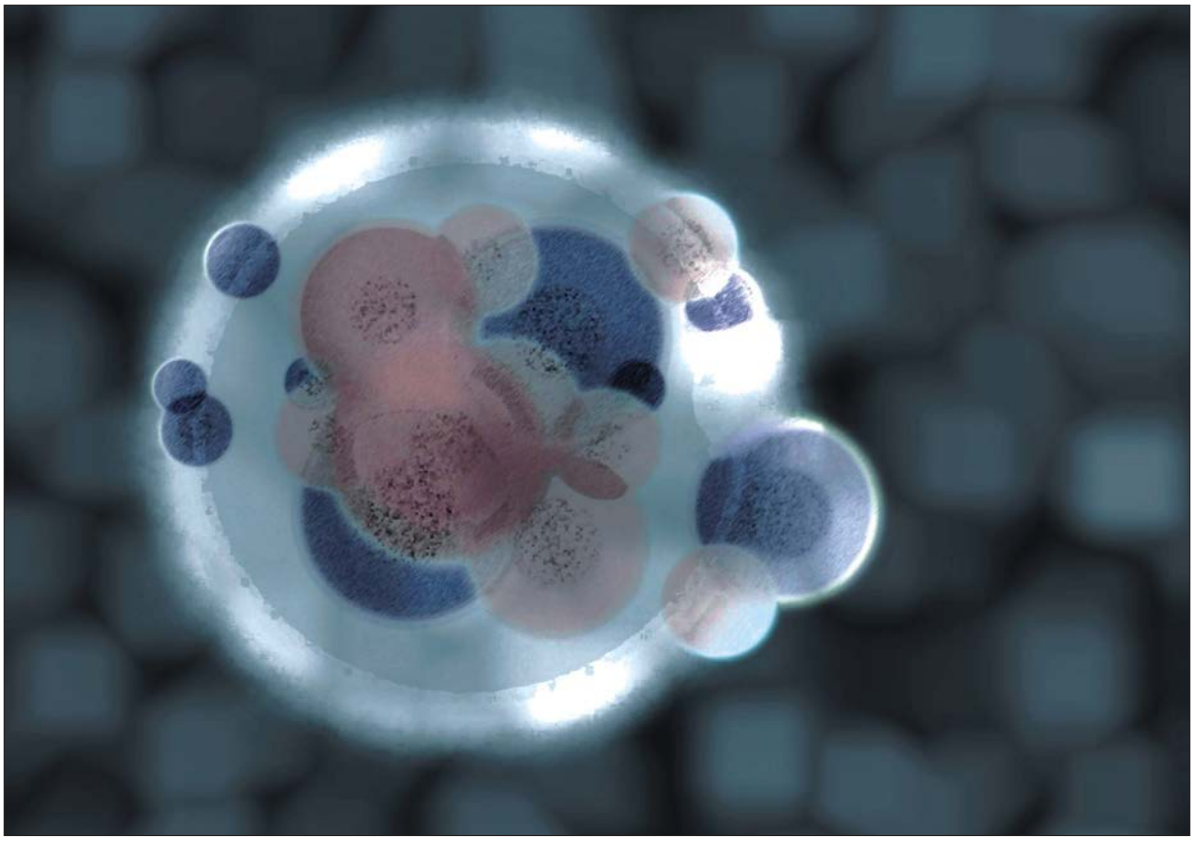

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Epiphytic Microflora on the Leaves of *Juniperus procera* from Aseer Region, Saudi Arabia

Saad A. Alamri
Department of Biology, College of Science, King Khalid University,
P.O. Box 10255, Abha 61321, Saudi Arabia

Abstract: Microflora including bacteria, actinomycetes, yeasts and filamentous fungi recovered from the leaves of *Juniperus procera* collected from two different altitudes at January and July 2007 from Aseer region, Saudi Arabia. Types and numbers of microflora varied according to the altitude and the month of collection. The number of microflora was higher on old leaves than young ones in most cases. Low altitude exhibited more microflora than high altitude. The relationship between meteorological factors and type and number of the recovered microflora was investigated. Inoculation of detached healthy leaves of *Juniperus procera* by predominant fungal isolates revealed that only *Alternaria alternata* as a pathogen of this plant. To confirm the pathogenicity of this fungus, scanning and transmission electron microscopic examination revealed the colonization of this pathogen inside the leaf tissue. Penetration of Juniperus leaves by the fungus occurred only through stomata and the invading hyphae were located in the intercellular spaces of leaf tissues. Bacteria also observed inside the intercellular spaces of leaf tissues of the host plant and not inside the leaf cells. Adjacent host cells to bacteria were also affected. Ultrastructural changes in the infected cells, from inoculated leaves, included changes in chloroplasts, nuclei and mitochondria.

Key words: Bacteria, fungi, leaf surface, microflora, phyllosphere, phylloplane

INTRODUCTION

Juniperus procera (Family Cupressaceae), commonly known as African Juniper or East African Juniper, is a coniferous tree native to the mountains of eastern Africa from eastern Sudan south to Zimbabwe and the southwest of the Arabian Peninsula. It is a characteristic tree of the Afromontane flora (Adams, 2004). *J. procera* communities often characterize altitudes between 2000 and 3000 m. The significance of these woodland ecosystems as a source of biodiversity, erosion protection and water storage is well known. In addition, it is an important source of durable timber in some countries (Negash, 1995).

Natural forests present a complex habitat that is inhabited by a rich and varied diversity of microbial organisms and communities (Farjon, 2005). The surfaces of the aboveground parts of plants are inhabited by various groups of microflora, which are defined as epiphytic microflora (Hirano and Upper, 2000). The epiphytic microflora occurs in this environment as transients, deposited on the surface of flowers or leaves with precipitation or carried there by wind or insects (Tukey, 1971).

The role of epiphytic microflora has not been fully elucidated. It is known that this group includes both plant pathogens and microflora which provides a protective barrier against them. These microfloras have profound effects on plant health and thus impact on ecosystem and agricultural functions (Baily *et al*., 2007). Several species of phyllobacteria have also been found to synthesize plant hormones and to play a role in stimulating plant growth (Beattie and Lindow, 1999). However, some of these microbes are deleterious to plants (Lindow and Leveau, 2002).

Microflora of the leaf surface (i.e., phylloplane) varies in size and diversity depending on the influence of numerous biotic and abiotic factors which affect their growth and survival (Bakker *et al*., 2002). These factors include leaf age, external nutrients, interactions between populations of different microorganisms (Blakeman, 1985), temperature, humidity, light intensity, wind speed and the presence of air pollutants (Dix and Webster, 1995). Many researchers as Lindow and Brandl (2003), Hemida (2004) and Rekosz-Burlaga and Garbolinska (2006) described the activity of microflora on leaf surfaces. The major groups of leaf surface microflora are present at any time of the year, but there are also evidences for seasonal successions (Blakeman, 1993). The colonization of leaf surfaces presents an interesting model for studying functional relationships between plants and microflora.

Aseer Mountains, located on the south western region of Saudi Arabia, with its high plateau (an elevation of almost 3000 m) and steep slopes provide an environment suitable to carry rich and varied vegetation. However, the *J. procera* trees have shown a significant

degradation in this area during the past decade. This current investigation is one of a series of coming studies concerning the reasons of the death of these trees. The aim of this research was to determine the main constituents of the microflora on the leaves of *Juniperus procera* relating to leaf age, time of collection and different altitudes.

MATERIALS AND METHODS

Sampling methods: Two different localities at two altitudes (2000 and 3000 m) in the Aseer region, Saudi Arabia, were selected for collecting plant samples. The observations were made on *J. procera* growing in a limited area. This is to ensure a uniform condition with respect to climate and air-borne distribution of spores. Bacteria, actinomycetes, yeasts and saprophytic fungi were isolated by a leaf washing technique (Pugh and Buckley, 1971). Young leaves (first fully expanded leaves) and old leaves (from the base of the plant) were sampled. Two observations (January represents the winter and July represents the summer) were made during 2007. Each sample included five leaves, in a similar state of maturity, from five plants at each location.

Media and isolation technique: Three selective media were used to isolate various types of microflora: Nutrient agar (NA; Difco laboratories, USA) for bacteria, chitin agar (CA; Lingappa and Lockwood, 1962) for actinomycetes and potato dextrose agar (PDA; Difco laboratories, USA) for fungi. The CA medium was supplemented with dextrose (10 g L^{-1}) and chloramphenicol (0.1 g L^{-1}) because the growth of recovered microbes on CA plates was sparse and the bacterial colonies overgrew on the actinomycetes colonies.

Dilution plating and colony counting: Culturable cell counts of leaf washes were carried out by serially diluting a 100 μL of the cell suspension in quarter strength Ringer's solution. Ten microliter aliquots of the appropriate dilution were pipetted, in triplicate, onto drop plates and allowed to dry thoroughly. The plates prepared were then incubated in the dark at 28°C. Microbes recovered on NA plates were counted 2 days after inoculation while those that appeared on the other media were counted one week after inoculation. Different organisms recovered on each medium were code-numbered and stored on suitable agar slants, PDA for fungi, oatmeal agar OMA (Difco laboratories, USA) for actinomycetes and NA for bacteria. Working cultures of fungi and actinomycetes were transferred to 6 cm PDA and OMA plates, respectively and exposed to diurnal light (12 h cycle, 37 μE m^{-2} sec^{-1}) from two 40 W cool-white fluorescent lamps suspended 45 cm above the plates to enhance sporulation. Identical looking colonies of the recovered microbes on different media were considered as the same microbe. Calculations of microbial numbers were carried out as colony-forming unit per mL.

Identification of microbes: For identification of fungal isolates, cultural characteristics and microscopic examination were carried out as described by Booth (1971), Ellis (1976), Samson (1979), Pitt (1985) and Hanlin (1990). Bacterial strains were identified according to morphological characteristics including pigment, colony form, elevation, margin, texture and opacity (Smibert and Krieg, 1981). In addition, bacterial strains were tested with respect to Gram reaction (Krieg and Holt, 1984).

Pathogenicity tests: Pathogenicity tests were carried out on healthy detached leaves of *J. procera* to determine the pathogens among the predominant isolated fungi (*Alternaria alternata*, *Cladosporium herbarum* and *Fusarium solani*). Detached leaves were surface sterilized using 0.1% mercuric chloride for 3 min followed by washing with sterile water and inoculation with fungal spores. The inoculated detached leaves were incubated at 22°C for 3-6 days and observed for symptoms development (Yu *et al*., 1984).

Electron microscopy: Only inoculated detached leaves by *Alternaria alternata* showed the disease symptoms. However, these diseased leaves were examined by scanning (SEM) and transmission (TEM) electron microscopy to confirm the pathogenicity and colonization of this fungus inside Juniperus leaves. Segments from healthy control leaves corresponding to approximately the same locations as those from diseased leaves were removed and similarly prepared for electron microscopic observations. The method adopted from Baka (1996) was used for SEM. Leaf segments were prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.0, washed in the same buffer and post-fixed in 1% OsO_4. Following this, leaf segments were dehydrated in a graded acetone series, dried and coated with gold. Samples were then examined and photographed using a JEOL JSM-6400 SEM. Pieces from diseased and healthy leaves were processed for TEM according to Baka and Lösel (1999). Leaf pieces (1.0 mm^2) were prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.0, washed in the same buffer, post-fixed in 1% OsO_4, dehydrated in a graded series of ethanol and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were cut using a Reichert

ultramicrotome, stained with 2% uranyl acetate followed by lead citrate. Sections were viewed and photographed using a JEOL 100-S TEM.

RESULTS

Bacteria and actinomycetes: The results indicated that, at high altitude, the numbers were lower in January than in July. At low altitude, the numbers were lower in July than in January. Old leaves showed high bacterial count than young leaves at both altitudes (Table 1). The morphological classification of 200 cultures, isolated in January and July from young and old leaves at the altitudes of interest are shown in Table 2. At high altitude, fluorescent pseudomonas, yellow-pigmented rods and non-pigmented rods exhibited the predominant groups in both months of collection. Lactobacilli are not detected at this altitude (Table 2). These three groups showed 64 and 79% of all isolates on young and old leaves in January and 68 and 90% on young and old leaves in July (Table 2).

At low altitude, fluorescent pseudomonas and yellow-pigmented rods, exhibited the predominant groups in both months of collection, but streptococci replaced the non-pigmented rods. These two groups showed 80 and 79% of all isolates on young and old leaves in January and 54 and 68% on young and old leaves in July (Table 2). At high altitude, the highest number of bacteria per cm^2 leaf area of 200 isolates is recorded on old leaves in July and represented by fluorescent pseudomonas, yellow-pigmented rods and non-pigmented rods (Table 3). At low altitude, the highest number recorded also on old leaves in July and represented by fluorescent pseudomonas, yellow-pigmented rods and streptococci (Table 3). Actinomycetes are more abundant at high altitude and their percentage is the same in January and July. Young leaves exhibited more actinomycetes than old leaves (Table 2).

Fungi: At high altitude, 22 fungal species were isolated from Juniperus leaves (Table 4). Seven species were found on young and old leaves during January and July. In January, 10 and 13 species were isolated from young and old leaves, respectively, while in July, 17 and 20 species were isolated from young and old leaves, respectively. *Alternaria alternata* was the most predominant species followed by *Fusarium solani* and *Cladosporium herbarum*. On the other hand, at low altitude, 29 fungal species were isolated (Table 4). Eight species were found on young and old leaves during January and July. In January, 20 species were isolated from both young and old leaves, while in July 20 and 23 species were isolated from young and old leaves, respectively. *A. alternata* was the most predominant species followed by *C. herbarum* and *F. solani* (Table 4). Generally, low altitude exhibited more fungal species than high altitude.

Electron microscopy: The examination by SEM revealed that rod-shaped bacteria inhabited the leaf surface of Juniperus (Fig. 1a) and they were observed in the intercellular spaces of Juniperus leaf tissue when examined by TEM (Fig. 1b, c). Moreover, SEM

Table 1: Plate counts of bacteria and actinomycetes from *J. procera* leaves isolated in January and July 2007 at two altitudes

	January		July	
Maturity of leaves	Young	Old	Young	Old
High altitude				
Number per g dry matter	7.5×10^6	11.2×10^6	13.0×10^6	25.0×10^6
Number per cm^2 leaf area	11.0×10^3	22.5×10^3	30.0×10^3	50.0×10^3
Low altitude				
Number per g dry matter	11.0×10^6	15.5×10^6	5.5×10^6	8.5×10^6
Number per cm^2 leaf area	21.0×10^3	40.5×10^3	12.0×10^3	20.0×10^3

Table 2: Distribution (in %) of 200 bacterial isolates from *J. procera* leaves collected from high and low altitudes

	High altitude				Low altitude			
	January		July		January		July	
Maturity of leaves	Young	Old	Young	Old	Young	Old	Young	Old
Fluorescent pseudomonades	20	23	22	34	50	60	31	43
Yellow-pigmented rods	24	33	30	36	30	19	23	25
Non-pigmented rods	20	23	16	20	1	5	6	9
Streptococci	20	12	11	3	15	10	21	18
Spore-forming rods	6	3	9	2	3	3	6	2
Actinomycetes	10	5	11	4	1	2	9	2
Others	0	1	1	1	0	1	4	1
Total	100	100	100	100	100	100	100	100

Table 3: Numbers of bacteria per cm^2 leaf area of 200 isolates from *J. procera* leaves collected from high and low altitude

	High altitude				Low altitude			
	January		July		January		July	
Maturity of leaves	Young	Old	Young	Old	Young	Old	Young	Old
Fluorescent pseudomonades	830	1022	3000	3600	1000	2100	4120	5200
Yellow-pigmented rods	940	1200	2500	2700	1000	3200	3100	4200
Non-pigmented rods	300	600	500	820	20	24	30	41
Streptococci	40	60	70	90	2100	920	2190	3175
Lactobacilli	0	0	0	0	50	70	120	140
Spore-forming rods	0	0	7	2	20	22	30	41
Actinomycetes	20	40	20	25	8	13	10	17
Others	0	0	0	3	2	3	1	4
Total	2130	2922	6097	7240	4200	6352	9601	12818

Table 4: Abundance (in %) of fungal species on *J. procera* leaves collected at high and low altitudes

	January		July		January		July	
Maturity of leaves	Young	Old	Young	Old	Young	Old	Young	Old
Alternaria alternata	16.2	20.1	24.0	30.3	42.2	60.1	36.4	44.1
A. solani	1.8	2.3	2.1	3.8	6.8	9.7	3.5	5.2
A. tenuissima	-	-	2.6	2.9	1.8	3.3	-	-
Aspergillus flavus	-	1.0	-	1.8	0.8	1.0	0.5	0.6
A. fumigatus	-	-	3.2	6.1	2.2	-	-	1.1
A. niger	0.9	-	-	0.9	-	-	0.4	0.6
A. niveus	-	-	-	-	0.9	1.4	1.2	2.9
A. oryzae	-	-	1.8	2.2	-	3.1	-	1.7
A. parasiticus	0.8	2.9	-	-	0.8	1.3	-	-
A. sulphureus	-	-	-	-	-	-	0.6	0.8
Cladosporium herbarum	6.6	9.7	11.8	19.5	22.2	30.1	15.4	19.5
C. cladosporioides	-	-	2.0	3.9	-	0.8	0.6	-
Curvularia siddiquii	-	-	2.8	3.4	1.3	-	0.4	-
C. vercuculosa	-	2.7	-	1.8	-	8.2	-	5.6
Drechslera sp.	-	-	2.5	4.2	6.2	8.4	-	6.5
Epicoccum nigrum	0.3	0.6	0.8	1.0	-	7.9	2.9	5.2
E. purpurasceus	-	-	-	-	1.8	-	1.5	1.8
Fusarium moniliforme	-	-	1.5	3.1	2.2	4.0	-	-
F. solani	17.5	18.1	18.2	23.4	22.9	25.8	17.1	20.2
Helminthosporium sp.	-	0.3	0.5	0.7	-	-	0.8	1.7
Humicola grisea	1.0	2.2	0.8	1.0	2.0	-	3.7	5.8
Penicilium citrinum	-	0.5	-	0.4	4.8	-	7.4	8.9
P. chrysogenum	-	-	-	-	2.9	6.9	-	9.5
P. commune	-	-	-	-	-	3.6	0.8	1.4
P. cyclopium	-	-	-	-	6.7	8.9	1.2	-
P. notatum	-	1.9	1.8	2.8	-	4.9	0.9	3.5
Rhizopus nigricans	4.8	-	3.9	-	3.8	7.9	1.5	3.1
Stachybotrys sp.	-	-	-	-	4.9	-	-	1.4
Yeasts	8.1	14.5	18.3	25.4	12.4	27.5	8.2	10.4
Total fungal isolates	122.0	147.0	138.0	188.0	138.0	189.0	239.0	360.0

-: Not recorded

examination revealed that after the colonization of *A. alternata* within leaf tissue, the branched conidiophores emerged from stomata (Fig. 1d-f). TEM examination revealed that the mycelium of *A. alternata* was located in the intercellular spaces of leaf tissue and characterized by the presence of two nuclei, mitochondria, vesicles, endoplasmic reticulum and a septum. The most striking feature is the presence of electron-dense material at the contact with host cell, which may acts as a cement to attach the mycelium with host cell wall (Fig. 2a).

The ultrastructure of cells from healthy leaves revealed the presence of normal chloroplasts, nuclei and mitochondria. The chloroplast is characterized by a well-organized membrane system of grana and intergranal lamellae, a well defined envelope, large starch grains and few plastoglobuli (Fig. 2b). Inoculation of Juniperus leaves by *A. alternata* spores caused major changes in the ultrastructure of chloroplasts. The disorganization of membrane system of the chloroplasts, the breakdown of chloroplast envelope, the disappearance of starch grains, the increasing of plastoglobuli are indicative of infection (Fig. 2c). Normal nuclei with double-membrane envelope, batches of electron-dense heterochromatin and electron-lucent euchromatin are recovered from healthy Juniperus

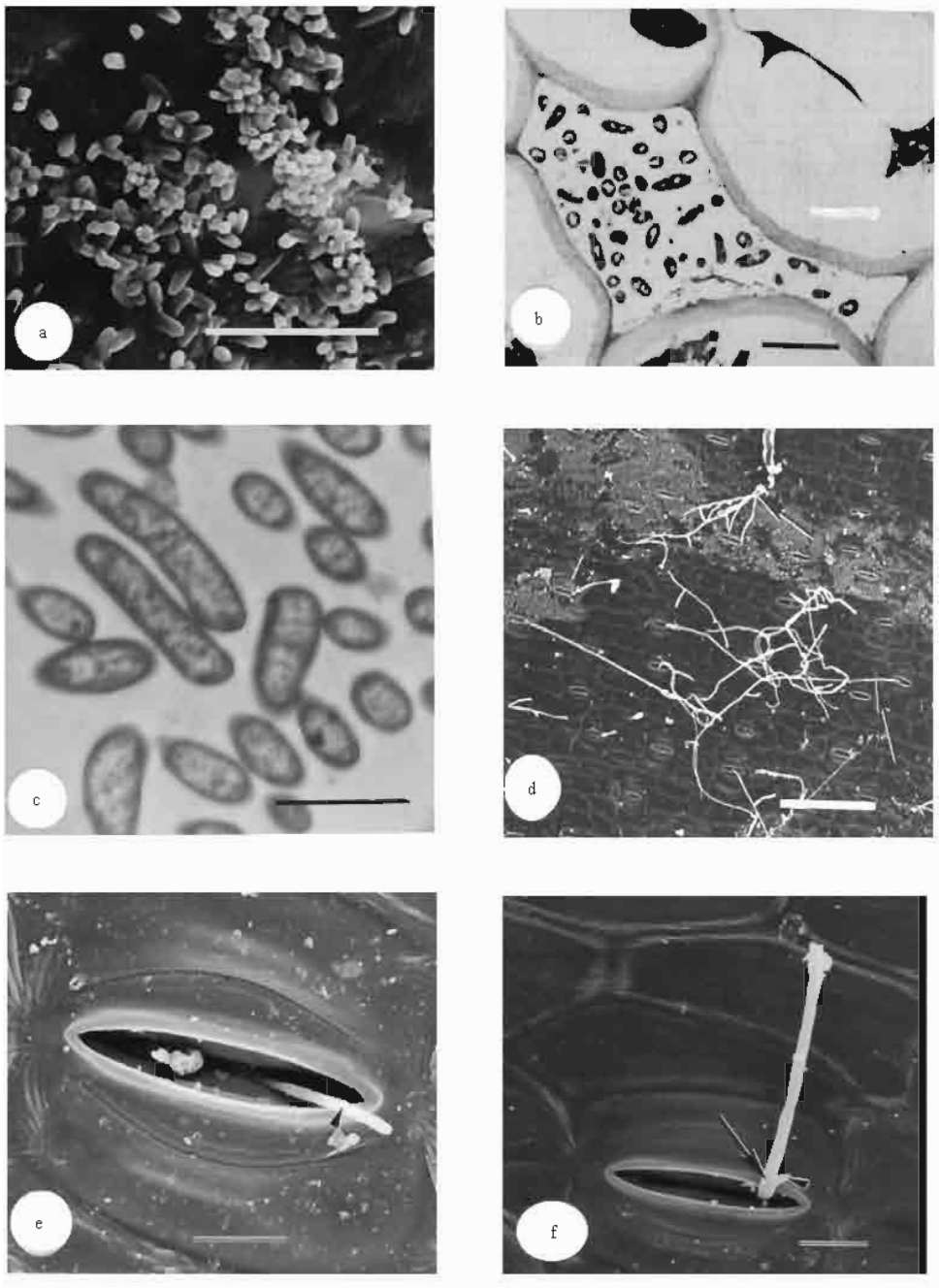

Fig. 1: (a) SEM micrograph showing rod-shaped bacteria on leaf surface. Scale bar = 10 μm, (b) TEM micrograph showing bacteria located in intercellular space of leaf tissue. Note dead cells adjacent to bacteria. Scale bar = 0.5 μm, (c) TEM micrograph showing magnified rod-shaped bacteria. Scale bar = 10 μm, (d) SEM micrograph showing the emergence of branched conidiophores (arrows) of *A. alternata* from stomata on leaf surface. Scale bar = 100 μm, (e) SEM micrograph showing the conidiophore (arrowhead) of *A. alternata* starts to emerge from stoma on leaf surface. Scale bar = 10 μm and (f) SEM micrograph showing a mature conidiophore (arrow) of *A. alternata* emerging from a stoma on leaf surface. Note the beginning of a new branch (arrowhead). Scale bar = 10 μm

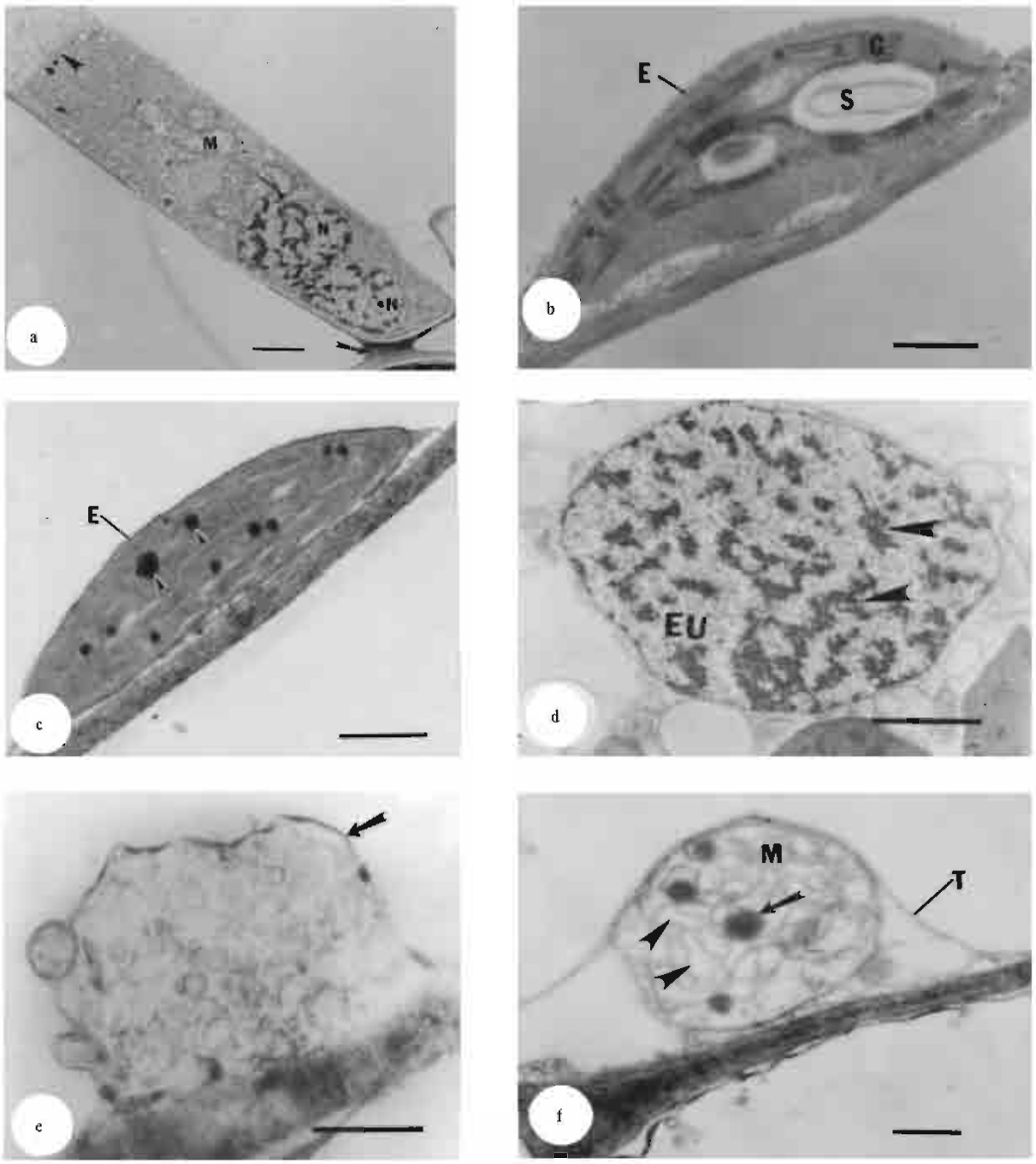

Fig. 2: (a) TEM micrograph of a hypha of *A. alternata* located in the intercellular space of Juniperus leaf. The hypha contains two nuclei (N), numerus mitochondria (M). Note the septum (arrowhead) and adhesive material (arrow) between cell wall and hypha. Scale bar = 1.0 μm, (b) TEM micrograph of a chloroplast from an uninoculated Juniperus leaf showing a well-organized membrane system of grana (G). Note the chloroplast envelope (E) and starch grain (S). Scale bar = 0.5 μm, (c) TEM micrograph of a chloroplast from a Juniperus leaf inoculated by *A. alternata* showing a disorganized membrane system. Note the increasing of plastoglobuli (arrowheads) and thickened chloroplast envelope (E). Scale bar = 0.5 μm, (d) TEM micrograph of a nucleus from an uninoculated Juniperus leaf showing well distribution of heterochromatins (large arrowheads) and euchromatins (EU). Note double nuclear membrane (small arrowhead). Scale bar = 0.5 μm, (e) TEM micrograph of a nucleus from Juniperus leaf inoculated by *A. alternata* showing the disturbance of chromatin materials. Note thickened nuclear membrane (arrow). Scale bar = 0.5 μm and (f) TEM micrograph of a mitochondrion (M) from Juniperus leaf inoculated by *A. alternata* showing the appearance of electron-dense bodies (arrow), swollen cristae (arrowheads). Note the tonoplast (T). Scale bar = 0.5 μm

leaf tissues (Fig. 2d). Infected leaf cells after the inoculation by *A. alternata* showed major changes in the ultrastructure of nuclei. The disorganization of chromatins, the appearance of many vesicles and the thickening of envelope are the most characteristics of these nuclei (Fig. 2e). Moreover, mitochondria from inoculated leaf cells by the fungus showed many ultrastructural changes such as the swelling of cristae and the appearance of electron-dense vesicles (Fig. 2f).

DISCUSSION

A comparative study of epiphytic microflora isolated from young and old leaves of *J. procera* at two altitudes in January and July 2007 was made. This study reveals that the highest total number of microflora was recorded in July at both altitudes and old leaves always exhibited higher number of microflora than young leaves. Microflora colonizing the above-ground parts of plants usually occurs in high numbers. It was reported that a 1 cm^2 surface of a leaf may contain 10^5 to 10^7 bacterial cells (Hirano and Upper, 2000; Mercier and Lindow, 2000; Lindow and Leveau, 2002). These values can also be expressed as the number of bacteria per 1 gram fresh or dry weight of leaves (Brighigna *et al*., 2000). In such cases, the number of bacteria per 1 gram fresh mass of leaves ranges from 10^5 to 10^8 bacterial cells.

The occurrence of microflora on the above-ground parts of plants depends on a number of factors such as weather variables, quality and quantity of spores in the air, nutritional substances on leaf surfaces, air pollution and species of the host plant (Thompson *et al*., 1993; Fahmy and Ouf, 1999). Competition between microbial species could also affect the types and numbers of microflora on leaves (Killham, 1999). Meteorological factors such as atmospheric temperature, relative humidity and rain are important for influencing the distribution of microflora on leaf surfaces. This study showed that the maturity and position of leaves are among the factors that influence the composition of epiphyte microflora. The gradually increasing number of microflora may reflect the increasing deposition from spores during prolonged exposure or it may be due to multiplication of microorganisms in the phylloplane of old leaves (Andrews and Harris, 2000).

Infected leaves of *Juniperus procera* after the inoculation with *A. alternata* led to disorganization of the chloroplast membrane system, breakdown of the chloroplast envelope, increasing of plastoglobuli and disappearance of starch grains. These results agree partially with the findings of Baka and Krzywinski (1996) and Alwadi and Baka (2001) who reported the disorganization of chloroplasts in host cells infected by different species of fungi. The disappearance of starch from chloroplasts and increasing the number of plastoglobuli due to infection with *A. alternata* coincided with the observations of Shabana *et al*. (1997) and Alwadi and Baka (2001). Decrease in starch is common in many foliar diseases (Wheeler, 1975). In addition, the ultrastructural changes of nuclei and mitochondria in infected Juniperus cells after the infection by *A. alternata* are similar to the observations of Baka (1987) and Mendgen *et al*. (1996). The remarkable damages of host cell organelles after the infection by fungi may be due to the toxins secreted by these fungi. Toxins play a significant role in a number of important diseases of plants caused by fungi and bacteria (Turner, 1984).

CONCLUSION

This study revealed that the number and distribution of microflora isolated from the phylloplane of *J. procera* varied according to altitudes, seasons and leaf ages. In addition, *A. alternata* was predominant between all fungi isolated. The pathogenicity of this fungus was confirmed by both scanning and transmission electron microscopy. Ultrastructural changes were noted in infected cells from inoculated Juniperus leaves by this fungus including changes in chloroplasts, nuclei and mitochondria. Further studies of biotic and abiotic factors are also needed to predict the reasons of the die-back of *J. procera* trees in Aseer region.

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