transversally disposed can be frequently observed (Fig. 7D). Usually, the width of the papillae is greater than their length and they are located above the average surface of the epidermis; the papillae density is 70-80 per mm^2 (CM 100). There are no hairs on the epidermal surface (Fig. 6I). Several epidermal cells are polygonal in shape, but frequently rectangular cells ca. 31 μm long by 15 μm wide can be observed. The epidermal cells are arranged in more or less well-defined rows running parallel to the long axis of the internode (Fig. 7C). The anticlinal walls of the epidermal cells are ca. 8 μm thick. The stomatal apparatuses are arranged in well-defined longitudinal

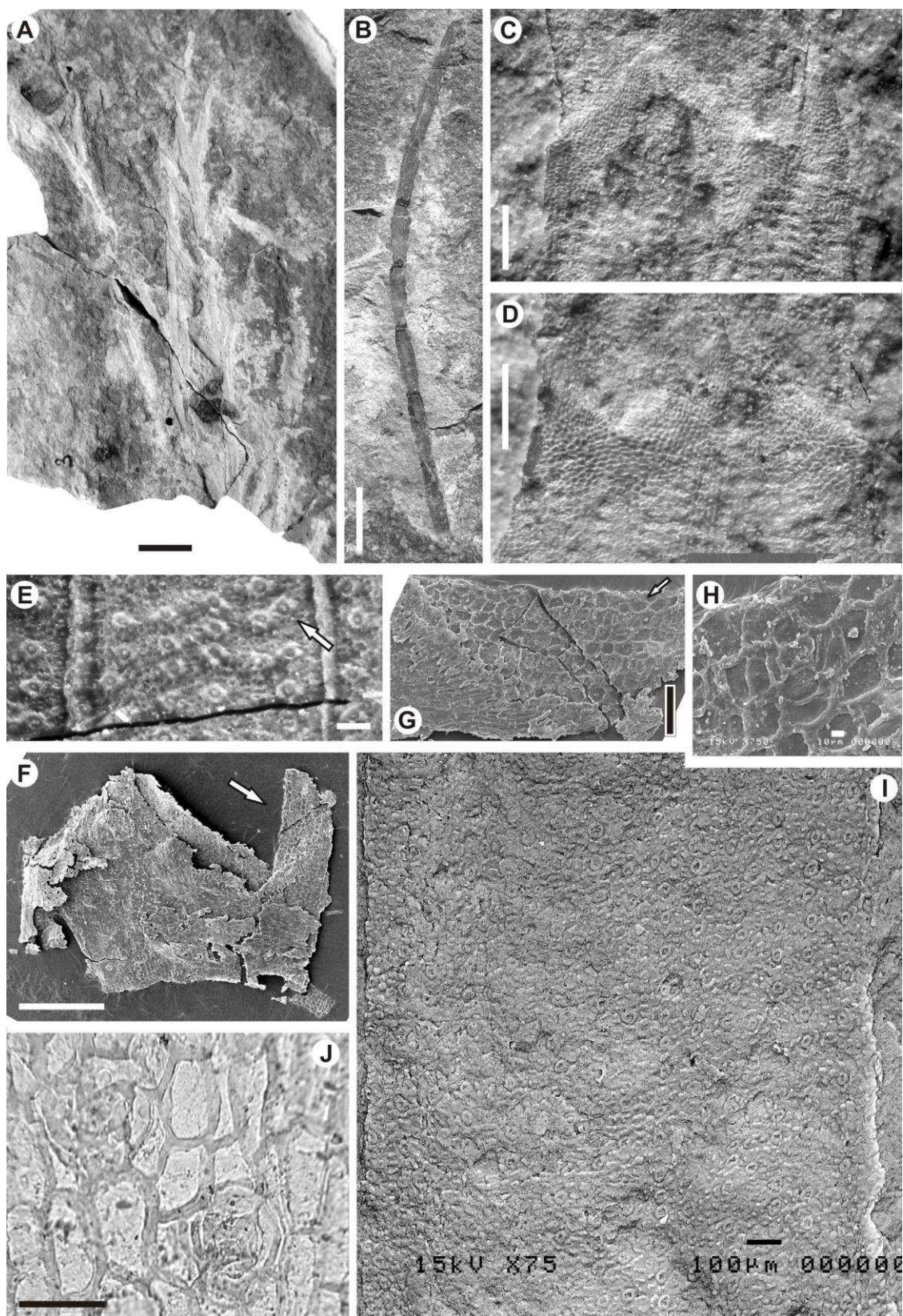


Fig. 6. Late Aptian fossil plants from Cusano Mutri: *Frenelopsis cusanensis* sp. nov. A, CM 3: impression of branched shoots. B-H, J, CM 100 (holotype): part and counterpart. B, shoot composed of eight internodes (part). C, impression of a distal part of an internode showing a whorl of three scale-like leaves. The upper part of the leaves is devoid of stomatal apparatuses (counterpart). D, impression of a distal part of an internode showing the marginal hairs (counterpart). E, typically exquisite impression of an internode area (part) with stomatal rows and two stomatal apparatuses directly connected without

interposed epidermal cells (arrow). F, fragment of internode cuticle (part) removed to demonstrate abaxial and adaxial surfaces. The arrow indicates the position of detail in G. G, inner side of abaxial surface with two stomatal rows (detail of F). The arrow indicates position of the detail in H. H, inner view of a dicyclic stomatal apparatus composed by four subsidiary cells (detail of G). I, CM 3: external view of the abaxial surface showing epidermal papillae and stomatal apparatuses arranged in rows (inverted image: negative). J, internode cuticle showing two stomatal apparatuses. The upper one focused on external papillae while the lower one shows the four subsidiary cells distinct in polar and lateral ones. C-E: SM observations; F-I: SEM observations; J: LM observation. Scale bar represents 10 mm in A, B; 500 μm in C, D, F; 100 μm in E, G, I; 10 μm in H; 50 μm in J.

rows (7-10 per mm) (Figs. 6I and 7C, E, I). The stomatal rows are separated by 1-2 (at times 3) epidermal cells. On the same stomatal row 1e2 (at times 3) epidermal cells occur between two stomatal apparatuses (Figs. 6G and 7C, I). Sometimes, the subsidiary cells of two adjacent stomatal apparatuses on the same row can be in contact without intervening epidermal cells (Figs. 6E and 7I). The stomatal density is 70-80 per mm^2 . Stomatal apparatuses are 40 μm in diameter on average (max. 62 μm in CM66, min. 30 μm in CM 22; 38 μm in CM 100). The stomatal apparatuses are haplocheilic and normally monocyclic (Fig. 6J); rarely, dicyclic stomatal apparatuses have been observed (Fig. 6H). The stomatal apparatuses are composed of four (Figs. 6I, J, 7A, C-E, H, I), sometimes five (Fig. 7G) subsidiary cells, more or less regularly disposed around the stomatal pit. The stomatal apparatuses are randomly orientated. From a morphological point of view, it is possible to distinguish two lateral and two polar subsidiary cells, usually with polar cells greater than lateral ones (Figs. 6J and 7E). Each subsidiary cell bears (1) one outer papilla protruding on the stomatal rim, partially covering the stomatal pit, and (2) one inner papilla which conceals the stomatal pit (Fig. 7A, E and F). The fusion of outer papillae results in a stomatal pit with a stellate outline and an aperture of ca. 14 μm in length and 6 μm in width (Fig. 7A). Normally, the median level of epidermis is prominent with respect to the stomatal apparatuses (Figs. 6I and 7A, D). The subsidiary cells are more or less rectangular in shape, being ca. 18 μm long and 12 μm wide. The subsidiary cells are more or less rectangular in shape, being ca. 18 μm long and 12 μm wide. The guard cells are sometimes preserved with well-cutinized dorsal plates underneath the stomatal pits (Fig. 7H). Occasionally, the inner cuticle surface is granulose (Fig. 7E and F).

Discussion. The studied specimens are typical *Frenelopsis* shoots on the basis of macro-morphological features such as the cyclic, decussate, leaf-whorl arrangement, and the absence of longitudinal sutures at the internodes. These characteristics exclude the attribution of the specimens to the genus *Pseudofrenelopsis* Nathorst, 1893 emend. Srinivasan, 1995. As Kunzmann et al. (2006) claimed, *Frenelopsis* consists of several

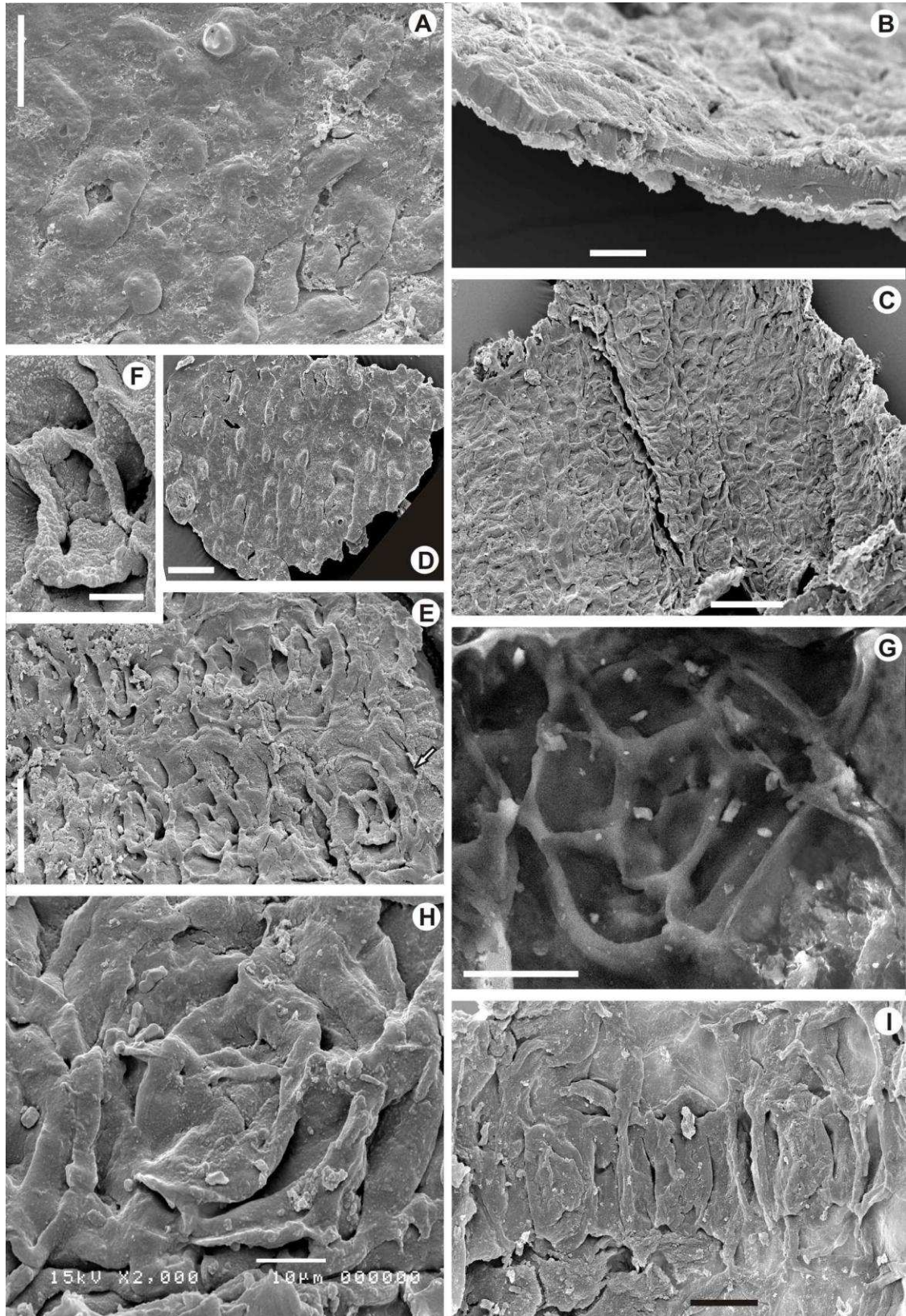


Fig. 7. Late Aptian fossil plants from Cusano Mutri. A-I, *Frenelopsis cusanensis* sp. nov., CM 100 (holotype). A, external view of the abaxial surface showing epidermal papillae and two stomatal apparatuses each composed of four subsidiary cells, each of these bearing one inner and one outer papilla. B, section of the cuticle internode through epidermal cells showing the thickness. C, internal view of the cuticle showing randomly orientated stomatal apparatuses in well-defined rows. D, external view of abaxial cuticle surface showing elongated papillae and four stomatal apparatuses.

E, internal view of the cuticle showing some stomatal apparatuses presenting fine granulosity (on the right). The arrow indicates position of the detail figured in F. F, internal surface of a stomatal apparatus bearing four inner papillae that are very closely linked (detail of E). G, internal view of a rare stomatal apparatus composed of five subsidiary cells. H, internal view of stomatal apparatus composed of four subsidiary cells showing the dorsal plates of the guard cells. I, internal view of cuticle showing three stomatal apparatuses (each composed of four subsidiary cells) of a stomatal row with no epidermal cells on the left and one on the right, between adjacent stomatal apparatuses. All figures: SEM observations. Scale bar represents 25 μm in A; 50 μm in D, E; 10 μm in B, F; 100 μm in C; 20 μm in G, I; 10 μm in H.

morphospecies typically bearing three leaves per whorl (Watson, 1988; Kvaček, 2000).

Among the species attributed to *Frenelopsis* (Bartirromo et al., 2009), the cuticles from Cusano Mutri are closest to *Frenelopsis ugunaensis* Gomez et al., 2002 from the upper Barremian of Spain and *Frenelopsis harrisii* Doludenko and Reymanówna, 1978 from the Cenomanian of Tadjikistan (Table 1). However, *F. ugunaensis* is different from the Cusano Mutri material in having longer (up to 3 mm) and wider (up to 2 mm) internodes. At a microscopic level, *F. ugunaensis* displays: (1) longer hairs in the upper margin of the leaf (up to 100 vs. up to 60 μm); (2) a greater thickness of the cuticle (15-20 vs. 7 μm); (3) ill-defined stomatal rows; (4) a lower stomatal density per mm^2 (40-55 vs. 70-80); and (5) greater size of stomatal apparatuses (55-60 vs. 40 μm). Moreover, *F. ugunaensis* has perfect conical hairs (up to 100 μm long) on the outer cuticular surface, whereas the Cusano Mutri specimens possess hemispherical papillae on the outer surface of each epidermal cell without hairs. *F. harrisii* is different from the Cusano Mutri material in having: (1) greater internodes measuring 8-12 mm in length (vs. 4-10 mm) and 1-4mm in width (vs. 1.3-2.4 mm); (2) a longer free part of the leaves (1.5 vs. 0.5-0.9 mm); (3) a greater thickness of internode cuticle (up to 100 vs. 7 μm); (4) stomatal apparatuses elliptical in shape; and (5) a smooth cuticle surface. Moreover, it is interesting to note that the specimens from Cusano Mutri display the smallest stomatal apparatuses of the genus *Frenelopsis*, with an average diameter of 40 μm as opposed to 107 μm for *Frenelopsis profetiensis* Bartirromo et al., 2009 from the lower-middle Aptian locality of Profeti, which is the *Frenelopsis* with the largest stomatal apparatuses (see Table 1). It is for these reasons that the new species, *Frenelopsis cusanensis*, is proposed for the material from Cusano Mutri.

Cheirolepidiaceae sp. 1

Fig. 8A-G

Material examined. CM 1

Description. Compression of branched stem 14 mm in length by 2mm in width made by nodes and internodes. The internode length is ca. 2.2 mm. The specimen (Fig. 8A) bears,

alternate and in one plane, three apparent buds probably representing lateral extension

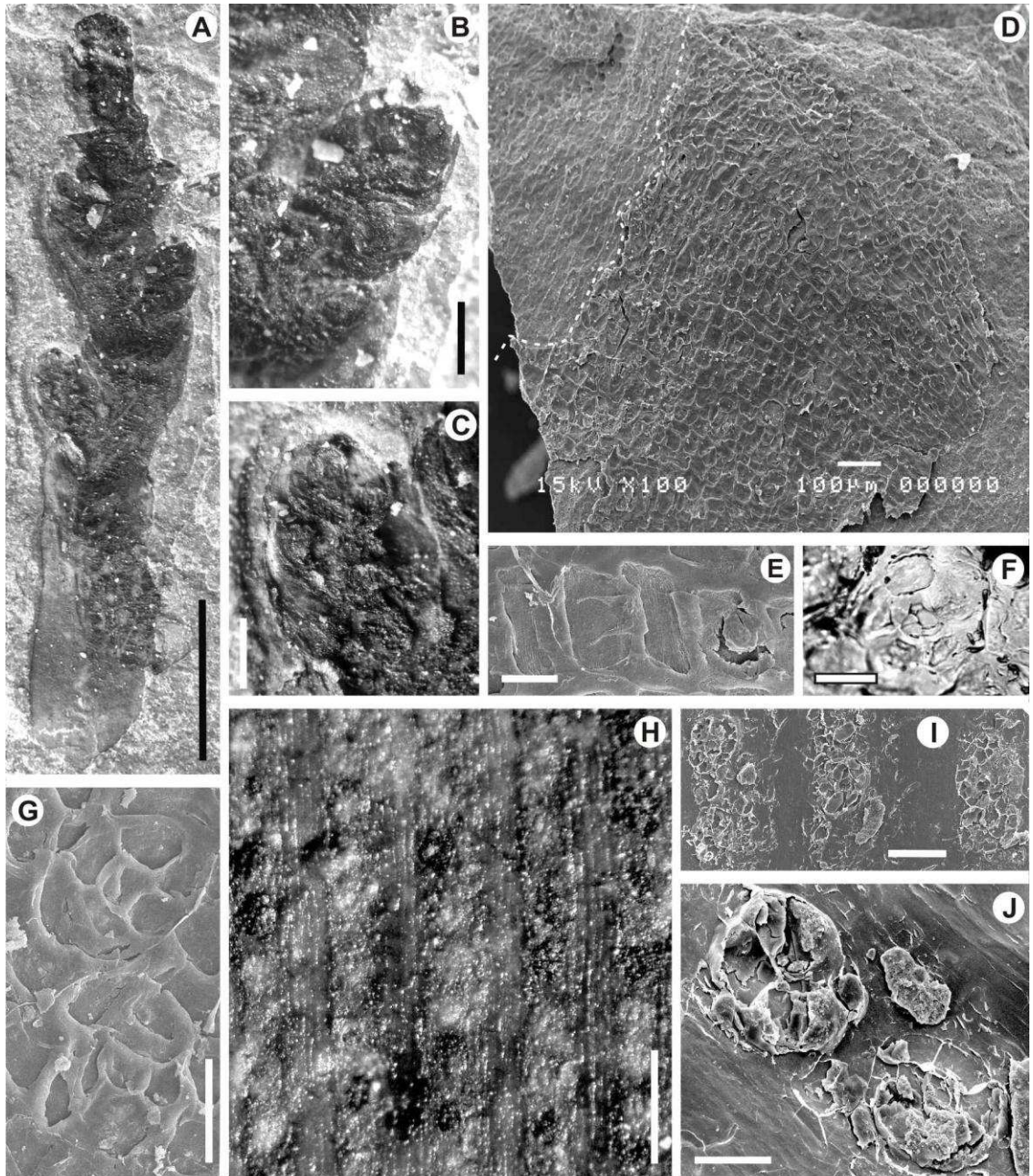


Fig. 8. Late Aptian fossil plants from Cusano Mutri. A-G, Cheirolepidiaceae 1, CM 1. A, portion of shoot consisting of a succession of internodes, bearing three lateral branches in the axils of their subtending leaves. B, C, buds possibly of lateral extensions shoot with *Brachyphyllum*-like leaves (details of A), respectively focused in the central and right part (B) and in the central and left part (C). D, in respect to the dashed line, impression (on the left) and compression (on the right) of a leaf showing stomatal apparatuses arranged in rows. E, internal view of cuticle surface showing a fine striation of periclinal walls of the epidermal cells. F, internal view of cuticle showing a stomatal apparatus formed by five subsidiary cells, each one bearing one papilla (collodion peel). G, two stomatal apparatuses composed of four and six (above) subsidiary cells (with subsidiary cells in contact) without interposed epidermal cells (detail of D). H-J, Cheirolepidiaceae 2, CM 91. H, poorly preserved cuticle fragment showing stomatal apparatuses and epidermal cells arranged in well-defined rows. I, poorly preserved stomatal apparatuses arranged in rows (collodion peel observed with the SEM). J, two stomatal apparatuses composed of six subsidiary cells with at least one interposed epidermal cell. A-C, H: SM observations; D, E, G, I, J: SEM observations; F: LM observation. Scale bar represents 3 mm in A; 0.5 mm in B, C, L; 25 µm in E; 50 µm in G, J; 30 µm in F; 300 µm in H; 100 µm in I.

shoots (two on the left and one on the right). Each bud bears a number (perhaps at least seven) of more or less *Brachyphyllum*-like leaves (*sensu* Alvin, 1983), which do not form a complete sheath around the axis (Fig. 8B and C). Each node bears one subtending leaf of “close type” (see Watson, 1977) at the axil of the node (Fig. 8A and D). There are no suture lines along the sheathing leaves. It was impossible to measure the cuticle thickness. The few stomatal apparatuses observed seem to be haplocheilic, monocyclic and made by four (Fig. 8D) or frequently five (Fig. 8F) or six (Fig. 8G) subsidiary cells. They have an ovoid to a rounded shape and measure on average 85 μm along the major axis and 56 μm along the minor one. The subsidiary cells measure on average 25 μm in length and 35 μm in width. Inner papillae are present (Fig. 8F). In external view no stomatal apparatuses have been observed. The few preserved cuticle fragments show epidermal cells of varying size having a polygonal (often isodiametric) morphology and disposition in more or less well-defined rows (Fig. 8D). The anticlinal walls of the epidermal cells are ca. 10 μm thick, sometimes exceeding 15 μm . The periclinal walls of the epidermal cells display a fine parallel striation (Fig. 8E). By means of collodion casts the hypodermis can be seen.

Discussion. The specimen presents a macroscopical architecture and micro-morphological characters that allow it to be placed within the family *Cheirolepidiaceae* Takhtajan, 1963. The macroscopical and especially the microscopical features differ from both *F. cusanensis* sp. nov. and *Cheirolepidiaceae* sp. 2. The gross morphology is comparable with *Pseudofrenelopsis parceramosa* (Fontaine) Watson, 1977 from the Wealden beds of the Isle of Wight (England), figured by Alvin and Hlušík (1979, fig. 15) and suggested again by Alvin (1983, Fig. 7H). The few observed stomatal apparatuses are probably related to the adaxial surface of the cuticle, in agreement with Watson (1977, p. 720): “Adaxial cuticle showing cells of variable shape and arrangement, often strongly papillate; few, scattered stomata, often abortive”. The micro- and macroscopic features together with the *Brachyphyllum*-type foliage are reminiscent of juvenile forms of *P. parceramosa* (Alvin, 1977, 1983; Watson, 1977). However, extensive morphological variation has been historically documented within and between assemblages of *P. parceramosa* (Axsmith, 2006). Unfortunately, the lack of well-defined microscopical features makes it difficult to identify it to genus level within the family *Cheirolepidiaceae* which, as Taylor et al. (2009) and others have claimed, certainly represents several different types of plants based on habit and ecology.

Table 1. Comparisons of the species described to date belonging to the genus *Frenelopsis* (updated and modified from Bartiromo et al. (2009)).

Characters/Taxa	<i>F. alata</i> (Feistmantel) Knobloch	<i>F. choshiensis</i> Kimura et al.	<i>F. elegans</i> Chow & Tsao	<i>F. harrisii</i> Doludenko & Reymanówna	<i>F. hohenneggeri</i> (Ettingshausen) Schenk emend. Reymanówna & Watson (type species)	<i>F. kaneviensis</i> Barale & Doludenko
Branching	moderate	moderate	rare	moderate	moderate	profuse
Internode length (mm)	up to 15	4-4.5	up to 23	8-12	8	8-10
Internode width (mm)	up to 5	1.5-2	2-3	1-4	3	1.7-3.5
Leaf number per node	alternating whorls of 3	2 opposite and decussate	alternating whorls of 3	alternating whorls of 3	alternating whorls of 3	alternating whorls of 3
Outer surface ridge number per leaf	12-13		?	absent	15	absent
Maximum length of free leaf (mm)	~ 1	0.5	~ 1	up to 1.5	1.5	1-0.7
Depth of sheathing base (mm)	up to 1		?	?	1	up to 1
Leaf upper margin	fringe of hairs	hairs up to 4 µm	?	?	scarious	short hairs up to 20 µm long
Internode and abaxial leaf cuticles thickness (µm)	30-40	20	?	up to 100µm	40	15-20
Stomatal arrangement	ill-defined rows	ill-defined rows	well-defined rows	well-defined rows	well-defined rows	ill-defined rows
Density of stomatal rows per mm	10-12		8-10	9-10	10-12	12-16
Density of stomata per mm²	90-100	200			90-100	
Diameter of stomatal apparatus (µm)	52-77	45-75	100	45×20 (elliptical)	60-70	35-50
Number of subsidiary cells	4-5, occasionally 6	4-6, usually 4	4-6	4-5	4-6, usually 4	4-6, usually 4
Orientation of stomatal aperture	? random	horizontal	transverse or oblique	?	transverse	?
Surface around pit		grooved			thickened ring	
Papillae in throat of stomatal pit	present	present	present	present	present	present
Cuticle surface	Irregularly ribbed	smooth	smooth	smooth	smooth	smooth
Rim of stomatal pit	stellate with lobed rim		elliptical	stellate, large pouch-like papillae	stellate, large pouch-like papillae	circular
Distribution	Czech Republic; France, Portugal, USA	Japan	East China	Tajikistan	Poland, Czech Republic	Ukraine
Stratigraphic range	Aptian, Senonian	Barremian	Aptian	Cenomanian	Hauterivian	Albian
Selected references	Feistmantel (1881); Knobloch (1971); Hlušík (1972, 1974, 1978, 1979); Hlušík & Konzalová (1976a,b); Alvin (1977); Watson (1977); Alvin & Hlušík (1979); Pons (1979); Kvaček (2000).	Kimura et al. (1985).	Chow & Tsao (1977).	Doludenko (1978); Doludenko & Reymanówna (1978).	Ettingshausen (1852); Schenk (1869); Reymanówna (1965); Reymanówna & Watson (1976); Hlušík (1979); Purkynová (1983).	Barale & Doludenko (1985); Doludenko & Pons (1986).

Characters/Taxa	<i>F. occidentalis</i> Heer emend. Alvin	<i>F. oligostomata</i> Romariz emend. Alvin	<i>F. pombetsuensis</i> Saiki	<i>F. ramosissima</i> Fontaine emend. Watson	<i>F. rubiesensis</i> Barale emend. Gomez et al.	<i>F. sifloana</i> Watson
Branching	sparse	moderate	?	profuse	profuse	?
Internode length (mm)	up to 16	9	at least 10	up to 20	6-13	up to 10
Internode width (mm)	up to 4	3-4.7	1.4	up to 20	3-5	1-3
Leaf number <i>per</i> node	alternating whorls of 3	alternating whorls of 3	2	alternating whorls of 3	alternating whorls of 3	3 (sometimes probably 2)
Outer surface ridge number <i>per</i> leaf	?	5-9		?	4-5	?
Maximum length of free leaf (mm)	?	up to 0.9	1.5	2	0.9	1
Depth of sheathing base (mm)	?	?		0.5	?	?
Leaf upper margin	?	small unicellular hairs		hairs up to 100 µm	¾ upper part scarious	entire
Internode and abaxial leaf cuticles thickness (µm)	up to 60	30	10	~ 30	30	?
Stomatal arrangement	well-defined rows	ill-defined rows	ill-defined rows	ill-defined rows	well-defined rows	ill-defined rows
Density of stomatal rows <i>per</i> mm	10-12	10-13	7-9	10-12 (sometimes less)	10-11	12-14
Density of stomata <i>per</i> mm ²			30-40		70-100	
Diameter of stomatal apparatus (µm)	75	75	65-80	50-75	70-75	80
Number of subsidiary cells	5-6	4-6, usually 5	4-5	4-6, usually 5	4-6, usually 4	4-5
Orientation of stomatal aperture	?	transverse	transverse	transverse	?	?
Surface around pit			thickened ring			
Papillae in throat of stomatal pit	massive papillae	short wide papillae	short wide papillae	no	present	large rounded
Cuticle surface	smooth	smooth	smooth	hairs up to 120 µm	smooth	papillae up to 10µm
Rim of stomatal pit	polygonal, bone or star- shaped	lobed	rectangular or polygonal	round: lobed or papillate	lobed	circular
Distribution	Portugal, Germany	Portugal, Spain	Japan	Maryland and Virginia (USA)	Spain	Sudan
Stratigraphic range	Berrasian – Aptian - Albian	Senonian	Albian	Barremian–Lower Albian	Upper Berrasian- Barremian	Lower Cretaceous
Selected references	Heer (1881); Alvin (1977); Watson & Alvin (1999).	Romariz (1946); Broutin & Pons (1976); Alvin (1977); Lauverjat & Pons (1978); Pons & Broutin (1978); Alvarez-Ramis (1981); Alvarez-Ramis et al. (1987).	Saiki (1997).	Berry (1910, 1911); Thompson (1912); Watson (1977); Upchurch & Doyle (1981).	Barale (1973); Gomez et al. (2002a)	Edwards (1926); Watson & Alvin (1976); Watson (1983)

Characters/Taxa	<i>F. teixeirae</i> Alvin & Pais emend. Mendes et al.	<i>F. turoloensis</i> Gomez et al.	<i>F. ugunaensis</i> Gomez et al.	<i>F. veneta</i> Gomez et al.	<i>F. profetiensis</i> Bartiromo et al.	<i>F. cusanensis</i> Bartiromo et al. sp. nov.
Branching	moderate	moderate	rare	moderate	?	moderate
Internode length (mm)	2.5-6	up to 42	up to 13	?		4-10
Internode width (mm)	1.5-2	up to 3.6	up to 2	up to 4	2-5	1.3-2.4
Leaf number <i>per</i> node	2 opposite decussate leaves	alternating whorls of 3	alternating whorls of 3	alternating whorls of 3	alternating whorls of 3	alternating whorls of 3
Outer surface ridge number <i>per</i> leaf	absent	?	?		?	?
Maximum length of free leaf (mm)	~ 0,7	up to 1.8	~ 1	?	1	0.9-0.5
Depth of sheathing base (mm)	?			?	?	?
Leaf upper margin	scarious	strip of hairs (20-40 µm)	hairs up to 100 µm	?	hairs up to 100 µm	hairs up to 60 µm
Internode and abaxial leaf cuticles thickness (µm)	50	20-30	15-20	20-30	10-20	~7
Stomatal arrangement	ill-defined rows	ill-defined rows	ill-defined rows	ill-defined rows	well-defined rows	well-defined rows
Density of stomatal rows <i>per</i> mm	10	11-13	7-9	8-11	6-7, at times 8	7-10
Density of stomata <i>per</i> mm²		140-200	40-55	70-90	60	70-80
Diameter of stomatal apparatus (µm)	75	55-75	50-60	60-90	~ 107	40
Number of subsidiary cells	5-6	usually 4	usually 4	4-5, usually 4	4, rarely 5	4, rarely 5
Orientation of stomatal aperture	?	?	?	variable	transverse	random
Surface around pit	slightly raised	slightly raised	slightly raised	papillate	papillate	papillate
Papillae in throat of stomatal pit	large	massive	present	present	no	present
Cuticle surface	smooth	smooth	hairs up to 100 µm		narrow grooves with papillae	papillae
Rim of stomatal pit	stellate or polygonal	stellate	stellate	stellate	stellate	stellate
Distribution	Portugal	Spain	Spain	Italy	Italy	Italy
Stratigraphic range	Hautervian or Barremian	Lower-Middle Albian	Upper Barremian	Cenomanian-Turonian	Middle Aptian	Late Aptian
Selected references	Alvin & Pais (1978); Mendes et al. (2010).	Gomez et al. (2002a).	Gomez et al. (2002a).	Gomez et al. (2002b).	Bartiromo et al. (2009).	herein

Cheirolepidiaceae sp. 2

Fig. 8H-J

Material examined. CM 91

Description. Fragmented specimen composed of one segment 9 mm long by 3.7 mm wide (Fig. 8H). Leaves not preserved. The haplocheilic stomatal apparatuses, apparently monocyclic, have a diameter of ca. 94 μm and are more or less round in shape; a few are elliptical with the main axis running parallel to the stomatal rows (Fig. 8J). The stomatal apparatuses are disposed in well-defined, longitudinal rows (Fig. 8H and I) and they are separated by 5-6 well-defined rows of epidermal cells and rectangular in shape (Fig. 8H). No stomatal apparatuses in between consecutive stomatal rows have been observed. It is difficult to quantify the number of the epidermal cells between adjacent stomatal apparatuses within the same stomatal row. The specimen displays a stomatal density of 35 per mm^2 . There are always more than six subsidiary cells, which are more or less well disposed around the stomatal pit.

Discussion. Cuticular features allow us to include the specimen in the family Cheirolepidiaceae. The specimen differs from those found in the Cusano Mutri succession and assigned to the genus *Frenelopsis* in the following characters: larger stomatal apparatuses, a higher number of rows of epidermal cells in between two stomatal rows, and a greater number of subsidiary cells. However, its fragmentary nature and the lack of clearly visible macroscopical characters (e.g. leaves and sutures) make it difficult to identify the specimen to genus level.

Parallel veined leaves

In the plant-bearing strata of Cusano Mutri, several parallel veined leaves have been found. Four representatives with different morphologies (CM 27, 76, 82, 85) are discussed briefly below: their macroscopical and microscopical features are summarised in Table 2. Specimens CM 76 (Fig. 9A) and CM 85 (Fig. 9E) are especially worth mentioning.

The former displays some poorly preserved epidermal features (Fig. 9B). The stomatal apparatuses show elongate stomatal pits. Subsidiary cells (number not clear) (Fig. 9C) have polar appendages extending outside the external rim. The anticlinal walls of the guard cells present wide cutinization. Papillae protruding from the subsidiary cells partially obstruct the stomatal pit (Fig. 9D). In the latter (CM 85), two small, damaged zones symmetrically

disposed on the left and right sides of the lamina (Fig. 9F) can be noted. Their size and morphology are in agreement with the damage caused by insects (Fig. 9G) and can be attributed to the Superfamily Coccoidea (C. C. Labandeira, pers. comm. to AB). Specimen CM 76 presents macroscopical (see Florin, 1936; Lundblad, 1957; Bose and Manum, 1990) and microscopical (see Florin, 1936; Watson and Harrison, 1998; Kiritchkova and Nosova, 2009) features reminiscent of the genus *Pseudotorellia* Florin, 1936 emend. Bose and Manum, 1990.

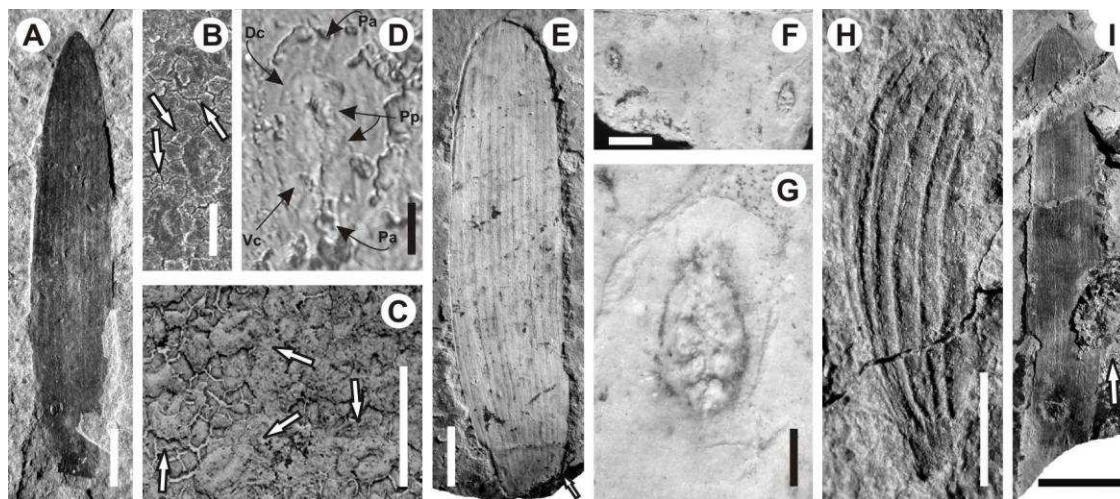


Fig. 9. Late Aptian fossil plants from Cusano Mutri. A-D, CM 76. A, lanceolate leaf which demonstrates 10 veins. B, CM 76s: detail of leaf impression showing stomatal apparatuses (arrows). C, impressions of stomatal apparatuses (arrows) (inverted image: negative). D, CM 76v: stomatal apparatus showing dorsal (Dc) and ventral (Vc) cutinization of guard cells, papillae (Pp) and polar appendages of the guard cells (Pa) (collodion peel). E-G, CM 85: part and counterpart. E, leaf with 15 veins and rounded apex. The arrow indicates the position of the detail figured in F and G (part). F, leaf base showing two bits of insect damage (counterpart). G, one of two bits of insect damage in the basal part of the leaf (detail of F). H, CM 82: entire petiolate leaf with five veins and rounded apex. I, CM 27: impression of lanceolate leaf showing ten veins, rounded apex and veins converging to the apex; the petiole is lacking. The arrow indicates a cluster of *Lepidotes* fish scales. A, E, H, I: SM observations; B, C: SEM observations; D: LM observations. Scale bar represents 5 mm in A, E; 50 µm in B; 100 µm in C; 10 µm in D; 1 mm in F; 250 µm in G; 2 mm in H; 1 cm in I.

The overall shape of specimens CM 82 (Fig. 9E) and CM 85 (Fig. 9H) together with their morphology and venation are comparable to those of the genus *Nageiopsis* Fontaine, 1889. *Nageiopsis* differs from *Podozamites* and *Lindleycladus* in having less convergent veins in the apical region of the lamina (Seward, 1919). By contrast, specimen CM 27 has veins that converge to the apex (Fig. 9I), which is reminiscent of *Podozamites* and *Lindleycladus*. The major difference (Knobloch and Kvaček, 1997) between these two genera is in the orientation of their stomata (Harris, 1979). *Lindleycladus* has stomata that are orientated longitudinal to the leaf margin, whereas in *Podozamites* they are transversely orientated (cf. Harris, 1935).

Ultimately, the poor state of preservation prevents precise assignment of the specimens; however, the publication of these leaves is noteworthy because it demonstrates the variability of gymnospermous remains at Cusano Mutri.

Table 2. Summary of macro- and micro-morphological features of parallel veined leale from Cusano Mutri.

	CM 27	CM 76	CM 82	CM 85
Dimension (length per width in mm)	55 x 9	36 x 6.6	7.7 x 2.2	38.5 x 9.1
Number of veins	10	10?	5	15
Veins converge or not to the apex	Yes	No	No	No
Apex	Rounded	Acute	Rounded	Rounded
Petiole present or not	Absent	Absent	Present	Absent
Length-width ratio of leaf	6.1	5.5	3.5	4.2
Dimension of epidermal cells (length per width in μm)	42 x 32	-	-	30 x 23
Dimension of stomatal apparatuses (length per width in mm)	81 x 57	45 x 30	-	-
Orientation of stomatal apparatuses	Longitudinally?	-	-	-

Reproductive structures and conifer woods

The flora includes other coniferous remains such as cones and pieces of wood. Associated with *Frenelopsis* shoots, poorly preserved microsporangiate cones (CM 28, 67, 80) have been found. These are ovoid in shape, 4.5-5.4 mm in length and 3-5 mm in width. They bear helically arranged sporophylls (0.8-1.3 mm long by 1-1.5 mm wide), which are deltoid in shape and have a rounded apex (Fig. 10A). In the basal part of specimen CM 67 the sporophylls probably continue into the sterile part of the shoot with leaves, possibly helically disposed. The single stomatal apparatus observed has a diameter of ca. 100 μm and is composed of six subsidiary cells. The epidermal cells are clearly visible: they are rectangular in shape and elongated with an average dimension (length vs. width) of 25-69 μm by 11.8-23.6 μm , with anticlinal walls 2.6-5.8 μm thick (Fig. 10B). The sporophylls differ from those found at the Profeti fossil site (see Bartiromo et al., 2009), which were assigned to the species *F. profetiensis* Bartiromo et al. The lack of both pollen-sacs and *Classopollis* pollen (see Alvin et al., 1978 diagnosis) does not allow us to assign them to the genus *Classostrobus*.

Numerous bedding surfaces are replete with dispersed fusainised tracheids most closely related to *Araucarioxylon*. They can be observed along a radial plane to have circular areolate pits (12-14 μm in diameter) on the tangential surfaces, disposed in vertical series (uniseriate pits) and more or less regularly spaced (Fig. 10C). According to views in a transverse plane, no tracheids have been observed. Araucarian-like conifer wood was a dominant, cosmopolitan element (Philippe et al., 2004) and in Europe it has sometimes been found to be associated with *Cheirolepidiaceae*. The material from Cusano Mutri resembles *Araucarioxylon* from the Aptian-Albian “Furoid Marls” of the Umbro-Marchigiano Apennine (Biondi, 1976).

Angiospermae

Family incertae sedis

Genus *Montsechia* Teixeira, 1954

Montsechia vidalii (Zeiller) Teixeira, 1954

Fig. 10D-H

Material examined. CM 9, 98, 106, 107, 145

Description. The material consists of fragmented compressions and small impressions of vegetative apparatuses. Hitherto only five specimens have been found at Cusano Mutri. The best preserved specimen (CM 145) is composed of part and counterpart measuring 21 mm in length (measured along the main axis) by 11.6 mm in width (Fig. 10D). The main axis is 0.7 mm thick at the base. This gradually narrows towards the distal part where it is 0.3 mm wide. The specimens consist of a succession of sub-opposite tufts referred to as “bouquets” by Blanc-Louvel and Barale (1983) (Fig. 10E). The branches of the last order depart from the area of bouquet-leaves (Fig. 10D and F). The leaves have an entire margin and a rounded apex; their length does not exceed 1.5 mm. The leaves curve upward and are a little narrower at the base. Owing to the poor preservation and brittleness of the cuticle, some pieces were directly mounted onto the stubs without chemical treatment for SEM observation, and peels were also taken to determine thoroughly the epidermal features. Two morphological types of epidermal cells in the leaves can be distinguished: (1) more or less isodiametric polygonal cells of no particular orientation (Fig. 10H); and (2) elongate cells disposed in longitudinal rows (Fig. 10G). The former have an average diameter of 15 μm , while the latter are on average 34 μm long and 15 μm wide. The anticlinal walls of the elongate epidermal cells are ca. 1.2 μm (max. 1.8 μm) thick. The rectangular cells are located in the central part of the leaves; the polygonal ones are situated in the apex. No veins or stomatal apparatuses were observed.

Discussion. The macro- and micro-morphology together with the size allow us to attribute the specimens from Cusano Mutri to *M. vidalii* (Zeiller) Teixeira, 1954. *M. vidalii* is an intriguing plant macrofossil from Barremian lithographic limestones in the Pyrenean Basin (“El Montsec de Rubiès”) and the Eastern Iberian Basin (“Las Hoyas”) (Daviero-Gomez et al., 2006) where it occurs abundantly (Diéguez et al., 2010). Crane and Upchurch (1987) and Crane (1988) suggested that its morphology bears some resemblance to *Drewria potomacensis* from the Early Cretaceous (cf. Aptian) Potomac Group of

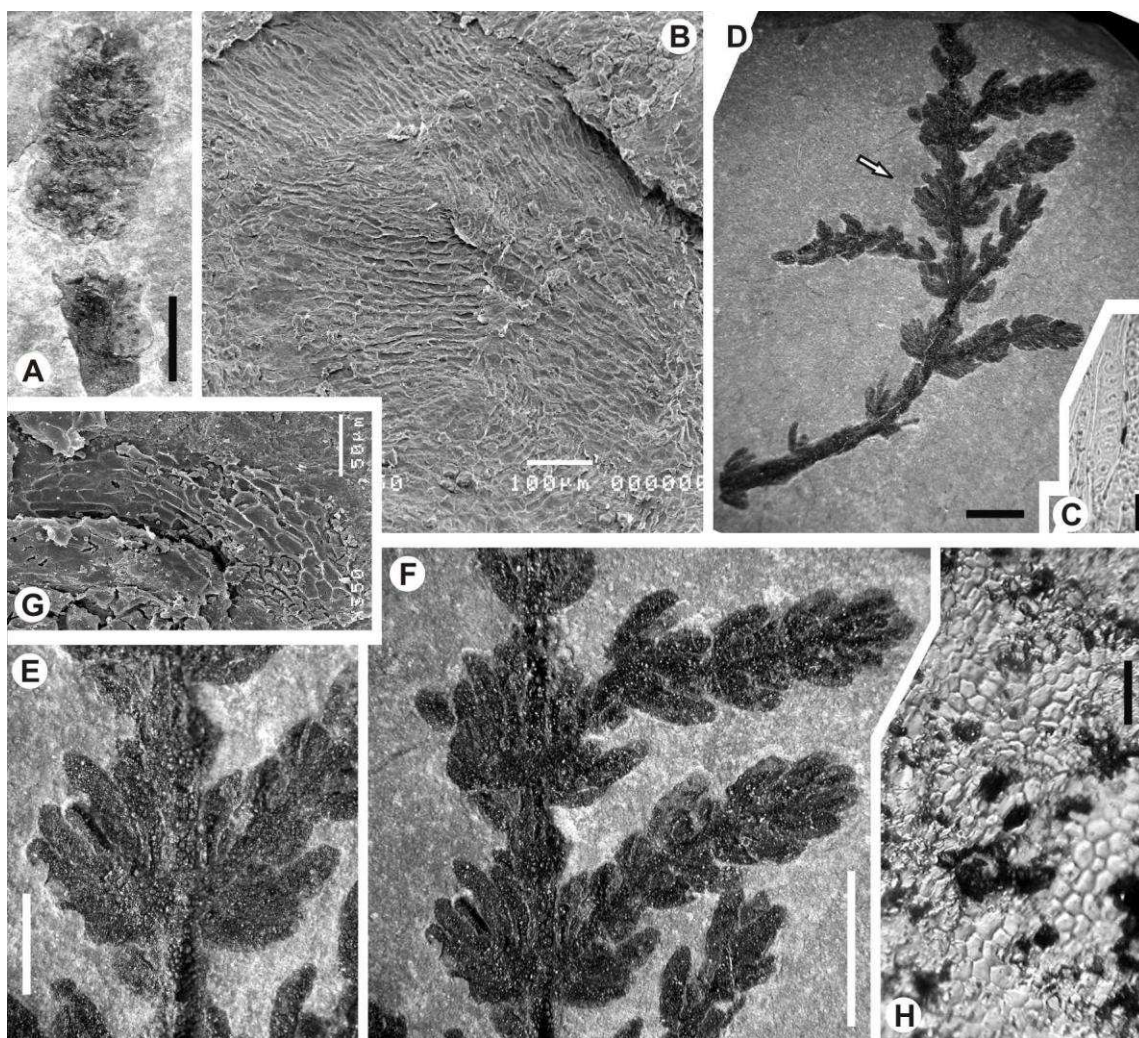


Fig. 10. Late Aptian fossil plants from Cusano Mutri. A, B, reproductive remains of conifers. A, CM 67: complete microsporangiate cone with sporophylls probably continuing into the sterile part of the shoot. B, CM 28: probably abaxial surface of a sporophyll showing the disposition of the epidermal cells. C, CM 108: fusainised tracheid showing circular areolate pits along a radial plane (collodion peel). D-H, *Montsechia vidalii*. DeF, CM 145. D, compression of vegetative apparatus. The arrow indicates the position of detail in E. E, “bouquet” (tuft) of leaves (detail of D). F, branches merging from the bouquet-area (detail of D). G, CM 107: elongate epidermal cells in the central part of a leaf. H, CM 106: polygonal epidermal cells without a particular orientation located in the apex of a leaf (collodion peel of an undulate surface). A, D-F: SM observations; B, G: SEM observations; C, H: LM observations. Scale bar represents 2 mm in A, D, F; 30 μ m in C; 1 mm in E; 100 μ m in H.

Virginia (Crane and Upchurch, 1987) and *Eoantha zherikhinii* described by Krassilov (1986) from Barremian-Aptian sediments in Mongolia and thus may also have gnetalean affinities (Osborn et al., 1993).

Although the specimens from Cusano Mutri display epidermal characters, the absence of stomatal apparatuses is worth noting. According to Daviero-Gomez et al. (2006), the slender axes, thin cuticles, and scarce stomata suggest that *M. vidalii* was linked to an aquatic habitat. The occurrence of *Montsechia* at Cusano Mutri is of great interest because (1) freshwater angiosperms are among the first angiosperms reported from the fossil record

(Martín-Closas, 2003) and (2) angiosperm-like fossils become fewer and harder to find below the Albian and seem to disappear below the Barremian or upper Hauterivian (Cornet and Habib, 1992). The oldest aquatic angiosperm assemblage is known from the Spanish Barremian with the species *Ranunculus ferreri*, *M. vidalii* and a few specimens related to the genus *Proteaphyllum* (Martín-Closas, 2003). This is the first Italian record of *M. vidalii* so far and the second oldest angiosperm found in Italy after those from the early-middle Aptian locality of Profeti (Bartirromo et al., 2009). Moreover, an SEM image of epidermal cells belonging to *M. vidalii* is published herein for the first time.

3.5. Taphonomic and palaeoecological remarks

Late Aptian Cusano Mutri plant remains occur in marly and calcareous strata. The marls contain impressions almost exclusively. The calcareous beds frequently reveal cuticle fragments, but these are poorly preserved.

3.5.1. Taphonomy

Plant macro-remains are usually of cm size. The absence of bioturbations allows us to suppose that the fragmentation is probably related to a highly dynamic syn-depositional environment and not to post-depositional reworking events. Throughout the stratigraphic column all macro-remains are set parallel to the bedding plane. In addition in marl deposits they are mainly isooriented, while they are sparse and randomly disposed in those of limestones. During episodes of freshwater influx (marl levels) the kinetic energy would have been raised, thus transporting and fragmenting the material prior to deposition. On the other hand, evidence of low energy phases is testified by the occurrence of random arrangement, sometimes associated with abundant small bivalves that are still articulated and mainly preserved as internal moulds with the concave surfaces facing upward (Fig. 3B and C).

Frenelopsis specimens are the best preserved plant remains. Branched specimens together with articulated and disarticulated internodes still bear cuticles. The other taxa are represented by isolated or fragmented leaves with poorly preserved remains of cuticle. The poor preservation of the cuticles could be explained by (1) prolonged flotation of plant remains before their final burial or (2) the slow sediment accumulation rate accompanied by bacterial and saprophagous degradation prior to burial. Differences in cuticle

preservation may also be explained as an intrinsic response to their different systematic affinity (see Möslé et al., 1998). It is not clear if shoot detachment in *Frenelopsis* is the result of a biotic (e.g. physiological) process or abiotic (e.g. storm damage) origin, or is owing to a combination of the two as suggested by Martín-Closas and Gomez (2004). However, Martin (1999) pointed out that in temperate climates conifers tend to shed more foliage in the autumn. The type and the state of preservation of the Cusano Mutri plants (see above) allow us to infer the distance between the sedimentary basin and the source area. Since only *Frenelopsis* is preserved as branched specimens and has the best preserved cuticle, it is considered to have suffered a minimum amount of transport (see Gomez et al., 2001, 2002a) and, therefore, possibly lived close to the sedimentary basin.

The surfaces of the marly strata frequently display many charred coniferous wood fragments (fusain) that vary from randomly arranged to orientated tracheid elements (Fig. 8C). The more or less constant lengths of the fusain fragments (on average 15 mm) seemingly are the result of sorting by wind (at least initially in subaerial environment) and water (e.g. marine currents) or both. The fusain probably derived from a neighbouring forest that was subjected to wildfires. The occurrence of fusain and fossil charcoal is accepted as a reliable indicator of the occurrence of wildfire (Watson and Alvin, 1996; Van Konijnenburg-Van Cittert, 2002), in most cases triggered by lightning (Sellwood and Price, 1993). The Wealden Cretaceous climate was characterized by frequent rainstorms leading to flash floods and lightning-generated wildfires (Haywood et al., 2004). During Jurassic and Cretaceous periods wildfires were increasingly important (Scott, 2000; Pausas and Keeley, 2009) to the extent that many taphofloras are explained by the occurrence of frequent wildfires (Martín-Closas and Gomez, 2004). The large amount of fusain throughout the whole Cusano Mutri succession, especially in marls, indicates that wildfires were rather frequent events.

3.5.2. Palaeoecology of the Cusano Mutri sedimentary basin

Freshwater influence on the Cusano Mutri Basin is testified by several lines of evidence. (1) The aquatic angiosperm *M. vidalii* is recorded in lithographic limestones deposited in freshwater lakes (Coiffard et al., 2007) and is considered to be a hydrophyte angiosperm (Teixeira, 1954; Martín-Closas, 2003). (2) Charophytes (Fig. 3E and F) are essentially freshwater algae (e.g. Martín-Closas, 2003). (3) The ostracod fauna is composed of strongly oligotypic to nearly monospecific associations, which is consistent

with a freshwater to brackish palaeoenvironment by comparison with extant assemblages (Giuseppe Aiello and Diana Barra, pers. comm. to AB: taxonomic study in process). The highest numbers occur in light-grey and red-brown marlstones, whereas they are generally scarce in grey and reddish limestones. (4) Several surfaces are densely crowded with disarticulated valves of estherids (conchostracans/ spinicaudatans). Today these usually inhabit ephemeral freshwater ponds, but they may also occur in brackish waters of coastal lagoons and tidally-influenced zones (Cunha Lana and de Souza Carvalho, 2002). The fact that extant spinicaudatans can withstand brackish water and many assemblages have been found associated with salt-water faunas led Webb (1979) to assume that some fossil counterparts inhabited at least brackish and possibly marine environments. Tasch (1961) attributed the association of spinicaudatan with marine fauna to the existence of temporary pools or lagoons affected by episodic seawater influx producing faunal mixing. (5) *Lepidotes* is the most abundant fish in Cusano Mutri; it is represented by whole and uncrushed skeletons suggesting parautochthonous conditions of deposition. Most species of this genus are supposed to have lived in freshwater, and, in Europe, the genus occurs in alluvial and lacustrine deposits (Buscalioni et al., 2008).

These sedimentological and taphonomic considerations lead us to the conclusion that the whole plattenkalk sequence is not the result of a “snapshot” of a mass mortality phenomenon (sensu Martin, 1999), but a continuous, long-lasting sedimentary decantation affected by tidal influx. The succession may correspond, therefore, to a tidal channel that was periodically influenced by freshwater with the constant supply of terrigenous material.

3.5.3. Xeromorphic adaptations of the plants

Most of the examined plants display, at both macroscopical and microscopical levels, xeromorphic adaptations, i.e. any plant structural feature reducing transpiration (Hill, 1998). Haworth and McElwain (2008, 2009) note that the presence of “xeromorphic” features in fossil plants is often used to infer aridity, although in extant plants these characters are not specifically restricted to plants growing in arid environments. They commonly occur also in environments with high precipitation as well as in those with high water availability. Retallack (2009) stated that xeromorphism is an adaptation related to dry climate and implies that in the past scleromorphic features (e.g. thick cuticle) were induced by dry climate. However, scleromorphy is also a result of other environmental hardships, such as nutrient shortage (e.g. soil poor in phosphorous; see Hill, 1998) and high

salinity. Therefore, there is no general consensus on the environmental meaning of the high frequency of “xeromorphic features” and, as Watson and Alvin (1996) suggested, the unavailability of water is probably related to soil loaded with salt as well as to an arid or semi-arid climate.

F. cusanensis nov. sp. displays a set of characters that are “classically” considered xeromorphic (sensu Watson and Alvin, 1996) such as (1) small leaves, (2) stems likely to be photosynthetic, (3) thick cuticle, (4) sunken stomata, and (5) papillate subsidiary cells. In addition this species shows marginal hairs and papillate epidermal cells. The marginal hairs present in *Frenelopsis alata* Knoblock (Feistmantell) have been compared (Pons, 1979; see also Watson and Alvin, 1996) with the extant North African conifer *Tetraclinis articulata* (Vahl) Masters where, in a semi-arid climate, the reduced leaves show similar marginal hairs that are used to capture condensing atmospheric moisture during the night. Moreover, the papillate epidermal cells serve to block sunlight and reduce the transpiration (Watson and Alvin, 1996). The massive presence of epidermal papillae on the external surface of *F. cusanensis* would have the effect of reducing the average leaf temperature and hence decreasing the rate of metabolic reactions associated with various harmful gases in and around leaves (Sharma, 1977). Jordan et al. (2005) claimed that trichomes or papillae create a glaucous surface to reduce the light reaching the mesophyll. Finally, the thick cuticle with the sunken guard cells may have provided sufficient protection under xeric conditions (Van der Ham et al., 2003).

Aside from stratigraphic and physiognomic indicators (Greb et al., 2006), isotopic studies on Cretaceous European fossil plant assemblages using $^{12}\text{C}/^{13}\text{C}$ analysis indicate that *Frenelopsis* in marginal marine facies had elevated ^{13}C relative to other genera in

more distal facies. This would suggest stress and possible saline influence in salt-water marshes (Nguyen Tu et al., 2002) or lagoons (Nguyen Tu et al., 1999).

3.5.4. Palaeoclimate and floral comparison

The specimens assigned to *Cupressinocladus* have diamond shaped, scale-like leaves partially covering one another. The leaves are small, inserted at an acute angle and adpressed against the axis. These features offer less surface exposure to solar radiation (Thévenard et al., 2005). The leaves spread wide apart, displayed by the specimens assigned to the genus *Pagiophyllum*, are similar to the relatively large, flattened, spreading leaves of *Brachyphyllum patens* from the Maastrichtian of Belgium (Van der Ham et al.,

2003), which might indicate a humid habitat. This would tie in with the presence of hydrophylous *M. vidalii* in the Cusano Mutri assemblage. On the other hand, the Profeti flora (Bartirromo et al., 2009) is characterised by (1) a lower variability of gymnosperm remains, (2) the absence of hydrophilous plants and (3) a significant lack of anatomical features that reflect moist conditions. These differences could be related to the progressive cooling trend that had been initiated during the Aptian (Srinivasan, 1994). According to Coiffard et al. (2007), the drought phases ended at about the middle Aptian, after which a wetter, cooler (by 5 °C) climate is evident from the occurrence of detrital quartz deposits in the Vocontian Trough (Wortmann et al., 2004).

Sedimentological and palaeoecological evidence (e.g. the frequent freshwater influence) together with the characters of the Cusano Mutri flora, would indicate, that growth of the vegetation in the region was influenced by a semi-arid climate, seasonally punctuated by wet phases on a regional to local scale. The wildfires suggested by abundant fusain in the Cusano Mutri plattenkalk succession could have been engendered by periods of aridity combined with high temperatures and lightning without accompanying rain (e.g. Watson and Alvin, 1996).

The Cusano Mutri and Profeti floras have in common the genera *Araucarites* and *Frenelopsis*. Although strictly not coeval, the resemblance between the Cusano Mutri flora and that of the Montsech (Spain) lithographic limestones (Barale et al., 1984; Lacasa and Martinez, 1986; Selden, 1989, 1990; Barale, 1991) is remarkable in that *Pagiophyllum*, *Araucarites*, *Frenelopsis* and *Montsechia* occur in both. Such an assemblage is typical of Euro-Sinian Province (Vakhrameev, 1991) and no Gondwanian representatives are recorded. It is suggested, therefore, that the Cusano Mutri and Profeti successions reflect relatively long, spatially limited exposed episodes of the Apenninic Carbonate Platform during the Aptian, providing further evidence that the Cretaceous climate was less stable than it was previously thought (Francis and Frakes, 1993).

CHAPTER IV

Plant remains from the Early Cretaceous Fossil-Lagerstätte of Pietraroja, Southern Italy, Benevento

4.4.1. Introduction

In Italy Cretaceous beds bearing plant macroremains have been known since the 19th Century. However, with the exception of some recent contributes in which the epidermal features have been treated (Gomez et al., 2002a; Bartiromo et al., 2008a, 2008b, 2009 and other works in progress), few palaeobotanical studies have been carried out and these have been scattered over a wide area. A summary of previous paleobotanical studies of the Italian Cretaceous has been made by Gomez et al. (2002a, tab. 1, p. 674). Although in the Campania Region Mesozoic rocks are exposed quite extensively, they only rarely host well-preserved compression floras (e.g. Bartiromo et al., 2009). Therefore, the purpose of this work is to contribute to the knowledge of the fossil plants and possibly to infer some palaeoecological considerations of the famous (Capasso, 2007) Early Cretaceous (Early Albian) locality of Pietraroja (Fig. 1(1)). This fossil site can be considered a “classic Konzentrat und Konservat” Fossil-Lagerstätte (Seilacher, 1970; Seilacher et al., 1985) for its numerous and exceptionally well-preserved animal (see below) and plant (Bartiromo et al., 2006a and b; Bartiromo et al., 2008a and 2008b) remains yielded. This “taphonomic window” (sensu Briggs, 2003) yields an extremely rich assemblage of invertebrate fauna (Costa, 1853-1864; D’Argenio, 1963; Bravi, 1996; Bravi and Garassino, 1998). Dasycladalean algae are also present (Bravi and Garassino, 1988; Carannante et al., 2006). Moreover, this fossil site is extraordinary for its findings of several well-preserved fossil vertebrates such as fish (Costa 1853-1864; Bassani, 1882, 1885; D’Erasmus, 1914-1915), amphibians (McGowan, 2002; McGowan and Evans, 1995) and reptiles (Costa, 1864; Estes, 1983; Evans, 2002; Evans et al., 2004; Barbera and Macuglia, 1988; Evans et al., 2004, 2006), with at least two specimens that can be ascribed to Mesosuchidae. It is worth mentioning the finding of a theropod dinosaur (Leonardi and Teruzzi, 1993), *Scipionyx samniticus* Dal Sasso and Signore, remarkable for its exceptionally well preserved internal organs (Dal Sasso and Signore, 1998; Dal Sasso and Maganuco, 2011).

As for Pietraroja fossil plants Costa (1865) mentioned “Quattro specie innominate” (Four nameless species). Some fossil plants collected from this site were assigned to the

genera *Zamites* and *Brachyphyllum* (Bravi and Garassino, 1998). Bartiromo et al. (2006a) pointed out to the presence of the genus *Frenelopsis* and later, Bartiromo et al. (2006b) discovered an impression of a matoniaceous fern assigned to the genus *Phlebopteris*.

The identification of fossil plants in the Pietraroja locality is an interesting result, as it: 1) represents the first most complete study of vegetal remains by means of epidermal features so far; 2) enriches the comprehension of palaeoecology of this fossil site and 3) enlarges the phytotaxonomic knowledge of the Apenninic Carbonate Platform during the Early Cretaceous.

4.2. Geological setting

Mesozoic carbonates are widely represented in the Campania-Lucania Apennines (Bosellini, 2004), the most shortened segment of the Southern Apennine Arc (Parotto and Pratlun, 2004). Peculiar terrains of this segment are the pelagic successions of the Lagonegro-Molise Basin, originally interposed between two carbonate platform/slope/basin systems. One corresponds to the future Apulian foreland and the other, more internal, corresponds to the Apennine Carbonate Platform unit, Late Triassic-Tertiary in age (Parotto and Pratlun, 2004). The latter includes the Matese Mountains in which the Pietraroja Fossil-Lagerstätte is located at its eastern edge. The Matese Mountains are part of the central southern Apennines, a thrust and fold belt originated during the Late Tertiary Period from the deformation of the continental margin of the Adria Plate (Carannante et al., 2006). This latter is interpreted as either an independent Cretaceous unit or as a part of the African Plate (Channel et al., 1979). During the Early Cretaceous the Matese Mountains was the site of shallow-water carbonate sedimentation



Fig. 1. 1. Map showing the location of the Pietraroja village in the Campania Region. 2. Sketch map showing the location of the exposure studied (***).

(Bosellini, 2004) characterized by an erosive mid Cretaceous gap, marked by bauxites cropping out in the north-western area of the southern Apennine (D'Argenio et al., 1987).

The plant-ichtyolithic, organic-rich beds of Pietraraja formed during OAE 1 (sensu Arthur and Schlanger, 1979) probably along the NE margin of the Apenninic Carbonate Platform (Ciarapica and Passeri, 2008) in a high energy marginal environments (sensu Ciarapica and Passeri, 2008). The Pietraraja locality (Fig. 1(2)) crops out at the top of a faulted monocline named “Civita di Pietraraja”, at “Le Cavere” quarry [I.G.M. (Italian Military Geographic Institute) map 162 III SW – Cusano Mutri sheet] and includes two plattenkalk levels (Catenacci and Manfredini, 1963; Bravi and Garassino, 1998; Carannante et al., 2006). Freels (1975) reported three Plattenkalk intervals.

The famous Lower (basal) Albian “Ichthyolitic level of Pietraraja”, yielding plant remains (Plattenkalk 2, sensu Bravi e Garassino, 1998) is about 8 m thick and its age assignment is controlled by foraminiferal data (Bravi and Garassino, 1998; Carannante et al., 2006) below and above the plant-bearing sequence (Fig. 2). The two Plattenkalks are overcome in paraconformity by trasgressive Miocene belonging to the Cusano Formation (Selli, 1957) with ostreids, bryozoans and lithothamnes (Bravi and Garassino, 1998). In the “Le Cavere” quarry, the rocks are well stratified, occurring in flat and even layers with an average thickness ranging between 2 and 10 cm. They mainly consist of more or

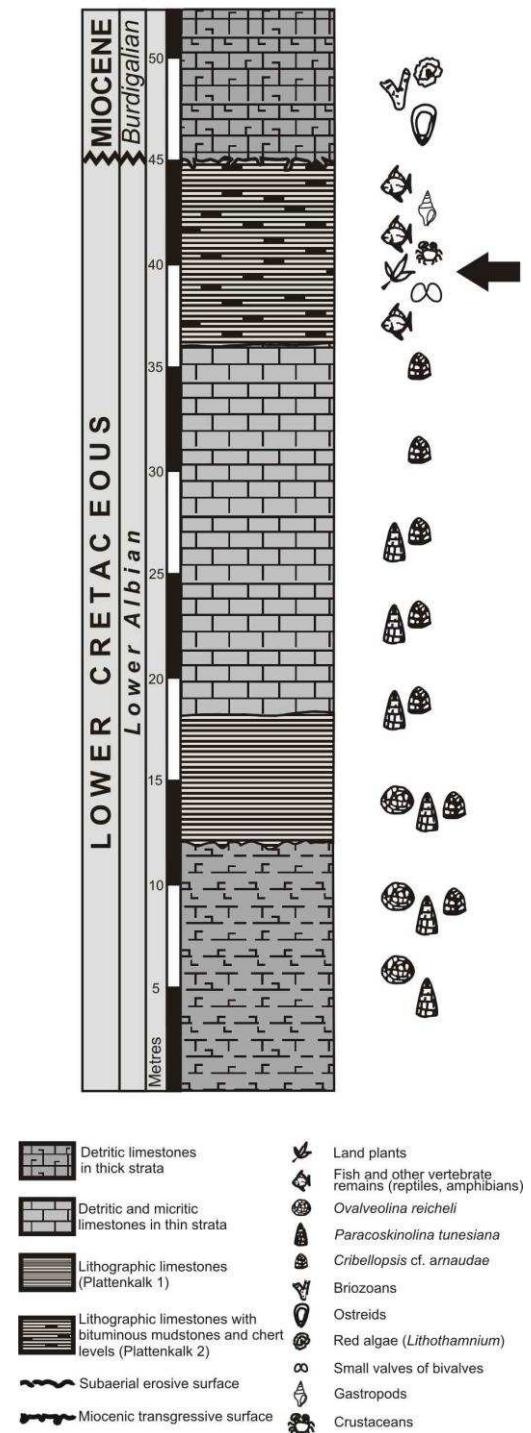


Fig. 2. Sedimentological stratigraphic section of the outcrop (modified from Bravi (1996)). The arrow indicates the levels studied.

less markedly micritic, avana to hazel or grey-coloured limestones (wackstones, mudstones and packstones). These are intercalated by frequent lenses, nodules and beds of grey or blackish cherts and marly-calcareous and sometimes clay and thin laminated horizons (Bravi and Garassino, 1998). Strata bearing plant remains are situated in the basal part of the “Le Cavere” quarry and they generally consist of packstones gradually turning into wackstones and mudstones.

4.3. Material and methods

The studied plant remains come from “Le Cavere”, a protected locality included in the “Geopaleontological Park” of Pietraroja, considered the depocentre area (Freels, 1975) of the outcrop. The core-material of this study is represented by specimens labelled with “M”, the result of an excavation effected in 1982 by the Museo Civico delle Scienze Naturali of Turin and the former Istituto di Paleontologia (actually incorporated in the “Dipartimento di Scienze della Terra”) of the “Federico II” University in Naples.

The marl and bituminous strata containing fossil plants led to the recovery of hundreds of specimens in a short time. The fossil plants are mostly impressions and most specimens are carbonaceous compressions without epidermal features. Even after the preliminary chemical treatments, observation of microscopical details was still difficult on most specimens. Not all of the adhering carbonaceous material was removed, because long processing times destroyed the cuticle. That is why some cuticle fragments have been mounted directly on the stubs for SEM (scanning electron microscope) observations. Cuticle fragments available were separated from the sediment surface with a pair of tweezers. They were cleaned in 37% hydrochloric acid (HCl), subsequently immersed in 40% hydrofluoric acid (HF) to remove adhering siliceous debris, and finally macerated in Schulze’s reagent ($\text{HNO}_3 + \text{KClO}_3$): the duration of the latter process depended on the degree of carbonization and preservation (Kerp, 1990). Some cuticle fragments have been immersed in ammonia for one hour. Subsequently, part of them was mounted in glycerine jelly on glass slides for light microscope (LM) examination. Some fragments were coloured with safranin before mounting. Treated and untreated cuticle samples were mounted on the stubs for SEM observations. Light microscope and stereo microscope (SM) observations were made by using Leitz microscopes. Photomicrographs with light microscope were taken with a Nikon Coolpix 990 camera. The cuticles were examined using a JEOL JSM-5310 SEM at the CISAG (Centro Interdipartimentale di servizi per

Analisi Geomineralogiche). All the quantitative measurements were made with tools in the ImageJ program (Abramoff et al., 2004). The leaf physiognomy of an angiosperm leaf was described using the terminology of the Leaf Architecture Working Group (Ash et al., 1999). Studied fossil plants come from: 1) a collection named “Fossili del giacimento di Pietraroja”, catalogued as “vegetali indeterminati” (unclassified vegetables) and stored in the Museo di Paleontologia of the Università degli Studi di Napoli “Federico II” (M and MPUN series); 2) those gathered by the author outside the protected area of the “Le Cavere” allowing the recovery of two specimens (PI 1-PI 2) actually housed in the “Dipartimento di Scienze della Terra, Università degli Studi di Napoli “Federico II”; 3) a private collection (PV 1); 4) a collection stored in the Museo Civico of Montefalcone in Valfortore (MF).

4.4. Systematic Palaeontology

Order CAYTONIALES

Genus *Sagenopteris* Presl in Sternberg, 1838

Sagenopteris? sp.

Figs. 3(1), 4(1-3)

Material examined – M 20802.

Description – Part and counterpart of a complete, rounded, petiolated and detached leaflet, with constricted base and entire margin. The specimen measures 13 mm in length and 11 mm in width. The lamina is symmetrical. The petiole is 0.8 mm wide. The leaflet shows a more or less distinct midrib ~ 0.2 mm in width at the base- departing from the petiole and disappearing shortly in the middle part. Secondary veins are hardly visible and arise from the rachis with an angle of $\sim 30^\circ$ (Figs. 3(1), 4(1)). Along the leaf margin and on the petiole area, poorly preserved epidermal features are visible. The cuticle is ~ 0.6 μm thick (Fig. 4(2)). The epidermal cells are more or less rounded. Numerous circular or ovate structures of ~ 50 μm in diameter of unknown function can be observed (Fig. 4(3)). No stomatal apparatuses have been found.

Discussion – Morphology and venation of the specimen resemble *Sagenopteris* Presl, 1838. In particular, the morphology and the length-width ratio (1.1) are comparable to *S. williamsii* described by Berry (1956, Pl. XXXIV, Fig. 1) from the Lower Cretaceous of Canada. At the microscopical level, observed circular-ovate structures are similar to those

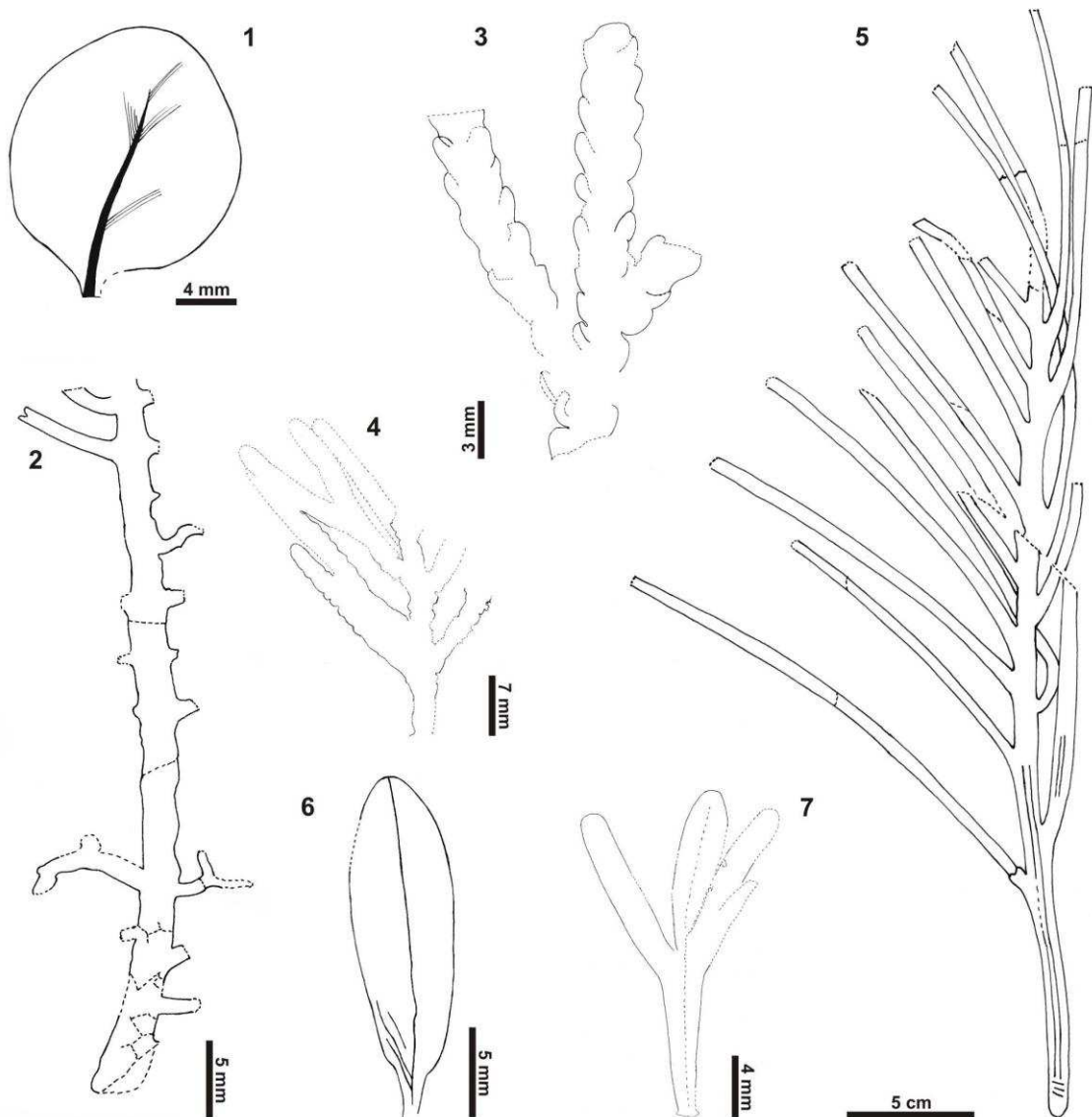


Fig. 3. Early Albian fossil plants from Pietraroja. Line drawings of selected specimens. 1. *Sagenopteris*? sp.. Showing primary and secondary veins (M 20802). 2. *Brachyphyllum* sp. 1. Showing branched specimen and poorly preserved leaf-scars in its basal part (M 20908). 3. *Brachyphyllum* sp. 2. Showing two-order branches and leaves tightly adpressed to the axis (M 20765). 4. *Brachyphyllum* sp. 2. Showing three-order branches (M 20909). 5. *Frenelopsis* sp.. Large branched specimen showing two-order branches and the central vascular bundle (M 20569). 6. *Angiospermae* 1. Showing the well-visible primary and secondary -in the basal part- veins (MF 1265). 7. *Incertae sedis*. Leaf showing three lobes and a distinct petiole (M 20796).

reported by Barbacka et al. (2006) for the Hettangian *S. pualensis* and interpreted by the authors as possibly glands or hair bases. However, the poor state of preservation of the specimen together with the unclear venation pattern, do not allow to assign the specimen with confidence to the genus *Sagenopteris*. In addition, the taxonomy of the genus *Sagenopteris* is still confused (Zaton et al., 2006) and a specific determination is

impossible judging from the macromorphology alone and needs to be supported by a cuticular study (Harris, 1964).

Class CONIFEROPSIDA (Endlicher, 1847)

Family incertae sedis

Genus *Brachyphyllum* Lindley and Hutton ex Brongniart, 1828 emend. Harris, 1979

Brachyphyllum sp. 1

Figs. 3(2), 4(4)

Material examined – M 20908.

Description – Part and counterpart of a branched shoot, 47 mm long and 3 mm wide at the base, gradually narrowing to the apex where it reaches 1.8 mm in width. The specimen is preserved as impression and presents fragments of organic matter. It is a shoot with opposite branches disposed to one plane and having a fixed distance of 3.5 mm (Fig. 3(2)). Branchlets are well visible in the distal and proximal parts of the specimen, while in the middle part, only their proximal parts are present. The inclination of the secondary branches in respect to the main shoot varies between 60 and 70°. The branchlets have a more or less constant width ~ 1 mm- along the whole length. In the basal part of the main shoot leaves are helically arranged and the leaf-base cushions are visible (Fig. 4(4)). The leaves are more or less rhomboidal in shape, but the bad preservation of the specimen does not allow to establish their phyllotaxis. No epidermal details have been preserved.

Discussion – Large leaf-base cushions helically disposed on the shoot and small free parts of the leaves allow to assign the specimen to the genus *Brachyphyllum* Lindley and Hutton ex Brongniart, 1828 emend. Harris, 1979. The studied specimen resembles *Brachyphyllum obesum* Heer, 1881 described from the Lower Cretaceous, Kitadani Formation, Tetori Group, Japan (Yabe and Kubota, 2004) and *B. obesum* described by Teixeira (1948) in his study concerning the Portuguese Mesozoic flora. The specimen coming from Pietraroja shares with *B. obesum* general morphology, dimension and branching modality. However, owing to the incomplete macroscopically diagnostic features and to the lack of the microscopical ones, it is impossible to assign the specimen specifically.

Brachyphyllum sp. 2

Fig. 3(3, 4), 4(5)

Material examined – M 20765, M 20909, M 20924, M 20925.

Description – Impressions of single (M 20924, M 20925: both part and counterpart) and branched (M 20765, M 20909) shoots (Figs. 3(3, 4)). The biggest specimen (M 20909; Fig. 3(4)) is 34 mm long and 2.3 mm wide. The smallest specimen (M 20925) is 11.9 mm long and 1.9 mm wide. The specimens bear leaf-base cushions and small leaves being 1 mm long and 1 mm wide. The leaves have rounded apex and are tightly adpressed to the axes (Fig. 4(5)). No epidermal features have been preserved.

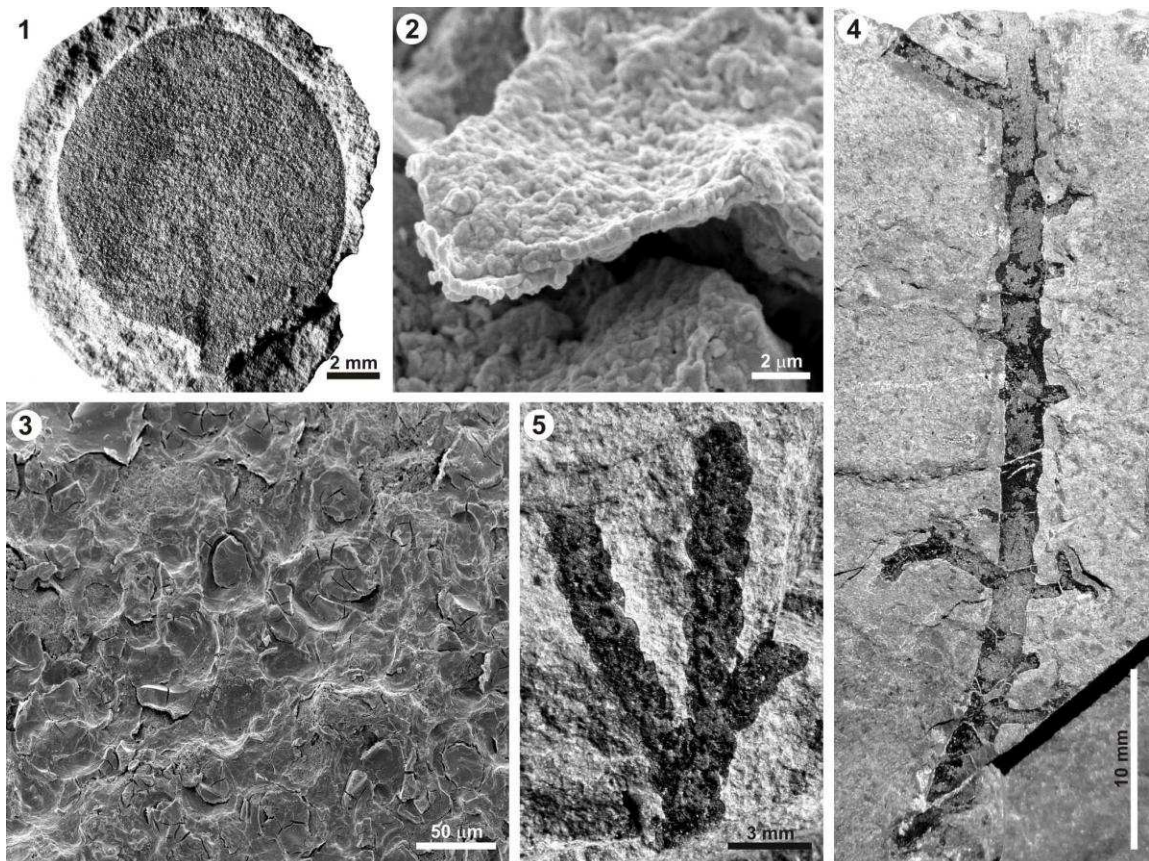


Fig. 4. Early Albian fossil plants from Pietraroja. 1–3. *Sagenopteris?* sp.. (M 20802). 1. Single leaflet. 2 Detail of transversal view of cuticle showing its thickness. 3 Detail of inner? view of cuticle showing circular-ovate structures of unknown function. 4. *Brachyphyllum* sp. 1. Carbonaceous compression of a branched stem: its basal part retains badly preserved leaf-scars and leaves (M 20908). 5. *Brachyphyllum* sp. 2. Branched shoot showing leaves helically arranged and tightly adpressed to the stems (M 20765). Figs. 1, 4, 5: SM observations. Figs. 2, 3: SEM observations.

Discussion – The specimens are typical *Brachyphyllum* shoots with large leaf-base cushions and a small free part of the leaf. This morphogenus refers to the Mesozoic conifer foliage with short and helically arranged leaves (e.g. Harris, 1979). The best preserved leaves can be observed in the apical part of the shoot (M 20765). Within the genus

Brachyphyllum, they are the smallest leaves observed from the Early Cretaceous plant localities of the Campania Region (Bartirromo et al., 2008b, 2009). The lack of cuticular details makes it impossible to determine the specimens at the species level.

Family CHEIROLEPIDIACEAE Takhtajan, 1963

Genus Frenelopsis (Schenk, 1869) emend. Watson, 1977

Frenelopsis sp.

Figs. 3(5), 5(1-7), 6(1-13)

Material examined – M 20563, M 20564, M 20566, M 20568, M 20569, M 20599, M 20762, M 20793, M 20798, M 20913, PI 1, PV 1, MF 1342.

Description – The material consists of impressions and compressions of articulated and disarticulated shoots. Large [from 220 mm (PV 1) to 550 mm (MF 1342)] profusely-branched shoots present a remarkable curvature (Figs. 3(5), 5(1, 2)). The branchlets, alternate and aligned, are disposed in one plane and are branched distichously. The angle between the branchlets and the main axes varies between $\sim 23^\circ$ on one side and $\sim 8^\circ$ on the other ones. The specimens show two orders of branching. The distance of the branchlets between two branching points is of 25-30 mm (Figs. 3(5), 5(2)). The shoots are segmented and the internodes measure on average 16 mm in length and 4 mm in width (Fig. 5(3)). There are no suture lines along the leaf sheath. The shoots show a central vascular bundle (Fig. 5(2)). The internodes bear whorls of two (opposite and decussate; see Fig. 5(1, 4)) or three (Fig. 5(3)) triangular free tips. The free part of the leaves is triangular in shape and measure ~ 1 mm in length. They have an acute apex and are adpressed to the axes (Fig. 5(1, 3)). The cuticle of the internode measures 11.5-19.8 μm in thickness, on average 15.7 μm (Fig. 6(1)). In outer view, the abaxial side of the internodes is densely covered by relatively long hairs (about 51-88 μm in length and 25-35 μm in width at the base) that tend to mask stomatal apparatuses. They depart from the epidermal cells and have an obtuse apex (Fig. 6(2, 3)). Sometimes, epidermal papillae can be observed (Fig. 6(3), arrow). In the middle part of the internodes hairs have not a preferential orientation (Fig. 6(2)), while tend to be oriented towards the distal part, especially in the leaf area (Fig. 6(4)). Sometimes, some less dense hairs areas within stomatal rows allow to observe hairs extending in the direction of the stomatal apparatus itself (Fig. 6(5)). Along the internode, epidermal cells of the sheathing leaves are hardly visible by means of LM and SEM due to the constant occurrence of hypodermis in the studied specimen (Figs. 5(6), 6(9, 13)). They

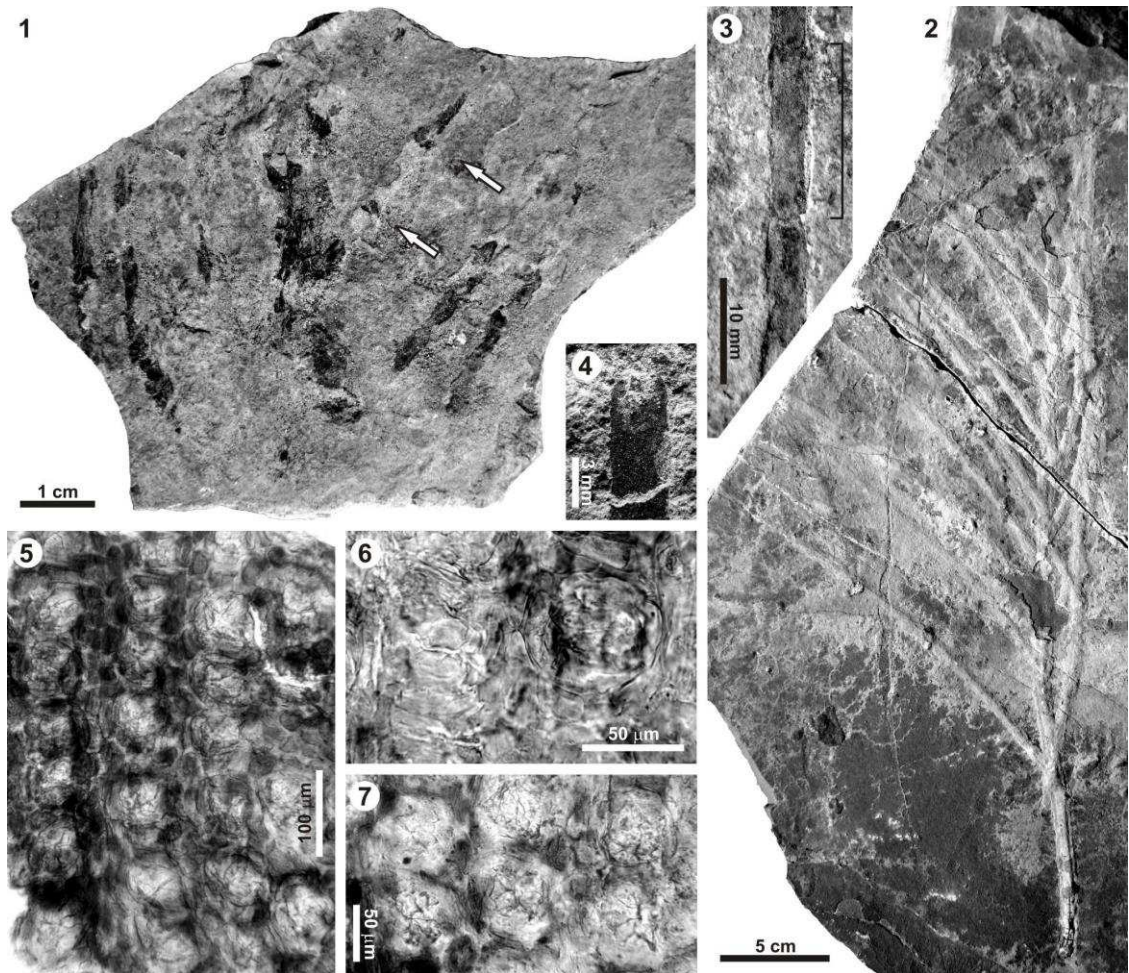


Fig. 5. Early Albian fossil plants from Pietraroja. 1-7. *Frenelopsis* sp. 1. Compression of the specimen showing two-order branches and leaves (arrows) in opposite, decussate pairs (M 20913). 2. Three-dimensional preservation (impression) of a large branched shoot showing two-order branches and a central vascular bundle (M 20569). 3. Compression of a branched specimen (detail) showing an internode (square bracket) with a whorl of three leaves, two leaves are visible (PI 1). 4. Detail of the upper part of an isolated internode with a whorl of two opposite leaves (M 20762). 5. Detail of internode cuticle showing stomatal apparatuses disposed in well-defined rows separated by two rows of epidermal cells (M 20913 4). 6. Detail of internode cuticle showing two stomatal apparatuses composed of four subsidiary cells bearing hypodermis. The dorsal plates of the guard cells are visible in the middle of stomatal apparatuses. 978 (M 20913 2). 7. Detail of internode cuticle showing stomatal apparatuses composed of four inner papillae (M 20913 2). Figs. 1-4: SM observations. Figs. 5-7: LM observations.

are arranged in more or less well-defined rows running parallel to the long axis of the internode. They are rectangular and sometimes polygonal in shape measuring on average ~ 55 µm in length by 29 µm in width. The anticlinal walls of the epidermal cells are on average 8-17 µm thick, usually more than 10 µm and sometimes attain 25 µm. The anticlinal walls, parallel to the long axes of the specimen, are thinner than the perpendicular ones (Fig. 6(6)). When the hairs are degraded, ordinary cells with thickly cutinized anticlinal walls form a ribbed irregular surface of the cuticle (Fig. 6(7)). Stomatal apparatuses are arranged in well-defined longitudinal rows (11-12 per 1 mm linear) (Figs. 5(5), 6(8, 9, 13)) and are transversally oriented to the main axes of the segments (Fig.

6(9)). On the same stomatal row, one to two epidermal cells occur between two stomatal apparatuses. It is not unusual to find two stomatal apparatuses on the same row in contact without interposed epidermal cells (Fig. 5(5)). Rarely, some stomatal apparatuses occupy an intermediate position within stomatal rows, thereby disturbing the linearity of adjacent rows (Fig. 6(9)). Stomatal rows are separated by 1 (sometimes 2) epidermal cell (Fig. 5(5, 7)); sometimes stomatal rows are tightly in contact between them in order to mask the interposed epidermal cells. The mean stomatal density is $\sim 130/\text{mm}^2$. The haplocheilic stomatal apparatuses are slightly elliptical in shape (probably owing to the diagenesis) measuring on average 80 by 69 μm . Measured from external view, stomatal apparatuses have a stomatal aperture of 196 μm^2 on average. The stomatal apparatuses are monocyclic and composed of four (Fig. 6(11)) and five (Fig. 6(10)) subsidiary cells, each of which bears one outer papilla on the rim of the stomatal pit, and one inner papilla in the throat of the stomatal pit. Stomatal apparatuses composed of four (equidimensionally) subsidiary cells (on average 46 μm long and 24 μm wide) are prevalent. Papillae give to the rim of the stomatal pit a stellate (Fig. 6(11)) or a polygonal (Fig. 6(8)) appearance. Surface around stomatal pit is slightly raised (Fig. 6(12)) and stomatal apparatuses are flanked by massive papillae protruding by encircling epidermal cells (Fig. 6(11)). On the inner surface of cuticle, well cutinized dorsal plates (31 (26-37) μm long and 15 (11-25) μm wide) of the guard cells which frequently mask inner papillae can be observed (Fig. 6(13)). The hypodermis is cutinized, the cells being polygonal or rectangular in shape, between 37-67 μm long and 14-25 μm wide (Fig. 6(9, 13)).

Discussion – The presence of a whorl of two or three leaves and the absence of longitudinal sutures along the internodes allow to ascribe the specimens to the genus *Frenelopsis* Schenk, 1869 emend. Watson, 1977. These characteristics exclude the attribution of the specimens to the genus *Pseudofrenelopsis* Nathorst, 1893 emend. Srinivasan, 1995.

The studied specimens do not well clarify the branching pattern, as a matter of fact it is difficult to understand if the branching points depart from the nodal or internodal region. Moreover, in the specimens from Pietraroja the number of leaf per node is unclear. To this end, an inconsistency in the leaf number per node was described by Watson and Alvin (1999), Alvin and Hluštký (1979) and Mendes et al. (2010) for *Frenelopsis occidentalis* Heer emend. Alvin, *F. alata* (K. Feistmantel) Knobloch (Daviero et al., 2001) and *F. teixeirae* Alvin and Pais emend. Mendes et al. 2010 respectively. As Watson and Alvin (1999) claimed, it is quite conceivable that at least some *Frenelopsis* species will

eventually prove to have had varied morphology and leaf number in different parts of the plant. As for the microscopical features, the specimens from Pietraroja resemble *F. teixeirae* in: 1) the presence of hairs on the margin of the leaves; 2) the abaxial internode cuticle thickness; 3) the diameter of the stomatal apparatuses; 4) the presence of hairs and papillae on the outer cuticle surface; 5) the morphology of the rim of stomatal pit. Moreover, the Italian material resembles *F. silfloana* Watson in: 1) the density of stomatal rows; 2) the diameter of stomatal apparatuses; 3) the number of subsidiary cells; 4) the presence of papillae in the throat of stomatal pit. However, at present, the unclear phyllotaxy at the nodes and the few and more or less poorly preserved cuticle fragments do not allow to understand well the epidermal architecture, and prevent the assignment of the specimens in a given species already described or their attribution to a new taxon and the analysis of further specimens is needed.

Cheirolepidiacean 1

Fig. 7(1-5)

Material examined – M 20914.

Description – Fragmented shoot of 20 mm long and 6 mm wide. It has short leaves of open-type arranged in a simple spiral that do not completely encircle the stem (Fig. 7(1)). A small cuticular fragment in inner view (Fig. 7(2)) allowed to observe few monocyclic stomatal apparatuses and partially to elucidate the thickness of poorly preserved cuticle: ~ 25 µm (Fig. 7(3)). The stomatal apparatuses are more or less rounded; they are 66 µm (61–71 µm) in diameter and are arranged in well-defined rows (Fig. 7(2)). Stomatal apparatuses are composed of six subsidiary cells (Fig. 7(4)). Some of them -especially those visible in the right part of the fragment shown in Fig. 7(2)- have preserved the guard cells. The subsidiary cells have anticlinal walls ~ 3 µm thick. The anticlinal walls of ordinary epidermal cells are ~ 6 µm thick. From the inner view, outer papillae are visible (Fig. 7(5)). The epidermal cells are difficult to observe but form one or two longitudinal rows in between the stomatal rows.

Discussion – The features listed above are comparable to some members of the family Cheirolepidiaceae, in particular to the genus *Pseudofrenelopsis* Nathorst, 1893 emend. Srinivasan, 1995. The two genera *Pseudofrenelopsis* Nathorst (1893) and *Frenelopsis* Schenk (1869) which have similar segmented shoots, differ mainly in the leaf number and phyllotaxis (Srinivasan, 1995). In *Pseudofrenelopsis* there are one (Watson, 1977) and two

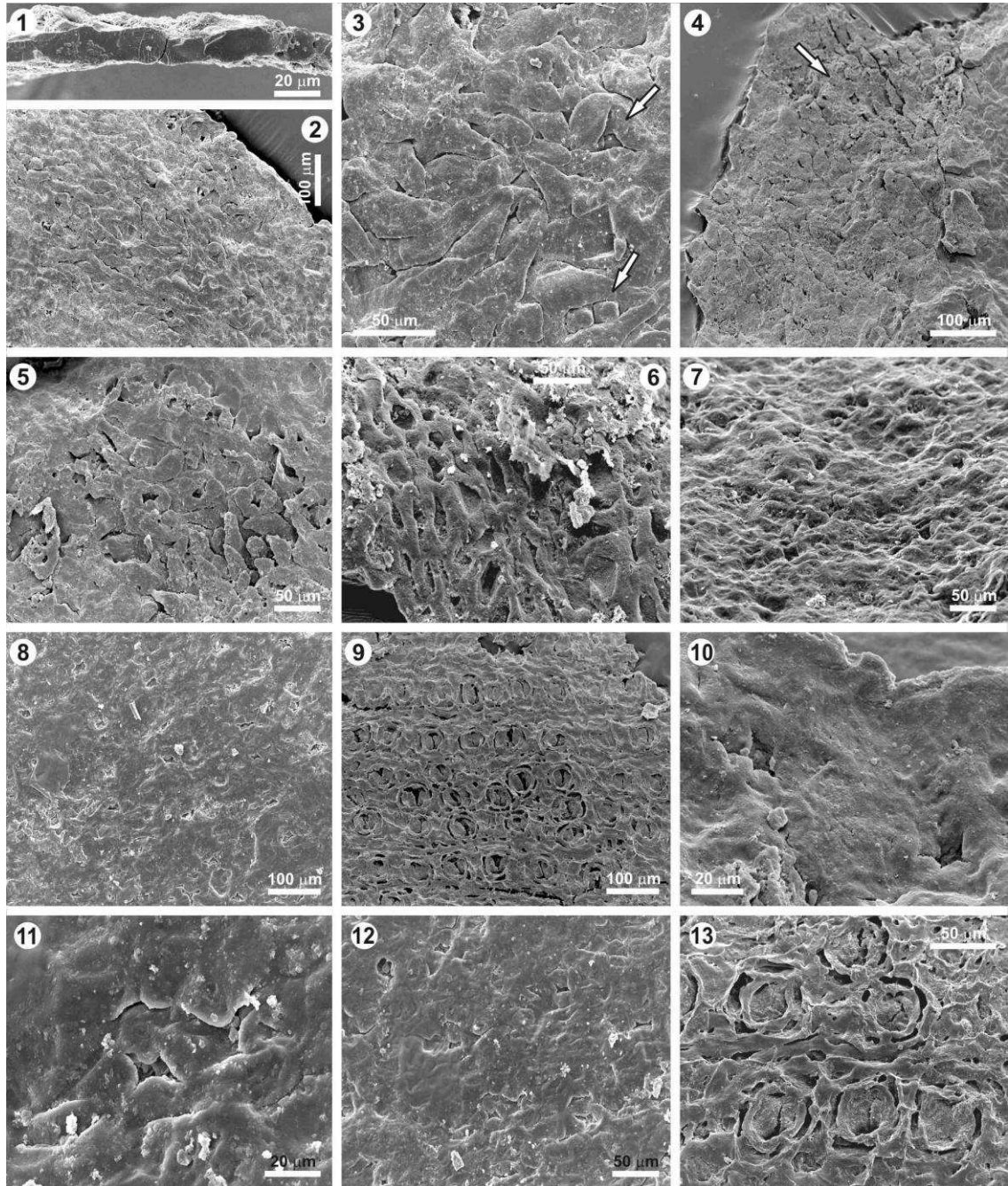


Fig. 6. Early Albian fossil plants from Pietraroja. 1-13. *Frenelopsis* sp. 1. Section of internode cuticle (M 20913). 2. Detail of outer view of internode cuticle showing crowded hairs without a preferential orientation (M 20913). 3. Detail of 2 showing hairs and epidermal papillae (M 20913). 4. Detail of outer view of leaf apex showing hairs oriented towards its distal part. The arrow indicates a stomatal apparatus composed of four subsidiary cells (M 20913). 5. Detail of outer view of internode cuticle showing hairs extending in the direction of the stomatal apparatuses composed of four (the right one) and five (the first two on the left side) subsidiary cells (M 20913). 6. Detail of inner view of abaxial? side of internode cuticle showing epidermal cells with thick anticlinal walls (M 20793). 7. Cuticle in outer view showing anticlinal walls forming a ribbed irregular surface of the cuticle (M 20793). 8. Cuticle in external view showing stomatal apparatuses which are disposed in well-defined rows and with polygonal pits (M 20793). 9. Inner view of cuticle showing transversally disposed stomatal apparatuses arranged in well-defined longitudinal rows (M 20913). 10. Outer view of cuticle showing two stomatal apparatuses made of five subsidiary cells showing stellate stomatal apertures (M 20793). 11. Detail of outer view of cuticle showing a stomatal apparatus with papillae boring from the neighbouring subsidiary cells and extending in direction of the stomatal apparatus itself (M 20793). 12. Outer view of cuticle showing the surface

around stomatal pit which is slightly raised (M 20793). 13. Inner view of cuticle showing two rows of stomatal apparatuses, the hypodermal cells undercovering ordinary epidermal cells and dorsal plates of guard cells (M 20913). All figures are made by means of SEM.

(Srinivasan (1995), for the species *Pseudofrenelopsis nathorstiana* Srinivasan, 1995) leaves per node spirally arranged and occasionally showing cyclic arrangement (Srinivasan, 1995). In *Frenelopsis*, the internodes have two or three leaves per node in an opposite decussate/cyclic arrangement and the internodes are only of the closed type (Srinivasan, 1995). However, the specimen from Pietraroja shows similarity to *Pseudofrenelopsis varians* (Fontaine) Watson, 1977 at both macroscopically (e.g. the shoot morphology) and microscopically (e.g. stomatal apparatuses) levels. As a matter of fact, the material from Pietraroja is comparable to the specimens figured by Watson (1977, Pl. 88, figs. 2, 4; the fig. 2 of the Pl. 2 is also suggested again in Watson 1988, fig. 9.19D). The specimen from Pietraroja differs from the species *P. intermedia* (Chow and Tsao) in having the thinnest cuticle and from the species *P. nathorstiana* Srinivasan, 1995 in having the greatest internode width. Moreover, the specimen is different from M 20752 by having 1) spirally arranged leaves and 2) greater stomatal apparatuses. Unfortunately, the lack of well-defined macro- and micro-features does not allow an identification at the genus level. However, the co-occurrence of *Frenelopsis* and *Pseudofrenelopsis* is not surprising because they are sometimes mixed in the same bed (Watson, 1977, 1983; Srinivasan, 1992; Yang et al., 2009).

Long parallel veined leaves

Material examined – M 20761, PI 2.

The plant-bearing strata of Pietraroja are full of leaves showing parallel venation which are difficult to determine. Herein, two representatives are presented. The specimen M 20761 is a compression of an isolated and petiolate leaf 18 mm long and 5 mm wide (Fig. 7(6)). The apex is rounded and the petiole measures 2 mm in length reaching the maximum width (2 mm) at the base. The veins converge to the apex (Fig. 7(7)). Structures resembling stomatal apparatuses (Fig. 7(8)) and transversally? oriented in respect to the long axes of the leaf can be seen. The general morphology, dimension and venation pattern are comparable to the genera *Lindleycladus* and *Podozamites*. Harris (1979) claims that *Lindleycladus* is distinguished from *Podozamites* for its longitudinally rather than transversally orientated stomata, which are placed in longitudinal rows. Unfortunately,

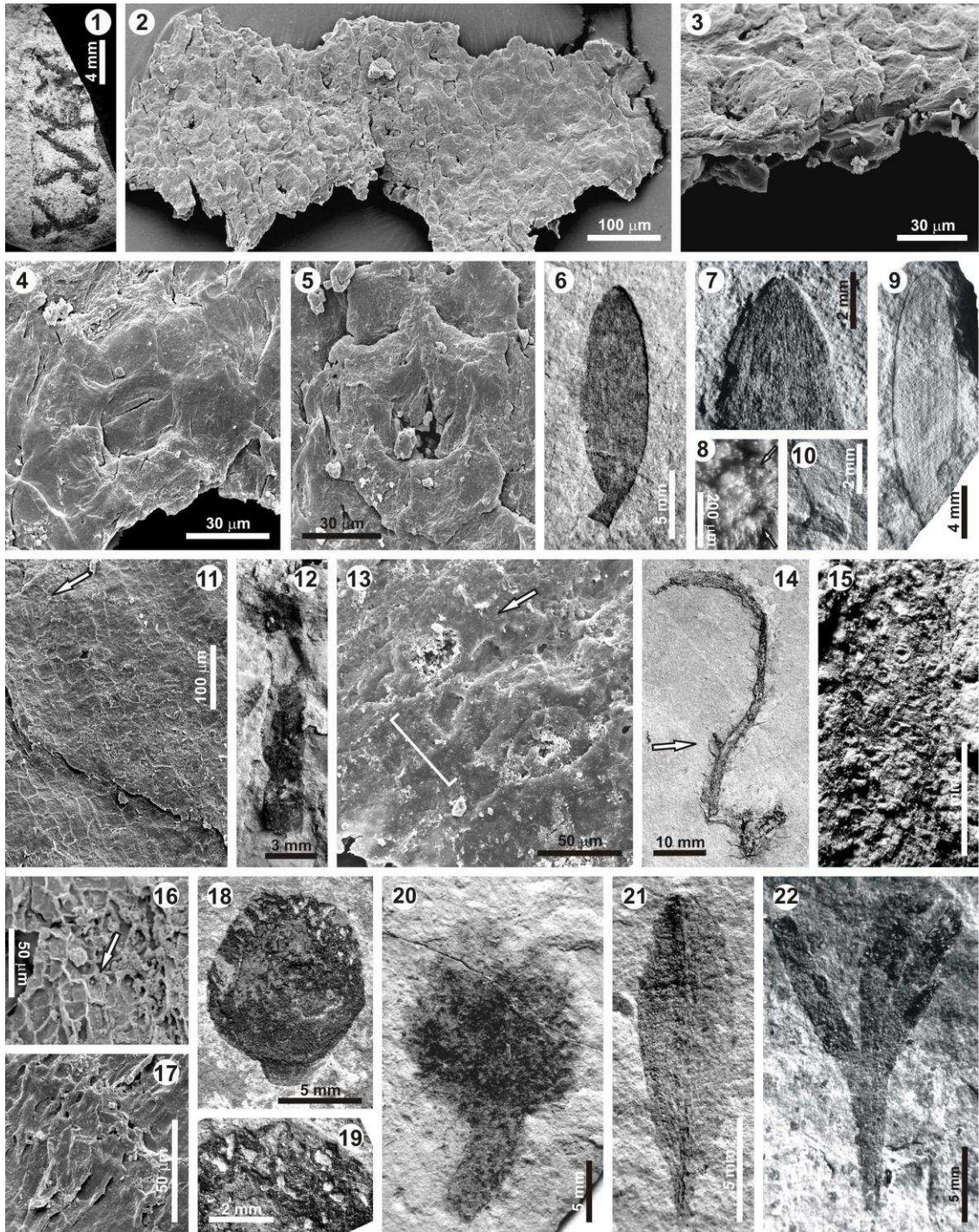


Fig. 7. Early Albian fossil plants from Pietraroja. 1-5. Cheirolepidiacean 1 (M 20914). 1. Fragmented shoot showing leaves in an open-type arrangement. 2. Cuticular fragment in inner view showing rows of stomatal apparatuses. 3. The section of cuticle internode showing its thickness. 4. Detail of inner view of cuticle showing a stomatal apparatus composed of six subsidiary cells. 5. Detail of inner view of cuticle showing a stomatal apparatus composed of six subsidiary cells each bearing one outer papilla. Guard cells have not been preserved. 6-8. Long parallel veined leaf (M 20761). 6. Complete leaf showing a well-developed petiole. 7. Detail of 6 showing the veins converging to the apex. 8. Possible stomatal apparatus (impression), transversally orientated, with encircling epidermal cells? (arrows). 9-11. Long parallel veined leaf (PI 2). 9. Fragmented leaf with parallel veins. 10. Counterpart: magnification of the short stalk. 11. Inner view of the cuticle showing epidermal cells disposed in rows with their anticlinal walls of a sinusoidal outline. Arrow indicates a structure resembling to a stomatal apparatus. 12-17. Sterile axes of conifer. 12-13. Conifer shoot (M 20752). 12. Stem showing a longitudinal suture. 13. Detail of outer

view of cuticle showing two stomatal apparatuses differently preserved with their interposed epidermal cells (square bracket). The stomatal apparatus on the right bears papillae overhanging the stomatal pit. 14-17. Conifer shoot (M 20906). 14. Branched shoot. The arrow indicates the small branch. 15. Leaf-scarres spirally arranged. 16. Detail of inner view of cuticle showing epidermal cells occurring in pairs (arrow). 17. Detail of inner view of cuticle showing a possible stomatal apparatus. 18-20. Remains of conifer reproductive organs. 18-19. Cone (M 20754). 19. Detail of 18 (upper part) showing amber remains. 20. Carbonaceous compression of a cone (MPUN). 21. Obovate leaf with midvein departing from the petiole and reaching the leaf apex. Secondary veins are also visible. (see also Fig. 3(6)) for the relative drawing. 22. Incertae sedis. Narrowly wedge-shaped petiolated leaf showing three lobes (M 20796). Figs. 1, 6-10, 12, 14-15, 18-22: SM observations. Figs. 2-5, 11, 13, 16-17: SEM observations.

poorly preserved epidermal features are scanty to place the specimen with confidence in either of two previously mentioned genera.

The specimen PI 2 (Fig. 7(9-11)) is made by part and counterpart of an isolated leaf, 20 mm long by 5 mm wide with a more or less deltoidal shape and an entire margin (Fig. 7(9)). In the proximal part of the leaf a short stalk is present (Fig. 7(10)). The leaf lamina is symmetric in relation to an ideal line joining the basal part to the apex. The leaf increases in width progressively from the stalk, reaching the maximum in the middle portion and then narrowing again towards the apex. There are 8? veins, unbranched, arising from the base and parallel to the whole lamina length. The veins are less convergent toward the apex. There are no visible dichotomies. The epidermal cells occur in rows and present anticlinal walls straight or slightly undulating, as a possible effect of diagenesis (Fig. 7(11)). The epidermal cells are 35 (20-49) μm long and 27 (18-37) μm wide. The average thickness of the anticlinal walls of epidermal cells is more or less regular ($\sim 2 \mu\text{m}$) from both longitudinally and latitudinally walls. A structure of $\sim 50 \mu\text{m}$ long and $30 \mu\text{m}$ wide resembling a stomatal apparatus has been observed (Fig. 7(11) arrow). The overall shape of the specimen, its morphology and venation, are comparable to the form-genus *Nageiopsis* Fontaine, 1889. This genus was established for vegetative shoots abundantly represented in the Potomac flora (Seward, 1919). *Nageiopsis* differs from *Podozamites* by having less convergent veins in the apical region of the lamina (Seward, 1919). The venation pattern and their number together with the length-width ratio (3.77) allow a comparison with some *Profeti* specimens (Bartirromo et al., 2009). Unfortunately, the poorly preserved epidermal features do not allow a precise attribution to the genus level.

Sterile axes of Conifers

Material examined – M 20752, M 20906.

In marl strata, vegetal debris is rich in sterile axes of conifer. Some of them present features comparable to the family Cheirolepidiaceae. The specimen M 20752 is such an

example. It is represented by an unbranched and fragmented shoot, 13.8 mm long by 2 mm wide with a longitudinal suture (Fig. 7(12)). SEM observations of a single, small and poorly preserved cuticle fragment, 2 mm long per 1.1 mm wide, allow to observe two stomatal apparatuses in outer view. They are $\sim 50 \mu\text{m}$ in diameter; the right one in Fig. 7(13) presents only two papillae preserved. These are $7 \mu\text{m}$ long, each one stemming from one subsidiary cell (Fig. 7(13)). The epidermal cells are squared off or rectangular in shape and are disposed in ill-defined rows. They are on average $\sim 30 \mu\text{m}$ long and $27 \mu\text{m}$ wide. The anticlinal walls are $2.4 \mu\text{m}$ thick. The presence of a longitudinal suture along the shoot together with the papillate stomatal apparatuses are comparable to the genera *Cupressinocladus* Seward, 1919 and *Watsoniocladius* Srinivasan, 1995. However, the poor state of preservation does not allow to place the specimen systematically. Moreover, the absence of the *Classopollis* pollen makes it difficult to place the specimen in the family *Cheirolepidiaceae*.

The specimen M 20906 is a branched shoot measuring 59 mm in length and 1.9 mm in width at the base. It gradually narrows towards the central part where it reaches a minimum width of 1.3 mm (Fig. 7(14)). In the distal part it reaches the maximum width of 2.7 mm. The specimen retains few small fragments of poorly preserved cuticle. Fragments of organic matter are also present. In the basal part of the shoot, a branch that at its base measures 3.4 mm in length and 1.5 mm in width, departs from the main axes at an angle of $\sim 60^\circ$. In the proximal and central parts of the shoot, spirally arranged and more or less rounded leaf scars have been preserved. They are $\sim 0.4 \text{ mm}$ in diameter (Fig. 7(15)). Because of the bad preservation of the specimen it is difficult to establish the phyllotaxis. The epidermal cells are disposed in longitudinal rows. They are rectangular and measure on average $22 \mu\text{m}$ in length by $18 \mu\text{m}$ in width. The ordinary epidermal cells sometimes occur in pairs usually longitudinally (Fig. 7(16)). The anticlinal walls of the epidermal cells are on average $2 \mu\text{m}$ thick. A structure resembling a stomatal apparatus ($58 \mu\text{m}$ long and $39 \mu\text{m}$ wide) has been preserved (Fig. 7(17)). The small dimension of the shoot could be related to the juvenile nature of the plant. Epidermal cells grouped in pairs are similar to the so-called "packets of cells" described and figured by Watson (1969, pl. 6, fig. 6) in English specimens of *Pseudotorellia* leaves and recognised in several other species (Watson and Harrison, 1998); each pair would represent a cell which underwent later division. However, bad preservation of the specimen does not allow to place it in a specific taxon.

Remains of conifers reproductive organs

Material examined – M 20754, MPUN.

Two small, isolated and egg-shaped poorly preserved cones. They measure (length per width) 10 by 9.4 mm (M 20754) (Fig. 7(18, 19) and 16.2 by 15.2 mm (MPUN) (Fig. 7(20)). Their small sizes suggest their microsporangiate nature. The cones bear an uncertain number of spirally-arranged sporophylls. The cones are positioned on a short (1.3 mm in M 20754) and relative long (9.1 mm in MPUN) leafy axis. Small fragments of amber remains have been found (Fig. 7(19)); no pollen grains were found. Poor preservation of the cones does not allow to define in detail their morphology. Thereby it is not possible to place them in any specific taxon.

Angiospermae 1

Fig. 3(6), 7(21)

Material examined – MF 1265.

Description – Impression of a leaf (nanophyll) or leaflet, thick, obovate, and marginally petiolate. The size (length per width) is 14 per 4.7 mm. The lamina is symmetrical with an entire margin and an acute apex (Figs. 3(6), 7(21)). The base is decurrent and slightly asymmetrical, the basal angle is acute. The midvein departs from the base of the fragmented petiole and reaches the leaf apex. The primary vein is pinnate and runs more or less straight along the lamina length. There are three secondary veins visible in the basal part of the lamina. The angle of divergence is more or less acute (ca. 22°). The epidermal features are not preserved. Some additional specimens coming from the Albian locality of Pietraroja have a poorly preserved primary vein and are characterised by similar shape and dimension.

Discussion – The Pietraroja specimen resembles those belonging to the genus *Sagenopteris*. However, this latter is characterized in having lanceolate leaflets with a main vein somewhat distal to the mid-line (Harris, 1969). In the material from Pietraroja, the lamina is obovate and the primary vein reaches the leaf apex. The specimen presents strong resemblance to the leaves named *Angiosperm 1* found in the early-middle Aptian locality of Profeti (Bartirolo et al., 2009: Plate V, Figs. 3, 6) and with *Dicothylophyllum pusillum* described for the Barremian-Aptian? of Northwestern Transbaikalia (Vakhrameev and Kotova, 1977). Unfortunately, the poor state of preservation of the material and the

absence of epidermal features does not allow an assignment of the material to any known species.

Incertae sedis

Figs. 3(7), 7(22)

Material examined – M 20796.

Description – Carbonaceous compression of a petiolated leaf that enlarges abruptly at its top to form lamina. The leaf measures 21 mm in length and 12 mm in width. The lamina is divided into 3 lobes (Figs. 3(7), 7(22)). The width of the lobes measured at the midshaft varies from 1.9 to 2.5 mm. The petiole is 8.8 mm long and 2 mm wide in its median portion; it narrows towards the base where it reaches 1.1 mm in width. The lobes form a basal acute angle that varies from 37.5° (between the first two lobes on the left) to 19.8° (between the central and the right lobe), with an average inclination of 28.6°. The lobes measure (length per width in mm) 12 x 1.7 (lobe on the left), 10.8 x 2.6 (central lobe), 10.8 x 2.3 (lobe on the right). The lobe apices are rounded. A single venation is visible in the central lobe, while two? venations are visible in left ones.

Discussion – The description made above is reminiscent of some Ginkgoales. Twentysix genera have been erected for Mesozoic and Cenozoic ginkgoalean leaves and shoots worldwide (Zhou, 2009), but the four most widely used, and in some ways well-defined, leaf-genera are *Ginkgo* L., *Ginkgoites* Seward, *Baiera* F. Braun and *Sphenobaiera* Florin (Watson et al., 1999). The specimen from Pietraroja resembles to the genera *Ginkgo* or *Baiera* (see Harris et al., 1974) but bad preservation of the material and the lacking of cuticular features do not allow to assign the specimen with confidence. In addition the attribution is difficult since, how Harris et al. (1974) claimed, leaves of Ginkgoalean species vary in form.

4.5. Taphonomic and palaeoecological implications

4.5.1. Taphonomy

The Early Cretaceous (Early Albian) plant remains of Pietraroja occur predominantly in the marly-calcareous strata of the “interval D” (sensu Bravi and Garassino, 1998). Bituminous mudstones strata bear plant remains randomly orientated, while in the clay-

marly interlayers evidence of isorientation have been noted. The density of plant-fossils on marl strata surfaces and especially in the bituminous layers is very high. Plant remains consist of either large specimens or isolated fragments, sometimes occurring as compressions, more commonly as impressions, but often as carbonaceous compressions. In calcareous beds plant remains are generally preserved as impressions in which, sometimes, epidermal features can be seen. In marl beds impressions to compressions of plants can be found, but cuticles are poorly preserved. Bituminous beds are full of carbonaceous compressions. The best preserved cuticles belong to *Frenelopsis*. Few isolated conifer reproductive organs are also found. Large *Frenelopsis* branches are preserved commonly as impressions both in the calcareous and marl strata, and branches in the form of compression are common in marly layers. These conditions of preservation could be due to a long-lasting flotation before burial, to a subaerial exposure in the leaf litter (Gomez et al., 2002b) or, in general, may be evidence of prolonged exposure to oxic environment before deposition in an anoxic environment. The different degree of preservation and a number of articles (e.g. *Frenelopsis*) of studied fossil plants would seemingly suggest a relative wide source area. Organic debris was supplied to the sedimentary basin either from the surrounding emerged lands or possibly from floating vegetation mats.

Most of specimens, especially *Frenelopsis* ones, have their original volume preserved, with segments of the thick and fleshy cylindrical leafy part attaining 9 mm in diameter. Probably, this feature is very close to the life value and could be due to their fast burial from the enclosing sediments after their detachment (e.g. Fig. 5(2)). Taphonomic evidence from *Frenelopsis* of the Early Cretaceous of Spain (Gomez et al., 2001, 2002b) and Late Cretaceous of Italy (Gomez et al., 2002a), suggests that large branches could not have endured long time under high energy transport without the shoots being fragmented. This species might have grown near the place of deposition and thus its habitat was probably under a brackish influence of a hypothesized lagoon (D'Argenio, 1963; Bravi and Garassino, 1998). In the Pietraroja Fossil-Lagerstätte, the large detached branches (Figs. 4(5), 5(1, 2)) are likely the result of mechanical (e.g. storms) or physiological processes. Actually, Gomez et al. (2002a) argue that shoots of the extant conifer *Cryptomeria japonica* (L.f.) D. Don are naturally shed during periods of drought. Alvin (1983) reconstructed one of the dominant conifers of the Wealden, *Pseudofrenelopsis parceramosa*, as a medium to large-sized tree with whorled branching and xeromorphic shoots that might have been shed during times of water stress.

Limestone and marly-limestone strata-surfaces of Plattenkalk 2 (sensu Bravi and Garassino, 1988) are full of connected small valves of bivalves, whose concavity is set upward, co-occurring with plant remains, especially large branches of *Frenelopsis*. Bravi and Garassino (1988) and Carannante et al. (2006) relate this mass mortality phenomenon to an overheating of a very thin water layer, or to water anoxia owing to the preservation of ligaments, respectively. As hypothesized for the plant fragments found in the Crato Formation (Martill et al., 2007), storm events could have detached the *Frenelopsis* branches found at Pietraroja. Moreover, storm events would have mixed anoxic or toxic bottom water with the surface as proposed for the Early Cretaceous Romualdo Member (Martill et al., 2008, Santana Fm., Brazil). These events could have caused the bivalve mass-mortality registered in the sedimentary basin. Actually, jumbled plant remains suggest deposition under stormy conditions (Watson and Alvin, 1996). It is worth noting that Baush and Bravi (1999) reconstructing Pietraroja palaeoenvironment revealed the sedimentary evidence of occasional storms. At “Le Cavere” no evaporite and no halite pseudomorph have been found, so it seems unlikely that hypersalinity was a factor of bivalve mass mortality. On the other hand, hyposalinity could be a possible candidate in case of oversupply of fresh-water to the sedimentary basin owing to the storms. However, mass mortality could be the result of multiple factors.

The exceptional degree of preservation of Pietraroja fossils (especially as to vertebrates) could be due to their fast burial, with high sedimentation rate especially for the plant-bearing strata, as is testified by preservation in volume of several plant remains (e.g. Fig. 5(2)). This could explain the paucity of the benthic invertebrates registered in the fossil site of Pietraroja.

In the Early Cretaceous of Pietraroja, *Frenelopsis* is the dominant, best preserved and well articulated plant. Sparse disarticulated internodes (Fig. 4(4)) without a preferential orientation are also present, but it is impossible to use them as paleocurrent indicators (e.g. Riera et al., 2010). The occurrence of large branches of this genus together with the well preserved terrestrial fauna allow to hypothesize a parautochthonous condition for plant and terrestrial animal remains so that a sedimentary basin was very close to the source area. *Frenelopsis* were probably not transported for a long distance for they lived seemingly in the surrounding land areas close to the sedimentary basin.

In contrast with what previously thought (Bravi and Garassino, 1998), during the Jurassic and Cretaceous the Apenninic Carbonate Platform was separated from continental areas (Ciarapica and Passeri, 2008). In particular, during the Albian an extensive regressive

event involved the Apennine Carbonate Platform (Carannante et al., 2009), therefore, the source areas of the plant debris were probably exposed portions of the Apennine Carbonate Platform with the formation of small and relatively ephemeral emerged lands surrounding the Pietraroja sedimentary basin.

4.5.2. Palaeoecology of the sedimentary basin

The paleoenvironmental interpretation of the Pietraroja plattenkalk has led to controversy hypotheses. The lagoonal (D'Argenio, 1963; Bravi and Garassino, 1998; Bausch and Bravi, 1999) versus slope/shallow basin paleoenvironmental (Catenacci and Manfredini, 1963; Freels, 1975) models have long been debated (Carannante et al., 2006). According to Carannante et al. (2006), both models are inadequate as they fail to take into account all sedimentological features of the Pietraroja Plattenkalk. Consequently they interpret it as abandon deposits of a submarine channel of a not specified water depth.

Moreover, according to Carannante et al. (2006, p. 573), "...the faunal assemblage of Pietraroja represents a mixture of organisms from different types of environments - but certainly not from a lagoonal environment at all.". Actually palaeobotanical and palaeozoological data contradict thereof. The most representative fish genera recorded at Le Cavere have been interpreted characteristic of reef to lagoon (Coelodus and Palaeobalistum: Kriwet and Schmitz, 2005; Nursall, 1996), non-marine habitat (Lepidotes and Clupavus: Martill et al., 2007; Gallo, 2005; Buscalioni et al., 2008; Maisey, 2000) and of back-reef lagoon and non-marine environments (Belonostomus: Maisey, 2000; Estes and Berberian, 1970). Moreover, a stratified water column could explain the reduced and the restricted benthos and the general lack of bioturbation advocated by Carannante et al. (2006). To sum up, the considerations made above, do not exclude a shallow-water hypothesis, e.g. a lagoon (see also 5.1, Taphonomy).

4.5.3. Palaeoclimate and comparison with other Albian floras

In the Albian of Pietraroja majority of plant remains are relatively fragmentary and it is difficult to identify them at the species level. However, it is worth noting that numerous plants display xeromorphic adaptations (see Thevenard et al., 2005; Watson and Alvin, 1996). The specimens assigned to the Family Cheirolepidiaceae (Frenelopsis and Cheirolepidiacean 1) show: 1) a probably photosynthetic stem, 2) small leaves and 3)

epidermal papillae that are indicative of true xerophytism (Mauseth, 1988) together with 4) a thick cuticle, 5) papillae protecting stomatal apparatuses and 6) sunken stomata indicating water stress (Wolfe and Upchurch, 1987) or being considered as extreme xeromorphic characters (e.g. Spicer et al., 1993). Xeromorphy can indicate either dry conditions or physiological difficulties to uptake or transport water internally (Watson and Alvin, 1996). Haworth and McElwain (2008) claim that xeromorphic features occur in plants growing both in arid and high water availability environments, suggesting that their ecological function may not be purely anti-transpirant. Retallack (2009) points out that xeromorphism is an adaptation attributed to dry climate and it implies that scleromorphic features (e.g. thick cuticle) were induced by dry climate. However, the environmental significance of a high frequency of xeromorphic features in vegetation is far from being clarified (Watson and Alvin, 1996). As Wing and Sues (1992) point out, the strongly xeromorphic attributes of many cheirolepidiaceus conifers and their world-wide abundance at low paleolatitude sites (<40 degree) with sedimentary indicators of aridity suggest they were dominant in dry-climate vegetation (Vakhrameev, 1970; Upchurch and Doyle, 1981; Alvin et al., 1978; Francis, 1983). According to Barale (1990) in the Coniferales two types of series can be distinguished: a wet series, Podozamites-Elatocladus; and a hot and dry series Pagiophyllum-Brachyphyllum-Cyparissidium and Cupressinocladus-Pseudofrenelopsis-Frenelopsis. The genus Frenelopsis with its small leaves is suspected to hold a photosynthetic stem (Watson, 1988; Alvin, 1982). The genera Frenelopsis and Pseudofrenelopsis exhibit extreme xeromorphism (Spicer et al., 1993) and morphological and anatomical features of Frenelopsis and Tomaxiella biforme from the Crato Formation are considered to be an adaptation to a warm and seasonally dry climate (Kunzmann et al., 2006). The apparent succulence, resembles that of some modern stem-succulent xeromorphic angiosperms (Watson, 1988). In large branches of Frenelopsis from Pietraroja, wide zones of parenchymatous tissue between the wood and the epidermis can be noted (Fig. 5(2)) and possibly indicates succulence (e.g. Axsmith and Jacobs, 2005; Kunzmann et al., 2006). As claimed by numerous authors (Watson, 1977, 1988; Alvin, 1982; Gomez et al., 2001, 2002b), frenelopsids were probably adapted to aridity. The middle Aptian flora of Profeti, with the species *F. profetiensis*, probably grew under a tropical-subtropical, somewhat arid climate (Bartiromo et al., 2009). However, Kunzmann et al. (2006) maintain that a high percentage and a large morphological variety of pollen within pollen spectra (Spicer et al., 1994) indicate that frenelopsids occupied a variety of ecological niches as also affirmed by Watson (1988) and Axsmith and Jacobs (2005).

Some *Frenelopsis* species favour costal habitats (Doludenko and Reymanówna, 1978; Pons and Broutin, 1978; Pons, 1979).

Therefore, it is possible that *Frenelopsis* grew in an arid or semi-arid climate. Locally, the deposition of the fossiliferous Upper Plattenkalk deposits (the Plattenkalk 2 sensu Bravi and Garassino, 1988) preceded the Albian regressive event associated with the formation of bauxites in the adjacent areas (e.g. Regia Piana, located only 2 km West of Pietraroja area) (Carannante et al., 2006). The broad climatic requirements for bauxitization are in general satisfied by consistently warm temperatures and high amounts of precipitation (Price et al., 1997). According to the palaeogeographic map (Dercourt et al., 1993; Schettino e Turco, 2011), between the Lower Aptian and the Late Cenomanian, the Apennine Carbonate Platform was located at 20-23° of latitude in the tropical belt to the north of equator.

According to Coiffard et al. (2007), the Lower Albian of Europe was a drier and semi-aride climate with a marked seasonality. As claimed by Vakhrameev (1991), during the Early Cretaceous extensive aride zones prevailed in both the Northern and Southern Hemispheres.

The flora of Pietraroja shows some similarities to that from the Escucha Formation, Spain (Gomez et al., 1999; Sender et al., 2005, 2008) and with the late Albian flora of the Ukraine (Doludenko and Teslenko, 1987) by sharing the genera *Brachyphyllum* and *Frenelopsis*.

Moreover, except for the genus *Brachyphyllum* (that occupies an ill-defined systematic position with some species included within the family *Araucariaceae* and others within the *Cheirolepidiaceae*: Gomez et al., 2002c) reported from the Tataouine Region in the south of Tunisie (Barale and Ouaja, 2002), it is worth noting that the Pietraroja flora does not present similarities with the Early Cretaceous Gondwanian floras (e.g. Barale and Ouaja, 2001). The Euro-Sinian character (Vakhrameev, 1991) of the Pietraroja flora excludes a proximity with Gondwanan continent as was previously thought (e.g. Evans et al., 2004).

GENERAL CONCLUSIONS

The first part of this research represents a contribution to the knowledge of biotic effect of volcanism on plant cuticular features permanently fumigated by toxic volcanic gases in a hostile environment.

The exposure of Aleppo pine to volcanic gases (Cap. I) induces an acceleration of needles ageing and produces changes in both epicuticular and epistomatal waxes. The change in tubular wax structure probably affects wettability favouring the ageing process by pollution. The injuries observed on the abaxial side of needle in cuticular surface and epistomatal chamber represent the effects of volcanic toxic compounds, directly (H_2S) or indirectly (SO_2 or/and H_2SO_4 formation) affecting *P. halepensis*. This would explain the deflection of vegetation succession in Pisciarelli area, where close to the main fumarole only *Erica arborea* grows in. The research shows that under the influence of volcanic gases: 1) the epicuticular and epistomatal waxes undergo degradation as fusion; 2) the thickness of cuticle membrane of *P. halepensis* increases; 3) the number of oxalate crystals deposits increases; 4) a different growth and equilibrium among the zones of the cuticle occur and 5) the fibrils of both B1 inner and B1 outer zones become more parallel to cell surface. All these features are interpreted as protection devices against volcanic gases and/or acids action. Moreover, fungal hyphae infection and reduction of fibrillar component -as well as the granular ones- have been noted in needles experiencing chronic fumigation.

The micro- and ultrastructural responses of *P. halepensis* to H_2S fumigation provide important insights into plant adaptation. However, further works are needed to confirm our results since this procedure (leaf cuticular ultrastructure using TEM) on fossil and extant plants should be extended to a higher number of taxa as it is still only insufficiently developed (Archangelsky and Taylor, 1986; Jeffree, 2006).

The main interest of this research sets in 1) a reliable comparison between not fumigated and fumigated needles in an extreme environment and 2) evidence that variations in cuticle ultrastructure in the extant conifer *Pinus halepensis* are environment-dependant and represent a sketch of how some fossil plants probably responded to global environmental change during LIP, CFBP and euxinic interval or however during changes in atmospheric chemistry.

The results could be applied: 1) to use extant and fossil plants as bioindicators through an intraspecific comparison of ultrastructural features of plant cuticles during periods of normal vs. volcanic activity; 2) to discriminate those situations of palaeoenvironmental misinterpretation in which the effects of volcanism mimic those of aridity (Harris and Van Couvering, 2005).

The exposure of *E. arborea* to volcanic fumigation (Cap. II) revealed alterations detectable with SEM and TEM approach. In outer view, on both adaxial and abaxial sides of leaves epidermis both wax degradation and overproduction have been found respectively together with numerous clusters

of rodlets. Wax overproduction could be linked to the high air and soil CO₂ concentration, while wax degradation could be the direct (H₂S) or indirect (SO₂ or/and H₂SO₄ formation) effect of volcanic toxic compounds. At ultrastructural level, the cuticle showed an outer A2 granular layer and a beneath B1 reticulate layer. The prevalence of the granular -amorphous- component in respect to the fibrillar ones can be noted. Under the effect of persistent fumigation the A2 layer undergoes a remarkable increase of thickening. Perhaps, in presence of volcanic toxic gases, the cuticle undergoes a carbon-based compound allocation within the cuticle thickness.

This study shows that the cuticle responds to adverse environmental conditions varying both in total thickness and in A2 and B1 layers. In particular, a good correlation between atmospheric CO₂ concentration and the variation in A2 thickness has been found.

However, the alterations noticed in Pisciarelli and Solfatara samples could be the result of different volcanic chemical compounds action (e.g. CO₂ or/and volcanic sulphurous compounds) also because as Paoletti et al. (2005) claimed, other gases virtually undetectable could have biological effects. For this reason it is herein better to speak of “volcanic gases action”, avoiding to attribute meso- and ultrastructural variations to a specific volcanic chemical compound, and further experiments with different plants will be necessary to discover the exact effect of rising [CO₂] on leaf surface structures (Paoletti et al., 1998).

Therefore, these observations could be useful to understand the behaviour and the adaptability of plants in ancient ecosystems characterized by volcanic gas emissions. The micromorphological variations noticed in *E. arborea* could be an example of what plants experience during long term volcanic degassing. However, the volcanic degassing vent-derived Pisciarelli and Solfatara populations of *E. arborea* shows adaptive resistance to toxic volcanic gases. The present study on the cuticle ultrastructure of *E. arborea* finds application in the determination of changes in atmospheric chemistry in present and past floras since, as Taylor et al. (1989) claimed, diagenetic factor do not disrupt the structural identity of fossil cuticles.

However, it is important that future assessments of *E. arborea* responses to volcanic gases consider better the physiological factors involved.

Fortunately, further similar researches are highly advisable in Campania Region because, with its numerous volcanic (e.g. Frondini et al., 2004; Chiodini et al., 2010a) and not volcanic (e.g. Chiodini et al., 2010b) localities emitting gases, represents a natural laboratory allowing experiments involving plant-volcano interactions.

The second part of this research represents a contribution to the knowledge of Cretaceous fossil plants found in the Fossil-Lagerstätten of Cusano Mutri and Pietraroja.

The late Aptian flora of Cusano Mutri (Cap. III) is dominated by conifers, with a rare angiosperm, *Montsechia vidalii*, in association. The lack of pteridophytes is noteworthy. The conifer shoots recovered belong to the genera *Cupressinocladus*, *Pagiophyllum*, *Araucarites*, and *Frenelopsis*. Coniferous cones are occasionally encountered. Most of the cuticle remains belong to a new

cheirolepidiacean species, *F. cusanensis* sp. nov., which has been erected on the basis of both macroscopic (e.g. the internode dimension) and epidermal (e.g. disposition, morphology and dimension of stomatal apparatuses) features. Its very small stomatal apparatuses have not been recorded previously in *Frenelopsis*. The occurrence of this new species in addition to *F. profetiensis* in the early-middle Aptian flora of Profeti testify to the importance of the family Cheirolepidiaceae in this part of the Tethys region during the late Early Cretaceous. Most of the conifer taxa are poorly preserved. *F. cusanensis* is the only well-preserved species. The occurrence of *M. vidalii* in the southern Apennines extends its geographical distribution beyond the Iberian peninsula. Palaeobotanical and sedimentological observations of the plant bearing strata indicate that the sediments were probably deposited under a tropical to subtropical climate in arid to semi-arid conditions, possibly with wet phases on a regional to local scale. Evidence of wildfire (fusain) suggests a periodic combination of arid periods, high temperatures and lightning strikes.

Early Cretaceous (Early Albian) plants from Pietraroja (Cap. IV) are known for a long time, but never studied systematically. The assemblage revealed a dominance of conifers, in particular the Cheirolepidiaceae, and the occurrence of rare angiosperms. The following floristic entities were documented: *Sagenopteris*? sp., *Brachyphyllum* sp. 1, *Brachyphyllum* sp. 2, *Frenelopsis* sp., Cheirolepidiacean 1, and Angiospermae 1. A specimen belonging to the genus *Phlebopteris* was previously found (Bartirromo et al., 2006b). However, the finding of numerous specimens belonging to the genus *Frenelopsis* is noteworthy because it demonstrates the importance of the Cheirolepidiaceae remains in this sector of the Tethys Ocean. *Frenelopsis* might have grown near Pietraroja lagoon under brackish influence. The hypothesis of small and transient emerged lands herein proposed for the Appenninic Carbonate Platform would be confirmed by the terrestrial fauna composed by animals of small size.

Previous sedimentological, palaeontological and palaeogeographical data provided from Pietraroja Fossil-Lagerstätte together with the palaeobotanical observations presented in this paper are reminiscent of a tropical-subtropical climate, probably exposed to relative long periods of aridity. Pietraroja taxa are typical of the Euro-Sinian Province (Vakhrameev, 1991) and no affinity with Gondwanan floras has been noted. After more than two hundred years (Breislak, 1798) of researches we are far from a common consensus on the palaeoecological setting for the Pietraroja Fossil-Lagerstätte which needs a more integrated approach.

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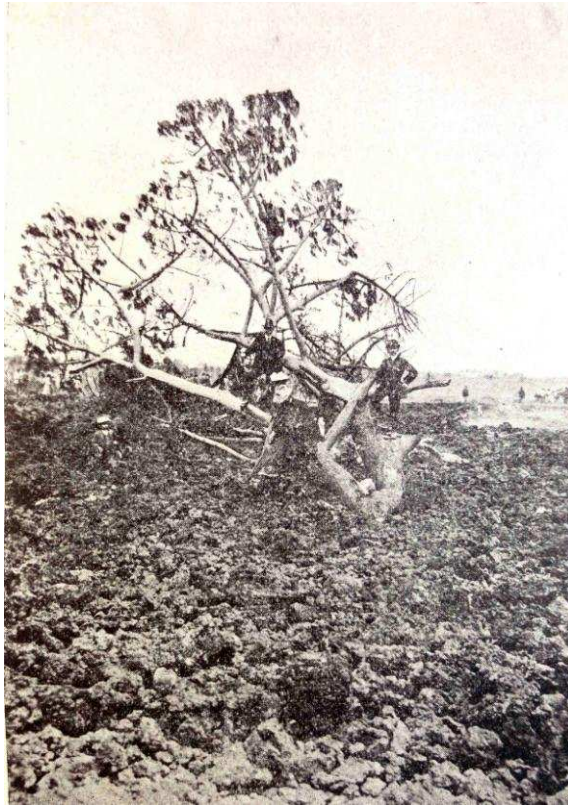
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Vesuvio: aprile 1906. Lava a superficie frammentaria che ha circondato una pianta senza abbruciarla, presso Boscotrecase (Fot. B. Friedlaender). Da: Mercalli G. 1907. I Vulcani attivi della Terra. Hoepli, Milano.



18/10/2010. Il sottoscritto alla Solfatara di Pozzuoli. Foto di Alfonso Tarallo.