The Physiological Function of Epicuticular Wax in Coastal Douglas-Fir (*Pseudotsuga menziesii* (Mirb.) Franco)

by

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ABSTRACT

The surface of conifer needles is covered by amorphous and crystalline epicuticular wax and, in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), the crystalline wax completely occludes the stomatal antechambers. It has been proposed that epicuticular wax limits cuticular water loss and provides an additional resistance to stomatal gas exchange. Under desiccating conditions, the wax may be a significant antitranspirant, but it may also act as a barrier to CO_2 uptake thereby limiting photosynthesis. Experiments were carried out to determine the effects of epicuticular wax on cuticular resistance and gas exchange through the stomata.

Experiments were performed during the summers of 1993 and 1994 on coastal Douglas-fir seedlings grown in pots under ambient conditions. Epicuticular wax was removed from entire branches by applying a fast-setting dental impression compound to the needles, heating to 32°C and then gently removing the compound with tweezers. Treated needles remained green and physiologically active and electronmicrographs showed that the wax could be removed from stomatal antechambers without damage to the underlying tissue. Further examination of treated needles showed that epicuticular wax was not regenerated over time. Measurements of photosynthesis (A), stomatal conductance (g_s), internal CO_2 concentrations (c_i) and instantaneous water use efficiency

(WUE, the rate of A to transpiration, E) were then made using a portable open gas exchange system. It was expected that the increase in g_s due to stomatal antechamber wax removal would produce two results: 1) an increase in A coupled with a decrease in WUE or 2) since stomata are very sensitive to changes in c_i, stomata would partially close to compensate for the increase in c_i. In other words, there would be no measurable change in the physiology of the treated needles. To determine the effect of changes in c_i on g_s, the gas exchange of a whole seedling was measured in a computer controlled whole plant cuvette.

Changes in cuticular resistance (r_c) due to wax removal were measured by suspending needles over saturated salt solutions of a known molality. Water loss over time was then determined gravimetrically.

Electronmicrographs showed that the wax removal technique produced two results. On some seedlings, greater than 50% of stomata were clear of stomatal antechamber wax, while on others, very little wax was removed. In the latter case, the wax crystals were smeared into an amorphous mass. When wax was removed from more than 50% of stomata, there was no significant change in g_s and A before and after treatment. In contrast, unsuccessful wax removal resulted in a decrease in both g_s and A.

The whole-plant measurements showed that Douglas-fir seedling regulated g_s in an attempt to maintain c_i constant as ambient CO_2 concentration (c_a) was increased (dc_i/dc_a was 0.4).

Epicuticular wax removal resulted in an overall decrease in r_c in all but one of the seedlings. Generally, needles with the greatest degree of wax removal from stomatal antechambers had the most significant reduction (17 to 40%) in r_c.

In conclusion, successful removal of stomatal antechamber wax has shown that the wax does not limit productivity in coastal Douglas-fir. In fact, it appears that the tree compensates for the loss of the physical resistance of the wax by adjusting its physiological resistance or by partially closing its stomata. However, the cuticular resistance measurements illustrate the importance of epicuticular wax, particularly amorphous wax, in limiting water loss through the cuticle.

Although it was not investigated in this study, the crystalline wax covering the rows of stomata may be essential for reducing wettability of needle surfaces thereby preventing adhesion and germination of fungal spores.

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LIST OF SYMBOLS AND ABBREVIATIONS

A	photosynthetic rate (μmol m^{-2} s^{-1})
a_l	leaf area (cm^2)
c_a	ambient CO_2 concentration (μmol mol^{-1})
c_e	CO_2 concentration entering leaf chamber (μmol mol^{-1})
c_i	intercellular CO_2 concentration (μmol mol^{-1})
c_o	CO_2 concentration entering leaf chamber (μmol mol^{-1})
e_a	vapour pressure of air (g cm^{-3})
e_s	saturated vapour pressure (g cm^{-3})
E	transpiration (mol m^{-2} s^{-1})
FW	fresh weight (g)
g_c	cuticular conductance (mol m^{-2} s^{-1})
g_{ca}	stomatal conductance to CO_2 (mol m^{-2} s^{-1})
g_s	stomatal conductance to water vapour (mol m^{-2} s^{-1})
J_v	flux density (g m^{-2} s^{-1})
M	mass (g)
M_d	dry weight mass (g)
M_t	mass at turgor (g)
r_c	cuticular resistance (m^2 s mol^{-1})
r_{cw}	resistance of wax covering cuticle (m^2 s mol^{-1})
r_i	intercellular resistance (m^2 s mol^{-1})
r_m	mesophyll resistance (m^2 s mol^{-1})
r_{sw}	resistance of wax occluding stomatal antechamber (m^2 s mol^{-1})
r_s	stomatal resistance (m^2 s mol^{-1})
r_v	resistance to diffusion (s m^{-1})
r_w	epicuticular wax resistance (m^2 s mol^{-1})
RWC	relative water content
SEM	scanning electron microscope
TEM	transmission electron microscope
u	flow rate (μmol s^{-1})
w	mole fractions of water in air (mol mol^{-1})
w_e	mole fractions of water entering leaf chamber (mol mol^{-1})
w_o	mole fractions of water leaving leaf chamber (mol mol^{-1})
WUE	instantaneous water use efficiency
Δc_v	vapour pressure difference (g m^{-3})
ψ	water potential (MPa)

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This thesis is dedicated to my husband, Shawn.

Thank you for listening.

1. INTODUCTION

1.1 The cuticle and epicuticular wax

The primary aerial surfaces of plants are bound by a thin continuous membrane, the cuticle, which acts as a protective and impermeable barrier between the plant and the atmosphere. The cuticular membrane is composed of cutin, a polymer of hydroxyfatty acids (Holloway 1982b), and a complex mixture of very long chain lipids or cuticular wax is embedded within the cutin matrix (Baker 1982).

Based on observations made using a transmission electron microscope, Holloway (1982a) gives a thorough review of plant cuticles. In many plant species the outermost region of the cuticle, commonly referred to as the cuticle proper, is composed of only cutin and cuticular wax. In transmission electron microscopy sections, this layer may appear either amorphous or lamellate with alternating electron dense and lucent bands. The cuticle proper is especially well defined in xerophytic and evergreen species. Beneath the cuticle proper is one or more cuticular layers which frequently form the bulk of the cuticular membrane. The cuticular layer is derived from the epidermal cell wall, but since it is incrusted with cutin and cuticular wax, it is included as part of the cuticle. Fibrillae may originate from the cell wall and form a network throughout the cuticular layer thus giving it a reticulate appearance. Finally, at the junction between the cuticle and the epidermal cell wall, there is often a pectin lamella that stains intensively with ruthenium red. Since this layer also contains cutin it, too, is considered part of the cuticle. Although the aforementioned layers are characteristic of the cuticles of many plant species, there is no typical plant cuticular membrane.

The outermost surface of the cuticle is often covered by another layer of wax referred to as epicuticular wax. Like the cuticle, this wax may provide a significant resistance to water loss through the leaf surface and, in some plants, through the stomata. It can also act as a physical or chemical barrier to insects and fungal pathogens, prevent leaching of nutrients and provide protection from UV radiation and air pollutants (Gates *et al.* 1965; Martin & Juniper 1970; Clark & Lister 1975; Dickson & Preece 1976; Juniper & Jeffree 1983; Turunen and Huttunen 1990).

Although it is sometimes difficult to separate the properties of cuticular from epicuticular waxes, most of the following discussion will focus on epicuticular wax. Examples will include many representative plant species, but emphasis will be placed on conifers.

1.2 Wax morphology

There are two morphologically distinct types of epicuticular wax; amorphous and crystalline. The amorphous wax is exuded as a structurally uniform coating, while the crystalline wax is frequently superimposed on this amorphous layer. The structure of crystalline wax varies considerably from species to species and even within the same plant. The most common crystalline waxes are plate waxes of varying size and shape and tubular waxes that may be partially embedded in the amorphous wax or project outward from the cuticle surface (Baker 1982). This latter type tends to predominate on the surface of conifer needles (Jeffree *et al.* 1976). Extremely delicate ribbons, rodlets and filaments have also been observed on a variety of plant species (Baker 1982), while *Brassica* spp. grown under high light and temperatures produce large interlocking dendrites (Hall *et al.* 1965; Baker 1974).

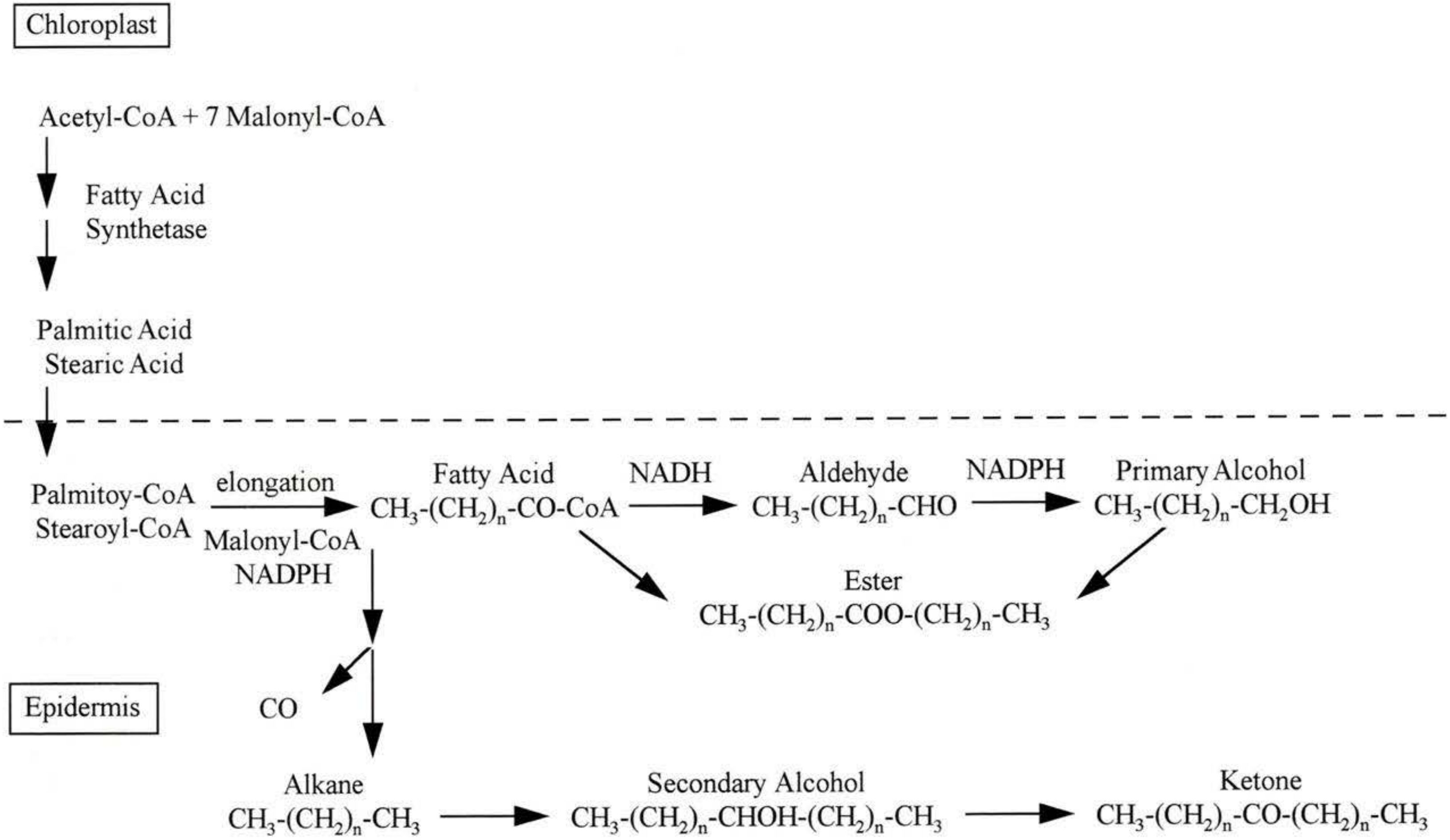
1.3 Wax chemistry

Epicuticular waxes are predominantly composed of homologous series of long-chain lipids including alkanes, alcohols, aldehydes, acetals, esters and fatty acids (Eglinton & Hamilton 1967; Kolattukudy 1980; Walton 1990; von Wettstein-Knowles 1993). However, analyses of waxes are often complicated by the occurrence of rare constituents, varying positions and numbers of functional groups and varying degrees of chain branching and unsaturation (Eglinton & Hamilton 1967). The chain lengths of the lipid homologs generally range from C_{21} to C_{37} with alkanes, ketones and secondary alcohols having mostly odd-carbon-numbered chains and free fatty acids, aldehydes and primary alcohols having a greater proportion of even-carbon-numbered chains (Eglinton & Hamilton 1967; Martin & Juniper 1970; Tullock 1976; Baker 1982). Other common wax lipids include terpenoid compounds and, in conifers, bifunctional lipids such as α-ω-diols and ω-hydroxyfatty acids that are free or in the form of estolides (polymers of four to six molecules) (Gülz 1994; Kolattukudy 1980). Gülz (1994) noted that there appears to be an evolutionary trend in wax chemical composition. Conifer wax is primarily composed of homologous series of long-chain fatty acids, the secondary alcohol, nonacosan-10-ol and bifunctional lipids. In contrast, angiosperms, which are more evolutionary advanced, appear to have a more diverse array of wax constituents including a number of compounds that are entirely absent in conifers.

1.4 Wax biosynthesis

The biosynthesis of the long-carbon skeletons of wax lipids involves the chain elongation of the fatty acids palmitic acid (C_{16}) and stearic acid (C_{18}) which originate in the chloroplasts of mesophyll cells (Ohlrogge *et al.* 1993; von Wettstein-Knowles 1993). The synthesis of these short-chain precursors takes place in the presence of light and an

Figure 1. Pathway of wax biosynthesis (adapted from Kolattukudy 1980).


Chloroplast
Acetyl-CoA + 7 Malonyl-CoA
Fatty Acid Synthetase
Palmitic Acid
Stearic Acid
Palmitoy-CoA
Stearoyl-CoA
elongation
Malonyl-CoA
NADPH
Fatty Acid
CH_3-$(CH_2)_n$-CO-CoA
NADH
Aldehyde
CH_3-$(CH_2)_n$-CHO
NADPH
Primary Alcohol
CH_3-$(CH_2)_n$-CH_2OH
Ester
CH_3-$(CH_2)_n$-COO-$(CH_2)_n$-CH_3
Epidermis
CO
Alkane
CH_3-$(CH_2)_n$-CH_3
Secondary Alcohol
CH_3-$(CH_2)_n$-CHOH-$(CH_2)_n$-CH_3
Ketone
CH_3-$(CH_2)_n$-CO-$(CH_2)_n$-CH_3

enzyme complex of elongases, soluble fatty acid synthetase, which promotes the repetitive joining of activated C_2 units to produce C_{16} and C_{18} (Stryer 1988; Ohlrogge *et al.* 1993; von Wettstein-Knowles 1993). The fatty acids are then passed on to the epidermal cells where, in the form of carbonyl acyl chains, they are elongated by membrane-bound elongases in the endoplasmic reticulum (Kolattukudy 1968; Kolattukudy & Buckner 1972; Cassagne & Lessire 1978). Elongation occurs through the sequential addition of two carbon units where malonyl-CoA and NADPH act as an elongating agent and reductant, respectively (Kolattukudy 1976; Kolattukudy 1980).

The aliphatic chains generated by the elongation process are further modified to form various compounds. A fatty acyl-CoA reductase reduces fatty acids to aldehydes using NADH, while an aldehyde reductase catalyzes a further NADPH-dependent reduction of the aldehyde to a primary alcohol (Khan & Kolattukudy 1975). The similarity between naturally occurring aldehydes and primary alcohols in plant waxes suggests that aldehydes are intermediates to alcohol synthesis (Kolattududy 1980). Primary alcohols may then be esterified with acyl-CoA to produce wax esters (Kolattukudy 1967). Aldehydes are also intermediates in alkane synthesis. A cobalt-porphyrin enzyme has been associated with the decarbonylation of an aldehyde to yield its corresponding alkane and carbon monoxide (CO) (Dennis & Kolattukudy 1992). Secondary alcohols and ketones occur with alkanes of similar size thus suggesting that oxygenation of the alkanes produces secondary alcohols, whereas oxidation of the secondary alcohols generates ketones (Kolattukudy *et al.* 1973).

1.5 Routes and mechanisms of wax formation

Once synthesis of the long-chain lipids in the epidermal cells is complete, the lipids are transported and deposited on the surface of the cuticle. As early as 1871, de Bary

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proposed that wax was exuded to the surface through pores that traversed the cuticle (Hallam 1982). Hall and Donaldson (1962, 1963) and von Wettstein-Knowles (1974) supported this theory and suggested that the number and types of pores would also influence the morphology and distribution of the wax. However, this theory is improbable for a number of reasons; pores that traverse the cuticle have not been clearly identified, a large pressure gradient would have to exist between the leaf surface and epidermal cells and the pores would have to be exceedingly complex to produce the elaborate wax crystals found on some plant surfaces (Hallam 1982). A more widely accepted explantation is the crystallization theory of wax origin (Jeffree *et al.* 1976; Hallam 1982). This theory proposes that as the wax components move across the plasmalemma of the epidermal cells, they are enveloped by lipo- or glyco-protein "shells" which, presumably, transport the waxes across the water phase of the epidermal cell wall. In TEM preparations made during wax development, these "shells"appear to be the electron-dense vesicles seen accumulating in the vicinity of the plasmalemma nearest the cuticle (Hallam 1982). When the vesicles reach the surface of the cuticle, they burst, thereby liberating the wax lipids, which then crystallize into characteristic shapes.

1.6 Chemical control of wax morphology

A number of studies have verified the fact that the chemical composition of the wax is the primary factor controlling the wax morphology. To illustrate this, Jeffree *et al.* (1975) performed an elagant experiment where epicuticular waxes were dissolved in organic solvents and then, using a wick feed method, the waxes were recrystallized on artificial porous surfaces. Waxes isolated from a wide range of species recrystallized into forms similar to those occurring naturally on plant leaves. This indicated that the size and shape of the wax crystals were influenced by the chemical composition of the wax and not by the physico-chemical properties of the cuticle.

Results from other recrystallization experiments have revealed a close correlation between discrete components of the wax and the crystal morphologies that they form. For example, observations of *Eucalyptus* and cereal waxes have suggested that primary alcohols crystallize as plates and β-diketones as thin tubes (Lundqvist *et al.* 1968). Von Wettstein-Knowles (1974) proposed that ketones produce the dendrite wax crystals on leaves of *Brassica* spp., while Gülz (1994) noted that at a concentration of 23 % or more, hentriacontan-16-one formed crystals in the shape of transversally ridged rodlets. Short stubby tubular projections, such as those observed on the surfaces of conifer needles, are evidently associated with the presence of nonacosan-10-ol in the wax (Jeffree *et al.* 1976; Günthard-Goerg 1986). In general, it appears that if there are several homologues within a particular class of wax lipids, there is a depression in the melting point of these wax constituents such that they have a fluid consistency typical of amorphous wax (Gülz 1994). In contrast, if a discrete component of the wax is present in greater concentrations or with few homologues (such is the case for nonacosan-10-ol in conifers), it will form wax crystals (Martin & Juniper 1970; Jeffree *et al.* 1976; Kolattukudy 1976; Baker 1982).

1.7 Environmental control of wax morphology

Although wax morphology is primarily a function of wax chemistry, there is substantial evidence that the environment exerts some control over the morphology and amount of wax deposition on leaves. The most important environmental factors are temperature and irradiance (Jeffree *et al.* 1976). For example, *Pisum sativum* leaves grown in darkness have smooth surfaces with very little wax development, whereas when grown under lights, the dimensions and overall density of wax crystals increases with increased irradiance (Juniper 1960). These results along with the observation that at least 20% of natural sunlight is required for normal wax development (Hallam 1970) are predictable since light is required for the production of stearic and palmitic acid, the precursors of wax synthesis.

In general, temperature changes appear to affect the dimensions of waxes and increased temperatures tend to augment the waxiness of leaves (Whitecross and Armstrong 1972; Baker 1974). An exception to this latter case are certain *Eucalyptus* species which require a mild frost in order to induce glaucousness (Barber 1955). Baker (1974) provided a very striking example of how the environment affects wax morphology. Under a moderate light and temperature regime, *Brassica oleracea* produced tubular waxes, but when the irradiance and temperature were increased the wax began to crystallize as dendrites over the tops of the underlying tubes. Other environmental factors which alter wax deposition include photoperiod, humidity, elevation, soil water and air pollutants (Hanover & Reicosky 1971; Baker 1974; Giese 1975; Jeffree *et al.* 1976; Bengston *et al.* 1978; Baker 1982; Hunt & Baker 1982; DeLucia & Berlyn 1984; Bacic *et al.* 1992).

While the environment exerts considerable control over the morphology and distribution of waxes, it appears to have a minimal effect on the chemical composition of the wax (Jeffree *et al.* 1976; Walton 1990). However, changes in light alter the chain lengths of wax constituents (Giese 1975) and even increase the proportions of some classes of homologs (Steinmuller and Tevini 1985). Moreover, it has been suggested that the effect of the environment is most evident in waxes composed of a complex mixture of wax compounds (Jeffree *et al.* 1976).

Little information is available regarding the influence of the nutrient status of plants on epicuticular wax production. Chiu *et al.* (1992) found that *Pseudotsuga* *menziesii* stands fertilized with nitrogen and potassium exhibited an increase in glaucousness due to altered wax morphology. Ylimartimo *et al.* (1994) found a significant reduction and morphological change in wax due to deficiencies in potassium, calcium and magnesium and excess nitrogen. Since waxes are secondary metabolites, their production is especially sensitive to both the environmental conditions and nutrient regime in which the plant is grown.

1.8 Seasonal and ontogenic changes in wax

The morphology and chemical composition of epicuticular wax tends to be a stable characteristic of plants, however there may be some seasonal and developmental changes. Von Rudloff (1959) noted that needles collected from *Picea pungens* during spring and summer months had a higher acidic fraction. Also, Schutt and Schuck (1973) observed seasonal changes in the wax composition of *Pinus sylvestris* needles and found variations in the proportions of major hydrocarbons, primary alcohols and esters. When studying the developmental variation of leaf surface wax, Freeman *et al.* (1979) found a series of changes in the major wax constituents of *Vaccinium ashei*. During leaf expansion, β-diketones gradually became the primary wax components, but once expansion was complete, there was a steady reduction in the β-diketones relative to a concommitant enrichment of terpenoid acids and hydrocarbons. These chemical changes correlated with changes in wax morphology starting with amorphous wax and then followed by extensive rodlet formation due to increased β-diketones during leaf expansion. Finally, the abundance of crystals declined as the β-diketones decreased. The changes observed in the chemical composition of the waxes are probably not the result of loss or degeneration of one wax component, but rather the increase or *de novo* synthesis of other wax compounds which alters the relative abundance of each. *De novo* synthesis of wax components has been documented in several plant species. For example, when 49% of epicuticular wax is composed of β-amyrenyl acetate, quadrangular rodlets form on the surface of *Tilia* leaves (Gülz *et al.* 1991). Initially, as the leaflets unfold, there is no β-amyrenyl acetate synthesis thus the leaves are covered by only amorphous wax, but within ten days, *de novo* synthesis of β-amyrenyl acetate begins and wax crystals are observed.

In conifer species, epicuticular wax deposition begins very early in needle development. Günthardt-Goerg (1987) studied the changing composition of wax during leaf expansion and suggested that wax was secreted in sequential layers each with a slightly different chemical configuration. Although it is unclear whether the amorphous wax appears first, both amorphous and crystalline waxes are evident in stomatal antechambers while buds are still expanding (Reicosky & Hanover 1976; Günthardt-Goerg 1986). Once a needle is exposed to the atmosphere, amorphous wax develops over the entire needle surface and crystalline wax concentrates in the stomatal regions where it often occludes stomatal antechambers.

It is apparent that several months following complete leaf elongation, epicuticular wax synthesis ceases (Hall & Jones 1961; Hallam 1970; van Gardingen *et al.* 1991). During the first vegetative period of conifer needles, there is an increase in wax coupled with changes in its chemical composition (Günthardt-Goerg 1986). However, from November to March there is no change in the wax composition thus indicating that by the onset of winter, wax synthesis is complete (Günthardt-Goerg 1987). These results are supported by the observation that there is no *de novo* synthesis of wax when it is damaged (Hall & Jones 1961; Reicosky & Hanover 1976; van Gardingen *et al.* 1991). If epicuticular wax is removed, it will only be regenerated if the leaf is still expanding, but it will never achieve the thickness of undamaged areas (Juniper & Jeffree 1983).

As leaves age, there is a gradual deterioration of crystalline waxes; crystals begin to fuse and become more amorphous. Of course the rate of the aging process varies from species to species and also greatly depends on environmental conditions. Factors such as wind abrasion, rain, pollutants and leaves bending and rubbing together help to accelerate wax degradation (Hall & Jones 1961; Hall 1966; Thompson 1974; Pitcairn & Grace 1984; Baker & Hunt 1986; Crossley & Fowler 1986; Crossley 1988; Hadley & Smith 1983). There have been studies indicating that the smoothed epicuticular wax of conifer needles recrystallizes (Bacic *et al.* 1992, 1994), however, this phenomenon appears to be most evident in younger needles and not rapid enough to counteract the natural aging process.

1.9 Physiological function of epicuticular wax

1.9.1 The reflection of light

The crystalline nature of the epicuticular wax is responsible for the light reflecting properties of the leaves of some plant species. The degree of glaucousness is a function of the structure, arrangment and density of the wax crystals and can vary considerably over the surface of a leaf (Hall *et al.* 1965; Martin & Juniper 1970). It has been suggested that glaucousness is important for those species growing in environments where high irradiance, including UV-B, can damage photosynthetic tissue. For example, Gates *et al.* (1965) noted that desert species exhibit greater light reflection than mesic species. Also, in conifers, increased glaucousness is correlated with increased reflection of light in the blue and UV spectra (Reicosky & Hanover 1978; Clark & Lister 1975). Not only would this provide protection from UV radiation, but it may also be important for conifers growing at alpine timberlines where intense sunlight can increase the temperature of needles and, thus, the evaporative demand (Marchland & Chabot 1978). In contrast, increased reflection in the photosynthetic action spectra may have a detrimental effect by limiting the light available for photosynthesis. For example, Clark and Lister (1975) found that in conifer species with a range of glaucousness, photosynthetic activity in the blue spectrum decreased with increasing blueness of the foliage. In addition, smoothing the epicuticular wax of *Eucalyptus* species resulted in higher photosynthetic rates under low irradiance (Cameron 1970). Therefore, the advantages and disadvantages conferred by light reflection are largely dependent on the plant's environment.

However, the leaves of many plant species only have glaucous wax on the abaxial surface. In this instance it seems unlikely that the wax reflects potentially harmful radiation.

1.9.2 Interaction with fungi

The problems associated with fungal infections have focused considerable attention on modes of resistance to fungal invasions in plants. The principle means of defense against infection are at the cellular level, but since fungi may penetrate through either the cuticle or stomata, the epicuticular wax on the surface of leaves could potentially act as an initial barrier to attack. For a fungal spore to adhere and then germinate on a leaf, it must be in a moist environment (Dickson & Preece 1976). However, depending on the chemical composition and ultrastructure of the epicuticular wax, the leaf surface may be very unwettable. For example, if the wax has a high proportion of alkanes and a crystalline structure that forms a rough surface, the contact angles of water droplets will be high and the water-holding capacity of the leaves very low (Martin & Juniper 1970; Turunen & Huttunen 1990). In conifer needles, the epicuticular wax in stomatal antechambers may act as an additional barrier to fungal penetration of the stomata. In fact, several authors have noted an increase in the numbers of fungal hyphae penetrating the cuticle and stomata of needles with wax that had been eroded and cracked by air pollutants (Huttunen 1984; Grill *et al.* 1987).

There is also some evidence that epicuticular wax contains fungal inhibitors (Martin & Juniper 1970; Lampard & Carter 1973; Hargreaves *et al.* 1982), however, the results are still varied and somewhat inconclusive.

1.9.3 Interaction with pollutants

Since the 1980's, an accumulation of experimental evidence has indicated a strong correlation between air pollutants and the decline of some coniferous species in Europe and North America (Crossley & Fowler 1986; Sauter & Voβ 1986; Cape *et al.* 1989; Hasemann *et al.* 1990; Turunen & Huttunen 1990). In order for damage to occur, the pollutants must first enter the leaves through either the cuticle or stomata and then interact with the mesophyll and guard cells (Weber *et al.* 1994). However, because the cuticle and epicuticular waxes provide a significant resistance to gas exchange, the pollutants tend to diffuse primarily through the stomata (Weber *et al.* 1994). Thus, differences in stomatal and cuticular conductances between conifer species may account for some of the variation in sensitivity to pollutants.

Exposure to air pollution often has a visible impact on epicuticular wax because the pollutants can cause accelerated degradation of the crystals in a pattern which is similar to aging (Cape & Fowler 1981; Cape 1983; Huttunen & Laine 1983; Riding & Percy 1985; Crossley & Fowler 1986; Percy & Baker 1990). The fusion of the tubular projections initially occurs in stomatal antechambers, but, over time, wax on the rest of the needle surface becomes eroded. Not only are these effects present in all age classes of needles (Huttunen & Laine 1983), but the severity of wax degradation is directly proportional to the concentration of pollutants (Turunen & Huttunen 1990). In some instances only the outer layers of crystalline wax are eroded suggesting that there is a direct interaction between waxes and pollutants (Percy and Baker 1988). However, exposed needles often exhibit depressed wax synthesis (Cape 1986) and altered wax composition (Trimble *et al.* 1982; Cape 1986; Percy & Baker 1990; Percy *et al.* 1990) due to the adverse effects of the pollutants on the general viability of the tree.

Modification of epicuticular wax may have several effects on conifer physiology. Smearing and fusing wax tubules in the stomatal antechambers could conceivably increase stomatal resistance by reducing the cross-sectional area of the stomatal antechamber and increasing the tortuosity of the pathway for diffusion (Barnes *et al.* 1988). As a result, there would be an increased resistance to diffusion of air pollutants into the leaves, however, there would also be less CO_2 uptake and lower photosynthetic rates (Riding & Percy 1985). Evidence for the latter case was presented by Sauter *et al.* (1987) who noted that in conifers treated with automobile emissions, photosynthesis was reduced by 30% when approximately one-third of the stomata were blocked. Unfortunately, these results were confounded by the possibility of direct damage to the leaf tissue by the pollutant. In contrast, severe degradation of the wax in stomatal antechambers may cause a decrease in stomatal resistance if the damage leads to cracking or complete erosion of the wax "plugs" or if the smeared wax prevents total stomatal closure (Riding & Percy 1985; Mengel *et al.* 1989).

The depression of wax synthesis together with the erosion of the epicuticular wax by air pollutants can result in decreased cuticular resistance (Cape & Fowler 1981; Mengel *et al.* 1989). This is particularly important in times of water stress (Huttunen & Laine 1983).

Air pollution damage to epicuticular wax is also detrimental if it results in increased wettability of the needle surfaces (Leyton & Juniper 1963; Cape 1983). An increase in water retention coupled with a decrease in cuticular resistance may lead to leaching of nutrients from needles especially when the pollutant is acid rain. For instance, a film of acid rain on a needle surface induces a complex process that involves the release of hydrogen ions in exchange for cations in the cuticle (Turunen & Huttunen 1990). The gradual loss of salts from the needles causes membrane damage through loss of buffering capacity and this , in turn, augments the leaching of nutrients from the leaves (Adams & Hutchinson 1984).

Increased wettability of a needle surface can also make a needle more susceptible to fungal invasion since water retention and leaching of nutrients on the surface creates a favourable environment for the adherence and germination of fungal spores (Huttunen 1984). Likewise, the cracking and erosion of the epicuticular wax in stomatal antechambers removes yet another physical barrier to infection (Huttunen 1984).

1.9.4 The control of gas exchange

In order to photosynthesize and grow, all plants must take up CO_2 and expose a large surface area to intercept sunlight. However, for terrestrial plants this poses a challenge since increased surface area and openings for gas exchange increase water loss to the surrounding air.

Gas exchange in leaves occurs through two parallel pathways; the stomata which provide a variable resistance since guard cells open and close in response to changes in turgor, and the cuticle which provides a fixed resistance and limits water vapour diffusion over the rest of the leaf surface.

The cuticle

Water loss through the cuticular membrane of a plant can be described in terms of an integrated form of Fick's first law:

$$J_V = \Delta c_V / r_V$$

where J_V is the flux density of water, r_V is the resistance to diffusion through the membrane and Δc_V is the difference in vapour concentration between the inside of the leaf and the surrounding air (Monteith & Campbell 1980). This equation assumes that the liquid-vapour transition of water occurs directly beneath the cuticle such that water vapour diffuses across the membrane as individual molecules. In this case, the resistance of the cuticle is independent of the vapour concentration.

If the resistance of the cuticle is a function of cuticle thickness, then the latter should be inversely proportional to water vapour flux, however, many studies have shown that this is not necessarily the case (Becker *et al.* 1986; Schönherr 1982). For example, Schönherr (1982) noted that despite *Allium* having the thinnest membrane of the species studied, it was the least permeable to water. Although there appears to be some correlation between cuticle ultrastructure and resistance (Gouret *et al.* 1993), research on isolated cuticles has indicated that the cuticular and epicuticular waxes are the primary barriers to water loss (Schönherr 1976, Haas & Schönherr 1979). Using isolated plant cuticles, Schönherr (1976) found that removal of these waxes resulted in a 300 to 500 fold increase in cuticular water loss.

It seems the chemical composition and arrangement of chemical components of the wax are largely responsible for the variation in cuticular resistance to water vapour diffusion between species. The long-chain lipids that are the primary constituents of waxes tend to be very hydrophobic. Studies on monolayers have shown that for every 2 -CH_2 group added to a lipid chain, the resistance of the monolayer doubles (Archer and LaMer

1955; LaMer *et al.* 1964). In fact, Holloway (1969) found that n-alkanes with no functional groups and high carbon numbers have the closest packing of surface methyl groups and are the least permeable to polar substances. However, orientation of the waxes is also important. In the case of the monolayers studied by LaMer *et al.* (1964), it was found that in order to achieve the relationship between lipid length and resistance, lipids must be oriented parallel to the surface of the membrane. In terms of structural epicuticular wax, Chambers and Possingham (1963) suggested that wax crystals with very complex shapes would also increase the cuticular resistance by increasing the tortuosity and thus the effective length of the pathway for diffusion.

There has been considerable interest in determining whether epicuticular wax, alone, provides a significant resistance to water loss through the cuticle. To date, most measurements of epicuticular wax resistance have been made on excised leaves using gravimetric measurements of water loss over time. However, it is important to ensure that stomata are fully closed. In order to achieve this, excised leaves are allowed to dehydrate in a controlled environment. Initially, stomata may be partially open, but as the tissue rapidly dehydrates, the stomata hydropassively close and evaporation rate declines and stabilizes as water loss occurs primarily through the cuticle (Hygen 1951).

Another issue associated with measuring epicuticular wax resistance is the removal of the wax without damage to the cuticle or epidermal cells. A number of methods have been employed including chemical and mechanical removal and comparative studies between glaucous plants and non-glaucous mutants. In 1957, Pfeiffer (see description in Martin & Juniper 1970) used trichloroacetate as a soil drench to inhibit the growth of surface waxes on *Pisum* leaves. This resulted in a significant increase in transpiration. Later, Grncarevic and Radler (1967) demonstrated that the addition of soluble surface waxes of sultana grapes to an artificial membrane increased the resistance of the membrane to water loss. Additional experiments have shown that removal of epicuticular waxes with organic solvents cause a substantial increase in cuticular conductance--as much as 50% to 25 times depending on the species and the solvent used (Chambers & Possingham 1963; Possingham *et al.* 1967; O'Toole *et al.* 1979). Unfortunately, these results are confounded by the fact that wax inhibitors and organic solvents also disrupt wax and lipoidal material in the underlying cuticle and epidermal cells.

Several studies of the effects of epicuticular wax erosion on cuticular conductance were prompted by the observation that plant leaves exposed to windy conditions had damaged epicuticular wax and increased cuticular conductance (Hall & Jones 1961; MacKerron 1976; Wilson 1984; van Gardingen *et al.* 1991). The pattern of wax destruction by wind is similar to that of aging--the wax crystals are flattened and fused around the periphery of the stomata and on the prominent ridges of the leaves. Hall and Jones (1961) tried to mimic the pattern of wind damage in *Pisum* by brushing the leaves with a camel hair brush. This resulted in a marked increase in the rate of cuticular water loss. In order to determine how wind causes damage to the wax, Grace (1974) placed *Festuca* plants in an artificial wind tunnel where he noted an increase in cuticular as well as stomatal conductance. He attributed the increase in cuticular conductance to gradual damage to the epicuticular wax and cuticle by windborne particles and the continual buffetting of the leaves against one another. To explain the increase in stomatal conductance, he suggested that the abrasion of leaf surfaces might rupture the epidermal cells around the guard cells and disrupt the proper functioning of the stomata. This hypothesis is supported by Pitcairn *et al.* (1986) who noted that compared to abrasion of *Festuca* leaves, rubbing the leaves caused a greater increase in cuticular conductance. They proposed that unlike the abrasion treatment, the rubbing made contact with the guard cells and caused partial stomatal opening. Thus, it is apparent that the cuticular conductance measurements made after mechanical destuction or removal of epicuticular waxes are often confounded by partially open stomata and abrasive damage to the cuticle.

Comparisons of the cuticular conductances of glaucous and non-glaucous lines of *Brassica oleracea* have shown that the glaucous lines have consistently higher cuticular resistances (Denna 1970). Although comparisons of this sort suggest that the crystalline nature of the epicuticular wax is important, it is equally likely that differences in the chemical composition of the wax are responsible for the variation.

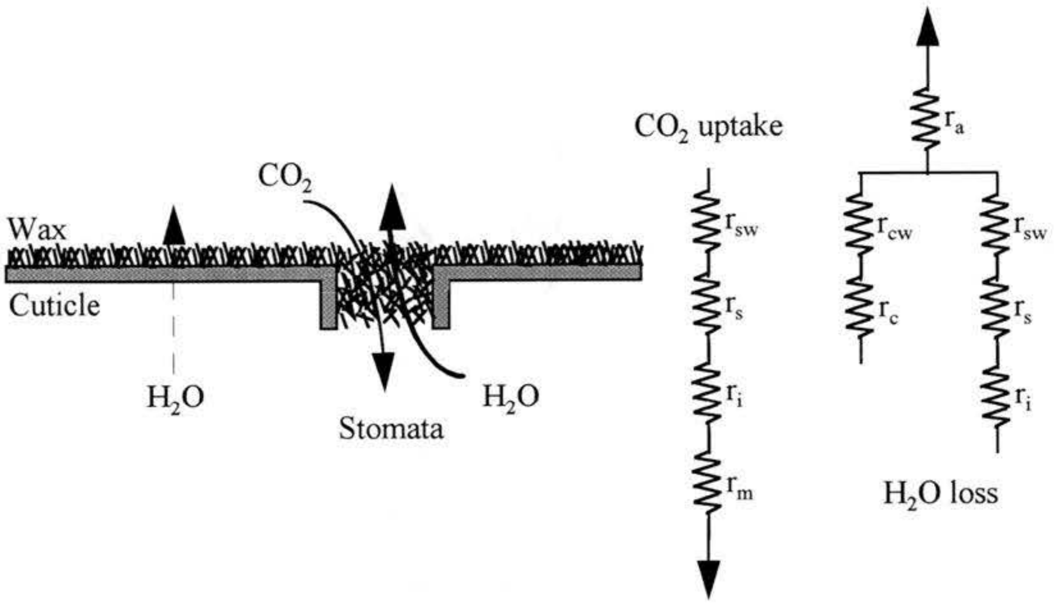
The maintainance of a high cuticular resistance is particularly important for evergreens that grow in desiccating environments where limited amounts of water are available. This is especially important for conifers that over-winter in high elevation and northern regions. Often, high solar irradiance coupled with a large vapour pressure deficit between the needle and the dry winter air will create a high evaporative demand (Marchland and Chabot 1978; Baig and Tranquillini 1980). However, because the soil is frozen, there is no water available to replace that which is lost by evaporation. There have been several studies (mostly on alpine trees) whose purpose was to determine whether epicuticular wax played a significant role in the cuticular resistance and over-winter survival of conifer needles. The results indicated that the first signs of needle damage generally occured on the windward sides of exposed trees (Tranquillini 1979; Hadley and Smith 1983) and was correlated with severe erosion of the epicuticular waxes and increased cuticular conductance (Hadley and Smith 1983, 1986; Grace, 1990). Since damage to the wax appeared to be due to needles rubbing together and bombardment by windblown particles and snow, there was probably additional damage to the cuticle and epidermal cells. Furthermore, van Gardingen *et al.* (1991) noted that conifer needles placed in an artificial wind tunnel had large cracks in the stomatal antechambers wax. These cracks coupled with partial stomatal opening due to damaged subsiduary or guard cells could also lead to substantial increases in water loss. Therefore, while it seems that epicuticular wax may be important in the prevention of over-winter needle desiccation, the results are not yet definitive.

The stomata

Although stomata may occupy only 0.5 to 5 % of the surface of a leaf, almost all the water transpired and all the CO_2 taken up for photosynthesis pass through these pores. A complex system of feedback and feedforward responses regulate the stomatal aperture such that the plant can optimize CO_2 uptake while maintaining the water status of the leaves. Guard cells repond rapidly to environmental and physiological conditions such as CO_2 concentrations, atmospheric humidity, temperature, changing leaf water potentials and irradiance.

Transpiration through the stomata occurs by molecular diffusion of water vapour where the rate is proportional to the vapour concentration difference between the inside of the leaf and the surrounding air and inversely proportional to a series of resistances (fig. 2). The first resistance is provided by internal air spaces. Water evaporates from the surface of mesophyll cells and then diffuses through the spaces between the cells. The more convoluted the air spaces, the greater the tortuosity and effective length of the pathway, and the higher the resistance to water loss. The next resistance is that offered by the stomatal pores. When the stomata are closed, this resistance is similar to that of the cuticle, but when the pores are open, stomatal resistance is several times lower. In many plant leaves, including conifers, guard cells are sunken below the surface and subsiduary

Figure 2. Resistances to gas exchange through the cuticle and stomata of a leaf. r_a, boundary resistance, r_{cw}, resistance of wax covering cuticle, r_{sw}, resistance of wax occluding stomatal antechamber, r_c, cuticular resistance, r_s, stomatal resistance, r_i, intercellular resistance and r_m, mesophyll resistance.


Wax
Cuticle
CO_2
H_2O
Stomata
H_2O
CO_2 uptake
r_{sw}
r_s
r_i
r_m
r_a
r_{cw}
r_c
r_{sw}
r_s
r_i
H_2O loss

cells arch over the stomatal aperture to form an external stomatal antechamber. This stomatal antechamber will also offer some resistance since it, too, increases the length of the pathway for diffusion. The final barrier to diffusion is provided by the boundary layer, a layer of moist, unstirred air at the leaf surface (Nobel 1991; Jones 1992).

Carbon dioxide diffuses across the same resistances encountered by water, but in the opposite direction. CO_2 must also enter the chloroplasts of mesophyll cells to be assimilated in photosynthetic reactions. Resistance to CO_2 diffusion is provided by the mesophyll cell walls, plasmalemma and cytosol as well as the chloroplast membranes and stroma. Since mesophyll cells offer a large resistance to CO_2 diffusion in the liquid phase, diffusion of CO_2 into the leaf is only about 62 % as efficient as water vapour diffusion out of the leaf (Nobel 1991; Jones 1992).

The observation that stomatal antechambers of most conifer species are completely occluded with crystalline epicuticular wax has prompted some speculation on how the wax affects gas exchange through the stomata. Jeffree *et al.* (1971) proposed that the complex matrix of interconnecting wax crystals would halve the cross-sectional area of the stomatal antechambers and thereby increase the length of the pathway for gas diffusion. They calculated that the wax reduces photosynthesis by 17%, and since the overall resistance to water vapour diffusion is much smaller, the wax has a proportionally greater effect on water loss. In fact, Jeffree *et al.* (1971) calculated that wax gives rise to a 36% reduction in transpiration. Although the hypothesis has yet to be confirmed, wax may be acting as an antitranspirant--a characteristic that may be very important for conifers growing in arid environments.

1.10 Thesis objectives

The purpose of my research was to determine whether epicuticular wax on the surface of conifer needles, specifically coastal *Pseudotsuga menziesii*, influences gas exchange through the stomata and cuticle. According to calculations by Jeffree *et al.* (1971), the resistance provided by wax "plugs" should have a proportionately greater effect on water vapour loss than on CO_2 uptake thereby increasing the water use efficiency of conifers growing in wet, warm environments. However, even though increased water use efficiency is not necessarily advantageous, these trees continue to produce copious quantities of stomatal antechamber wax. Not only does this wax production appear to be energetically inefficient, but the wax "plugs" may significantly reduce productivity by impeding CO_2 uptake through the stomata.

Accordingly, my first objective was to devise a technique to non-destructively remove epicuticular wax from the surface of *P. menziesii* needles. Previously published wax removal treatments are unsuitable because they cause damage to the underlying cuticle and epidermal cells.

My second objective was to quantify the total cuticular resistance of *P. menziesii* needles. Wax removal would provide a means of determining epicuticular wax resistance since r_{cw} is the difference between the total cuticular resistance and the resistance of the cuticle alone.

My third objective was to measure the resistance to water vapour and carbon dioxide exchange provided by the epicuticular wax in stomatal antechambers of coastal provenances of *P. menziesii*. I hypothesized that removal of the wax "plugs" from the stomatal antechambers would effectively increase the overall stomatal conductance to both water vapour and CO_2. However, stomata are very sensitive to the CO_2 mole fraction in the intercellular spaces and c_i is maintained surprisingly constant over a wide range of conditions and photosynthetic rates (Wong, Cowan & Farquhar 1979). Therefore, the elevated c_i that would result from increased stomatal conductance might be balanced by either increased CO_2-assimilation or partial stomatal closure. To determine the sensitivity of *P. menziesii* stomata to increased c_i, I proposed to measure changes in A and g_s at increased ambient CO_2 concentrations.

2. MATERIALS AND METHODS

2.1 Plant material

In the spring of 1993, seven one-year old container grown Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings were potted in a soil mix consisting of 70% peat and 30% perlite and vermiculite. The stock originated from coastal provenances and had been kept in cold storage prior to planting. The following year, measurements were made on six coastal Douglas-fir seedlings selected from two-year old stock that was grown in the Forest Biology greenhouse at the University of Victoria, British Columbia and then repotted in the spring in the same manner as the previous year. All seedlings were grown outdoors in the University of Victoria, Forest Biology compound.

Plants were watered to maintain adequate soil water and periodically fertilized. Measurements were made on current, fully expanded foliage during the summers of 1993 and 1994 when the seedlings were approximately 16 and 28 months old respectively. In addition, several of the 1993 seedlings were selected for further measurements in 1994.

2.2 Wax removal

A treatment was devised to non-destructively remove epicuticular wax from stomatal antechambers. Initially, a number of techniques were tested including chloroform and xylene rinses and physical removal of the wax using silicone rubber and cosmetic face masks (Rachel Rushforth, unpublished data). However, all were unsuccessful since they either failed to remove the wax or caused needle damage. Epicuticular wax was removed by coating the abaxial surface of individual needles with a low viscosity, fast setting (10 minute) dental impression material, "Light Bodied Permlastic ®" (Kerr Manufacturing

Company, Romulus, MI). Approximately 0.2 g of dental impression material was applied to each needle using a flattened toothpick. Once the needles of an entire branch (typically 100 to 150 needles) had been coated and the material had set, the treated branch was inserted in a cuvette and heated to 32°C for 30 minutes. The dental impression material was then gently peeled away with Teflon coated tweezers. Without heating the needles, wax removal was unsuccessful, however, a control experiment performed on an extra *P. menziesii* seedling indicated that the heating process alone had no effect on gas exchange or cuticular conductance.

Following treatment, needle surfaces were examined under a scanning electron microscope (JOEL model JSM-35) to look for damage and to estimate the amount of wax removal. Five needles were randomly selected from one of the treated branches of each seedling. The needles were mounted abaxial side up on aluminum stubs using double-sided adhesive tape, sputter coated with gold in an atmosphere of argon (35mA, 4×10^{-2} kPa, twice for 1.5 minutes) in an Edwards S150B specimen preparation chamber and then observed under the SEM at a beam accelerating voltage of 15 kV. The amount of wax removal was determined by the numbers of clean versus occluded stomata where a stomate was considered clean if more than half of its stomatal antechamber was free of wax. Between 400 and 1000 stomata per needle were counted.

To determine if success of wax removal changed with the season, two 1993 seedlings were treated again in January 1994. These seedlings were chosen on the basis of having either primarily clean or occluded stomata. Treated needles were also examined periodically for signs of wax regeneration or recrystallization.

2.3 Gas exchange measurements

In late July of 1993 and June of 1994, four to five healthy fully-exposed branches near the apex of each seedling were selected for gas exchange measurements. Measurements were made on each branch once per day for several days. Two branches served as the controls, while the remaining branches were used for wax removal. Following treatment of the branches in late August and early July of 1993 and 1994 respectively, gas exchange measurements were continued over a period of about one month. To determine the long term effects of wax removal, control and treatment branches of three 1993 seedlings were measured again in August and September of 1994.

Most gas exchange measurements for both years were made on relatively clear days when irradiance exceeded 1000 μmol m^{-2} s^{-1}. In 1993, measurements were made over the whole day, however, the following year measurements were restricted to the morning hours when irradiance was still saturating, but the vapour pressure deficit was low.

Needle water vapour and CO_2 flux were measured with an open gas exchange system (Analytical Development, Co., Hoddesdon, U.K.). In 1993, measurements were made using the LCA-3 differential porometer. Air flow through the cuvette was maintained at 300 μmol s^{-1} and air entering the chamber was dried to ensure that the transpiring branch inside the chamber was exposed to vapour pressures similar to ambient conditions. In 1994, a LCA-4 was used. Again, ambient air was drawn in and, depending on the size of the branch, air flow through the leaf chamber was set at 360 to 410 μmol s^{-1} to prevent excessive increases in water vapour due to the transpiring branches.

To guarantee consistent placement of the leaf chamber and to reduce damage to the branches, several needles were removed from the area where the leaf chamber was clamped on to the branch. This also ensured that the same needles were measured each day. The CO_2 and relative humidity of the inflow air was kept relatively constant by drawing air from several meters away. Likewise, heating of the chamber was kept to a minimum by shading the porometer, influx hoses and the leaf chamber when not in use.

Gas exchange measurements were recorded when the system had reached a steady state and readings for apparent internal CO_2 partial pressure and stomatal conductance were relatively constant. This usually took two to four minutes depending on the photosynthetic activity of the branch. Transpiration (E) was calculated from the following formula:

$$E = \frac{u_o w_o - u_e w_e}{L} \ [\text{mmol m}^{-2} \text{ s}^{-1}]$$

where u_e and u_o were the flow rates (μmol s^{-1}) and w_e and w_o were the mole fractions of water in air entering and leaving the chamber (mol mol^{-1}). L was the projected leaf area (m^2). Similarly, photosynthesis was obtained from the difference in flow of CO_2 into and out of the chamber:

$$A = \frac{u_e c_e - u_o c_o}{L} \ [\mu\text{mol m}^{-2} \text{ s}^{-1})$$

where c_e and c_o were the CO_2 concentrations. Stomatal conductance to water vapour was determined by:

$$g_s = \frac{E}{(e_s - e_a) \cdot a_l} \ [\text{mol m}^{-2} \text{ s}^{-1}]$$

where $(e_s - e_a)$ (g cm^{-3})was the the vapour pressure deficit between the inside of the needles and the surrounding air in the chamber. Stomatal conductance to CO_2 (g_{ca}) was then $g_{ca} = g_s / 1.6$, where 1.6 is the ratio of diffusivities of CO_2 and water vapour in air. Intercellular CO_2 concentration could then be calculated as:

$$c_i = \frac{\left(g_{ca} - E/2\right) c_a - A}{\left(g_{ca} + E/2\right)} \ [\mu\text{mol mol}^{-1}]$$

where c_a was the ambient CO_2 concentration (von Caemmerer & Farquhar 1981). Instantaneous water use efficiency (WUE) was determined as the ratio of net photosynthesis to transpiration.

Once the gas exchange measurements were complete, the projected leaf area of the branches was measured using a Li-3100 leaf area meter (Li-COR Inc., Lincoln, NB). However, for seedlings measured in 1993 and again in 1994, leaf area was not determined until after the 1994 measurements.

2.4 Whole-plant measurements

To determine whether stomata in coastal *P. menziesii* respond to changes in ambient CO_2 concentrations and maintain internal CO_2 partial pressure at a constant level by negative feedback, measurements were made in a computer-controlled whole plant cuvette system (Livingston *et al.* 1994). Due to the large size of the seedlings used in the wax removal experiments, a smaller 6 month-old greenhouse grown coastal Douglas-fir seedling was used. The seedling was transplanted into a PVC cylinder filled with fine sand, watered to field capacity and allowed to equilibrate in the whole plant cuvette for five days.

During the experiment, the seedling was enclosed in a cuvette (0.14 m O.D. and 0.2 m long) that allowed good control of environmental conditions and high resolution of measurements. Daylength was set to 8 hours with a photosynthetic photon flux density of 1160 $\mu mol\ m^{-2}\ s^{-1}$ ($\pm$ 40 $\mu mol\ m^{-2}\ s^{-1}$), while temperature and vapour pressure were held at 20°C (± 0.5°C) and 1.2 kPa (± 0.05 kPa), respectively. When the lights were switched on at 09.00 h., the CO_2 concentration was 200 $\mu mol\ mol^{-1}$. After approximately 30 minutes, the CO_2 concentration was increased by 100 $\mu mol\ mol^{-1}$. The CO_2 concentration was increased in a step-wise manner to 800 $\mu mol\ mol^{-1}$ and then decreased in the same manner to 200 μmol mol^{-1}. Each step was 30 minutes not including the time allowed for the CO_2 concentration to stabilize. Meanwhile, continuous measurements of whole plant photosynthesis, transpiration, and CO_2 concentration were logged and internal CO_2 partial pressure and stomatal conductance were derived from the previous calculations.

2.5 Cuticular conductance measurements

Upon completion of the gas exchange measurements, soils were watered to field capacity and left over-night to ensure equal water status of seedlings. Control and wax removal branches were then excised, their ends submerged in water and placed in the dark at 5°C for 12 hours to allow complete needle hydration. A total of 30 needles per seedling were randomly selected for cuticular conductance measurements; 15 needles from the control and 15 from the treated branches. Immediately following removal, the ends of the needles were sealed with paraffin wax, weighed on a balance (AE240 Mettler Instrument Corp., Hightstown, New Jersey, USA) with a precision of 10 μg and then suspended in 30 mL vials above saturated salt solutions of known molality. The vials are described by Livingston and de Jong (1988, 1991) with the exception that they were partially filled with 2 mL of a saturated salt solution of K_2CO_3, which is equivalent to -110 MPa or 41.9% relative humidity at 5°C (see calculations by Lang 1967). The vials were lined with filter paper to maintain a constant Ψ throughout and the needles were suspended in the vials in modified Eppendorf microcentrifuge tubes where the tapered ends had been cut and replaced with nylon mesh (see Livingston *et al.* 1992). The vials were then sealed and placed in a dark, recirculating water bath at 4°C (± 0.1°C) to eliminate temperature gradients and minimize weight loss due to respiration. Initially, the needles were weighed twice per day, but after about 60 hours, the needles were weighed once daily for a total of

120 hours. When the change in needle weight was plotted over time, two distinct zones were evident; in the first 60 hours, a period of rapid, but declining conductance when stomata were not completely closed and, next, a period of slow and steady water loss when it was assumed that stomata were closed, but the inside of the needle was still at saturated vapour pressure (Hygen 1951; Sinclair & Ludlow 1986). The slope of this latter zone was used to calculate the cuticular conductance.

Cuticular conductance (g_c) was calculated as:

$$g_c = \Delta FW/t \cdot 1/\text{area} \cdot 1/(e_s - e_a) \qquad (\text{cm s}^{-1})$$

where ΔFW was the change in fresh weight (g) of the needles between 60 and 120 hours, area was the projected needle area (cm^2) and e_s - e_a was the vapour pressure deficit (3.88 g cm^{-3}) between the inside of the needles and the surrounding air in the vials. Cuticular resistance was expressed as:

$$r_c = 1/g_c \qquad (\text{s cm}^{-1})$$

and at sea level and 25°C, an approximate conversion for resistance is:

$$2.5\ r\ (\text{s cm}^{-1}) = r\ (\text{s m}^2\ \text{mol}^{-1})$$

Several assumptions were made; the air in the vial was at vapour equilibrium with the salt solution, the temperature of the needle was equal to that of the surrounding air and the boundary layer resistance was similar for each needle and was included in the estimate of r_c.

Very accurate estimates of needle area (± 0.005 mm^2) were obtained by projecting images of individual needles on to a desk top scanner (HP ScanJett IIc) and then processing the information using a digital image analysis system (NIH "Image").

A two-factor ANOVA was used to test for differences between seedlings and to determine whether the wax removal treatment resulted in a significant reduction in g_c. A two-tailed student t-test was used to test for significant differences between control and treatment needles within a seedling.

2.6 Verification of cuticular conductance measurements

Artificial needle experiment

To determine the consistency of the cuticular conductance measurement protocol, rates of water loss were measured from artificial needles. Three types of artificial needles were designed from 12 rectangular copper rods (24 x 4 x 4 mm). Each type had four, six and eight 0.5 mm holes. Squares of filter paper were tightly rolled, inserted into the rods and then wetted with distilled water. Both ends of the rods were dipped in paraffin to limit water loss and immediately weighed and placed in vials in the same manner as the needles. The artificial needles were then reweighed 8 hours later. This procedure was repeated four times.

Measurements of water loss from the copper rods showed that those with 4, 6 and 8 holes lost 8.0, 11.0 and 12.3 μg of water per minute, respectively. Although I had expected that the copper rod with 8 holes would dehydrate twice as quickly as one with 4 holes, there was a significant ($P<0.05$) and consistent difference in rates of water loss between the different types of artificial needles.

The use of copper rods to simulate true needles was somewhat limiting since water loss was lower than expected for rods with 6 and 8 holes. This was probably due to higher evaporation rates which quickly dried the filter paper in the vicinity of the holes. As a result, the assumption that the absolute humidity gradient was equal to the difference between saturated vapour pressure at the filter paper surface and the vapour pressure of the surrounding air was probably true for rods with 4 holes, but not those with 6 and 8 holes. Despite this limitation, measured rates of water loss were consistant for each copper rod and this suggested that the protocol generated repeatable results.

Relative water content measurements

To test the assumption of saturated vapour pressure inside the needles during the 120 hour cuticular conductance measurements, relative water content (RWC) of the needles was measured over time. Relative water content was calculated as:

$$\mathrm{RWC} = \frac{(M - M_d)}{(M_t - M_d)}$$

where M is needle mass, M_d is needle dry weight and M_t is weight of needle at full turgor. Since needles were assumed to be at full turgor just before drying, M_t was equivalent to needle weight at time 0. M_d was determined by drying the needles until there was no further weight loss; approximately 48 hours at 65°C.

The results indicated that even after 120 hours of drying at 3.88 $\mu g\ cm^{-3}$, the relative water content of the needles never dropped below 85%. Thus, it was likely that the vapour pressure inside the needle was close to saturated vapour pressure.

3. RESULTS

3.1 Wax removal

The needles of *P. menziesii* are hypostomatic with two parallel bands of stomata located on either side of an abaxial midrib. Amorphous wax covers the entire needle surface and tubular crystalline wax is located in isolated clusters on the adaxial surface and on the midrib and peripheral areas of the abaxial surface. However, crystalline wax is most evident along the length of the rows of stomata where the wax forms a dense covering that completely obscures and occludes the underlying stomatal antechambers (fig. 3 (a, b)).

Observations of needles after treatment with dental impression material and heat showed that the wax removal treatment gave two results. In some seedlings, the treatment successfully removed all crystalline wax covering the cuticle and occluding stomatal antechambers (fig. 3 (c, d)). Examination of the dental impression material after treatment showed stomatal antechamber wax adhering to the material in a pattern similar to that on the intact needle (fig. 3 (e)). However, the same treatment used on other related seedlings sometimes appeared to remove very little wax and caused smearing of the wax tubules into an amorphous mass (fig. 3 (f)). There was some variation in the degree of wax smearing in these needles, however, the severity of wax destruction was not quantified. Wax removal of a seedling was only considered to be successful if greater than 50% of stomata were free of stomatal antechamber wax. Approximately half the seedlings treated had successful wax removal (Table 1).

Figure 3. Scanning electron micrographs of the abaxial surface of a *Pseudotsuga menziesii* needle. (a, b) Surface view of stomata showing crystalline epicuticular wax occluding stomatal antechambers. (c, d) Surface view of stomata after successful wax removal. Wax crystals have been removed from stomatal antechambers and the surrounding leaf surface. (e) Surface view of the dental impression material after treatment showing wax from stomatal antechambers adhering to the surface. (f) Surface view of stomate after unsuccessful wax removal. Crystalline epicuticular wax has been smeared into an amorphous mass. (a, c, e) bar=10 μm; (b, d, f) bar=3 μm.

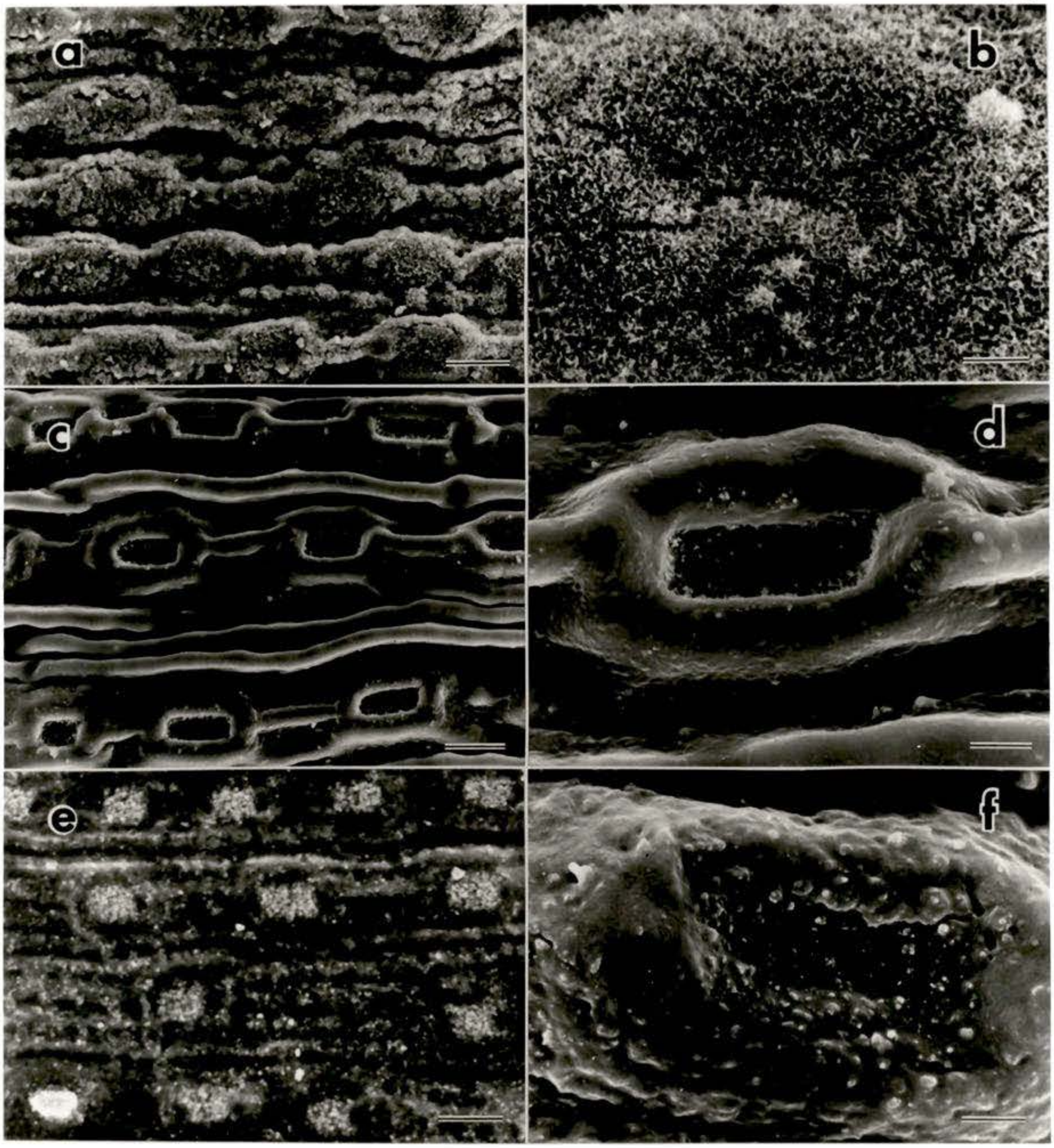

a
b
c
d
e
f

Table 1. Success of wax removal and cuticular resistances for seedlings treated in 1993 and 1994[a].

Seedling	Success of wax removal	Average cuticular resistance		Change in r_c after wax removal
	clean stomata %	Control (m^2 s mol^{-1})	Treated (m^2 s mol^{-1})	%
1993-1	86	1848 (±130)	1473 (±73)	20*
1993-2	85	1820 (±113)	1733 (±135)	5
1993-3	78	2505 (±113)	2090 (±90)	17*
1993-4	38	1588 (±134)	1375 (±116)	13
1993-5	13	2065 (±86)	1663 (±103)	20*
1993-6	5	1693 (±124)	1453 (±87)	14
1994-1	82	2080 (±150)	1253 (±135)	40*
1994-2	78	2170 (±134)	1520 (±80)	30*
1994-3	58	2138 (±91)	1545 (±73)	28*
1994-4	26	2195 (±120)	2368 (±152)	+8
1994-5	19	1608 (±33)	1523 (±69)	5
1994-6	6	1940 (±71)	1563 (±59)	19*

[a] All plus and minus values are SE

* Values are statistically significant ($P<0.05$)

Needles examined periodically for several months after the wax removal treatment showed no evidence of wax recrystallization or further wax production . Furthermore, the needles were green and healthy with no signs of necrosis and chlorosis.

To determine if success of wax removal changed with the season, adjacent, untreated branches on two 1993 seedlings were treated in January 1994. The success of wax removal on these newly treated branches was similar to that on branches previously treated in July 1993.

3.2 Gas exchange measurements

For branches treated in both 1993 and 1994, measurements of stomatal conductance, photosynthesis, internal CO_2 partial pressure, and instantaneous water use efficiency were normalized by showing the response of treated branches as a proportion of the control branches (figs. 4 and 5). Upon first analysis of the data, the results are not obvious. However, when the data is separated into seedlings with successful (> 50% antechamber wax removal) and unsuccessful wax removal (< 50% antechamber wax removal), two trends become very apparent. In the following discussion, these trends are illustrated by representative trees.

Successful wax removal

Of the seedlings treated in 1993, three were considered to have had successful wax removal since 78 to 86% of stomatal antechambers were free from wax (Table 1). The results show that despite fluctuations in absolute g_s, there was a relative increase in

Figure 4. Time course of normalized stomatal conductance and photosynthesis of *P. menziesii* seedlings treated with heat and dental impression material. Measurements were made from July 9, 1993 (day 1) to October 4, 1994 (day 452). Arrows indicate time of wax removal on treated branches. Data points are the average response of the treated branches as a proportion of control branches. Each symbol represents a different tree.

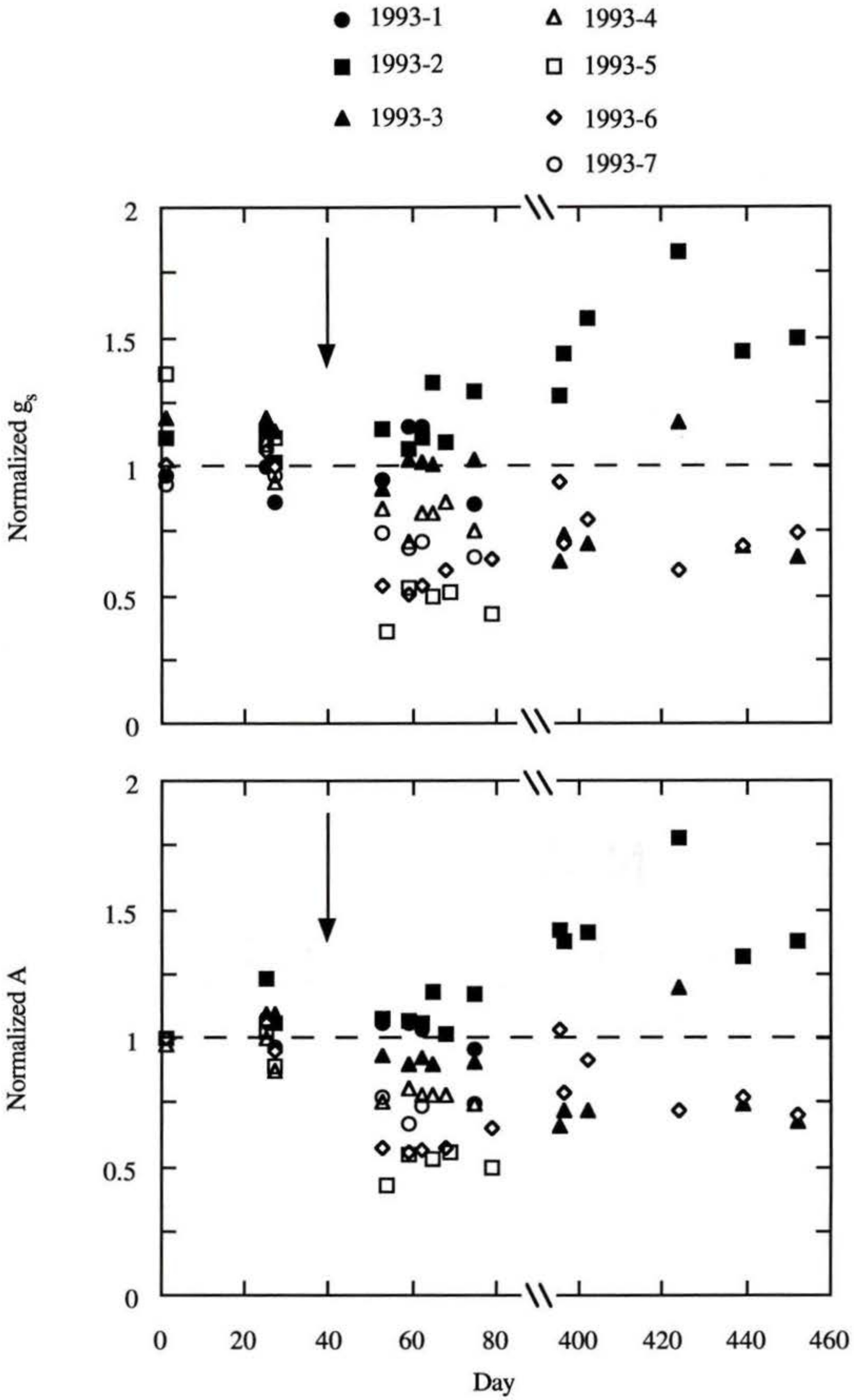
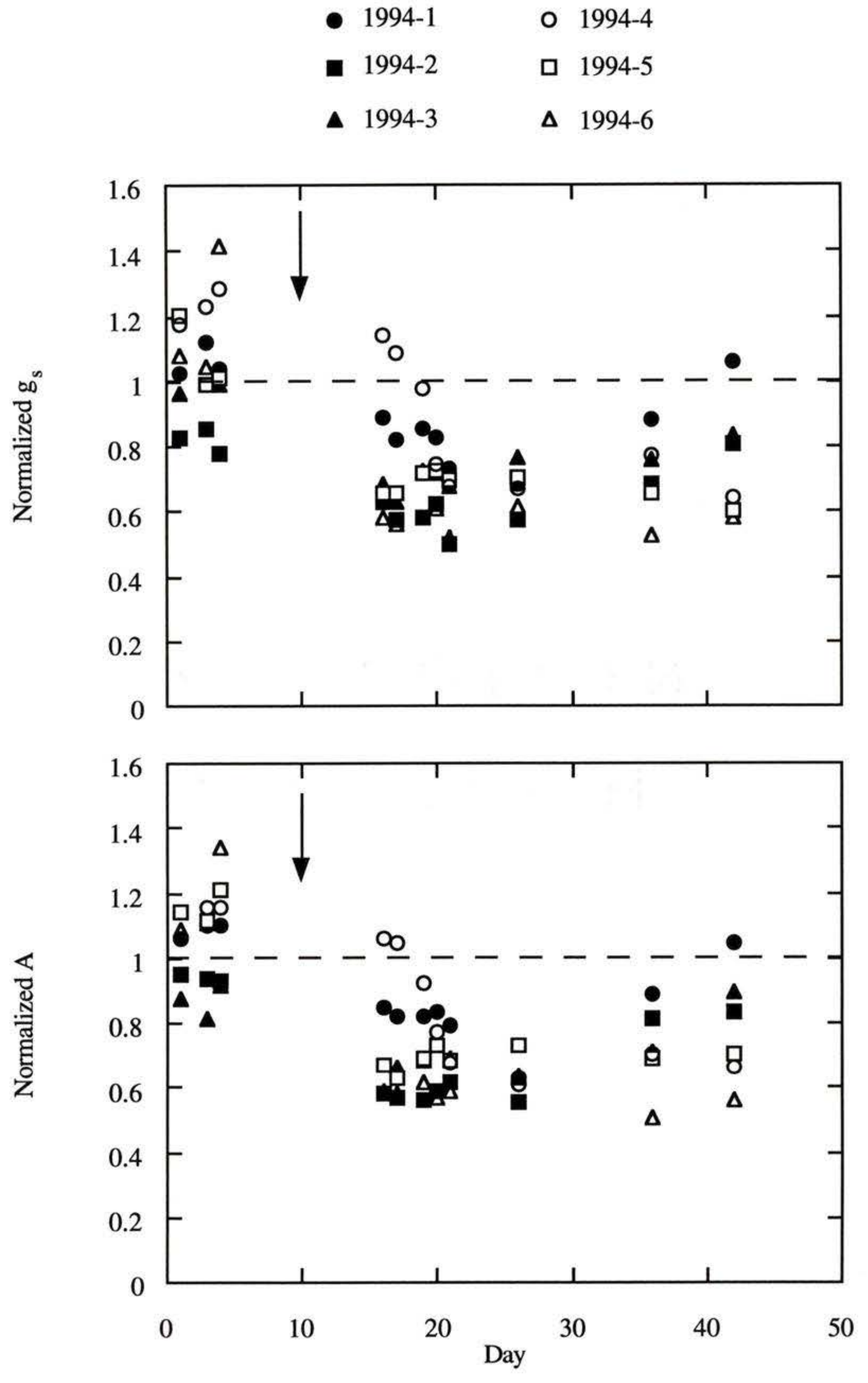

● 1993-1
■ 1993-2
▲ 1993-3
△ 1993-4
□ 1993-5
◇ 1993-6
○ 1993-7
Normalized g_s
Normalized A
Day
0
0.5
1
1.5
2
0
20
40
60
80
400
420
440
460

Figure 5. Time course of normalized stomatal conductance and photosynthesis of *P. menziesii* seedlings treated with heat and dental impression material. Measurements were made June 27 (day 1) to August 15 (day 42) of 1994. Arrows indicate time of wax removal on treated branches. Data points are the average response of the treated branches as a proportion of control branches. Each symbol represents a different tree.


● 1994-1
■ 1994-2
▲ 1994-3
○ 1994-4
□ 1994-5
△ 1994-6
Normalized g_s
Normalized A
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Day

g_s of treated branches of seedling 1993-2 following wax removal (fig. 6). This is indicated by the increase in normalized g_s from 1.1 on day 1 to 1.3 on day 75. However, the g_s of the other two successfully treated seedlings (1993-1 and 1993-3) remained constant before and after treatment (fig. 4). The photosynthetic rate of treated branches of 1993-2 also increased (normalized A; day1=1.0, day 75=1.2) although, once more, this trend was not reflected by the other two seedlings where A remained constant. Finally, for all three seedlings c_i and WUE remained relatively stable throughout the measurements.

Further measurements in 1994 on two successfully treated seedlings from 1993 showed inconsistent results (fig. 4). Treated branches of 1993-2 exhibited an increase in g_s (normalized gs; day 1=1.1, day 452=1.5) and A (normalized A; day 1=1, day 452=1.4) while those of seedling 1993-3 appeared to be less physiologically active than the previous year (normalized g_s; day 1=1.2, day 452=0.7; normalized A; day 1=1, day 452=0.7).

Three of six seedlings treated in 1994 also showed successful wax removal (58 to 82% clean stomata, Table 1). Results from the representative seedling, 1994-1, illustrated that immediately following wax removal treatment there was a decline in g_s (normalized gs; day 1=1, day 16=0.9) and A (normalized A; day 1=1.1, day 16=0.8) (fig. 7). This decline was coupled with an increase in c_i and somewhat depressed WUE. However, approximately 35 days after treatment, seedlings recovered such that there was no change in the physiology of treated branches before and after treatment (normalized values for day 42; g_s=1.1, A=1.0, c_i=1.0, WUE=1.0).

Figure 6. Time course of normalized stomatal conductance, photosynthesis, internal CO_2 partial pressure and instantaneous water use efficiency of seedling 1993-2 with successful wax removal. Measurements were made from July 9 (day 1) to September 23 (day 75) of 1993. Arrows indicate time of wax removal on treated branches. Solid data points represent treated branches as a proportion of the control branches. Open data points show averaged absolute values for control branches.

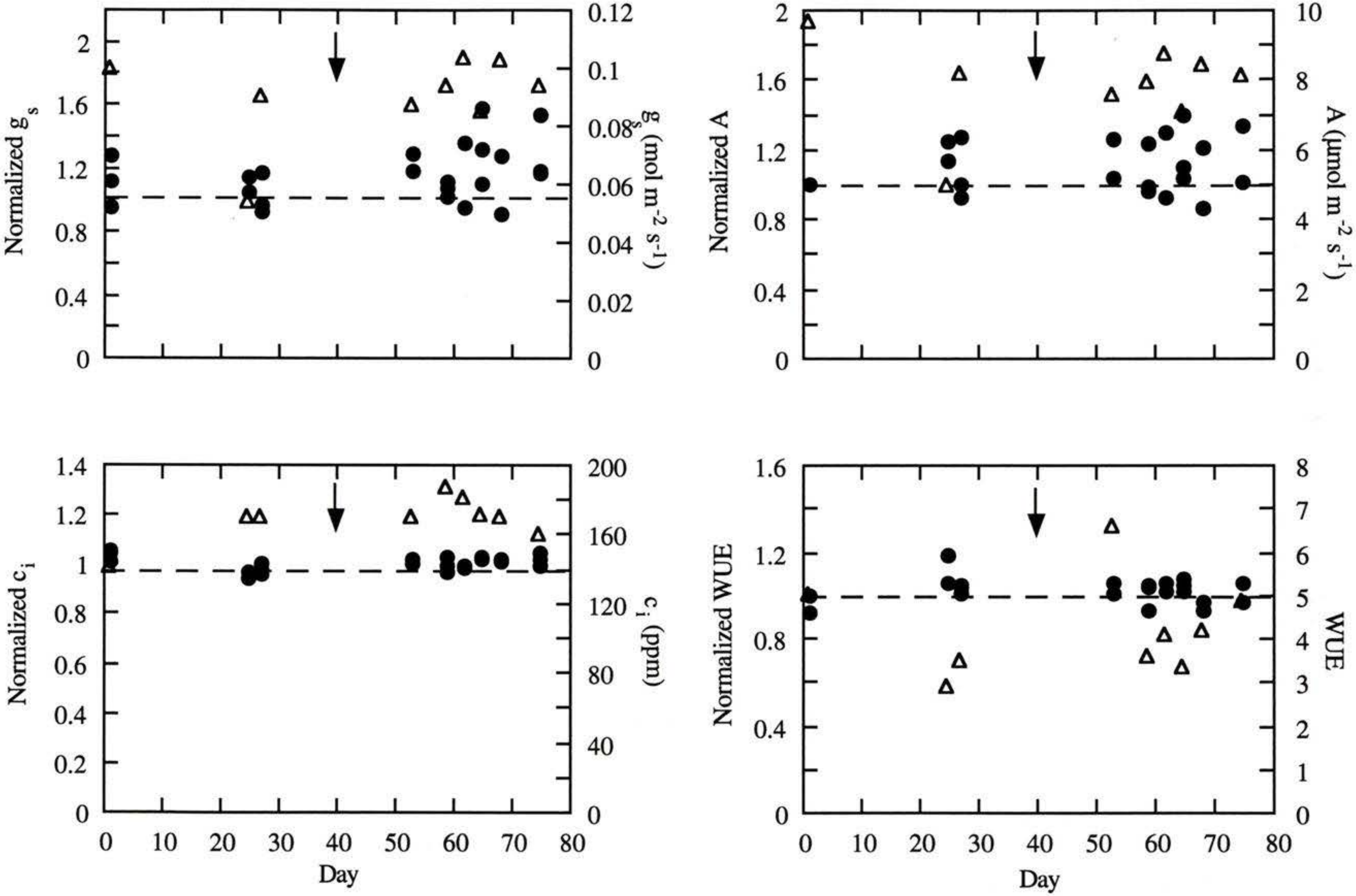
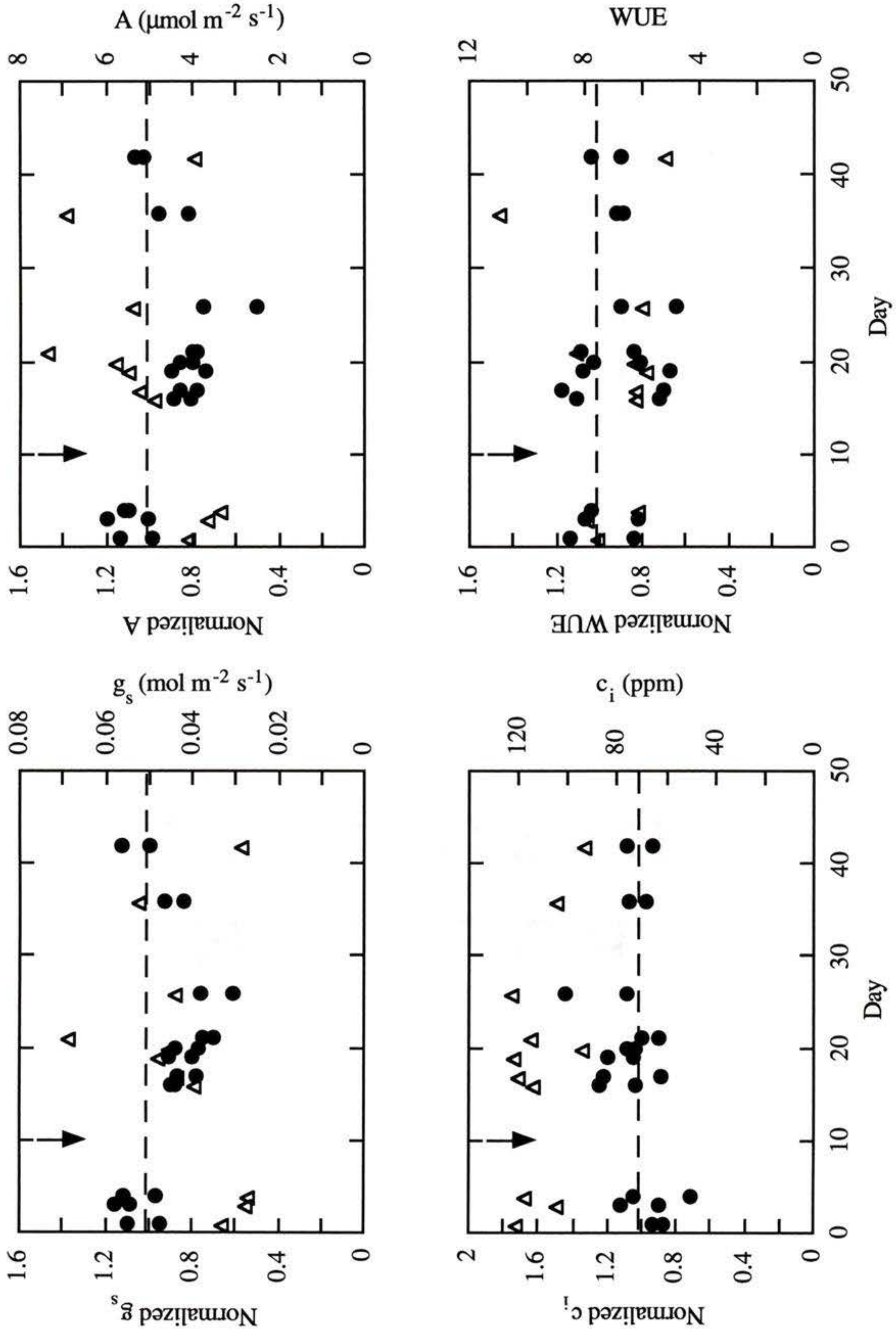

Normalized g_s
g_s (mol m^{-2} s^{-1})
0
0.4
0.8
1.2
1.6
2
0
0.02
0.04
0.06
0.08
0.1
0.12
Normalized A
A (µmol m^{-2} s^{-1})
0
0.4
0.8
1.2
1.6
2
0
2
4
6
8
10
Normalized c_i
c_i (ppm)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
40
80
120
160
200
Normalized WUE
WUE
0
0.4
0.8
1.2
1.6
0
1
2
3
4
5
6
7
8
Day
0
10
20
30
40
50
60
70
80
Day
0
10
20
30
40
50
60
70
80

Figure 7. Time course of normalized stomatal conductance, photosynthesis, internal CO_2 partial pressure and instantaneous water use efficiency of seedling 1994-1 with successful wax removal. Measurements were made from June 27 (day 1) to August 15 (day 42) of 1994. Arrows indicate time of wax removal on treated branches. Solid data points represent treated branches as a proportion of the control branches. Open data points show averaged absolute values for control branches.


Normalized g_s
g_s (mol m^{-2} s^{-1})
Normalized A
A (µmol m^{-2} s^{-1})
Normalized c_i
c_i (ppm)
Normalized WUE
WUE
Day
0
10
20
30
40
50
0
0.4
0.8
1.2
1.6
2
0
0.02
0.04
0.06
0.08
0
2
4
6
8
0
40
80
120
0
4
8
12

Unsuccessful wax removal

A different trend was observed in the remaining 1993 seedlings which were considered to have had unsuccessful wax removal with only 5 to 38% of stomata clean (Table 1). These seedlings, as represented by seedling 1993-7, showed a substantial decrease in g_s (normalized g_s; day 1=1.0, day 79=0.6) and A (normalized A; day 1=1.0, day 79=0.7) following treatment with the dental impression material (fig. 8). Likewise, there was some depression in WUE (normalized WUE; day 1=1.0, day 79=0.9), but no apparent change in c_i. Seedling 1993-7 was measured again in 1994 and, except for variable c_i, the results were consistent with 1993 measurements (fig. 4).

The pattern of response of unsuccessfully treated 1994 seedlings was similar to that observed in the unsuccessful wax removal seedlings of 1993 (fig. 9). Both g_s (normalized g_s; day 1=1.1, day 42=0.6) and A (normalized A; day 1=1.1, day 42=0.6) of treated branches declined significantly after treatment. However, c_i and WUE remained relatively stable throughout.

3.3 Whole-plant measurements

Measurements in the whole-plant cuvette system indicated that there was a slight decline in g_s with increasing ambient CO_2. This is illustrated by a comparison of c_a to apparent c_i where dc_i/dc_a is about 0.4 (fig. 10). If there had been no stomatal control of c_i, then dc_i/dc_a would have been 1.

Figure 8. Time course of normalized stomatal conductance, photosynthesis, internal CO_2 partial pressure and instantaneous water use efficiency of seedling 1993-7 with unsuccessful wax removal. Measurements were made from July 9 (day 1) to September 27 (day 79) of 1993. Arrows indicate time of wax removal on treated branches. Solid data points represent treated branches as a proportion of the control branches. Open data points show averaged absolute values for control branches.

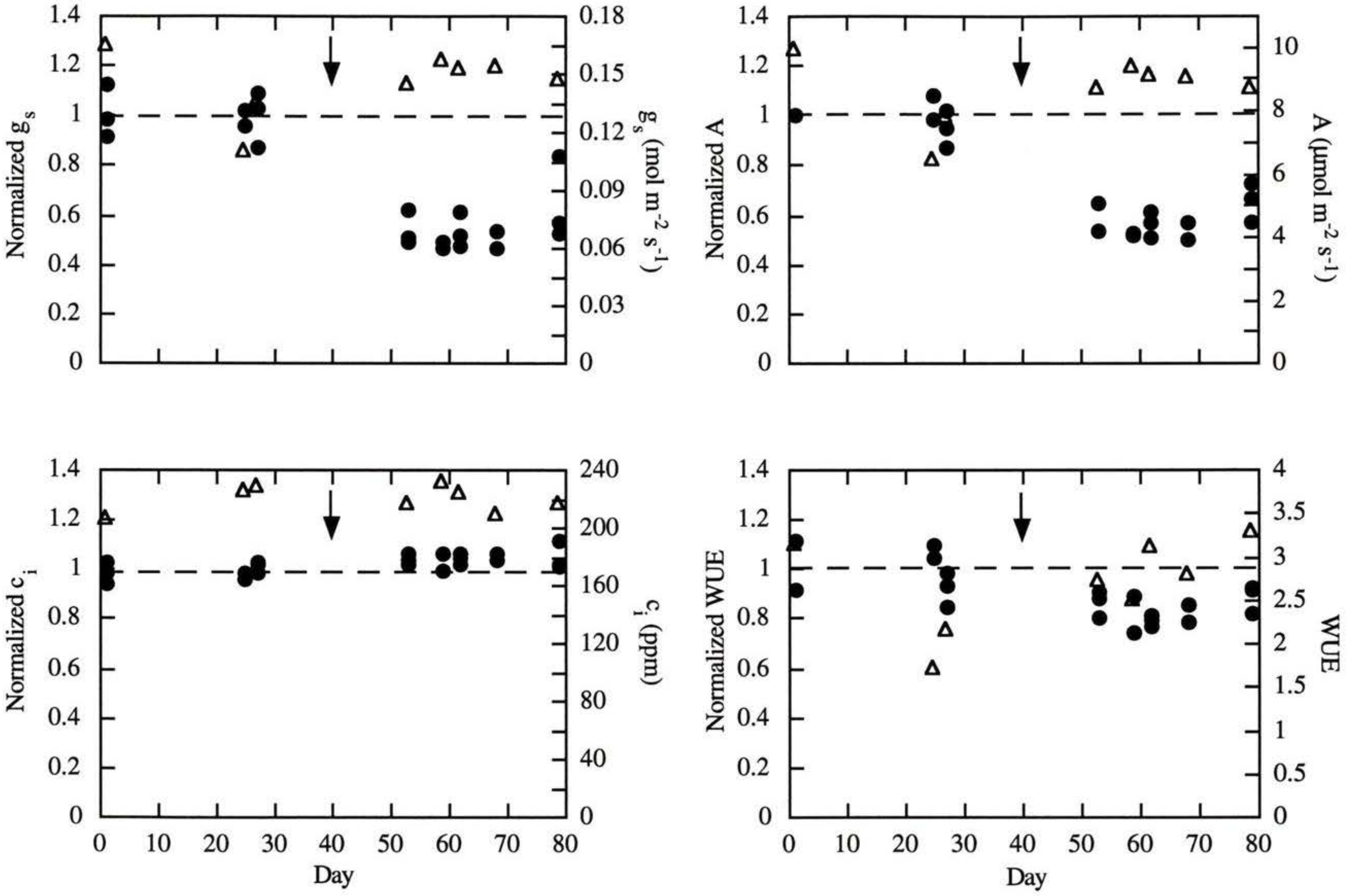

Normalized g_s
g_s (mol m^{-2} s^{-1})
0, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4
0, 0.03, 0.06, 0.09, 0.12, 0.15, 0.18
Normalized A
A (μmol m^{-2} s^{-1})
0, 2, 4, 6, 8, 10
Normalized c_i
c_i (ppm)
0, 40, 80, 120, 160, 200, 240
Normalized WUE
WUE
0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4
Day
0, 10, 20, 30, 40, 50, 60, 70, 80

Figure 9. Time course of normalized stomatal conductance, photosynthesis, internal CO_2 partial pressure and instantaneous water use efficiency of seedling 1994-6 with unsuccessful wax removal. Measurements were made from June 27 (day 1) to August 15 (day 42) of 1994. Arrows indicate time of wax removal on treated branches. Solid data points represent treated branches as a proportion of the control branches. Open data points show averaged absolute values for control branches.

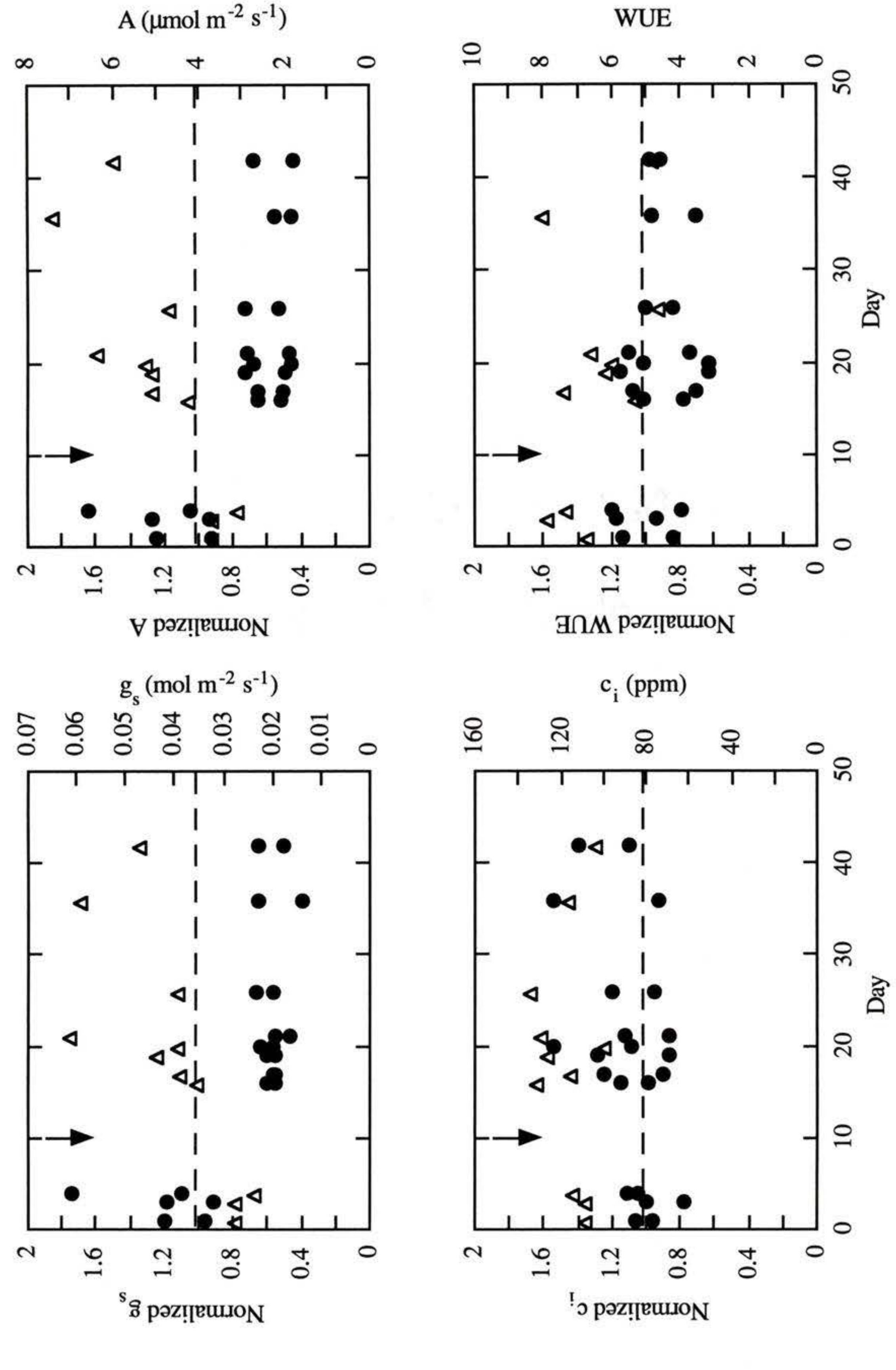
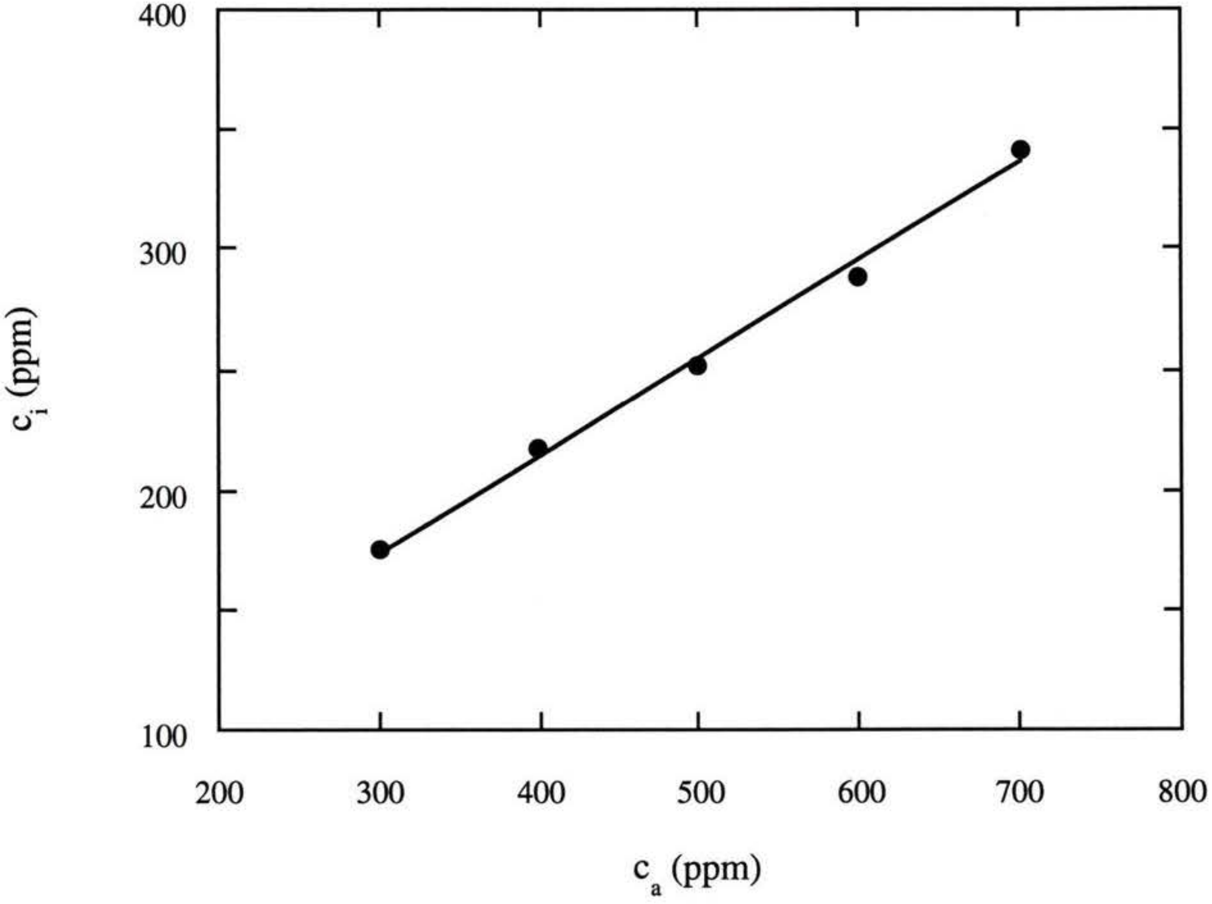

Normalized g_s
g_s (mol m^{-2} s^{-1})
Normalized A
A (µmol m^{-2} s^{-1})
Normalized c_i
c_i (ppm)
Normalized WUE
WUE
Day
0
10
20
30
40
50
0
0.4
0.8
1.2
1.6
2
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0
2
4
6
8
0
40
80
120
160
0
2
4
6
8
10

Figure 10. Relationship between c_a and apparent c_i for *P. menziesii* seedling measured in the whole-plant cuvette system.

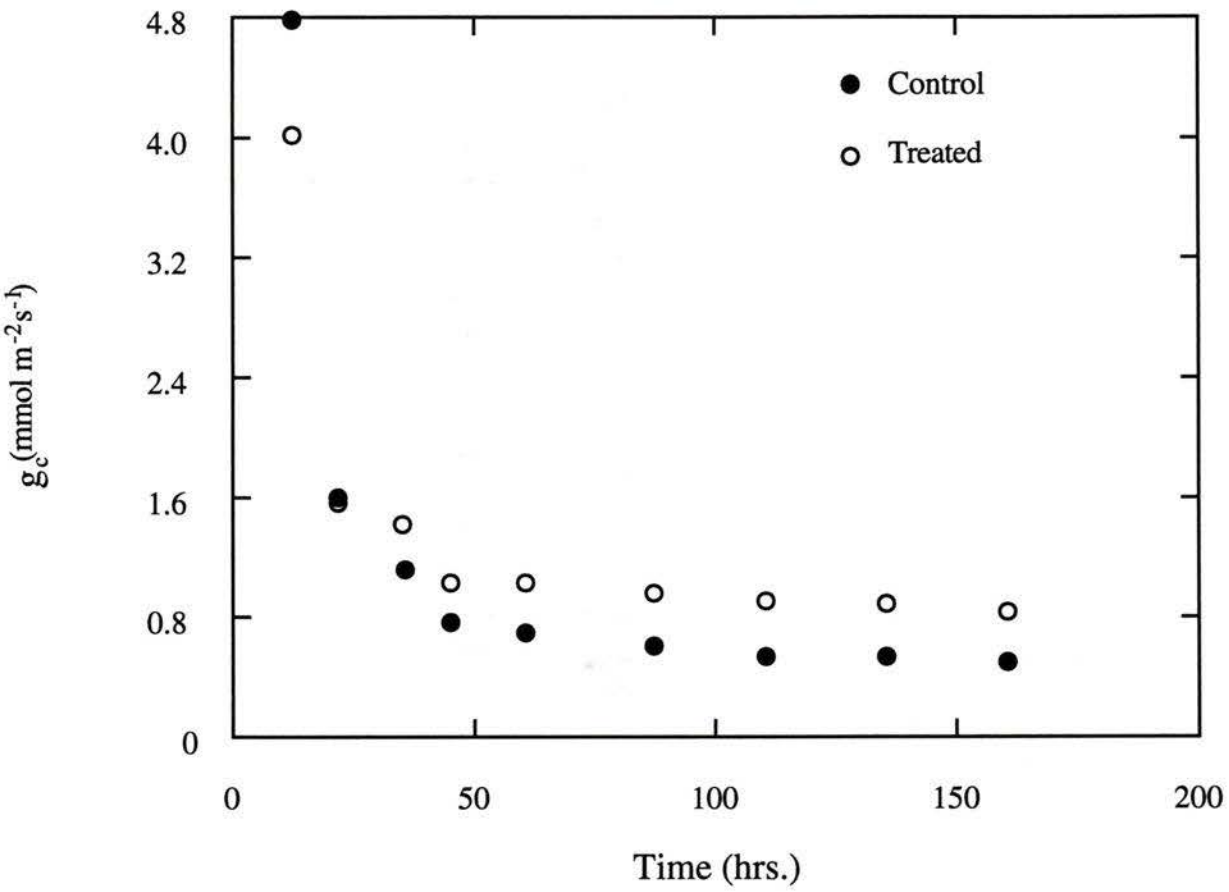

400
300
200
100
200
300
400
500
600
700
800
c_i (ppm)
c_a (ppm)

3.4 Cuticular conductance measurements

Prior to treatment with dental impression material and heat, the cuticular resistance of *P. menziesii* needles ranged from 1600 to 2500 s m^2 mol^{-1} (Table 1) and was significantly different ($P<0.05$) between seedlings. Following treatment, there was a reduction in needle r_c in all seedlings except seedling 1994-4 which showed an 8% increase. Generally, needles with successful wax removal had the most significant reduction (17 to 40%) in r_c with the exception of seedling1993-2 (5% reduction). Furthermore, although cuticular resistance values were highly variable within seedlings (SE=33 to 152), there was no further increase in within seedling variation following wax removal.

The cuticular conductance versus time curves illustrate the time course required for partially open stomata to respond to declining tissue water status and close (fig. 11). For all seedlings, stomata on treated and control needles were equally responsive such that both groups of needles reached the cuticular phase of water loss or minimal conductance at approximately the same time (60 hours).

Figure 11. Cuticular conductance versus time curves (from seedling 1994-1) illustrating the typical pattern of water loss from control and treated needles.


4.8
4.0
3.2
2.4
1.6
0.8
0
0
50
100
150
200
Time (hrs.)
g_c(mmol m^{-2}s^{-1})
● Control
○ Treated

4. DISCUSSION

Scanning electron microscope observations of treated needles indicate that wax removal treatment using the dental impression material and gentle heating can successfully remove epicuticular wax without causing damage to the underlying cuticle and epidermal cells. In addition, the fact that needles remained green with no signs of necrosis or chlorosis for up to a year after treatment provides further evidence for the non-destructive nature of the treatment.

There was considerable variation in the amount of stomatal antechamber wax removed despite the fact that seedlings treated within a particular year were genetically related. Although there is no obvious explanation for this variation, it is conceivable that the success of wax removal treatment is dependent upon the chemical composition of epicuticular wax. Since numerous studies have indicated that the tubular wax of gymnosperms is composed of crystallized nonacosan-10-ol (Jeffree *et al.* 1976; Gunthard-Goerg 1986), it is more likely that the underlying amorphous wax layer is the primary source of variation in wax chemistry evident between plants of the same species (Eglinton & Hamilton 1967). Since heat is necessary for successful wax removal and because amorphous wax has a lower melting point than crystalline waxes (Gülz 1994), use of heat in the treatment probably softened the amorphous wax such that it and the superimposed crystalline wax adhered to the dental impression material. Conversely, subtle differences in the chemistry of amorphous wax may increase the melting point of wax beyond 32°C and, thereby account for the relatively poor wax removal observed on some seedlings. Unfortunately, because amorphous wax is featureless under the SEM, the absolute amount of amorphous wax removed could not be measured.

Once needles are fully expanded, epicuticular wax production ceases and there is little or no change in the chemical composition of wax beyond this point (Hall & Jones 1961; Hallam 1970; Günthardt-Goerg 1986; van Gardingen *et al.* 1991). This agrees with the observation that the success of the wax removal treatment on a particular seedling did not change with season or increasing needle age.

Bacic *et al.* (1994) recently studied *Pinus sylvestris* needles after fumigation with various pollutants and observed wax rodlets recrystallizing on the surface of amorphous wax crusts. A similar phenomenon was noted by other researchers who isolated and recrystallized conifer epicuticular wax on artificial surfaces (Jeffree *et al.* 1976; Gunthard-Goerg 1986). However, in the present study, there was no sign of wax recrystallization on successfully treated needles, nor was epicuticular wax replaced in stomatal antechambers after it had been removed. This is interesting since the results indicate that the treatment completely removed the component of epicuticular wax, presumably nonacosan-10-ol, that normally forms crystalline wax.

Successful wax removal

According to calculations by Jeffree *et al.* (1971), the porous wax plugs in the stomatal antechambers of *Picea sitchensis* reduce transpiration and photosynthesis by 36 and 17% respectively. Coastal provenances of *P. menziesii* have similarly occluded stomata and it was expected that removal of crystalline wax from stomatal antechambers of *P. menziesii* needles would promote stomatal gas exchange. An increase in stomatal conductance would then lead to either higher CO_2-assimilation rates at the expense of decreased water use efficiency or partial reduction in stomatal aperture in response to increased internal CO_2 mole fractions.

In both the 1993 and 1994 gas exchange measurements, the wax removal treatment had no definite effect on the stomatal conductance or photosynthesis of treated branches. However, when the treatments were separated into successful (>50% clean stomata) and unsuccessful wax removal (<50% clean stomata), those branches with successful wax removal showed no significant changes in stomatal conductance before and after treatment. Since the results from the cuticular conductance measurements suggest that epicuticular wax does provide a resistance to gas exchange, it appears that stomata on treated needles compensated for the loss of the physical resistance of the wax by increasing their physiological resistance or by reducing their stomatal aperture. This response is not entirely surprising since it suggests that the assimilation rate of *P. menziesii* needles is conservative and, as suggested by Wong *et al.* (1979), the stomatal conductance may be a function of the photosynthetic capacity of the tree rather than vice versa.

In the whole-plant chamber experiment, the response of the *P. menziesii* seedling to increased ambient CO_2 concentration supported the conclusion that stomata were sensitive to increased internal CO_2 concentrations. As c_i increased with increasing c_a, the stomatal aperture was reduced in an effort to maintain c_i constant. However, the slope of the dc_i/dc_a curve (0.4) suggests that the feedback response of the stomata was not sufficient to keep c_i absolutely stable. Thus, stomata on successfully treated needles may be maintaining a constant g_s by responding to metabolites of photosynthesis as well as changes in c_i.

Branches of seedlings treated successfully in 1993 and measured again in 1994 showed that stomatal conductance of treated needles was relatively high in one seedling, but low in the other. However, despite the inconsistent results, the facts that treated needles continued to be physiologically active one year later and that treated branches of seedling 1993-2 have higher g_s and A than adjacent control branches indicate that the treatment non-destructively removed the epicuticular wax. The reduction in both photosynthesis and stomatal conductance was presumably due to needle aging.

Jeffree *et al.* (1971) calculated that the resistance of the wax in the stomatal antechamber of *P. sitchensis* would be 1.8 s m^2 mol^{-1}. Unfortunately, this estimation could not be verified in the present study since a different species was studied and it appears that in *P. menziesii*, stomatal aperture is reduced to compensate for loss of the physical resistance of stomatal antechamber wax. Furthermore, it is erroneous to compare the resistance of the crystalline wax in stomatal antechambers to the resistance of wax covering the cuticle since the latter is composed of both crystalline and amorphous wax.

To my knowledge, no other study has been conducted to measure the effect of stomatal antechamber wax removal on gas exchange in conifer needles. Krause & Houston (1983) observed variations in the epicuticular wax structure of SO_2-tolerant and -sensitive *Pinus strobus* clones and noted that sensitive clones frequently had cracks in the stomatal antechamber wax. Although their hypothesis was not supported by actual measurements of stomatal gas exchange, they suggested that increased sensitivity may be due to the cracks which enhance stomatal conductance and allow increased influx of SO_2.

Unsuccessful wax removal

On seedlings where the treatment caused smearing of stomatal antechamber wax, there was a reduction in stomatal conductance and photosynthetic rates of treated needles. This was interesting since the pattern of destruction of crystalline epicuticular wax in stomatal antechambers was similar to that found on conifer needles exposed to various air pollutants. Riding & Percy (1985) studied the effects of air pollutants on epicuticular wax structure in *Pinus strobus* and *Pinus banksiana* needles. They proposed that fusion of the tips of wax tubules would further decrease the cross-sectional area available for gas diffusion through stomata and increase water-use efficiency. In contrast, severe degradation would block stomata open such that guard cells could no longer regulate gas exchange and, consequently, water-use efficiency would decrease. Neither of these possibilities appear to account for the results obtained after smearing the wax with the dental impression material. First, evidence from cuticular conductance measurements showed that seedlings with >50% occluded stomata had the least reduction in cuticular resistance after treatment; a situation which would not exist if severely degraded wax blocked stomata open. Moreover, rather than occlusion of stomatal antechambers causing an increase in WUE, there was an equal or even slightly greater reduction in assimilation rate relative to stomatal conductance. Although there is no obvious explanation for these results, it is evident that smeared wax forms a continuous film in many stomatal antechambers. Thus, the wax may be acting like an antitranspirant and, as most studies indicate, antitranspirants tend to reduce both transpiration and photosynthesis (Solárová *et al.* 1981). Also, due to the greater molecular weight of CO_2, even the best film forming antitranspirants tend to be about four times more permeable to water vapour than CO_2 (Solárová *et al.* 1981).

Unfortunately, there are few air pollutant studies that correlate gas exchange measurements with observations of stomatal antechamber wax degradation. Sauter *et al.* (1987) noted that complete occlusion of 30% of stomata on *Picia abies* needles reduced both photosynthesis and transpiration by approximately 30%. While these results are in agreement with our own, the pollutants may also have a direct effect on the physiology of the trees.

Measurements made in 1994 on 1993 unsuccessfully treated branches showed that the needles continued to have reduced stomatal conductance and photosynthesis relative to the control. Therefore, it appears that smearing stomatal antechamber wax caused long-term reductions in stomatal conductance, yet did not lead to needle senescence.

The cuticular resistance values of coastal *P. menziesii* were consistent with those reported for *Picea sitchensis* and *Pinus sylvestris* (Grace 1990; van Gardingen *et al.* 1991), but were somewhat lower than resistances measured in alpine *Pinus albicaulis* (Sowell *et al.* 1982) and *Picea engelmannii* (Hadley & Smith 1983, 1986). The large variation in cuticular resistance within individual trees was also evident in several of these studies (Hadley & Smith 1983; van Gardingen *et al.* 1991).

Wax removal clearly resulted in a decrease in cuticular resistance indicating that epicuticular wax acts as a resistance in series with that of the cuticle. However, because there was no means of determining whether all crystalline and amorphous wax had been removed, it was not possible to assign an absolute resistance value to epicuticular wax.

In general, needles with successful wax removal showed the greatest reduction in cuticular resistance. Due to the upright orientation and porous nature of crystalline wax, it is unlikely that this wax layer offers a significant resistance relative to the cuticle. Therefore, the reduction in cuticular resistance may be primarily due to amorphous epicuticular wax removal. Limited amorphous wax removal may also explain why the removal of tubular wax from 85% of stomatal antechambers of seedling 1993-2 reduced cuticular resistance by only 5%. Conversely, although there must have been some wax removed by the treatment, seedlings with more than 50% occluded stomatal antechambers appeared to have more wax and hence, less reduction in cuticular resistance.

Other studies have shown a decrease in cuticular resistance when epicuticular wax was damaged or removed. For example, a 2 second dip in chloroform reduced the cuticular resistance of rice (*Oryza sativa*) leaves by 50 to 60% (O'Toole *et al.* 1979) whereas a 30 second exposure of *Vitis vinifera* leaves to a light petroleum vapour resulted in a 25-fold increase in cuticular water loss (Possingham *et al.* 1967). In addition, removal of wax from *P. menziesii* needles using a 2 second chloroform dip doubled cuticular conductance (unpublished results). Wind erosion of epicuticular waxes of several alpine conifers caused a 30% to 2-fold reduction in cuticular resistance (Hadley & Smith 1983; van Gardingen *et al.* 1991), while exposure of current year *Pinus sylvestris* needles to SO_2 polluted air resulted in epicuticular wax degradation and a 25% reduction in cuticular resistance. However, results obtained after any wax removal technique should be interpreted with care. Use of chemical solvents can confound results by removing lipoidal material from the cuticle and epidermal cells. Furthermore, processes that mechanically remove or erode waxes can damage underlying tissue and guard cells and create "leaky" or partially open, dysfunctional stomata that lead to substantial increases in apparent cuticular water loss (Pitcairn *et al.* 1986).

Although our wax removal treatment caused a significant decrease in cuticular resistance in some seedlings, the results seem to indicate minimal damage due to the treatment. Firstly, there was no increase in variation of r_c due to the wax removal treatment as would be expected if the treatment had caused variable damage to the cuticle, epidermal cells or guard cells of needles. Secondly, in his study on alpine timberlines, Grace (1991) noted that stomata damaged by wind abrasion tended to be less responsive to decreasing water status of needles during cuticular conductance measurements. However, in the present study, stomata from both treated and control needles were equally responsive to the gradual drying process and reached minimal rates of conductance at approximately the same time.

Finally, although the possibility of minor damage due to the treatment cannot be entirely excluded, it is apparent that even the largest reduction in cuticular resistance (40%

in seedling 1994-1) would not have a significant effect on transpiration rates when the stomata were open. Assuming that stomatal conductance averages about 0.2 mol m^{-2} s^{-1}, the 40% reduction in cuticular resistance measured in seedling 1994-1 would increase transpiration by only 0.6%.

Finally, based on SEM observations of treated needles and results from cuticular conductance and gas exchange measurements, the wax removal technique had been improved from 1993 to 1994. Therefore, more emphasis was placed on 1994 results.

5. SUMMARY

The purpose of this work was to determine the physiological function of epicuticular wax and its significance in terms of cuticular resistance and stomatal gas exchange in coastal *P. menziesii*. However, in order to attempt such measurements, a technique had to be devised to non-destructively remove the epicuticular wax. These efforts were realized with the use of a fast-setting dental impression compound. Treated needles remained green and physiologically active and review of the current literature suggested that of all the methods employed, this treatment caused the least damage. Also of interest was the fact that after removing the crystalline epicuticular wax, it never grew back.

Successful wax removal, where greater than 50% of stomata were clear, had no appreciable affect on stomatal conductance or photosynthesis. In addition, whole-plant measurements indicated that stomata of *P. menziesii* were sensitive to changing internal CO_2 mole fractions. Thus, it appears that if there was an increase in stomatal conductance caused by removal of the physical resistance of the wax it was offset by the partial reduction of stomatal aperture. This suggested that the assimilation rate of *P. menziesii* was conservative and that wax in the stomatal antechambers did not limit productivity.

Smearing the crystalline wax in stomatal antechambers into an amorphous mass led to an overall reduction in both stomatal conductance and photosynthesis. The smeared wax appeared to form a continuous wax film over the stomata such that the wax acted like an antitranspirant that was almost equally resistant to both CO_2 and water vapour diffusion.

Although we were unable to quantify the resistance of the epicuticular wax, its removal clearly resulted in a decrease in cuticular resistance thus indicating that the wax acted as a significant resistance in series with that of the cuticle.

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Title of Thesis: The Physiological Function of Epicuticular Wax in Coastal Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco)

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