

Mediterranean forest tree decline in Italy: relationships between drought, pollutants and the wax structure of leaves

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ABSTRACT - After presenting the situation of forest decline in Italy and analyzing the factors that play a contributing role, this paper studies the response of the epicuticular wax structures and the stomata in ten broadleaf species and one conifer to fog-like treatments with acids and/or surfactants and to severe water stress. The main results are that wax structure alterations vary in intensity in the different species studied and that the microstructural alterations observed in field conditions cannot be attributed only to severe drought, since sample trees put through water stress simulations do not differ significantly from controls. In the artificial surfactant treatment, a positive relationship between structural damage to the stomata and transpiration suggests possible synergies between the effects of drought and those of pollutants in inducing stress conditions in Mediterranean vegetation.

KEY WORDS - Forest decline, acid rain, surfactant, water stress, Mediterranean species, wax structure

INTRODUCTION

Forest decline was defined as a disease of forest ecosystems (Schütt, 1988), which includes a simultaneous and rapid decline in the health and vigor of both coniferous and broad-leaved forests with symptoms classified as growth-decreasing, abnormal growth and water stress. Recently, Skelly and Innes (1994) observed that the concept of a general forest decline, as described by Schütt and Cowling (1985), is untenable and that forest decline is a complex problem involving a range of stress factors, including pollution, and argue that no single hypothesis can explain the forest decline phenomena observed in different locations on different species. Each species decline is characterized by a specific suite of symptoms. Moreover, crown transparency, the most widely used index of forest health in surveys, was recently considered non etiologically specific (Innes, 1993). Further, in Europe the parameters of defoliation and discolouration are calibrated on *Picea abies*, but this seems insufficient in the Mediterranean area (Cadahia *et al.*, 1992). Forest damage assessments need more careful diagnostics and the use

of all the knowledge of the fields of forest pathology, entomology, stress physiology, silviculture before reaching conclusions about forest health. It is very difficult to evaluate the vigor of a tree with the classical morphological characters utilized now in forest surveys.

Because of the great debate on the evaluation methodology of tree health, since 1988 our research has examined the wax leaf structures of trees in forest and of young stressed trees. Because the symptomatology of forest decline is often non-specific and similar to a generic response to stress, especially water stress, the main goal of our research programme was to artificially reproduce the alterations of the leaf wax structures by treatments with pollutants and by water stress actions and to compare among them the alterations caused by these two treatment types and those found in forest.

STATUS OF RESEARCH IN ITALY

Table 1 shows that the percentage of damaged trees has been continuously increasing from 1985 to 1991, both in the Northern, Central and Southern Italy. An analysis of the yearly survey reports carried out between 1987 and 1991 in Tuscany (Central Italy) along a vegetational transect including coastland Mediterranean vegetation (evergreen sclerophylla), sub-Mediterranean vegetation (deciduous oakwoods) and mountain Mediterranean vegetation (beechwoods), has allowed Bussotti *et al.* (1995a,b,c) to ascertain the following: 1. the crown status is worse in the more markedly Mediterranean areas (coastlands) because of the influence of winds and sea aerosol, as well as the presence of large urban and industrial concentrations; 2. unlike mountain conifers (*Abies alba* and *Pinus nigra*), the crown status of Mediterranean pines is worse in the younger trees, probably because their reduced root system does not allow them to withstand water stress as well; 3. crown status is affected by the meteorological trends of the previous year, especially as far as total rainfall is concerned; 4. mountain trees (especially European beech) recover very slowly even when rainfall returns to abundant levels after a period of drought; 5. Mediterranean zone trees, on the other hand, react excellently to variations in the rainfall level and (especially *Quercus ilex*) appear to be more sensitive to low temperatures and to winter droughts than they are to summer water stress.

The stress factors which affect the status of Italian forests are many and varied. An element frequently recorded is the acidification of precipitation (Pantani *et al.*, 1984, Cossu *et al.*, 1989, Udisti *et al.*, 1990, Fuzzi *et al.*, 1990, MA, 1992), which occurs especially in winter months, although only rarely does the acidity reach values below the damage threshold as demonstrated experimentally (acid mist at pH < 3.5, e.g. Morrison, 1984). Among the gaseous pollutants, ozone is the most widespread and is frequently found in concentrations higher than the toxicity threshold for forest trees (50 $\mu\text{g m}^{-3}$; Bergmann, 1992). Damage caused by sulphur dioxide is closely related to proximity to industrial estates (Rosini and

TABLE 1
 PERCENTAGE OF DAMAGED TREES IN THE WHOLE OF ITALY AND INS OME REGIONS WHICH CARRIED OUT
 THEIR OWN INVENTORIES BETWEEN 1985 AND 1991

		1985	1986	1987	1988	1989	1990	1991
Alto Adige	Td	8.3	7.5	9.7	14.6	14.6	17.1	22.8
<i>Northern Italy</i>	Ed	0.9	0.8	1.8	3.3	3.5	4.6	8.6
Trentino	Td	38.8	21.8	44.5	42.4	46.4	43.6	47.4
<i>Northern Italy</i>	Ed	10.8	3.7	14.7	16.8	15.3	13.0	12.9
Friuli	Td	55.7	55.2	55.4	74.4	74.8	77.1	78.2
<i>Northern Italy</i>	Ed	17.4	17.6	15.3	33.8	36.1	34.8	33.2
Lombardia	Td	—	—	—	—	—	50.9	—
<i>Northern Italy</i>	Ed	—	—	—	—	—	25.6	—
Toscana	Td	—	—	51.1	54.0	—	67.0	64.4
<i>Central Italy</i>	Ed	—	—	19.4	20.9	—	33.0	33.6
Sardegna	Td	—	—	41.6	—	—	—	—
<i>Southern Italy</i>	Ed	—	—	16.2	—	—	—	—
Italy	Td	5.8	5.8	10.4	9.1	24.0	38.6	41.6
	Ed	—	—	—	—	9.0	14.8	16.4

Td = Total damage (the sum of slight, moderate, severe and lethal damage)

Ed = Evident damage (the sum of moderate, severe and lethal damage)

Sources: reports of local administrations

Nucciotti, 1969, AA.VV., 1992). Also atmospheric nitrogen concentrations do not seem to be very important in Italy: nitrogen depositions higher than load levels have been found only in the north-western regions of the country (Tartari *et al.*, 1994). Italian forests tend to present a chronic deficiency of this important nutrient (Bussotti *et al.*, 1995a). Variations in the N/K ratio are never due to an increase of N (Flückiger *et al.*, 1986), but rather to a marked deficiency of K (Bussotti *et al.*, 1995a), a trait common to most of the highly anthropized Mediterranean region where soil is very poor due to intense exploitation.

Italy has more than 7,500 Km of coastland where the vegetation sometimes displays acute damage caused by surfactants (Bussotti *et al.*, 1992). Surfactant substances become part of the chemical composition of the surface layer of seawater and of seaspray which is transported by the wind and deposited on the vegetation. Normally the damage caused by surfactants in sea aerosol affects only vegetation growing at most one Km from the coast, although on occasions of particularly strong windstorms it has been recorded in trees growing up to several dozen Km from the coast. In Italy the decline of coastal vegetation dates back to the beginning of the sixties and has been the subject of several studies. Gellini *et al.* (1983, 1985) demonstrated that artificial treatment with anionic surfactants and

NaCl caused the same symptoms in the needles of *Pinus pinea* and *P. pinaster* as those observed in the pines along the Italian coastlines.

Climate changes and fluctuations also appear to exert a harmful influence on Mediterranean vegetation, especially on the mesophyll beechwoods in the mountainous areas (Bussotti *et al.*, 1995c). Lastly, one of the main manifestations associated with oak decline in Italy is the increase of weak parasites (Vannini and Scarascia Mugnozza, 1991; Scortichini *et al.*, 1993; Ragazzi *et al.*, 1993). These attacks are related to water stress conditions and can therefore be considered a consequence of climate changes. In any case, the contribution of water stress to the appearance of forest decline in Italy has not yet been studied in sufficient depth.

MATERIALS AND METHODS

Artificially treated seedlings

The effects of drought and some atmospheric pollutants on the epicuticular wax structures and stomata of a variety of common Italian tree species, 3-year old seedlings of ten broadleaf and one conifer species (20 seedlings per species) were examined. The broadleaf species were: common maple (*Acer campestre* L.), tree of heaven (*Ailanthus altissima* L.), Italian alder (*Alnus cordata* Loisel), chestnut (*Castanea sativa* Mill.), European beech (*Fagus sylvatica* L.), manna ash (*Fraxinus ornus* L.), walnut (*Juglans regia* L.), London plane (*Platanus acerifolia* Willd.), holm oak (*Quercus ilex* L.) and European white elm (*Ulmus laevis* Pall.). The conifer species was Italian stone pine (*Pinus pinea* L.). Seedlings were planted directly in the soil, in a special shelter with open sides and a roof that closed automatically when the relative humidity in the air exceeded 95% in order to exclude rain.

Water stress treatment

For an entire growing season (15 April - 15 October), 15 seedlings per species were regularly irrigated from below with water; the remaining 5 seedlings were protected by specially designed structures ensuring that no lateral water penetration occurred and were irrigated only whenever the leaves showed signs of wilting.

Spraying with acidified solutions and/or surfactants

Of the 15 irrigated seedlings, 5 per species and per treatment were artificially sprayed with either demineralized water or acid solution (pH 3.5, H₂SO₄:HNO₃ in a mass ratio of 5:2) or with demineralized water acidified as above with the addition of 50 mg/L of the surfactant ABS (sodium dodecylbenzene sulphonate). European beech and stone pine seedlings were sprayed only with demineralized water or 50 mg/L of ABS. All sprays were applied twice a week as a fog up to the drip-point.

Measurements

Samples for SEM observation were collected at the first instances of wilting and at the end of the growing season on seedlings subjected to water stress, and only at the end of the growing season in the case of artificially irrigated seedlings. Five leaf samples were collected from each seedling. Only current

year leaves were gathered from evergreen species. Samples were removed from the central part of needles and from the central section (halfway between the main vein and the outer edge) of leaves. All the samples were air-dried, sputtered with gold film using an S105A Edward sputter coater, and then observed with a Philips 505 SEM at 15-15.5 kV. Parameters observed were: morphology of the stomata, of the epicuticular and epistomatal wax structures; status of covering trichomes and glandular hairs (where present).

In the case of European beech seedlings, 20 stomata per leaf were classified according to the arbitrary damage rating scale defined by Moricca *et al.* (1994) and on the basis of this rating the stomatal damage index (SDI) for each seedling was calculated according to Raddi *et al.* (1994) and Moricca *et al.* (1994). In addition, about 40 and 10 days before the end of the sprayings, transpiration was measured with a steady state porometer LI-COR 1600 (5 leaves from each European beech seedling) equipped with the standard broadleaf aperture. The readings were done on the fifth leaf of a lateral shoot, on the central portion of the leaf, over the rib. All measurements were done between 11.30 a.m. and 1 p.m. on windless and overcast days. The mean values of photosynthetically active radiation (PAR), of relative humidity (RH), and of cuvette temperature (T_c) were the following:

8 September:	PAR 1020 $\mu\text{E m}^{-2} \text{s}^{-1}$; RH 58%; T_c 24.1°C
6 October:	PAR 875 $\mu\text{E m}^{-2} \text{s}^{-1}$; RH 30%; T_c 20.2°C

Analysis of the data

The transpiration and SDI data in European beech seedlings sprayed with demineralized water with or without the addition of ABS were then compared using analysis of variance.

Declining trees in the open field

Samples were collected in the open field in order to examine the alterations affecting the leaf surface microstructures by means of SEM observations. The species observed were: a deciduous broadleaf (European beech), an evergreen broadleaf (holm oak) and a conifer (Italian stone pine). Five sun leaves were collected from each of 5 adult trees, declining but from no apparent biotic cause. Only current year leaves and needles were gathered from the evergreen trees (holm oak and pine). Sampling was performed in sites in Central Italy which are known to have been suffering for some time the effects of atmospheric pollution (Barbolani *et al.*, 1986, 1988; Enel, 1991; AA.VV., 1992). Beech leaves were collected at Pian di Novello near Pistoia, holm oak leaves at San Rossore near Pisa and pine needles from the pinewoods at San Vitale near Ravenna. The samples were prepared according to the methodology described above.

RESULTS

Artificially treated seedlings

Seedlings subjected to artificial water stress

Cultivation in a state of water stress caused the wilting and subsequent death of many leaves (or needles) which were retained on the tree for a long time. Samples which had withered and dried because of drought showed no significant difference as compared to controls in either the stomata or the epicuticular wax,

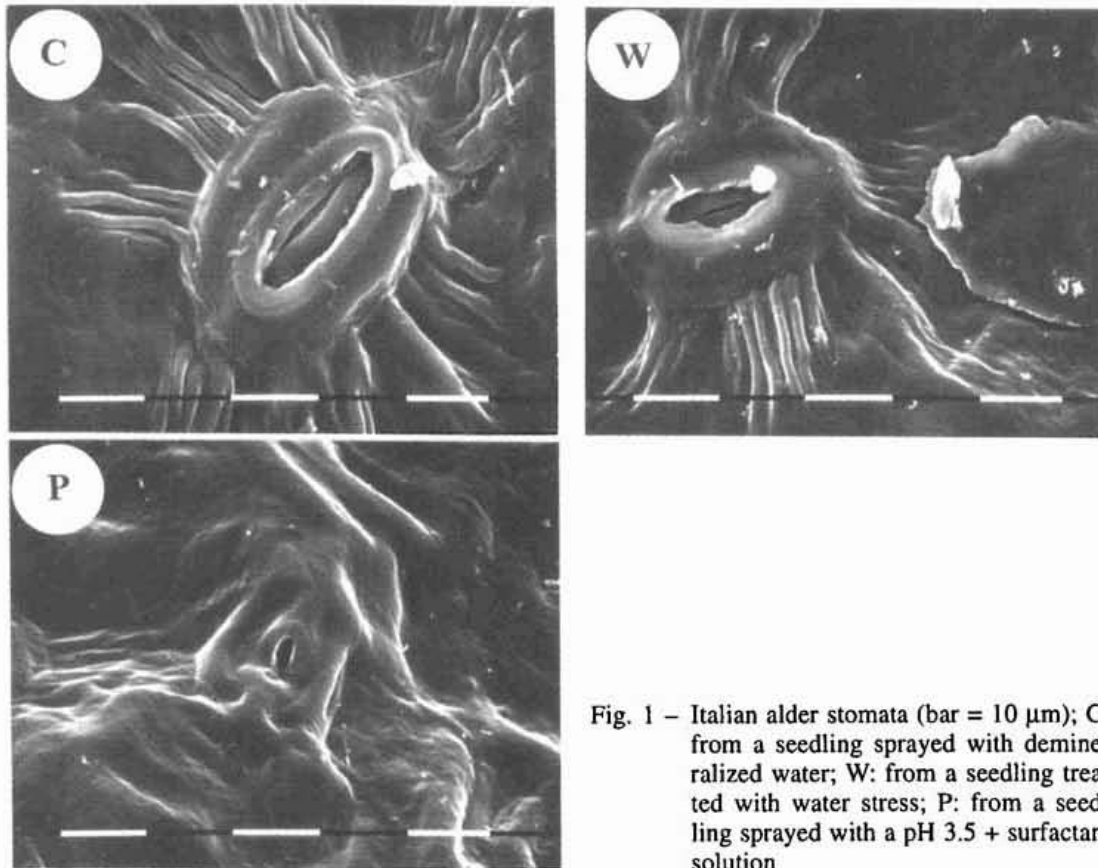


Fig. 1 – Italian alder stomata (bar = 10 μm); C: from a seedling sprayed with demineralized water; W: from a seedling treated with water stress; P: from a seedling sprayed with a pH 3.5 + surfactant solution

except for Italian alder samples. This species displayed occasional detachments of the surface wax layer, although in these samples, too, the stomatal morphology was identical to that of the controls (Figure 1).

Seedlings sprayed artificially with acid pollutants and/or surfactants

Macroscopically, only two species displayed evident damage after treatment with surfactants and/or acidified solutions: pine needles displaying chlorotic stains and holm oak leaves with yellow patches and early leaf loss. SEM observations of leaf surfaces showed that acid rain treatment caused damage in all species and that the addition of surfactants made the damage more severe. The effects noted in broadleaves were: erosion of the epicuticular wax structures and their migration, e.g. into the stomata; alteration of the stomata with deformation of the rimas; in the most severe cases the rima was partially closed due to the collapse of one or both guard cells or because of the migration of fused wax; small fractures of the epicuticular wax layer, often located near the stomata, the trichomes, the glandular hairs and the vascular tissues; abscission of the trichomes, or at any rate alteration of their morphology; collapse of the secretory head or disarticulation of the glandular hairs; hyperplasia and hypertrophy of the mesophyll in the walnut leaves treated with acid + ABS solution; development of fungine hyphas; insect attacks. The damage

TABLE 2
 TRANSPIRATION ($\mu\text{g cm}^{-2} \text{s}^{-1}$) AND STOMATAL DAMAGE INDEX (SDI) IN EUROPEAN BEECH SEEDLINGS
 SPRAYED WITH DEMINERALIZED WATER (CONTROL) OR WITH SURFACTANTS (ABS)

Treatment	Transpiration September 8	Transpiration October 6	SDI October 15
Control	6.33 \pm 0.21	4.18 \pm 0.12	1.06 \pm 0.13
ABS	5.70 \pm 0.45	5.16 \pm 0.18	2.40 \pm 0.09
	NS	*	**

Differences among treatments were significant at 1% (**); at 5% (*) and not significant (NS)

decreased in the following order: walnut, manna ash, common maple, chestnut, London plane, *Ailanthus*, European white elm, holm oak, Italian alder. Beech samples were only treated with surfactants, yet the damage they presented was fairly similar to the other samples: degeneration of the surface wax layer with the appearance of wax lumps, occasionally so numerous as to give the leaf surface a granular or irregular appearance; irregularities in the stomatal rimas; loss of fullness in the guard cells; occlusion of the stomatal rima by wax granules. The effects noted on pine needles sprayed with surfactant were: disactivation of the stomata due to the degeneration of the epistomatal wax microtubule network, often caused by the formation of amorphous plaques of fused wax and occasionally by the development of fissures; presence of lumps of fused wax inside the stomatal cavities.

In the case of European beech samples, stomatal damage indicated a considerable and statistically significant increase of SDI after treatment with surfactants (Table 2). Transpiration in seedlings sprayed with ABS increased significantly as compared to controls, but only on the occasion of the second measurement (Table 2). In the October survey, transpiration was lower than that recorded in September.

Trees declining naturally in the field

In the case of the three species (holm oak, European beech and stone pine) for which samples were collected from trees presenting symptoms of decline, we noticed the presence of marked alterations both in the stomata and in the epicuticular wax structures. The symptoms observed were tendentially similar to those described above and caused by artificial treatment with pollutants, although they were both more intense and more severe (Figures 2, 3 and 4). In addition to the symptoms caused by artificial treatments, in European beech leaves we also observed an irregular depression of the epidermal layer; in holm oak leaves the presence of large quantities of particulate matter stuck between the hairs covering the abaxial surface; and on the pine samples the formation of a surface layer of amorphous wax as well as the complete deformation of the stomata.

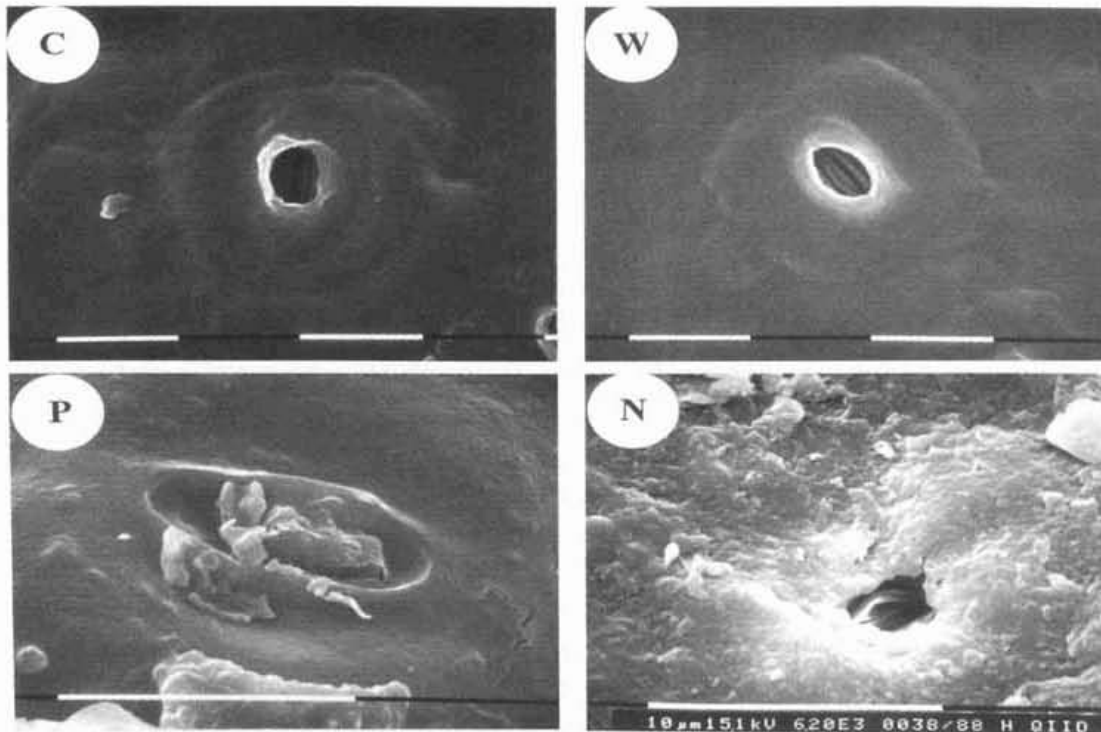


Fig. 2 – Holm oak stomata (bar = 10 μm); C: from a seedling sprayed with demineralized water; W: from a seedling treated with water stress; P: from a seedling sprayed with a pH 3.5 + surfactant solution; N: from a declining tree in the open field

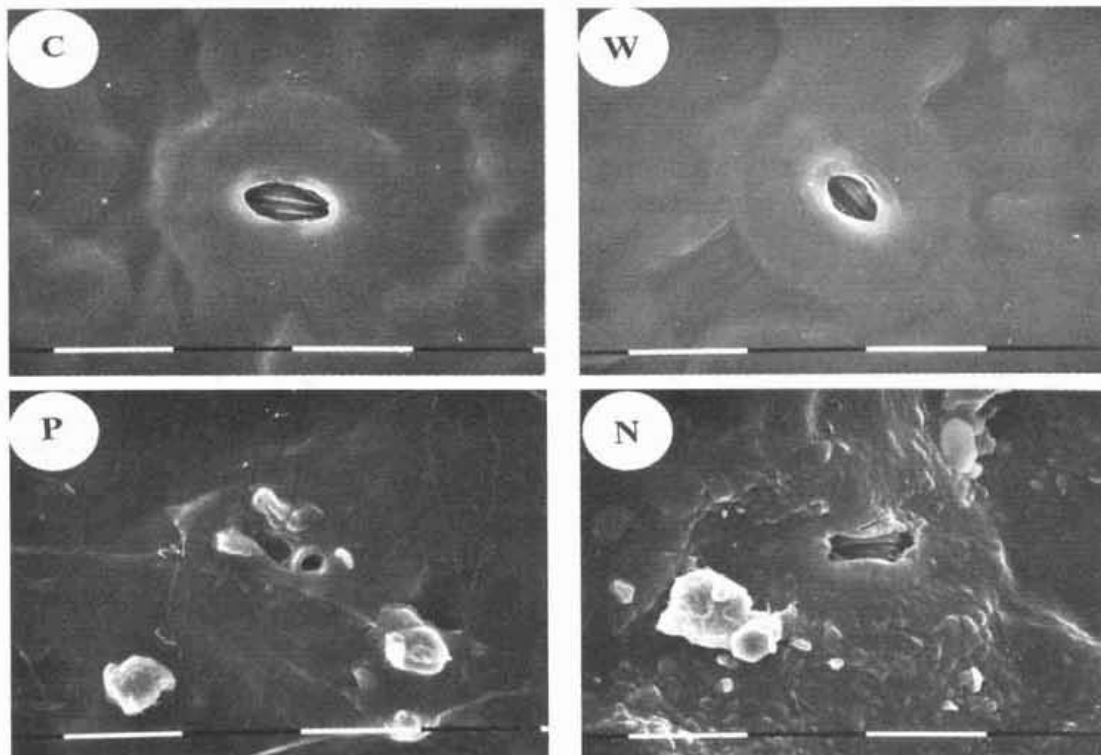


Fig. 3 – European beech stomata (bar = 10 μm); C: from a seedling sprayed with demineralized water; W: from a seedling treated with water stress; P: from a seedling sprayed with a surfactant solution; N: from a declining tree in the open field

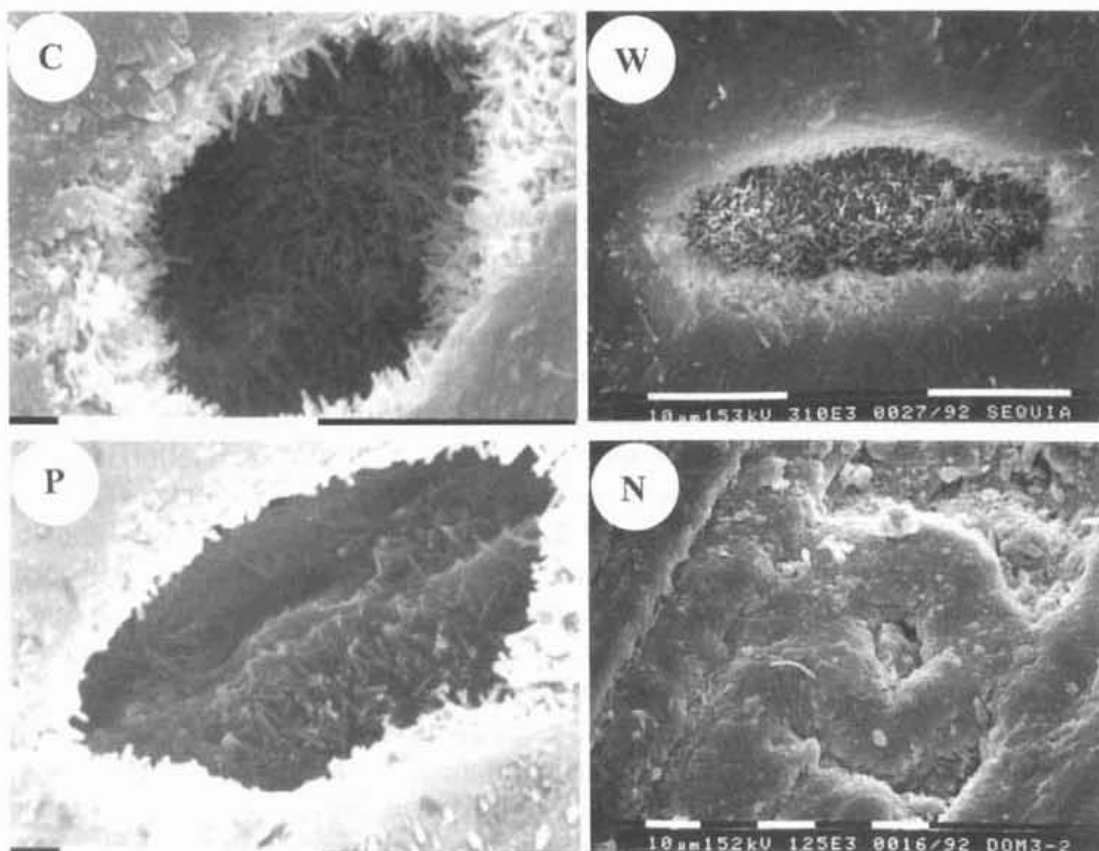


Fig. 4 – Italian stone pine stomata (bar = 10 μm); C: from a seedling sprayed with demineralized water; W: from a seedling treated with water stress; P: from a seedling sprayed with a surfactant solution; N: from a declining tree in the open field

DISCUSSION

There are many stress factors which may affect the crown status of Mediterranean region trees. We chose to study the effect of drought and of some atmospheric pollutants (acid rain and surfactants) on the structure of epicuticular wax and stomata. And in fact, Turunen and Huttunen (1990) considered the condition of epistomatal wax structures a good indicator of atmospheric pollution, although Gunthardt-Georg *et al.* (1994) suggested that atmospheric pollution is only one of the many factors involved in wax structure degeneration. Since there is a lack of studies on the specific sensitivity of broadleaf wax structures (Gellini *et al.*, 1987; Rinallo and Raddi, 1989a,b; Moricca *et al.*, 1993, 1994; Neinhuis *et al.*, 1994), we felt the need for further investigations on this topic.

Seedlings of all species studied, sprayed with acid pollutants and/or surfactants displayed evident structural alterations in the epicuticular wax and the stomata, and this was in keeping with the observations of other authors for conifer species (Turunen and Huttunen, 1990). But seedlings forced to grow in conditions of severe water stress did not present any visible alterations, except for some fissures in Italian alder, a species which has a very thick wax layer. Our observation of samples

collected from declining trees growing in the open field revealed severe damage in the surface structures. This damage was not totally identical to that reproduced with artificial treatments, but this was only to be expected since there are very complex interactions at play in nature and the various stress factors can act synergistically. Clearly the sampling carried out in this study cannot be taken as a monitoring survey, and the microstructural damage observed in the leaves of declining trees in no way authorizes us to suggest that decline in the Mediterranean area is always associated with alterations of the surface wax. However, as far as stone pines are concerned, alterations comparable to those observed by us and those reported by Turunen and Huttunen (1990) have also been reported in the case of several *Pinus pinea* Mediterranean pinewoods: they are normally attributed to the action of atmospheric pollutants (Manes *et al.*, 1988; Bussotti *et al.*, 1991; Grossoni *et al.*, 1991; Altieri *et al.*, 1994), of marine salt (Bussotti *et al.*, 1995d) and surfactants contained in sea aerosol (Gellini *et al.*, 1983, 1985; Raddi *et al.*, 1994; Bussotti *et al.*, 1995d). Bussotti *et al.* (1995b) have shown that micromorphological alterations in European beech wax structures can also occur as a result of the development of new layers, and not as a consequence of erosion processes, as is the case in atmospheric pollution (Turunen and Huttunen, 1990): the end result is an increased thickness of the epicuticular wax layer which can be interpreted as a xeromorph adaptation.

The findings relating to the transpiration in the ABS-treated seedlings appeared to indicate an increase as temperature and RH were lower, i.e. in the October measurements. Artificial treatments with pollutants, such as acid rains (Hogrebe and Mengel, 1989; Leonardi and Flückiger, 1989), O₃ (Keller and Hasler, 1984; Barnes *et al.*, 1990), or SO₂ (Mansfield and Freer-Smith, 1984), have been proved to induce an increase of transpiration or conductance in forest trees, although contradictory findings have also been reported (Darrall, 1989). Koch and Maier-Maercker (1986) carried out a long-term experiment with low concentrations of SO₂ and showed that, after an initial stage in which transpiration is suppressed, there is another stage when transpiration is high but erratic (i.e. the response varies according to the prevalent humidity in the air); this, in turn, is at times followed by a third stage with low rates of transpiration and visible symptoms of damage. This can explain because a significant difference between our control and pollutant-treated seedlings is evident only under the less favourable environmental conditions.

The quantification of stomatal damage and the transpiration measurements indicated an increase in transpiration as stomatal damage increases, accordingly with the findings on conifer species artificially treated with air pollutants (e.g. Mengel *et al.*, 1989). Data recorded on silver fir growing in the open field also indicated an increased transpiration in the more declining trees (Paoletti and Gellini, 1992).

CONCLUSIONS

Among the various factors capable of exerting a negative influence on the crown status of Mediterranean region trees, the frequency of droughts may contribute to the decline ascertained in field surveys. It is already an accepted fact

that there is no well-defined main stress factor solely responsible for forest decline (BMFT-Journal, 1987). It is therefore unlikely that forms of decline will ever be explained as being caused by a single mechanism of action, although a variety of attempts have been made to do so. To date, about twenty or so mechanisms have been suggested as being responsible for this phenomenon (Tributsch, 1992), but none appears capable of functioning within the context of all the manifold causes involved, which furthermore depend also on time, location and species.

Restricting the field to the effects of drought and surfactant or acid rain pollution on the surface leaf structures, based on the results of this study, it is in any case possible to state that the appearance of micromorphological alterations of the stomata and the epicuticular wax in 1-year old leaves of trees in the field cannot be considered an effect due exclusively to severe drought. Further studies will be needed in order to verify the response of epicuticular wax to recurring moderate droughts and also to ascertain whether water stress can act in synergy with other stress factors in inducing physiological modifications. The water-cohesion-tension insufficiency syndrome, for example, states that forest decline is the product of dry pollution precipitation (particulate matter, ozone, hydrogen peroxide, acidic gases) and water stress of trees (Tributsch, 1992). Our finding showing that transpiration tends to increase as stomatal damage increases stresses once again the need for studies on the combined action of drought and pollutants on Mediterranean trees.

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