Monitoring environmental features using leaf stomatal characteristics

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by

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Declaration

The material contained within this thesis has not been previously submitted for a degree at the University of Durham or any other University. The research reported within this thesis has been conducted by the author unless indicated otherwise.

Michelle Metcalfe
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Abstract

It is recognised that atmospheric CO_2 concentrations are increasing, with concerns raised as to the global impact continued rises may have. Plant stomatal parameters have been suggested as a means of monitoring changing CO_2 levels. In order to assess their suitability for use, a thorough examination of leaf characteristics within a variety of surroundings was undertaken. Environments examined included both natural variations in CO_2, which incorporated a variety of environmental influences and artificially enhanced ones where it was possible to monitor specific responses to variable CO_2 levels. A short term study of *Ranunculus ficaria, Sambucus nigra* and *Hedera helix* revealed considerable variation in stomatal parameters, with inconsistent responses observed between and within species. These variations could not be attributed to CO_2 changes and were assigned to a combination of other factors. *R. ficaria* grown in an artificial environment at ambient and elevated CO_2 produced significant and consistent changes in stomatal parameters. Stomatal density and index were found to be reduced at elevated CO_2 concentrations, with a decrease in guard cell dimensions. *Salix herbacea*, growing along a naturally reduced CO_2 partial pressure gradient further highlighted the extent of variation in stomatal parameters in extant material within a species. No consistent stomatal trends associated with changing CO_2 levels were observed. This inherent variation must be considered before any conclusions can be made regarding stomatal parameters obtained from fossil material. The novel application of molecular biology techniques to identify *S. herbacea* leaves was partially successful. However, identification of leaf macrofossils was not possible, due to the inferior preservation of DNA. However, the technique will provide a useful tool for identification if suitably preserved macrofossil fragments were available. *S. herbacea* leaf macrofossils revealed no consistent correlation to past changes in CO_2 levels. The use of $\delta^{13}C$ as an additional tool with which to monitor environmental change once again showed variation, and was not associated closely with changes in CO_2 levels or stomatal parameters. In light of this work it would appear that the use of stomatal parameters is of limited value as a model to monitor environmental change, in the absence of information concerning other variables, and as such must be treated with caution.

Abbreviations used in text

aDNA	ancient deoxyribonucleic acid
ABA	abscisic acid
ATP	adenosine triphosphate
bp	base pair
BP	before present
BSA	bovine serum albumin
C_3	plants which dominantly utilise Rubisco to metabolise carbon
C_4	plants which dominantly utilise PEP to metabolise carbon
^{12}C	carbon of isotopic mass 12
^{13}C	carbon of isotopic mass 13
ca.	approximately
CAM	those plants which adopt Crassulacean Acid Metabolism
CH_4	methane
cm	centimetres
CO_2	carbon dioxide
CTAB	mixed alkyltrimethyl ammonium bromide
CTP	cytosine triphosphate
°C	degrees Celsius
$\delta^{13}C$	the change in ratio of ^{13}C to ^{12}C
DEC	decrease
DNA	deoxyribonucleic acid
ECD	epidermal cell density
EDTA	ethylenediamine tetra acetic acid
FAA	Formyl-Aceto-Alcohol
GTP	guanosine triphosphate
IAA	isoamyl alcohol
INC	increase
kb	kilo base
m	meters
ml	millilitres
mm	millimetres
mm^{-2}	square millimetres

n.s	non-significant
p value	probability
PCR	polymerase chain reaction
PDB	PeeDee Belemnite (used as a carbon isotope standard)
PEP	phosphoenolpyruvate carboxylase
p_a	the ambient partial pressure of carbon dioxide
p_i	the partial pressure of carbon dioxide within the leaf vacuole
PL	pore length
p.p.m.v	parts per million by volume
PW	pore width
%	percent
‰	permille
r value	correlation coefficient
Rubisco	ribulose-1,5 -bisphosphate
rDNA	ribosomal deoxyribonucliec acid
rpm	revolutions per minute
SD	stomatal density
SEM	scanning electron microscopy
SI	stomatal index
SW	stomatal width
sig	significance
TAE	tris-acetate-EDTA buffer
TBE	tris-borate-EDTA buffer
TE	tris EDTA
Tris	tris (hydroxymethyl) methylamine
TTP	thymidine triphosphate
µm	micrometers
UV	ultra-violet
V	volts
v/v	volume for volume
WUE	water use efficiency
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

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Chapter 1.

1. Introduction.

1.1 Environmental Change.

Increases in atmospheric 'greenhouse gases' since the Industrial Revolution and the effects these may have on global ecology have attracted considerable attention in recent years (Sundquist, 1993). The rapid rise in concentration of these gases, including carbon dioxide (CO_2) and methane (CH_4), have been brought about by the burning of fossil fuel, deforestation and changes in agricultural practices and are predicted to continue to increase in the future (Houghton *et al.,* 1990). The earth's mean global atmospheric CO_2 concentration is predicted to increase from the current ambient level of 450 parts per million by volume (p.p.m.v) to 520 by the year 2100, assuming the present pattern of fossil fuel burning (Mitchell *et al.,* 1990). This corresponds to an increase of approximately 1.8p.p.m.v per year (IPCC, 1990; Watson, 1992). It is further predicted that the resulting 'greenhouse effect' will cause an accompanying rise in mean surface temperature of 1.5-4.5°C (Mitchell *et al.,* 1990). The changes in global temperature over the last 40 years do not correlate closely with CO_2 changes, suggesting the relationship between the greenhouse effect and increased CO_2 is not simple (Beerling & Woodward, 1994). However, the speed at which CO_2 levels will actually change is dependent on both the scale of human activity and the response of the natural carbon cycle. The Intergovernmental Panel on Climate Change (IPCC) has investigated some of the atmospheric and terrestrial processes that influence the climate system (Houghton *et al.,* 1990, 1992). The future climate will be influenced by a range of factors and feedback mechanisms, many of which are not yet fully understood. Therefore there are major limitations of using model projections such as General Circulation Models (GCMs) to make adequate predictions about the future climate (Wigley & Raper, 1992).

It is recognised that variations in atmospheric CO_2 concentrations have occurred throughout the World's history (Gammon *et al.,* 1985). Measurements of changes in the atmospheric concentration of CO_2 have shown dynamics over many time scales, from

months, years and decades (Bacastow & Keeling, 1981), and centuries (Neftel *et al.*, 1985), to millennia (Neftel *et al.*, 1982; Barnola *et al.*, 1987). Changes in CO_2 levels over millennia have been implicated in triggering the onset and completion of ice ages (Hays *et al.*, 1976), whereas changes over centuries, such as the period from the 18th century to the present, indicate the increased anthropogenic influence on the atmosphere (Friedli *et al.*, 1986). Continuous monitoring of CO_2 concentrations in the atmosphere at Mauna Loa, Hawaii provide data post-1958 (Keeling *et al.*, 1976; 1989), before this CO_2 levels are obtained from ice core records (e.g. Barnola *et al.*, 1987).

Changes in atmospheric CO_2 concentrations have already been implicated in exerting effects on plant growth (Houghton *et al.*, 1990). Many plants have shown marked increases in growth rates, size and biomass when grown at elevated CO_2 (LaMarche *et al.*, 1984; Graumlich, 1991; Hunt *et al.*, 1991; Woodward *et al.*, 1991). As plants play such a major role in the global carbon budget through the photosynthetic fixation of CO_2 (Brown & Lugo, 1982), many investigations have been carried out examining the effects of elevated atmospheric CO_2 concentrations on plant growth, morphology and physiology (as reviewed by Beerling & Chaloner, 1992; Woodward *et al.*, 1991).

To predict how plants may respond in the future, with respect to an anthropogenically elevated atmospheric CO_2 concentration, and the resulting altered climate (so-called 'global warming') requires an understanding of both the 'long-term' (Beerling *et al.*, 1993), and 'short-term' responses. The 'long-term' response incorporates the changes observed over hundreds, thousands or millions of years, whereas 'short-term' studies report on time scales from brief periods of exposure (e.g. minutes) to a few hundred years.

Stomatal density (expressed as the number of stomata per unit area of leaf) is a physiological parameter of major importance, as it underlies the productivity of all the Earth's terrestrial vegetation and is thought to be related directly to environmental features such as CO_2 levels (Beerling *et al.*, 1993). Stomatal density is able to affect both the rate of carbon uptake and water use efficiency (WUE) (defined as the number of units of CO_2 fixed in a given time per unit of water lost in the same time period (Friend & Woodward,

1990). A consideration of stomatal characteristics could therefore give an indication of past and potential future changes within the environment.

1.2. Stomata.

Some 400 million years ago plants left the aquatic environment and invaded land. In order to colonise land and survive plants had to develop features which would prevent excessive water loss, whilst allowing CO_2 to enter the plant in order for photosynthesis to proceed. A hydrophobic waxy outer layer or cuticle evolved that prevented desiccation of the plant. However, the necessity of the plant to lose water by evaporation was inhibited by such a layer and was detrimental to gaseous exchange Without the development of stomata in the epidermis, the supply of photosynthetic substrate would have been insufficient to maintain plant life (Martin *et al.,* 1983). It is generally considered that the cuticle, stomata and vascular tissue developed simultaneously leading to the emergence and development of large terrestrial plants (Chaloner, 1970).

Stomata are pores formed by a pair of specialised 'guard' cells of the epidermis, and are found in the surface cellular layers of aerial parts of most higher plants. The terms stoma and stomata refer to the pores, the guard cells and the adjacent cells that form the stomatal complex. Stomata allow atmospheric CO_2 to diffuse into the intercellular air spaces of the leaf tissue where it dissolves in the aqueous phase allowing photosynthetic reduction to take place. During this diffusion water is lost through the stomatal pores to the atmosphere by transpiration. As evolution progressed, stomata became more versatile, developing opening and closing mechanisms. This allowed sufficient CO_2 to enter the leaf for photosynthesis to proceed, whilst maximising water conservation.

Guard cells are observed in two basic forms, the elliptical (kidney-shaped) type, which are found in most plants, and the graminaceous (dumbbell-shaped) type which, as the name suggests are characteristic of grasses (Sack, 1987).

The adjacent cells of guard cells are also categorised. If they are found to be morphologically distinct they are termed 'subsidiary cells'; if there is no distinction of morphological appearance, they are referred to as 'neighbouring cells' (Weyers & Meidner, 1990). In addition, stomatal complexes can be classified on the basis of the number and positions of the subsidiary cells and on the ontogeny of the cell types as described in Baranova (1987).

Distribution of stomata.

The leaves of most herbaceous plants contain stomata on both the upper (adaxial) and lower (abaxial) surface and are termed 'amphistomatous'. They usually possess fewer stomata on the upper surface, with the exception of some Graminaeae species. However, this becomes less clear when considering grass leaves that are often twisted, so that the true adaxial surface may be on the underside (Weyers & Meidner, 1990). Leaves with stomata on the abaxial leaf surface only, including virtually all tree species, are termed 'hypostomatous' and account for approximately two-thirds of species (Slavik, 1974). Aquatic plants with floating leaves, e.g. water lilies are found to have epistomatous (or hyperstomatous) leaves, where the stomata are only found on the adaxial epidermis. Submerged leaves of aquatic angiosperms generally have no stomata at all (Weyers & Meidner, 1990).

Formation of stomata.

Stomata arise through differential divisions in the protoderm, which becomes secondarily meristematic, forming guard mother cells. The governing mechanisms are not clearly understood, but placement of division planes in parent cells (Palevitz, 1981), leads to the formation of stomata. How stomatal density is controlled during leaf growth is still unknown. Giles & Shehata, (1984), found cell division and elongation in *Zea mays* were related to plastochron index and that guard mother cell differentiation was linked to the potassium pump. Friend & Woodward, (1990), hypothesised that the responses observed in stomatal densities and indices were under the mechanistic control of a substance such as

ATP. This theory has since been questioned. It is clear however, that the formation of both the guard cell initials and mature guard cells are subject to influence by environmental factors.

1.3 The functional response of stomata to environmental factors.

The leaf surface positioning of stomata means that they are ultimately situated to control the influx and efflux of gases between the interior of the leaf and its environment. The guard cells are usually only connected to neighbouring cells via their dorsal walls, and, at maturity, these walls do not possess functional plasmodesmata. Therefore, as they are relatively isolated from the rest of the plant body, stomata are ideally situated for sensing and responding to environmental factors (Willmer & Fricker, 1996). A wide range of environmental variables influence stomata indirectly or directly. Two factors affecting stomata on a daily basis are light and CO_2. However, temperature, water relations, humidity, pollutants and the availability of mineral nutrients are all known to influence the guard cell response and thus produce a net stomatal response. In addition stomata are under the control of a 'biological clock' which regulates stomatal circadian rhythms, which is thought to reside in the guard cells (Willmer & Fricker, 1996).

The effect of light on the functional response of guard cells.

Light is probably the most important factor in determining stomatal behaviour. Stomata respond to both light quality and quantity and are influenced by the visible spectrum range ($\cong$ 400-700nm) and ultra violet radiation. In most plants (with the exception of CAM plants) stomata open during the day and close at night, being under the influence of a circadian rhythm (Willmer & Fricker, 1996).

The effect of CO_2 on the functional response of guard cells.

CO_2 concentration is probably detected by the plant guard cells either on the external surface of the plasma membrane or within the cells themselves. Stomata normally close as the ambient CO_2 concentration increases, and open as it decreases.

Intercellular CO_2 (C_i) levels are also thought to affect stomatal activity (Mansfield, 1985; Willmer & Fricker, 1996). The response of stomata to CO_2 appears to be species dependent as indicated by Morison, (1987).

The effect of temperature on the functional response of guard cells.

Guard cell metabolic activities will tend to increase with increased leaf temperature until an optimum level is reached, at which stage down regulation occurs to avoid cell damage. The result of increased activity will tend to stimulate opening of the stomatal pore and thus temperature is able to affect C_i, (Willmer & Fricker, 1996).

The effect of water availability on the functional response of guard cells.

Water deficits occur when the rate of water loss from the plant exceeds the rate of water uptake. Guard cells are able to regulate water loss by opening and closing the pore aperture. Substantial water deficit has the general effect of depressing daytime stomatal conductance (Davies *et al.,* 1981). The sensitivity of stomata to a decrease in leaf water potential varies between species and is influenced by age and growth conditions (McCree, 1974; Turner & Begg, 1981).

The effect of environmental change on stomata.

As can be seen, stomata are highly specialised structures that possess an array of cellular and metabolic responses, enabling a plant to adjust to prevailing environmental conditions. However, in addition to regulating guard cell behaviour, plants can alter stomatal patterning and density in response to environmental change. Thus, whilst specific stomatal numbers have been reported for many plant species, their values are not always fixed under different environmental conditions. In fact, numerous studies have found that there is considerable plasticity in the formation of the stomatal complement in leaf tissue (Willmer & Fricker, 1996).

In previous studies emphasis has been placed on studying stomatal density, as it is a major ecophysical parameter which directly influences the productivity of the world's vegetation. It is therefore important to understand its response to environmental changes. Considerable attention has focused on a palaeological perspective using changes in stomatal density to determine atmospheric CO_2 concentrations through geological time, with a view to predicting the response of plants to the forecasted future increases in CO_2 concentrations (Beerling *et al.,* 1993, van der Burgh *et al.,* 1993; McElwain & Chaloner 1995).

1.4 Stomatal density and index.

Stomatal density is 'the number of stomata per unit area of one leaf surface', and is established during the early stages of leaf development (Dale, 1982*a*). Stomatal density (of a stomata-bearing epidermis) varies from 20 to 2000 pores mm^{-2}, among species with typical values falling between 40 and 350 (Weyers & Meidner, 1990). It can vary significantly within leaves, plants or individuals of a single species within a community and can be modified by environmental factors, leaf morphology and genetic composition levels (Weyers & Meidner 1990; Weyers *et al.,* 1996). Large differences in stomatal density is seen among species, cultivars (Salisbury, 1928; Meidner & Mansfield, 1968), height of leaf insertion and leaf area (Tichá, 1982). Within an individual plant, the fully expanded leaves exhibit ontogenetics in the spacing of stomata (Tichá, 1982). The shortcoming associated with using stomatal density as a monitoring parameter is that it is subject to variation related to changes in epidermal cell size and expansion (Meidner & Mansfield, 1968). Indirect changes in stomatal density in response to environmental stimuli, can be mediated by effects on epidermal cell expansion and size. For example, if an environmental factor caused an increase in leaf expansion, and the total number of stomata within the leaf remained the same, the stomatal density would effectively decrease. Therefore, it could be incorrectly assumed that the environmental factor was actually reducing stomatal initiation. To overcome this problem stomatal index (SI) is adopted as a measured parameter. Stomatal index is considered to remain fairly constant within leaves of the same plant (Salisbury, 1928), and relates the number of stomata per

unit area to the number of epidermal and guard cells per unit area within the leaf. Therefore stomatal index can be used to determine whether an environmental parameter is exerting a direct effect on stomata, since in order to have an effect the actual proportion of stomata initiates, or the ratio of stomata to epidermal cells must change. It is thought that stomatal index remains stable, except under extreme experimental conditions. However, some studies have reported changes in stomatal index associated with environmental change (Beerling, 1993).

The influence of environmental factors on stomatal density and index.

Environmental factors known to influence stomatal density and index include light intensity, water availability, and CO_2. Before considering the effect of changing CO_2 concentrations on stomatal parameters, the aforementioned will be briefly considered.

The influence of light.

Stomatal density is usually found to be higher in plants grown under higher light intensity (Salisbury, 1928; Wild & Wolf, 1980). There is often a change in leaf area associated with a change in leaf irradiance, indicating that light intensity is one of the factors determining leaf area, by influencing both cell enlargement and cell division (Rahim & Fordham, 1991). However, it has been argued that the overall stomata numbers per leaf and stomatal index may not be significantly different (Kubinová, 1991). The observed responses of leaf area to light intensity are found to vary between studies. Friend & Pomeroy, (1970), reported an increase of leaf area in low irradiance, whereas Dale *et al.*, (1972), found no change and Lichtentaler, (1985), found a decrease. The reasons for these apparently conflicting results are not clear, but could be due to species specific differences (Willmer & Fricker, 1996). As the growth of the new leaf is likely to be controlled by previous leaf growth (Dale, 1982*b*), in some species the light intensity and quality received by mature leaves can affect the stomatal index of developing leaves as demonstrated by Schoch *et al.*, (1980). The response of stomata index to light intensity is also not consistent (Schoch

et al., 1980; Rahim & Fordham, 1991; Poole *et al.,* 1996). The red to far-red ratio can also effect stomatal parameters (Mitchell & Woodward, 1988).

The influence of water availability.

Water availability is known to effect both stomatal behaviour and development. In fact, the effect of drought on plants causes an extensive range of responses in which almost every growth parameter is changed if the stress is applied for a long enough time period, at a sufficient magnitude (Hsiao, 1973). Long term exposures to water deficits can have developmental effects on guard cell size (Spence *et al.,* 1986). Limited water availability was found to increase stomatal densities and decrease leaf areas (Ciha & Brun, 1975; Rawson & Craven, 1980). An opposite response is exhibited on leaves growing under conditions of high water availability which are found to have lower stomatal densities (Tichá, 1982). Stomatal indices are also effected by water availability, with a decrease reported in response to a reduction in water availability (Ferris, 1991; El-Hashani 1996).

The influence of CO_2 concentrations.

Atmospheric CO_2 has often been found to have a direct effect on stomatal density and index in both short and long-term studies. However, the recorded changes in stomatal parameters are conflicting. Pioneering studies of Bristow & Looi, (1968), showed that stomatal density increased as CO_2 concentration decreased. This was further investigated using present day, herbarium and fossil specimens. Woodward, (1987), first demonstrated the response of stomatal density to ambient CO_2 concentration in herbarium specimens of leaves of several native trees. He found that stomatal densities had declined by about 40% over the last 200 years. The atmospheric CO_2 concentration over the same period, established from the Siple Station ice core record (Friedli *et al.,* 1986), increased by 60 p.p.m.v . Woodward therefore, suggested that atmospheric CO_2 changes could be directly affecting stomatal density. This decrease in stomatal density was associated with a drop in stomatal index, suggesting that there was a definite change in stomatal initiation, and not simply a leaf expansion response. Peñuelas & Matamala, (1990), also documented a

decrease in the stomatal density of leaves of plants from temperate and Mediterranean ecosystems in response to rising CO_2 levels over the past three centuries. Other studies have since confirmed this response in extant species (Beerling & Chaloner, 1993*c*; Beerling et al., 1993).

These results were corroborated by the controlled environment experiments of Woodward & Bazzaz, (1988), which again showed an inverse relationship between stomatal density and CO_2 concentration. In addition, Beerling & Woodward, (1995), also found that the stomatal densities and indices of variegated leaves on unpigmented and pigmented leaf areas grown at 350 and 700 μmol mol^{-1} decreased following increased CO_2 exposure. Variegated leaves allow any potential effects of mesophyll photosynthetic capacity to be uncoupled from the responses of stomatal density to changes in atmospheric CO_2 concentration. Stomatal densities and indices on homobaric variegated leaves showed a greater sensitivity to CO_2 on green portions, whereas heterobaric leaves showed a greater sensitivity on white areas. The results also indicated that structure might play an important role in determining the magnitude of stomatal densities and indices in response to changing CO_2 concentrations.

In spite of the importance of studying short-term responses in controlled environments, studies were extended to investigate the long-term responses of stomatal parameters to environmental change. By studying fossil leaves it was thought possible to measure stomatal parameters changes in response to CO_2 concentrations through many thousands of generations. Decreases in stomatal densities associated with increased atmospheric CO_2 concentrations has been reported using fossil leaves (Beerling & Chaloner 1993*b*; Van de Burgh *et al.*, 1993; Van de Water *et al.*, 1994; McElwain & Chaloner, 1995).

Responses shown by fossil leaves to the fluctuations in atmospheric CO_2 through such time periods offers the possibility of providing an independent complement at low latitudes, to the ice core records of the Polar regions.

The ice core record at present is limited to the last two glacial cycles, whereas the fossil leaf records offers a potential indicator of past CO_2 concentrations at least as far back as extant species can be recognised (Beerling & Chaloner, 1994). For this reason palaeo-botanists have attempted to use stomatal characters to determine palaeoclimates (Beerling *et al.,* 1993, van der Burgh *et al.,* 1993; McElwain & Chaloner 1995).

In addition to the implications of these findings from palaeological perspectives, suggestions were further made that plants have modified their stomatal density to enhance water economy, whilst maintaining their productivity (Beerling & Chaloner, 1993*c*). Improved water economies of plants grown under elevated CO_2 levels has been confirmed by other studies which do not refer to stomatal density (e.g. Morison & Gifford, 1984; Morison, 1985; Eamus & Jarvis, 1989), suggesting that such an adaptation would be an evolutionary advantage to plants in an elevated CO_2 environment. The potential disadvantage of reducing stomatal density in response to increasing CO_2 levels is that of a rise in leaf temperature, due to reduction in evapotranspirational cooling, with the added possibility of small but significant reductions in photosynthesis.

However, such investigations and suggestions formulated from these results have often failed to acknowledge contrasting results in the field. The response of stomatal parameters to increased CO_2 concentrations is not always apparent. Some species have been reported to actually show no effect of CO_2 enrichment on stomatal density, stomatal index or both. Körner, (1988), found no consistent response on Austrian plants to atmospheric CO_2 concentration increases, comparing published stomatal density figures (1864-1919) with corresponding fresh material and finding no differences in stomatal parameters. Although, Peñuelas & Matamala, (1990), had reported a decrease in stomatal density with elevated CO_2 levels, no significant reduction in stomatal index was observed, suggesting CO_2 was not exerting a direct effect on stomata. Both studies concluded that any global increase in CO_2 appeared to affect only epidermal cell expansion. Other species also found to record no response have included French beans (O' Leary & Knect, 1981, Radoglou & Jarvis, 1992); chestnut seedlings (Moussea & Enoch, 1989); poplar clones (Radoglou & Jarvis,

1990); perennial ryegrass (Ryle & Stanley, 1992); oak trees (Miglietta & Raschi, 1993), and steppe grasses (Morgan *et al.,* 1994).

In addition, mixed responses in stomatal density and indices were found in plants exposed to elevated CO_2 levels grown either in open top chambers or controlled environment growth chambers (Thomas & Harvey, 1983; Ferris & Taylor, 1994; Knapp *et al.,* 1994). Ceulemans *et al.,* (1995), found that two poplar clones, reduced their stomatal densities and indices under elevated CO_2 in expanding leaves in the upper portion of the plant, but showed no reduction on the mature leaves from the middle/lower portion of the plant. This indicates that interactions with leaf age and/or position often confounded the CO_2 effect.

Clearly there are conflicting observations regarding the effect of changing CO_2 concentrations on stomatal parameters. Previous studies on both modern and fossil leaves have rarely considered the natural variations found within and between leaves of the same species. If realistic and accurate attempts are to be made predicting the response of plants to environmental change, and in particular rises in atmospheric CO_2 concentrations, it is postulated that a number of other factors need to be taken into account. These include the natural variation in stomatal parameters inherent within and between plant species; the variation which may be observed when a plant is subjected to growth in a situation which allows other environmental factors to influence stomatal parameters, and the variation introduced by collection methods. Assessment of these factors will provide a better model to predict future responses more accurately, and would indicate the extent to which bias can be introduced from fossil sampling. Pioneering work in light of these considerations has been reported by Poole *et al.,* (1996).

This study examines a variety of plant species in both artificial and natural environments to assess the potential of using plant stomatal characteristics as a tool to monitor environmental change. Because fossil material was to be used in this study it was deemed important to accurately identify leaf samples, due to the variations observed in stomatal parameters between species as discussed. Identification using microscopy has been utilised in the past, however in this study a novel approach using ancient DNA molecular biology techniques was attempted. In addition to stomatal analysis, another technique,

utilising the carbon isotope, ^{13}C was attempted to monitor environmental change. Careful examination of extant species will hopefully provide information as to the reliability and potential use of using fossil material to study environmental changes.

Chapter 2.

The effect of both natural and artificial changes in CO_2 concentration on plant stomatal characteristics.

2.1 Introduction.

Changes in atmospheric CO_2 concentration have already been implicated in exerting effects on plant growth (Houghton *et al.*, 1990). Much of the literature has indicated a fall in leaf stomatal density in response to rising atmospheric CO_2 concentration (Woodward, 1987; Beerling & Chaloner, 1992; Peñuelas & Matamala 1990). Experiments using artificial environments (growth chambers) have also shown that increasing CO_2 concentration causes a reduction in stomatal density (Thomas & Harvey, 1983; Woodward, 1987), and, additionally, it has been suggested that CO_2 may exert different effects between species (Woodward & Bazzaz, 1988; O'Leary & Knecht, 1981). These experiments led to the idea that it may be possible to monitor changes in environmental CO_2 levels using plant stomatal characteristics. However, implicit in these analyses is the assumption that the CO_2 concentration is the principal causal factor responsible for the observed changes in stomatal parameters. It has not been determined to what degree, if any, other environmental factors could be contributing to the change. In addition, previous studies rarely took into account the differences in stomatal parameters found among species (Salisbury, 1928; Meidner & Mansfield 1968), and mainly reported the response of an individual species. Changes in stomatal parameters have also been attributed to specific responses by individual species to changing atmospheric CO_2 concentrations (Malone *et al.*, 1993).

In this study the response of three species found growing within a defined natural area will be described. The aim being to investigate whether there was a consistent response of stomatal parameters in different species to changing environmental parameters. Criticisms have been made of experiments in which plants were exposed to short-term controlled experimental elevated CO_2 regimes because of the poor coupling and the balance between energy supply and water loss from leaves (Morison, 1987). Criticism has also been made of long-term studies which report on the

response of stomatal density over centuries, which incorporate extensive changes of CO_2 levels (Beerling *et al.*, 1992; Beerling & Chaloner, 1993*b*; 1994; van der Burgh *et al.*, 1993; McElwain & Chaloner, 1995). For these reasons, this study aimed to investigate the response of natural vegetation growing *in situ* that was well coupled with the atmosphere. The responses of stomatal density recorded would therefore be the product of a combination of environmental factors including CO_2, temperature, light, and water availability.

The Italian Offanto valley is a volcanic region and was chosen for the collection of plants since it possesses naturally elevated levels of CO_2. This enabled the detection of variations in plant responses to CO_2 as a consequence of natural selection of repeated generations of individuals. The CO_2 gradient prevalent at the Offanto valley offers the potential for recording the response of the species to a more realistic change in CO_2 level in comparison to previous artificial studies. CO_2 levels are known to vary by 20p.p.m.v between the crater region and the valley due to volcanic influence (Dr. J.A. Pearson, personal communication). At the present annual rate of CO_2 increase of 1.8 p.p.m.v (Houghton *et al.*, 1990), this difference is approximately equivalent to an increase over 11 years.

The species studied, *Ranunculus ficaria, Sambucus nigra* and *Hedera helix* are all well established in the region. Analysis of these plants will provide novel information as to the response of stomatal parameters to environmental change in a true environment. However, as controlled environmental chambers are known to induce changes in stomatal initiation (Woodward, 1987), it is important to additionally consider individual species responses under the influence of a much elevated CO_2 gradient. This will provide information as to whether conclusions drawn about the effects of CO_2 on plants and populations in controlled environments were equally apparent in a natural environment. This would assess the feasibility of using stomatal parameters to predict responses to environmental change.

2.2 Materials and Methods.

2.2.1 Species used to assess the effect of naturally enhanced CO_2 levels on plant stomatal parameters.

***Ranunculus ficaria* (lesser celandine).**

R. ficaria is a herbaceous terrestrial or marsh plant which can reach up to 25cm in height. It has fibrous roots and numerous fusiform or clavate root-tubers. The leaves are glabrous and all stalked with sheathing bases. The lowest leaves are very broad with the basal leaves being 1-4cm long and arranged in a rosette. These are long stalked and are cordate, bluntly angled or crenate. The stem leaves are similar but smaller and shorter stalked; all fleshy and glabrous (Clapham *et al.,* 1989).

***Sambucus nigra* (elder).**

S. nigra is a small tree-like-shrub which reaches heights of up to 10m. The leaflets are 3-9cm in size and are usually found in groups of 5-7. They are more or less ovate-elliptical in shape, acuminate, with the lower veins being sparsely hairy with serrated margins (Clapham *et al.,* 1989).

***Hedera helix* (common ivy).**

H. helix is an evergreen woody root climber. The leaves are arranged spirally and are simple, leathery, glabrous, exstipulate and dark green on the above surface. They often have pale veins that are sometimes tinged purple. The leaves beneath are paler. The leaf is palmately shaped possessing 3-5 lobes that are more or less triangular entire lobes (Clapham *et al.,* 1989).

2.2.2 Leaf material collection and storage.

R. ficaria, S. nigra and *H. helix* were kindly collected in Italy by Dr. J. Allen. They were sampled from the Lago Grande Major (LGM) Grande di Monticcho, which is the larger of two maar lakes of the western flanks of Monte Vulture (*Monte san Michele*) 1262m, Basilicata, southern Italy. The Monte Vulture is forested, with *Fagus sylvatica* and *Quercus cerris* the dominant species present above the lakes, and *Ilex aquifolium, Corylus avellana, Hedera helix* and *Rucus aculeatus* forming the shrub layer. An outflow stream is present below the lakes.

The modern climate is winter-wet with 510mm of the annual 815mm rain falling between October and March. The mean monthly temperature exceeds 20°C in June, July and August and falls below 5°C in January (Dr J. Allen, personal communication).

Leaves of each species were collected randomly when available from three sites within the Offanto valley region. The CO_2 levels at these sites were known to vary as shown below. The exact CO_2 level at the outflow is unknown, but included in this study as *R. ficaria* is not found at the crater.

No.	Site	Text label	CO_2 Level
1	The surrounding Offanto valley	Valley	Ambient 360 p.p.m.v
2	From the Lago Grande Major Crater lake	Crater	Raised by 20 p.p.m.v
3	From the outflow stream	Outflow	Variable

Two separate collections were made in October (1993) and April (1994). These are described simply as 1993 and 1994 respectively.
Material collected was preserved at the site in formyl-aceto-alcohol (FAA) (50% 100ml ethanol, 37-41% 6.5 ml formalin, concentrated 2.5ml glacial acetic acid).

2.2.3 The effect of artificially enhanced CO_2 levels on stomatal parameters of *R. ficaria.*

Growth at controlled CO_2 levels.

Experimental manipulation of the plant growth conditions were performed using the Solardome facility at The Bangor Research Unit, Institute of Terrestrial Ecology, Centre for Ecology and Hydrology University of Wales, Bangor, Gwynedd. *R. ficaria* plants were kindly donated from the Botanical Gardens, University of Durham. This species was selected for use in Solardome experiments as it is relatively easy to grow, produces leaves regularly and was also being studied in its natural environment.
Solardomes (see plate 2.1) provide large-scale exposure facilities that are designed to study the effects of predicted increases in carbon dioxide and temperature on

vegetation. They provide an excellent facility for monitoring the effects of artificially enhanced CO_2 levels on plant species.

Their features include :-

- Computer-controlled CO_2 regulation. Levels of approximately 340 p.p.m.v were used for experiments at ambient CO_2 levels and 680 p.p.m.v for experiments at elevated CO_2.
- Computer-controlled maintenance of differential temperatures between domes, e.g. ambient and 3°C tracked above ambient.
- Air monitors for recording levels of carbon dioxide, sulphur dioxide, ozone, nitrogen oxides and water vapour.
- Glass that transmits solar ultra-violet light thus maintaining a more realistic radiation conditions than silica glass.
- Computer-controlled variable speed fans to maintain good ventilation of the chambers.

Air is drawn through a particulate filter and over a heat exchanger. For high carbon dioxide treatments, computer-operated mass flow controllers release measured amounts of carbon dioxide into the air streams, which are ducted underground and emerge just above ground level in the centre of the Solardomes. A bulk liquid supply tank with a vaporiser is used as the source of carbon dioxide. The air is deflected across the test plants and exits from the Solardomes via peripheral vents. Air samples from within the Solardomes are pumped to analysers and levels of carbon dioxide and water vapour are computer logged. The Solardomes operate four complete volume air changes per minute. To counteract the solar heat gain for experiments at ambient temperature, air is cooled under computer control by a heat exchanger linked to a refrigeration unit.

Twenty *R. ficaria* plants were used in the study and grown in individual plant pots using soil taken from the collection site at the Botanical Gardens, University of Durham. Ten plants were placed at ambient (340 p.p.m.v) CO_2 levels and ten at elevated levels (680 p.p.m.v). The plants were watered daily and left to acclimatise

for three months, at which point any leaves or leaf buds present were removed. They were then left for a further two months. At this point four plants from each CO_2 environment (eight total) were removed transported to Durham. Two plants from each CO_2 level were placed back at their site of origin at the Botanical Gardens, University of Durham for a further two months. The plants were watered daily and exposed to normal fluctuations in light intensity and temperature. Since these plants were returned to their original habitat they were considered as being in a 'control' environment. The leaves from the other four plants were removed and stomatal parameters examined. The remaining 12 plants were left in the Solardomes for a further two months before sampling.

2.2.4 Stomatal counts and guard cell measurements.

Leaf surface sampling.

Leaf surface replicas were used to determine stomatal characteristics. The area of the *R. ficaria, S. nigra* and *H. helix* leaves studied was located midway between the tip and the base of the leaf, in order to avoid trichromes and large veins.

Micro-relief methods.

A permanent record of the leaf epidermal features and details of stomatal and epidermal cell density, and guard cell dimensions were achieved using cellulose acetate (Hsiao & Fischer, 1975, Jones 1983). A thin layer of colourless cellulose acetate (nail varnish) was applied to the adaxial and abaxial leaf surfaces of each species. The cellulose acetate was allowed to dry for between 20 and 30 minutes and the layer formed removed with forceps, placed on a microscope slide and covered with a cover slip.

Microscopy methods.

Replicas were examined by light microscopy (C. Baker light microscope) to obtain the stomatal density, epidermal cell density and hence stomatal index for each leaf replica sampled. The cell densities (number of stomata and the number of epidermal cells) were counted (unless otherwise stated) from ten fields of view (area 0.091mm^2) from each of the individual leaf surface replicas (usually total 50 microscopic fields of view per sample) at x400 magnification.

Cell densities.

Stomatal/epidermal cell numbers were recorded as raw data and later expressed as stomatal/epidermal cell densities (the number per unit area). Raw data was converted to cell densities per square millimetre using Microsoft Excel.

Stomatal index.

The stomatal index relates the number of stomata per unit area to the number of epidermal cells per unit area. Stomatal index is essentially independent of leaf size once the cells are differentiated.

Stomatal index is given by:

SI = [S / (E + S)] x 100

Where **SI** is the stomatal index

S is the number of stomata per unit area (stomatal density, **SD**)

E is the number of subsidiary and epidermal cells per unit area (epidermal cell density, **ECD**)

Stomatal guard cell dimensions.

Guard cell lengths were measured under the same magnification and field of view as cell densities. For each field of view one stoma was measured along its long axis using an eye piece graticule. A calibrated microscope slide was then used to convert measurements to micrometers (μm).

In parallel an attempt was made to measure pore length. It was unfeasible to measure stomata aperture using this method due to the fact it is difficult to distinguish between the "throat" of the stomatal pore and the eisodial aperture between the cuticular ledges of the guard cells (Weyers & Meidner, 1990). Micro-relief peels can fail to show

changes in the relevant pore dimension. The distance between the opposing guard cell ridges may appear to remain relatively unchanged, when in fact the throat of the pore may have changed considerably (Jarvis & Mansfield, 1981). Pore widths and lengths were determined to the nearest graticule unit. At low apertures, the accuracy of such measurements is limited by the resolution of the light microscope (about 0.2μm) and by light scattering effects (Weyers & Meidner, 1990). An eyepiece graticule pre-calibrated with a stage micrometer was used to calculate the area of the field of view. Fields of view were sampled at random.

2.3 Results.

2.3.1 The effect of naturally enhanced CO_2 levels on recorded stomatal parameters of *Ranunculus ficaria, Sambucus nigra* and *Hedera helix*.

The stomatal responses of each species will be given separately.
Error bars in all figures show standard error.

2.3.1.1 *Ranunculus ficaria.*

R. ficaria has elliptical type guard cells. The adjacent cells did not appear to be morphologically distinct and thus can be referred to as neighbouring cells ("anomocytic"). Stomatal distribution in *R. ficaria* was amphistomatous, as stomata were present on both surfaces.

R. ficaria was collected in both 1993 and 1994 from the crater and the outflow sites. *R. ficaria* was not present at the valley site. Stomatal parameters were recorded on both surfaces.

The effects of sampling site on stomatal parameters of *R. ficaria* are illustrated in Fig.2.1 and 2.2. Fig. 2.1 demonstrates the variation observed in cell densities and stomatal index of both 1993 and 1994 samples between the crater and the outflow site. Fig. 2.2 shows the differences recorded in guard cell dimensions.

Statistical analysis of data.

The results illustrated in Fig. 2.1 and Fig. 2.2 were analysed statistically using one-way analysis of variance (ANOVA) utilising Microsoft Excel. Comparisons were made between physiological surface responses of *R. ficaria* sampled at the crater site with those at the outflow site for each sample year (1993 and 1994), (Table 2.1). The differences between sample responses between years is then reported (Table 2.2). Data was statistically analysed at the 95% ($p<0.05$) significance level (Sig.). F crit values (F) are given as according to Bailey (1981). Abbreviations used in the results tables to illustrate the response (Res) are: INC, a significant higher result is recorded; DEC, a significant lower result is recorded; n.s. there is no significant difference.

Table 2.1. ANOVA analysis of stomatal parameters of *R. ficaria* collected at the crater and outflow. (F crit = 3.94)

	Abaxial surface						Adaxial surface					
	1993			1994			1993			1994		
	Sig	F	Outflow Res	Sig	F	Outflow Res	Sig	F	Outflow Res	Sig	F	Outflow Res
SD	p<0.05	70.7	DEC	n.s	0.25	---	p<0.05	5.76	INC	p<0.05	6.29	DEC
ECD	n.s	0.92	---	p<0.05	10.04	INC	p<0.05	17.7	INC	p<0.05	5.02	INC
SI	p<0.05	59.2	DEC	n.s	1.51	---	n.s	1.29	---	p<0.05	10.3	DEC
SL	p<0.05	113	INC	n.s	0.9	---	p<0.05	90.1	INC	n.s	3.27	---
PL	p<0.05	48.3	INC	n.s	0.48	---	p<0.05	4.27	INC	n.s	0.02	---
SW	p<0.05	228	INC	n.s	0.0	---	p<0.05	98.7	INC	p<0.05	6.26	DEC

Abaxial surface response.

1993 sample.

As outlined in Table 2.1, *R. ficaria* collected at the outflow showed significantly lower stomatal density and stomatal index than specimens collected at the crater. Guard cells were found to be significantly larger at the outflow with respect to all recorded dimensions.

1994 sample.

Results obtained for the abaxial surface of 1994 were inconsistent with those of 1993. Epidermal cell density was significantly higher in outflow specimens, however, there was no significant difference in stomatal density or stomatal index. Guard cell dimensions showed no significant differences between habitats.

Adaxial surface response.

1993 sample.

The adaxial surface of plants collected at the outflow site showed significantly higher cell densities than at the crater. However, there was no significant difference in stomatal index. Guard cell dimensions were significantly higher in plants collected from the outflow.

1994 sample.

A similar response to 1993 was not seen in 1994. A significantly lower stomatal density was found in outflow specimens along with a significantly higher epidermal

cell density. The resulting stomatal index was found to be lower in outflow plants. When compared with the guard cell dimensions of 1993, stomatal width was lower in outflow plants and no significant difference was recorded in either stomatal or pore length.

Comparison of *R. ficaria* stomatal parameters between 1993 and 1994 at the crater and outflow sites.

Data obtained in the two successive years were compared with each other in order to assess whether stomatal parameters varied between sampling periods.

Table 2.2. Statistical analysis of recorded stomatal parameters of *R. ficaria* in 1993 and 1994.

	Crater						Outflow					
	Abaxial			Adaxial			Abaxial			Adaxial		
	Sig	F	1994 Res	Sig	F	1994 Res	Sig	F	1994 Res	Sig	F	1994 Res
SD	p<0.05	252	DEC	p<0.05	12.12	DEC	p<0.05	68.8	DEC	p<0.05	57.2	DEC
ECD	n.s	2.12	---	p<0.05	166	DEC	p<0.05	29.8	INC	p<0.05	228	DEC
SI	p<0.05	264	DEC	n.s	n.s	---	p<0.05	105	DEC	p<0.05	16.3	DEC
SL	p<0.05	185	INC	p<0.05	189	INC	p<0.05	21.1	INC	p<0.05	20.9	INC
PL	p<0.05	105	INC	p<0.05	145	INC	p<0.05	48.3	INC	p<0.05	31.3	INC
SW	p<0.05	406	INC	p<0.05	258	INC	p<0.05	45.1	INC	n.s	0.15	---

Crater samples.

As outlined in Table 2.2, the abaxial surface of leaves collected at the crater showed significant differences between those collected in 1993 and those in 1994. A significant decrease in stomatal density and index was recorded between 1993 and 1994 samples. In addition guard cell dimensions were found to increase significantly in 1994.

The adaxial surface showed a similar pattern with minor discrepancies. Significant decreases in cell densities occurred in 1994, although stomatal index did not differ. Guard cell dimensions on the adaxial surface were all significantly higher in 1994.

Outflow samples.

R. ficaria collected from the outflow showed a significant decrease in stomatal density and stomatal index (with an increase in epidermal cell density) on the abaxial surface between 1993 and 1994. Guard cell dimensions were also found to have increased significantly in 1994. The same response was recorded on the adaxial surface, except a significant decrease in epidermal cell density occurred between samples and stomatal width did not differ significantly.

2.3.1.2 *Sambucus nigra.*

S. nigra was observed to have elliptical type guard cells. The adjacent cells did not appear to be morphologically distinct. Stomatal distribution was hypostomatous with stomata only present on the abaxial surface. Leaves of *S. nigra* were not present on the trees at the outflow site in 1993. The effect of sampling site on stomatal parameters of *S. nigra* is illustrated in Fig. 2.3. One-way ANOVA was carried out on 1993 and 1994 samples to test if there were significant differences in stomatal parameters recorded between the valley and crater sites. The results are shown in Table 2.3.

Table 2.3. Statistical analysis of recorded stomatal parameters of *S. nigra* collected in the valley and crater.

(F crit = 3.94)

	Abaxial surface					
	1993			1994		
	Significance	F Value	Crater Response	Significance	F Value	Crater Response
SD	p<0.05	21.9	DECREASE	p<0.05	101	DECREASE
ECD	p<0.05	127	INCREASE	p<0.05	30.6	DECREASE
SI	p<0.05	68.3	DECREASE	p<0.05	50.2	DECREASE
SL	p<0.05	11.37	INCREASE	p<0.05	64.8	INCREASE
PL	p<0.05	11.6	INCREASE	p<0.05	34.4	INCREASE
SW	n.s	3.43	----------	p<0.05	14.2	INCREASE

1993 sample.

S. nigra collected at the crater showed significantly lower stomatal density and stomatal index than at the valley. In comparison epidermal cell density, stomatal and pore length were found to be significantly higher at the crater site.

1994 sample.

S. nigra collected in 1994 at both the valley and crater sites was found to show a similar response. Cell densities and stomatal index decreased significantly between the two sites and guard cell dimensions were significantly higher at the crater site.

Comparison of stomatal parameters recorded from the crater and outflow sites (1994 only).

As mentioned previously, samples of *S. nigra* could not be taken in 1993 so results are presented only from 1994. Statistical analysis was performed using ANOVA as previously (Table 2.4).

Table 2.4 Statistical analysis of recorded stomatal parameters of *S. nigra* (1994) collected in the crater and outflow.
(F crit = 3.94)

	1994		
	Significance	F Value	Outflow Response
SD	p<0.05	8.69	INCREASE
ECD	p<0.05	8.47	INCREASE
SI	n.s	2.11	----------
SL	n.s	0.04	----------
PL	n.s	2.16	----------
SW	p<0.05	11.65	INCREASE

As illustrated in Table 2.4, significantly higher cell densities were found on *S. nigra* leaves collected at the outflow when compared with those collected at the crater site. There were no significant differences in stomatal index between the samples. The guard cell width was the only stomatal cell dimension to show a significant

difference, recording a greater width of stomata on leaves collected from the outflow site.

The comparison of *S. nigra* stomatal parameters recorded between 1993 and 1994 samples at the valley and crater sites.

The stomatal parameters recorded in 1993 were compared with those of 1994 at both the valley and crater sites where it had been possible to make leaf collections at both sampling times. The statistical analysis of the data is shown in Table 2.5.

Table 2.5. Statistical analysis of recorded stomatal parameters of *S. nigra* 1993 sample compared with those of 1994.

	1993 v 1994					
	Valley			Crater		
	Significance	F Value	1994 Response	Significance	F Value	1994 Response
SD	p<0.05	26.1	INCREASE	n.s	1.95	----------
ECD	p<0.05	8.58	DECREASE	p<0.05	646	DECREASE
SI	p<0.05	41.05	INCREASE	p<0.05	30.81	INCREASE
SL	p<0.05	17.32	DECREASE	n.s	0.36	----------
PL	n.s	0	----------	n.s	0.75	----------
SW	n.s	2.97	----------	n.s	0.67	----------

Valley samples.

Stomatal density was found to increase between 1993 and 1994 samples. The epidermal cell density behaved in an opposite manner, with a significant decrease observed in 1994. The stomatal index showed an increase between 1993 and 1994. Stomatal length was the only guard cell dimension that showed a significant change, being reduced in 1994 leaves.

Crater samples.

The epidermal cell density showed a significant decrease in 1994 compared with the valley region. The stomatal index increased in 1994 in the crater collection sites in comparison with 1993. Non-significant differences were recorded in the stomatal density and guard cell dimensions between 1993 and 1994.

2.3.1.3 Hedera helix.

H. helix is a hypostomatous species which possesses elliptical type guard cells. The cellulose acetate micro-relief peels revealed sunken guard cells, which resulted in guard cells appearing to possess a double membrane like structure. These were particularly apparent in the 1993 sample and made it impossible to determine guard cell dimensions. The thick waxy cuticle of *H. helix* made visualisation of epidermal cells difficult. *H. helix* was not present at the outflow site at the 1993 collection, but was taken from all three collection sites in 1994. Fig. 2.4 shows the effect of collection site on all recorded stomatal densities. Fig.2.5 illustrates the responses of guard cell dimensions of 1994 samples to the three collection sites. The statistical analysis of all stomatal parameters recorded is shown in Table 2.6.

Table 2.6 Statistical analysis of recorded stomatal parameters of *H. helix* collected in the valley and crater.

	Abaxial surface					
	1993			1994		
	Significance	F Value	Crater Response	Significance	F Value	Crater Response
SD	p<0.05	7.56	DECREASE	n.s	2.53	----------
SL	N/A	N/A	N/A	p<0.05	19.8	DECREASE
PL	N/A	N/A	N/A	p<0.05	8.09	DECREASE
SW	N/A	N/A	N/A	n.s	2.34	----------

H. helix collected from the valley region in 1993 showed a significantly lower stomatal density in comparison with the crater site. The stomatal density of 1994 did not show a similar response, with no significant difference between the two sample sites. Guard cell measurements taken on material collected in 1994 at the crater site showed significantly lower stomatal and pore lengths.

Comparison of *H. helix* stomatal parameters at the crater and outflow sites.

It was only possible to record dimensions of guard cells on the abaxial surface of 1994 *H. helix* leaves.

Table 2.7 Statistical analysis of recorded stomatal parameters of *H. helix* (1994) collected in the crater and outflow. (F crit = 3.94)

	1994		
	Significance	F Value	Outflow Response
SD	p<0.05	30.2	DECREASE
SL	n.s	1.62	----------
PL	n.s	0.32	----------
SW	n.s	0.437	----------

Table 2.7 revealed that there was a significantly lower stomatal density on *H. helix* leaves collected from the outflow when compared with those from the crater.

Comparison of *H. helix* stomatal parameters recorded from the valley and outflow sites.

Fig. 2.4 illustrates that there was a lower stomatal density on *H. helix* collected in the valley than collected at the outflow in 1994. A non-significant difference in stomatal density has been previously reported between the valley and the crater site (Table 2.6). One-way ANOVA was carried out to statistically analyse this observation. ANOVA revealed a significantly ($p<0.05$) lower stomatal density between these two sites. (F crit = 3.94, F value = 90.99).

Comparison of stomatal parameters recorded between 1993 and 1994 samples at the valley and crater sites.

It was only possible to analyse differences between the stomatal densities of 1993 in comparison with 1994 at the valley and crater sites.

Valley

H. helix collected in the valley showed a significant ($p<0.05$) increase in stomatal density between 1993 and 1994 samples. (F crit = 3.94, F value = 163.3).

Crater

Significantly higher stomatal density in 1994 was once again observed on *H. helix* leaves collected at the crater site (F crit = 3.94, F value = 75.4).

2.3.2. The effect of artificially enhanced CO_2 levels on recorded stomatal parameters of *R. ficaria*

R. ficaria were removed from the Solardomes two months after the acclimation period and leaves removed immediately. Stomatal parameters on both the abaxial and adaxial leaf surfaces of the plants were recorded. Unfortunately due to circumstances beyond our control, the plants that were to be left in the Solardomes for a further two months died.

R. ficaria leaf growth.

It was observed that elevated CO_2 levels resulted in a great increase in the number of leaves produced by *R. ficaria.* Plate 2.2 illustrates the number of leaves produced from one *R. ficaria* plant grown in an elevated CO_2 level for 2 months after the acclimation period, compared with two plants grown at ambient CO_2 levels.

2.3.2.1 The comparison of stomatal parameters of *R. ficaria* grown at artificially enhanced CO_2 and ambient CO_2 levels.

Fig. 2.6 and 2.7 illustrate the effect of artificially enhanced CO_2 levels on stomatal parameters. There was a significant change observed in all stomatal parameters on both leaf surfaces between those plants grown at ambient CO_2 levels in the Solardome and those at elevated CO_2. Statistical analysis of the data is complicated because of different sample sizes. In order to illustrate the effect of moving *R. ficaria* between ambient and elevated CO_2 levels the results are presented as a % change in parameter relative to recordings obtained from the ambient Solardome environment. Results from three experiments are given. "Ambient-control" indicates the plants were grown at ambient conditions in the Solardome and then returned to the control environment. Likewise "elevated-control" indicates that the plants were grown at elevated CO_2 and then returned to the control environment. Finally, "elevated only" indicates plants that remained growing in the Solardome at elevated CO_2 levels and not returned to the control environment. Results are presented in Table 2.8.

Table 2.8. The percentage difference between plants sampled at elevated CO_2, elevated CO_2 to 'control' and ambient CO_2 to 'control' where 100% parameters are found at ambient CO_2 (no move).

		Abaxial						Adaxial					
		SD	ECD	SI	SL	PL	SW	SD	ECD	SI	SL	PL	SW
1	ambient-control	1.5 DEC	0.3 INC	5.4 INC	3.4 DEC	5.8 INC	0 ---	0.3 INC	7.8 INC	10.8 INC	1.8 INC	3.5 INC	2.8 INC
2	elevated-control	24.3 DEC	7.7 DEC	22.3 DEC	13.5 INC	15.6 INC	18.1 INC	18.3 DEC	7.7 INC	10.3 DEC	22.6 INC	30.4 INC	23.4 INC
3	elevated only	66.7 DEC	52.3 DEC	26.9 DEC	35.6 INC	34.0 INC	16.5 INC	59.3 DEC	40.2 DEC	18.8 DEC	41.6 INC	56.5 INC	26.9 INC

The results illustrated in Figs. 2.6 and 2.7 and Table 2.8 demonstrate that after two months there was little change in stomatal parameters on the abaxial surface when plants were taken from the ambient CO_2 dome and transferred to the 'control' environment. The adaxial surface responds in a similar way although an approximate 10% increase is recorded in stomatal index.

However, when plants were grown in elevated CO_2 alone a significant response was recorded in all stomatal parameters. On both surfaces, cell densities and stomatal index were reduced, whereas guard cell dimensions were found to have increased.

With respect to the plants which had been removed from elevated CO_2 and placed in the 'control' environment, these appeared to record stomatal parameters some what in between those found in ambient and elevated CO_2 levels.

Plate 2.1 Solardome plant growth facilities.

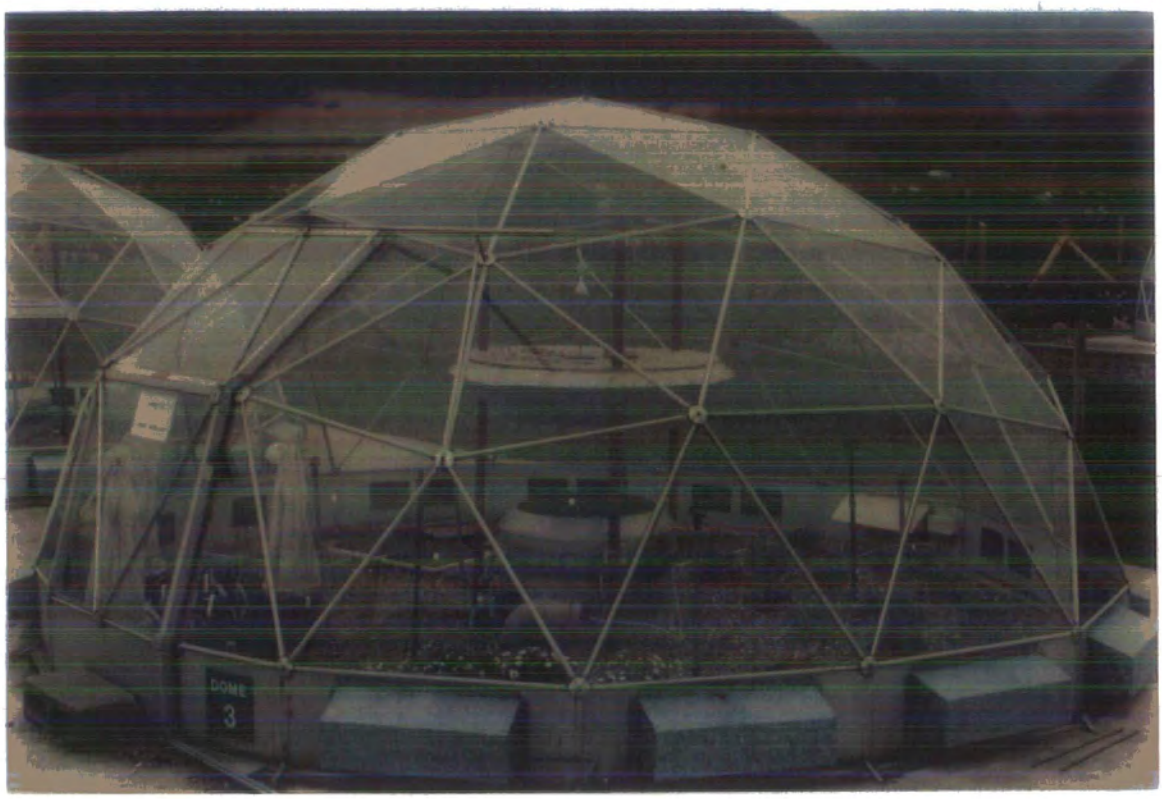


Plate 2.2 *Ranunculus ficaria* grown at different CO_2 concentrations.

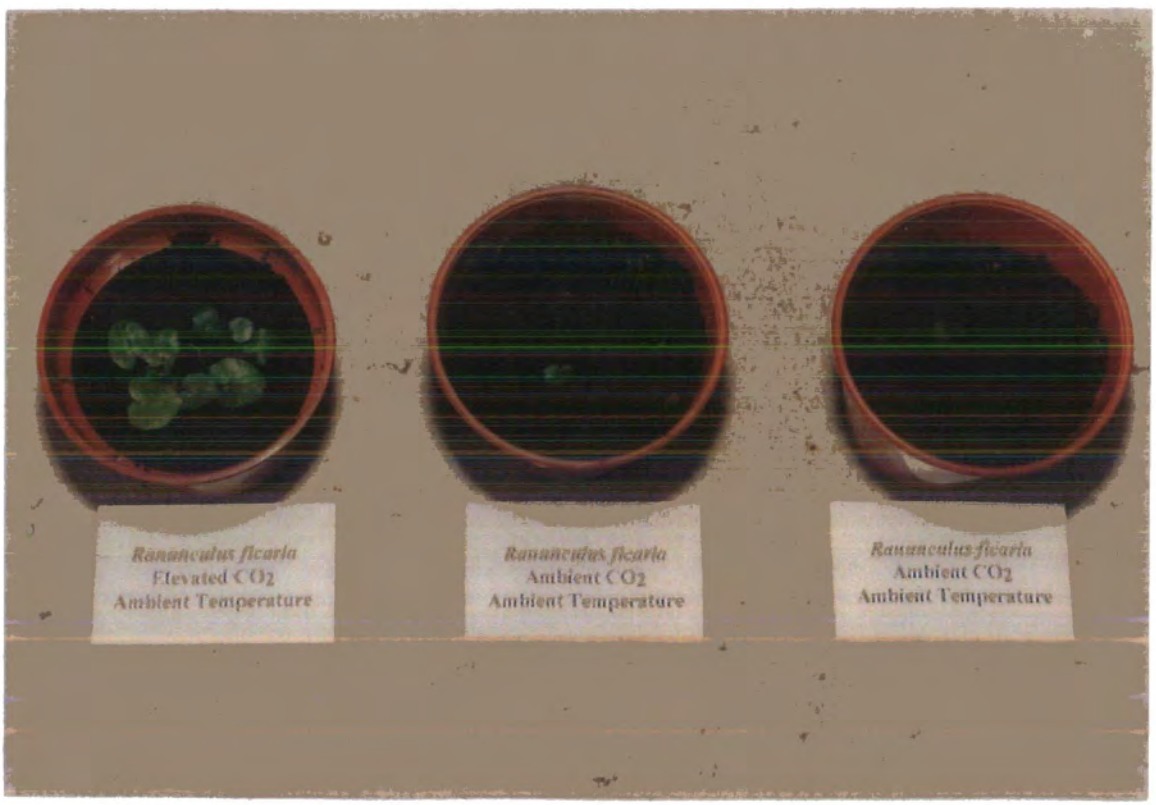


Figure 2.1 Differences in the stomatal density, epidermal cell density and stomatal index of *Ranunculus ficaria* between sampling sites.

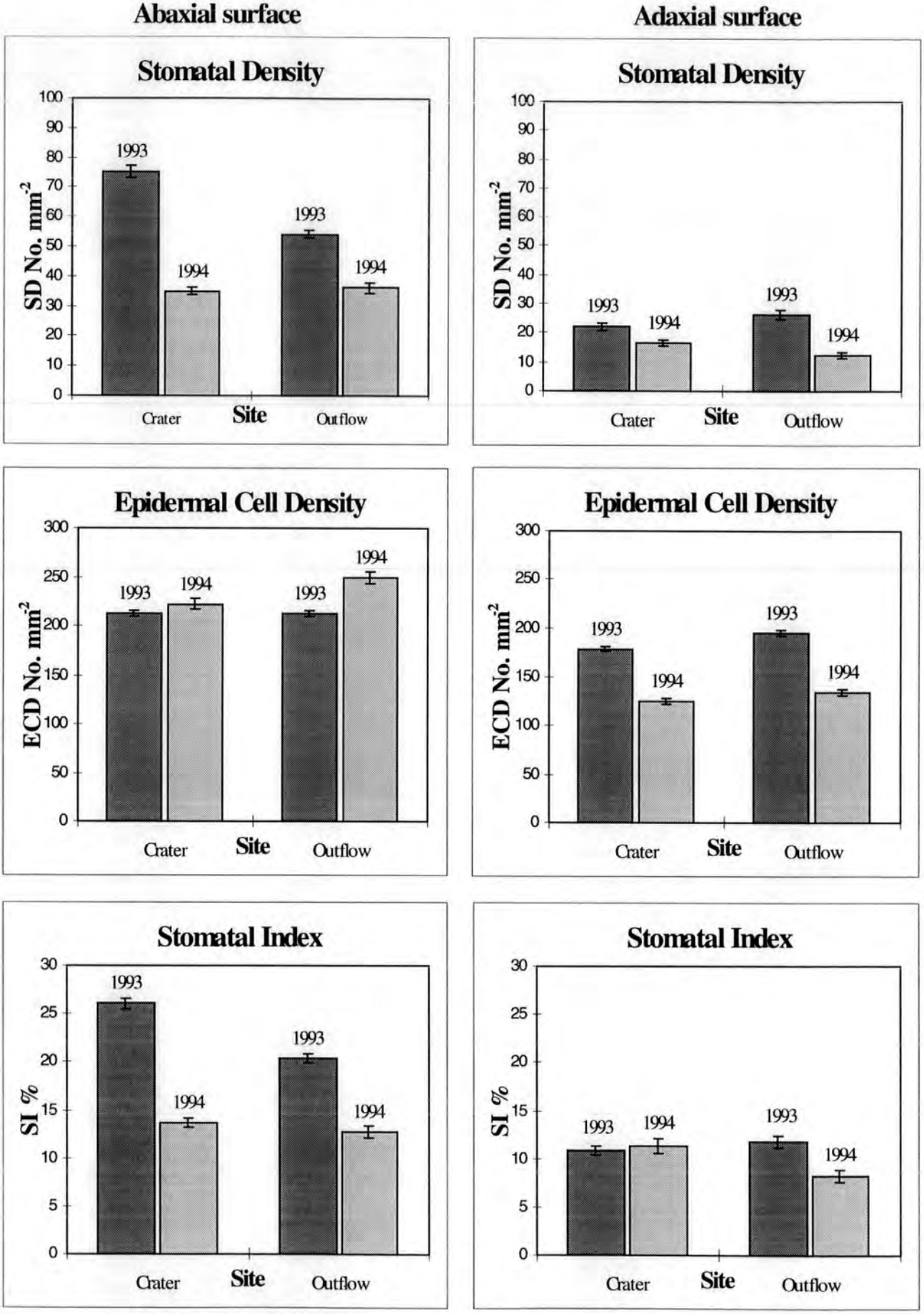


Figure 2.2. Differences in guard cell dimensions of *Ranunculus ficaria* between sampling sites.

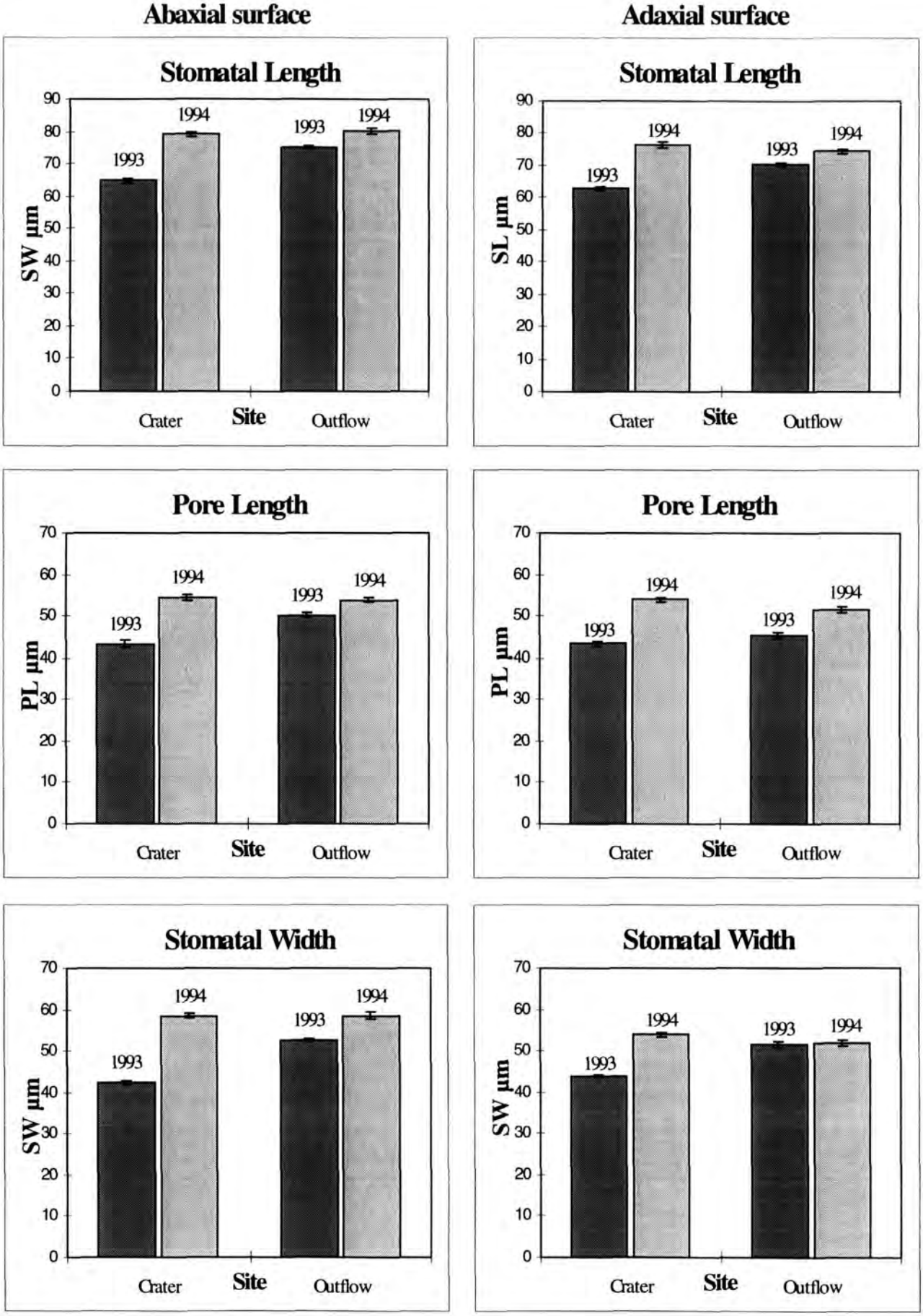


Figure 2.3 Differences in *Sambucus nigra* stomatal parameters between sampling sites (Abaxial surface only).

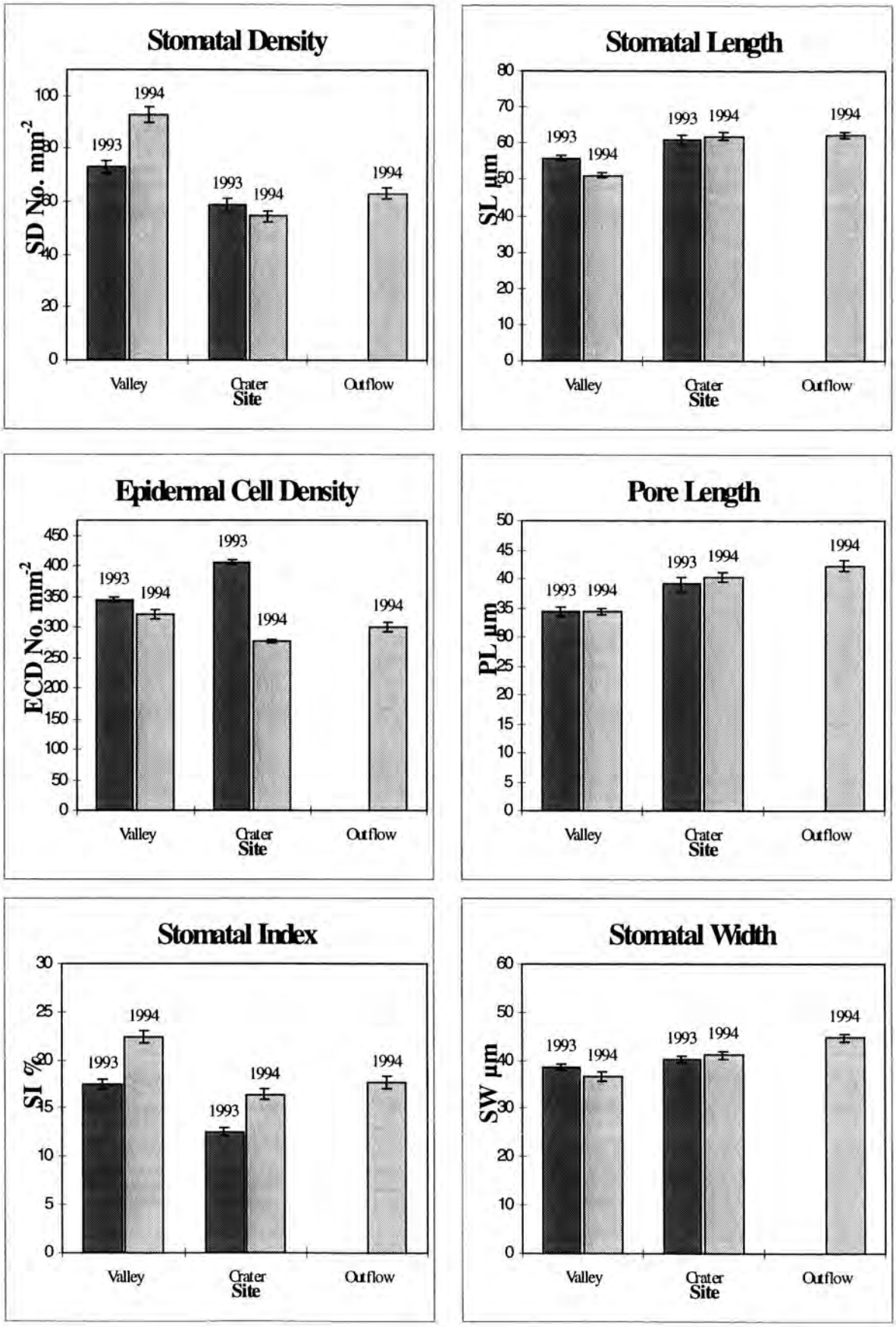


Figure 2.4. Differences in stomatal densities of *Hedera helix* between sampling sites.

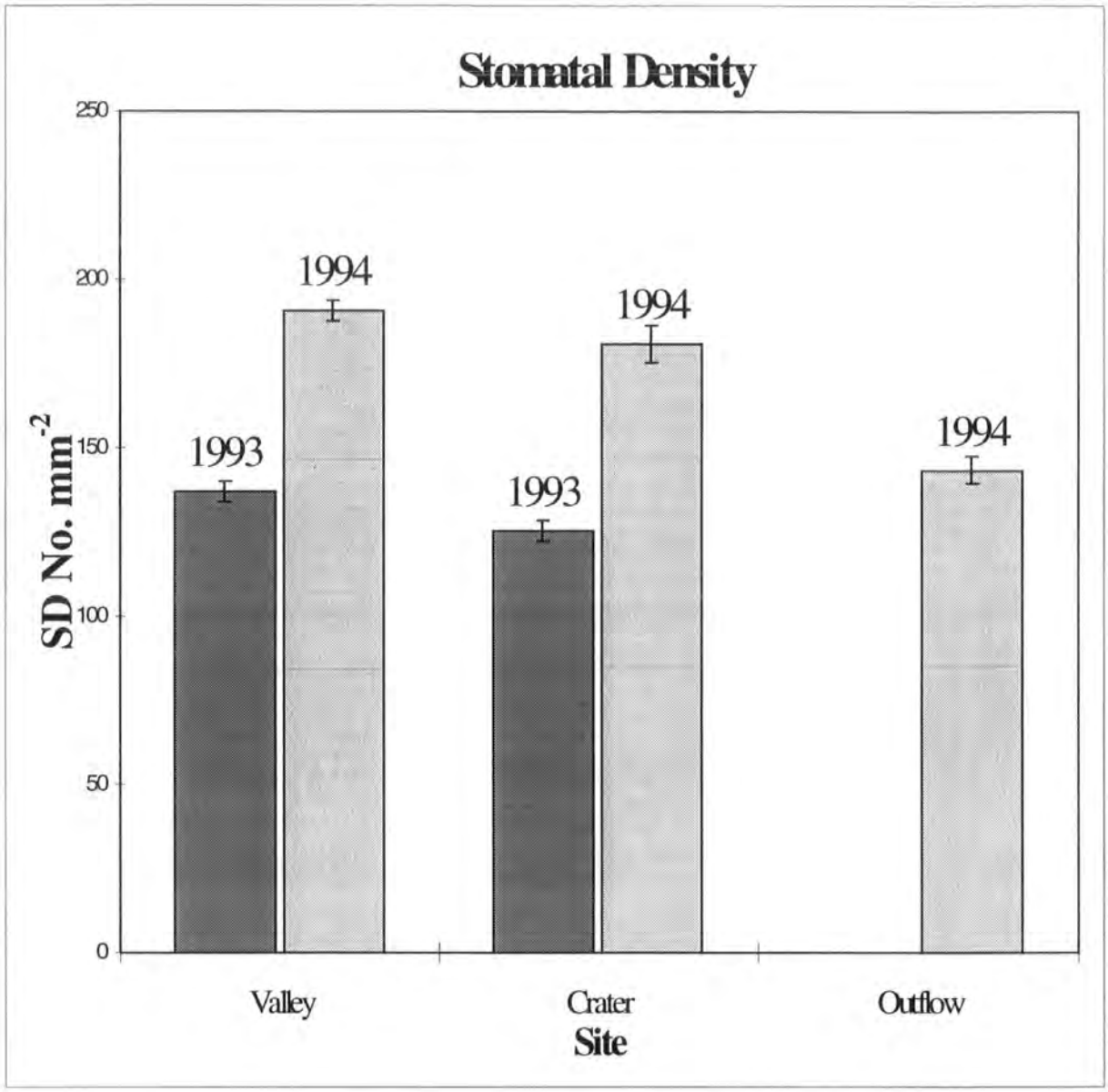
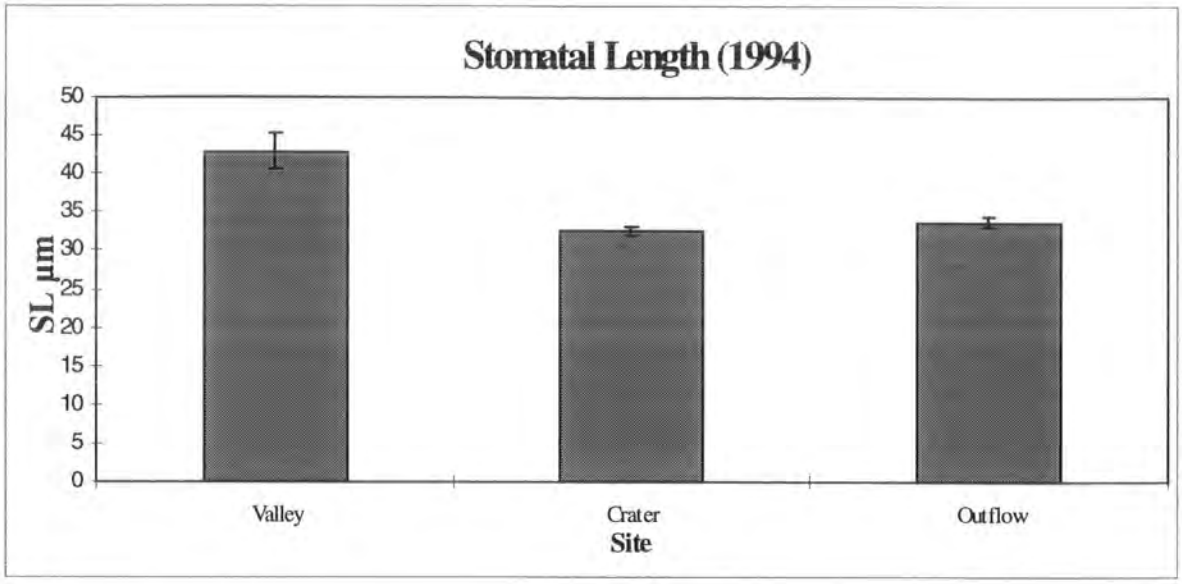
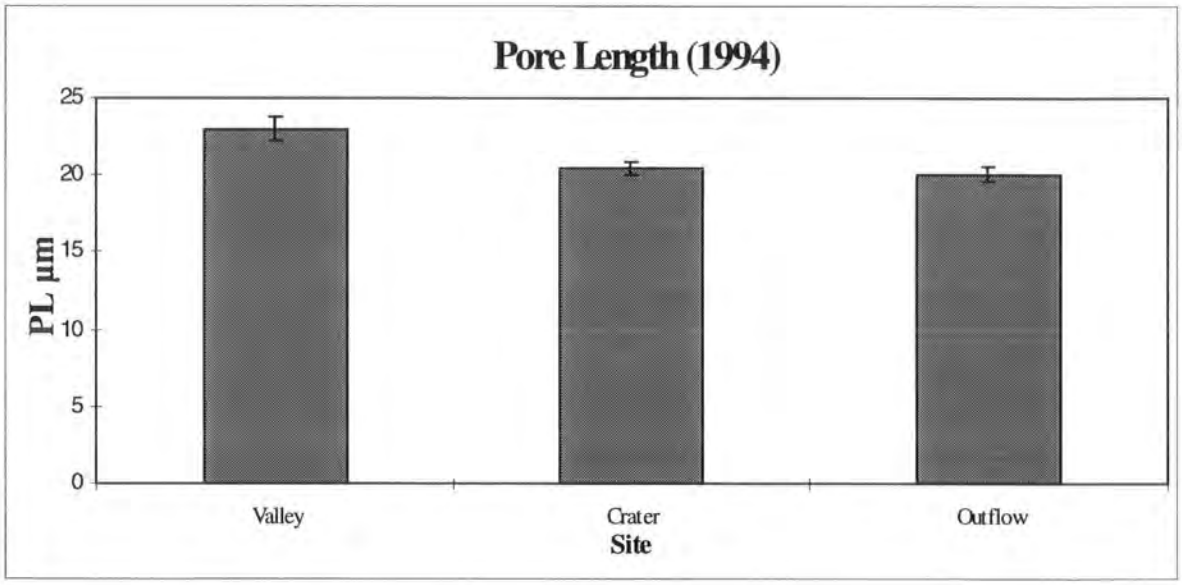


Figure 2.5. Differences in guard cell dimensions of *Hedera helix* between sampling sites in 1994.





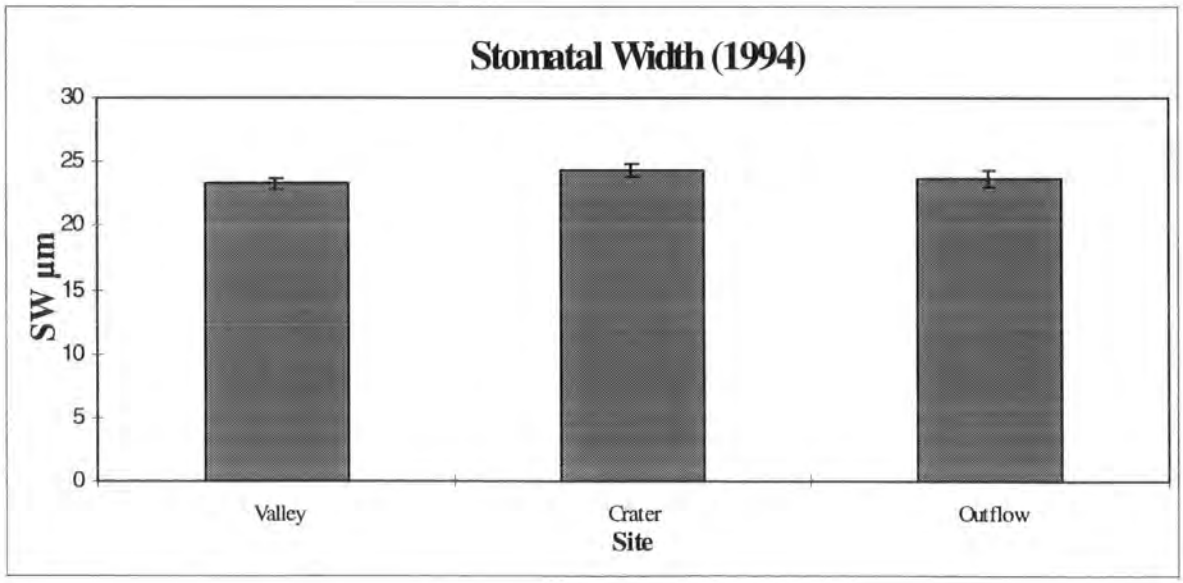
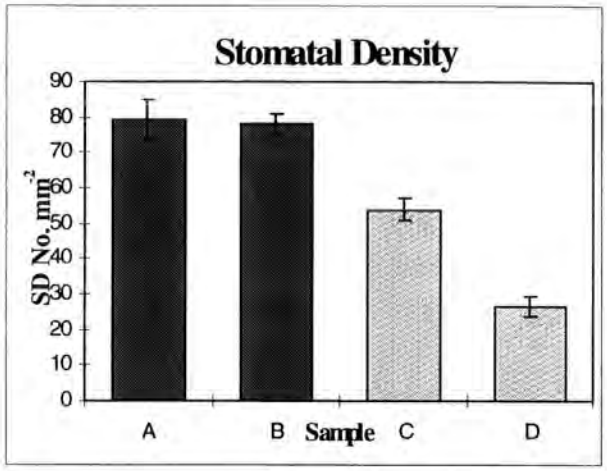
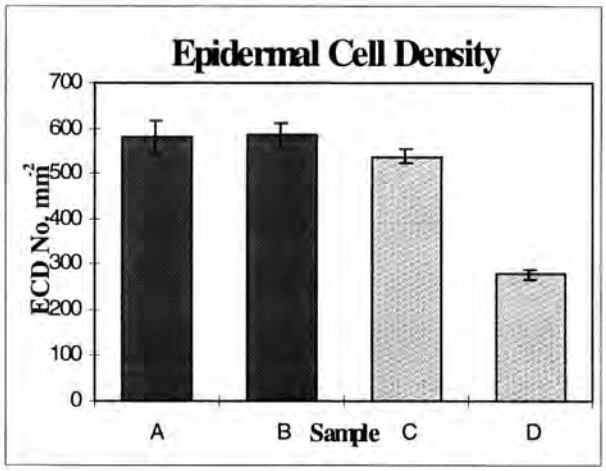
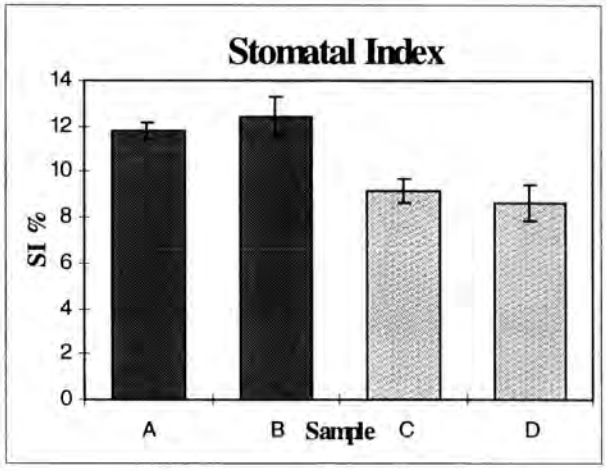


Figure 2.6. The effect of artificially enhanced carbon dioxide levels on Stomatal Density, Epidermal Cell Density and Stomatal Index of *R. ficaria.*

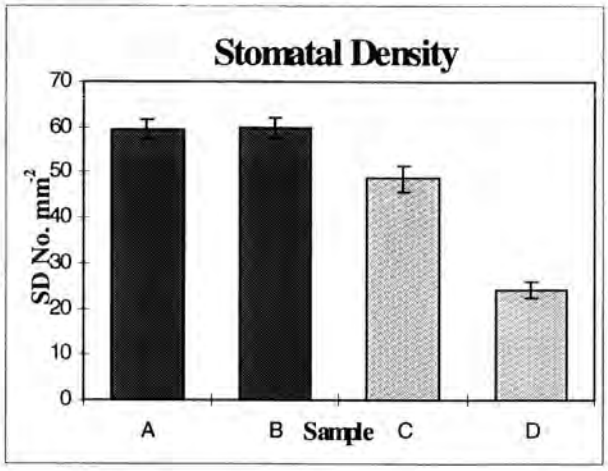
Abaxial surface

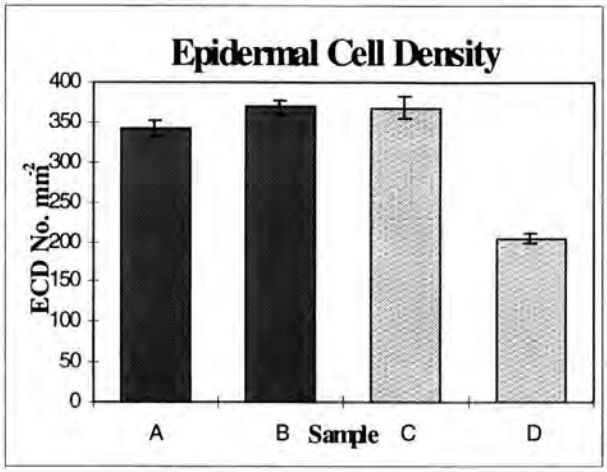


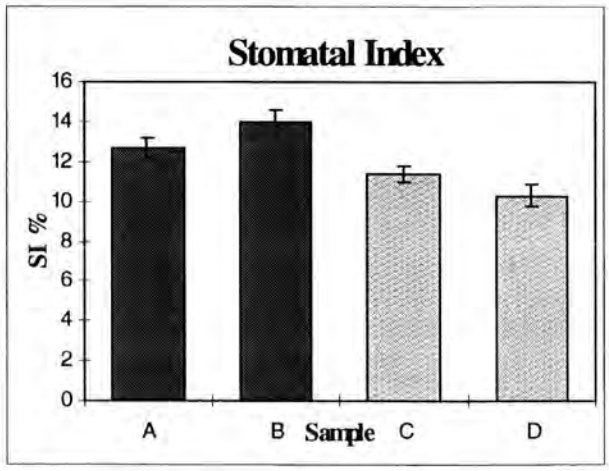




Adaxial surface



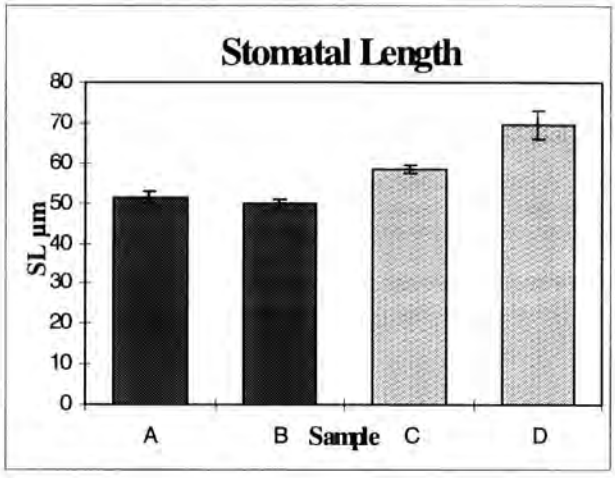


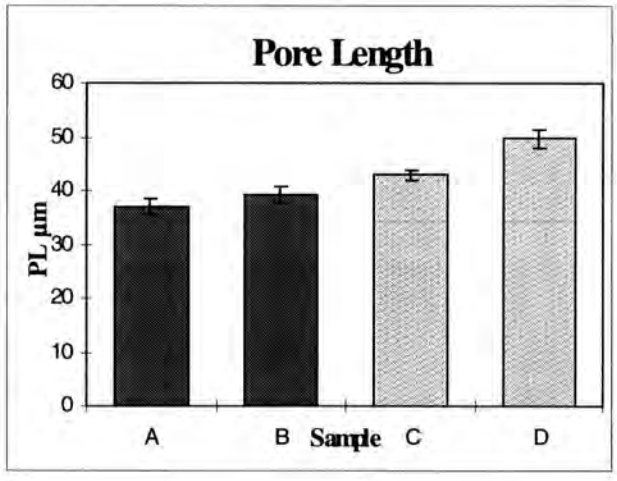


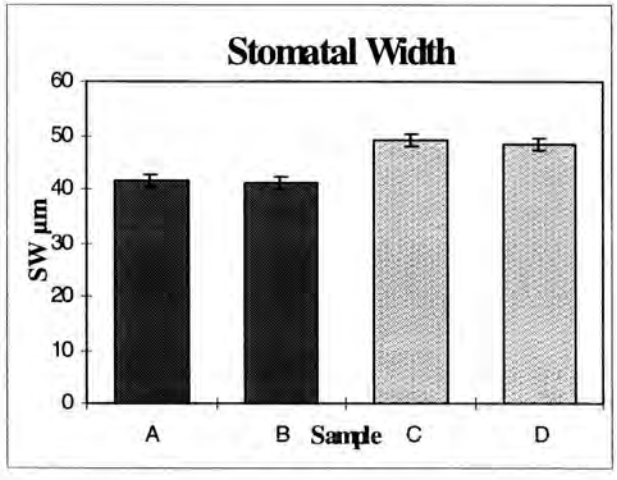
A = Ambient carbon dioxide
B = Ambient carbon dioxide to control
C = Elevated carbon dioxide to control
D = Elevated carbon dioxide only.

Figure 2.7. The effect of artificially enhanced carbon dioxide levels on Stomatal Length, Pore Length and Stomatal Width of *R. ficaria.*

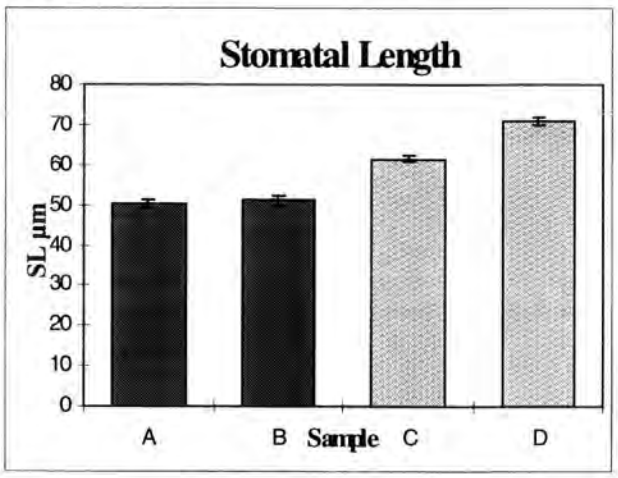
Abaxial surface

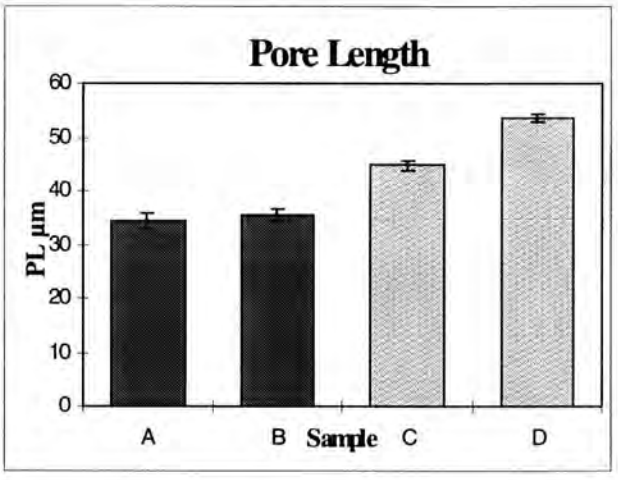


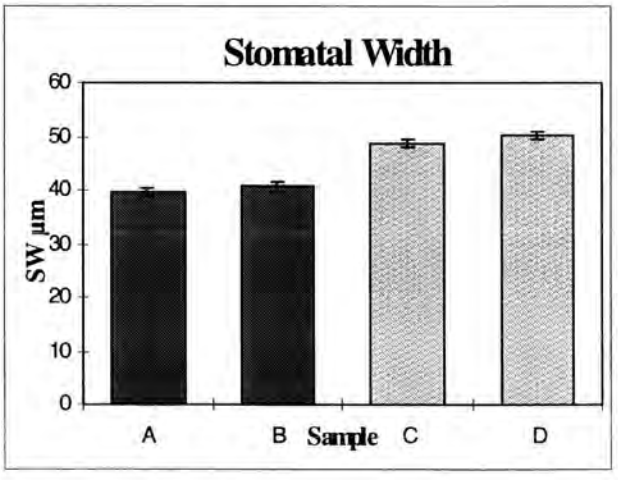




Adaxial surface







A = Ambient carbon dioxide
B = Ambient carbon dioxide to control
C = Elevated carbon dioxide to control
D = Elevated carbon dioxide only.

2.4 Discussion

2.4.1 Variations in stomatal parameters of *R. ficaria.*

Stomatal characteristics.

Amphistomatous species, with the exception of some Gramineae show a greater abaxial surface stomatal density than adaxial (Weyers & Meidner, 1990). Such species are also known to show considerable differences in leaf characteristics such as hairiness, stomatal size and density (Turner, 1979). In this investigation both leaf surfaces were studied and confirmed that stomatal density was indeed consistently greater on the abaxial surface at all sample sites. Turner, (1979), reported differences in stomatal response on the two leaf surfaces observed under a wide range of conditions. It is thought that a higher density may be expected when the irradiance source is normal to the leaf surface, since the light reaching the abaxial surface would be of a lower intensity and reduced in wavelengths absorbed by leaf pigments. However, when light reaching the two surfaces is the same, it is found that the abaxial stomata are more responsive than the adaxial stomata (Weyers & Meidner, 1990). In addition, it is also common to find a disparity of stomatal apertures between the two surfaces (de Silva *et al.,* 1986), and non-parallel responses to light, temperature and water stress have been reported for the adaxial and abaxial stomata of many species (review Pospísilová & Solárová, 1980). Guard cell dimensions typically show little difference between abaxial and adaxial stomata on the same leaf.

Variations between *R. ficaria* stomatal parameters at the crater compared with the outflow.

Assessing differences in stomatal parameters due to different CO_2 concentrations between sample sites is difficult since the actual CO_2 level at the outflow in unknown. However, it is still possible to determine whether differences in stomatal parameters exist between sites. Considerable variation was observed in *R. ficaria* stomatal parameters according to the leaf surface, site and sampling date.

The stomatal density and stomatal index of the abaxial surface on the 1993 sample was found to be significantly lower at the outflow site compared with the crater. As stomatal index is affected it would appear that the abaxial surface of *R. ficaria* in 1993 was responding to an environmental factor, since stomatal index reflects changes in stomatal initiation and is independent of cell expansion (Salisbury, 1928). It is known that the crater lake has enhanced CO_2 levels compared with the surrounding valley area (approx. 20 p.p.m.v), however, the exact level at the outflow site is unknown. Stomatal density has been observed to decrease as CO_2 levels increase (Woodward, 1987; Thomas & Harvey, 1983; Beerling & Chaloner, 1992; Willmer & Fricker, 1996), which could imply that *R. ficaria* is responding to a different CO_2 level between the crater and outflow sites. Increased CO_2 levels could occur at the outflow habitat due to an increased circulation of water rich in CO_2 (due to volcanic effect) mixing with air. This phenomenon often occurs at outflow water environments. However, when the abaxial leaf surface was analysed in 1994 no significant differences were recorded with respect to stomatal density or index. Therefore if CO_2 levels were previously causing the change in these parameters it would suggest that the levels at both sites were very similar in this year. Potentially, this could arise due to insufficient water mixing occurring within the outflow to raise CO_2 levels. Alternatively, stomatal development may have changed as a result of another factor(s) not recorded. For example, weather conditions may have been wetter during stomatal development at the outflow that could have influenced the formation of stomata (chapter 1). Guard cell dimensions on the abaxial surface were found to be significantly greater when stomatal density decreased, which is in accordance with that reported by Willmer & Fricker, (1996), but contradicts Beerling & Woodward, (1995), who found an overall decrease in guard cell length with elevated CO_2. In 1994 no differences in either guard cell dimensions or stomatal density were observed. Therefore, when considered together, results obtained in 1993 and 1994 suggest that there maybe a relationship between guard cell dimensions and stomatal density.

The adaxial surface also showed variation in stomatal characteristics between sites. However the observations did reflect those of the abaxial. The increased cell densities in 1993 were unexpected, given the response of the abaxial surface. However, the increase in these parameters did not result in a significant difference in the stomatal index, thus it could be related to a plant developmental stage. For example, it could be

suggested that the epidermes did not arrest development at the same time (Dr A. Pearson, personal communication). Samples taken in 1994 showed a decrease in stomatal density on the adaxial surface and index between the crater and outflow sites, which again would indicate that an environmental factor was influencing stomatal initiation. However, this response contrasts with that of the abaxial surface in 1994 which revealed no significant difference, and thus the theory that in 1994 the atmospheric CO_2 concentration between the two sites was similar should be reviewed if CO_2 is considered the only factor affecting stomatal parameters.

Variation in *R. ficaria* stomatal parameters recorded between sampling years at the crater and outflow sites.

R. ficaria leaves sampled at both the crater and the outflow showed a significant reduction in stomatal density and index on both surfaces (with the exception of the adaxial crater site) in 1994 compared with the 1993. This reduction could be due to influences such as the growth status of the plant, a change in the CO_2 levels between sampling times or other environmental factors. Stomatal parameters are strongly affected by growth conditions. Important influences include light intensity, (Gay & Hurd, 1975), water availability (Gindel, 1969: Rawson & Craven, 1980), humidity, nutrient availability as well as CO_2, these factors have been discussed in chapter 1. The fact that the variations observed in stomatal parameters could be attributed other environmental factors makes it difficult to draw conclusions relating to a specific response to changes in CO_2 levels.

2.4.2 Variations in abaxial stomatal parameters of *S. nigra.*

Variations in *S. nigra* stomatal parameters between the valley and the crater.

The significant decrease observed in stomatal density and index of plants collected in both years at the crater site when compared with those from the valley, is in accordance the work of Woodward, (1987); Beerling & Chaloner, (1992; 1993*c*), and Beerling *et al.,* (1993), who all found that stomatal density decreases with increased CO_2 concentration. The significant increase also recorded in guard cell dimensions at the crater site is in agreement with reports that guard cell dimensions are enlarged as stomatal density decreases (Willmer & Fricker, 1996).

Samples taken in 1994 exhibited a significant decrease in stomatal index at both sites compared with 1993. Once again, a variety of environmental factors could be attributed to this change.

Variations in *S. nigra* stomatal parameters between the crater and the outflow.

In contrast to the observed response of *R. ficaria* between the crater and outflow sites, *S. nigra* was found to show an increase in cell densities with no significant difference in stomatal index on the abaxial surface, indicating that the response is more likely to be associated with reduced cell expansion (Salisbury, 1928). It would therefore appear that *S. nigra* is responding in a species specific manner (Malone *et al.*, 1993).

2.4.3 Variations in abaxial stomatal parameters of *H. helix.*

H. helix was also found to show variation in stomatal density between the three collection sites.

Variations in *H. helix* stomatal parameters between the valley and the crater.

Stomatal density is seen to be significantly reduced on leaves sampled at the crater in comparison to those from the valley in 1993. As the valley area was known to have a lower ambient CO_2 level when compared to the crater site, it could be suggested that the reduction in stomatal density is once again in response to the increase in CO_2 concentration, and would be consistent with the results obtained for *S. nigra* and those of Woodward, (1987); Beerling & Chaloner, (1992; 1993*c*); Beerling *et al.*, (1993). However, the stomatal density recorded in 1994 shows a non-significant reduction at the crater. This suggests that either the CO_2 gradient was less prevalent in 1994, some other factor had influenced the result, or a species specific reaction was being observed. In contrast again to *S. nigra,* reduced *H. helix* stomata and pore lengths were found on crater leaves. If this observation is linked to changes in CO_2 between the two sites then it is in agreement with Woodward, (1995), who reported that stomatal dimensions actually decrease in response to increasing CO_2.

Variations in *H. helix* stomatal parameters at the crater lake and the outflow.

Stomatal density decreased significantly in *H. helix* leaves collected at the outflow site. This is similar to the response seen in leaves of *R. ficaria*, but in contrast to those of *S. nigra*. Guard cell dimensions did not differ in contrast to the response recorded between valley and crater leaves, which are similar to the results obtained for *S. nigra* but in contrast to those for *R. ficaria*. Stomatal density also recorded significant variation between 1993 and 1994 samples. The decrease in stomatal density between 1993 and 1994 samples at both the valley and crater sites contrasted with the temporal response observed in *R. ficaria* and *S. nigra*.

2.4.4 Consideration of all species studied.

The variations observed when the three species are considered together, suggest that component species of a plant community differ in their response to elevated CO_2, as suggested by Ferris & Taylor, (1994). The variation in stomatal parameters is found to occur both within and between species. Within species, parameters varied depending on the leaf surface, the time of sampling and the sampling site. Between species parameters varied in the same extent, but also revealed no consistent trend between sites of differing CO_2 levels. Although there is known to be a CO_2 gradient between the valley and crater, the reduction of stomatal density in response to an increase in CO_2 level was not consistent. It may be that the differences in CO2 levels were not extreme enough to induce a response in all cases. In addition, the three species may have a different 'threshold' of response, implying that some are more sensitive to small fluctuations in CO_2 than others. The parameters recorded between the crater and the outflow sites revealed further variation even when sampling was carried out at the same time, thus making it impossible to relate CO_2 levels solely to stomatal parameters.

Guard cell dimensions were also found to vary with respect to stomatal density. It is known that stomatal characters are often linked to each other (Jones, 1987), and it was reported that stomatal density is inversely related to guard cell size (Pallardy & Kozlowsky, 1979), which would appear to be in agreement with results presented from *R. ficaria* and *S. nigra*. *H. helix* did not to show this response, but the comparisons between the valley and the crater samples were in agreement with the

work of Miglietta & Raschi, (1993). They reported oak tree leaves at sites near and far way from a localised gas vent area in Central Italy, which were known to produce enhanced CO_2 levels, showed a reduction in guard cell size without a change in stomatal density and index at elevated CO_2.

2.4.5 The effect of artificially enhanced levels of CO_2 on *R. ficaria.*

Leaf growth responses.

Apart from the stomatal parameters results, it was noted that *R. ficaria* grown in elevated CO_2 increased leaf production. It would therefore appear that elevated CO_2 is enhancing plant growth. The study of Ceulemans *et al.,* (1995), further corroborates this suggestion that elevated CO_2 might affect photosynthesis at the level of the photosynthetic apparatus, either by increasing the number of reaction centres or by improving the turnover status of the photosynthetic apparatus, which results in higher leaf initiation and growth rates.

The effect of artificially enhanced CO_2 levels on recorded stomatal parameters of *R. ficaria.*

In order to investigate the response of *R. ficaria* stomatal parameters to elevated CO_2, and eliminate as far as possible the influences of other environmental factors, plants were grown in Solardomes at elevated and ambient CO_2. This facility enabled factors such as water availability, light availability, temperature and humidity to be kept constant. Growing plants under elevated CO_2 regime in controlled environmental chambers has been reported to decrease leaf stomatal density (Thomas & Harvey, 1983; Woodward, 1987). The results of *R. ficaria* reported here were found to be in agreement with this work. A significant reduction of stomatal density was recorded on both the abaxial ($\approx$ 67%) and adaxial ($\approx$ 60%) leaf surfaces grown under elevated CO_2 when compared with those leaves grown under ambient CO_2. In addition, stomatal index was significantly reduced at elevated CO_2, suggesting that elevated CO_2 was influencing stomatal initiation as well as stomatal density. The stomata of *R. ficaria* are therefore able to respond to elevated CO_2 levels, however it is not clear whether this effect is permanent or transitory.

It is known that the stomatal densities of land plants are able to respond to changes in CO_2 concentration within weeks or months (Eamus & Jarvis, 1989), as was recorded in this study. It was not possible to extend the period that the leaves had been in the Solardomes past five months (acclimation period three months, leaves sampled two months after this period). Previous studies of exposing plants to elevated levels of CO_2 for relatively short periods (less than 12 months) were thought to be reporting short term acclamatory responses, rather than long term physiological adaptation (Jarvis, 1989). In order to investigate this possibility and to ascertain if the response was permanent *R. ficaria* plants from elevated and ambient CO_2 conditions were transferred to a 'control' situation (that is, returned to the environment from which they were taken). The aim of this was to determine whether *R. ficaria* was able to regulate leaf stomatal parameters after just two months in a different environment. Plants removed from the ambient condition and placed in the 'control' environment were found to show little difference in stomatal parameters with the exception of an increase in stomatal index. This suggests that transport of the plant to the control resulted in an environmental factor having an effect on stomatal initiation, which could be attributed to changes in light intensity and temperature. This response tends to support the criticisms which have been made of experiments exposing plants to elevated CO_2 regimes in small scale glass houses and field enclosures because of poor atmospheric coupling and the balance between energy supply and water loss from leaves (Morison, 1987). In contrast natural vegetation is well coupled with the atmosphere both in terms of its energy budget and environmental feedback mechanisms which may also have caused this discrepancy. *R. ficaria* which were moved from elevated CO_2 to the 'control', demonstrated a reduction in stomatal density and index with associated greater guard cell dimensions in comparison with those grown at ambient CO_2. However, the response was not as pronounced as if the plants had been grown at elevated CO_2 only. This suggests that the stomatal parameters are able to respond to the reduction in CO_2 concentration in the 'control' environment within a two month period. The mechanisms causing the changes taking place are still not clearly understood (Willmer & Fricker, 1996).

It is known that stomata arise through differential divisions in the protoderm which becomes secondarily meristematic, forming guard mother cells but the governing mechanisms are not clearly understood (Palevitz, 1981).

By producing a greater stomatal density and index in response to the decrease in CO_2, the stomata of *R. ficaria* appear to be re-acclimatising. However, they are unable to increase parameters in this short time period, which are comparable to those found on plants that have adapted to ambient CO_2 levels. The guard cell dimensions illustrate a similar response indicating that in this case they are closely related.

2.4.6 The comparison of stomatal parameters of *R. ficaria* recorded from plants grown at artificially enhanced CO_2 and ambient CO_2 levels with those described from Italy.

Simulated experimental environments are often found to be inadequate models for predicting long term responses of plants to slow environmental changes. This is because the duration of controlled environmental experiments is often too short to induce significant stomatal density response particularly to greatly elevated concentrations of atmospheric CO_2 (McElwain & Chaloner, 1995). However, Solardome facilities were used in this case to make a comparative study with a much reduced natural CO_2 gradient as described from the Offanto valley region, Italy, and findings are not considered in isolation. Thus, the results presented here are novel in this research area as extensive literature searches have revealed little information as to the stomatal parameter response of other species to growth in both naturally elevated, artificially elevated, naturally ambient, artificially ambient and 'transferred' experiments.

As previously stated it was difficult to determine the effect of CO_2 levels on stomatal parameters of *R. ficaria* recorded in a naturally enhanced CO_2 environment where levels were only known to be raised by 20 p.p.m.v and other non-tangible factors are able to influence results. However, when CO_2 levels are much raised by 340 p.p.m.v and other factors are controlled stomatal parameters are found to respond in a specific

manner in agreement with other controlled environment work (e.g. Thomas & Harvey, 1983; Woodward, 1987). The results presented here therefore suggest that plant responses to relatively small increases in CO_2 in a natural environment where other environmental factors are able to influence plant growth differ significantly from those observed in enhanced controlled environments as would be predicted from shortcomings associated with Solardome experiments. It would be interesting to grow *R. ficaria* in a controlled environment using similar CO_2 gradients to the Offanto region, to see if stomatal parameters were directly affected by smaller changes in CO_2 in the absence of other environmental stimuli. Unfortunately, the Solardome equipment cannot simulate such small changes in CO_2 concentrations. However, these experiments do indicate the potential for *R. ficaria* to respond to small changes in CO_2 levels.

2.5 Conclusion.

The results obtained in this chapter illustrate the problems associated with predicting the response of species to environmental change using plant stomatal parameters. It is found that even when three species are considered within an area, the variation in response to a realistic change in natural CO_2 level of 20 p.p.m.v. is great. These variations may be due to a number of reasons, which include: the influence of other environmental factors, the surface sampled, the time of sampling, and the species sampled and illustrate that the response of a species is not uniform and neither is the response among species.

Growth of *R. ficaria* at controlled elevated CO_2 levels produced significant changes in stomatal parameters, however this is not observed in the field. The changes in CO_2 concentration used in the Solardome experiments are far greater than would occur in the natural environment. Therefore, the prediction of environmental change in particular relation to changes in CO_2 levels using plant stomatal parameters is difficult.

Since the study highlighted the variation observed in three species it was decided to concentrate on the response of one species to changes in CO_2 levels. This would enable an extension of investigations to consider variations within and between sampling sites and to further the study to incorporate longer term experiments. This would further assess the feasibility of using stomatal parameters to monitor environmental change.

Chapter 3.

The effect of natural CO_2 concentration gradients on stomatal parameters of *Salix herbacea.*

3.1 Introduction.

In order to assess the significance of changes in stomatal parameters observed in the fossil record, it is essential to firstly determine the inherent variability within extant examples of a species. In nature, plants are normally exposed to gradual fluctuations in atmospheric CO_2 concentration rather than abrupt ones. Chapter 2 illustrated that the effect of naturally elevated CO_2 levels on stomatal parameters using three species and demonstrated the extent of variation both within and between species. Chapter 2 also concluded that the response of a plant in artificially elevated CO_2 might not follow those observed in the field. In order to further investigate the potential use of stomatal parameters to monitor environmental features it is necessary to concentrate on the response of a single species. The natural variability within the chosen species should be studied in both the short and long-term responses.

One situation where CO_2 levels are known to vary naturally is from sites along an altitudinal gradient in the montane environment. Plants growing along an altitudinal gradient are known to have been formed under progressively reduced CO_2 partial pressures at higher altitudes (Gale, 1972). Therefore, the study of stomatal parameters along this type of gradient offers the potential of recording contemporary responses to changes in CO_2 levels. Previous studies observed that the leaves of the arctic-alpine, *Salix herbacea* in such an environment responded by reducing abaxial stomatal density with increasing altitude whilst the adaxial density remained constant (Beerling *et al.*, 1992). These results were in agreement with the work of Woodward, (1986), who had demonstrated the same trend on the abaxial leaf surface of the shrub *Vaccinium myrtillus* L. Woodward, (1986), additionally confirmed the results using artificially reduced CO_2 environments. However, these studies did not investigate the potential for differences in stomatal parameters within an altitudinal site or assess variation across individual leaves. Knowledge of intra-site and intra-plant variation is essential, for if significant variation is

found to occur this would have serious implications when considering the suitability of using stomatal parameters of fossil leaves to monitor environmental change.

This study incorporates consideration of these factors and extends the previous work of Beerling *et al.,* (1992). *S. herbacea* is known to be established along altitudinal gradients and is abundant in many full-glacial and late glacial habitats in N.W. Europe, (Godwin, 1975). It is relatively common in British fossil deposits over time periods when CO_2 levels began to rise rapidly (Beerling & Chaloner, 1994). Therefore, consideration of both long and short-term changes in stomatal parameters can be made in this species. Therefore, *S. herbacea* was used in this study as a suitable species to be found in the fossil and extant state. Firstly an extensive study of stomatal parameters was carried out using modern material to assess the variation within the species under the influence of a CO_2 gradient. Ideally plants should have been monitored following exposure to artificially enhanced CO_2 concentrations in the controlled and defined environments of Solardomes. Difficulties in this respect were encountered with *S. herbacea,* as it did not establish. In addition further complications were envisaged given the perennial nature of the species and the fact that leaf stomatal patterns could have been established within a bud of the previous year. The study will then assess long-term changes by analysing stomatal parameters of fossil leaves with a view to using them as an environmental change indicator.

3.2 Materials and Methods.

3.2.1 *Salix herbacea* L.

S. herbacea (the dwarf willow) is the smallest of the British willows. It is typically less than 6cm high and forms loose, flattened mats with a large number of aerial shoots arising from an extensive rhizome system. The leaves are 'broadly and bluntly' ovate or suborbicular, sometimes rather broader than long. They are very variable in size, ranging from 0.3-2 cm (or exceptionally 3 cm) in length with a similar width. Leaves are thinly white-pilose above and below at first, but soon become glabrous and dark shining green. The venation is prominently reticulate. The leaf margins are rather evenly crenulate-serrate and rarely entire. The leaf apex is rounded or shallowly emarginate and occasionally sub-acute. The leaf base is rounded, subcordate or very broadly cuneate. The petiole is very short, usually less than 4mm long and channelled above with minute 'stipules' (as described by Meikle, (1984). *S. herbacea* is characteristic of alpine snow-bed communities on acidic soils (Caseldine, 1989). The restriction of *S. herbacea* to harsh environments such as exposed rock ledges and summits may be the result of its low competitive ability:- it is classified as a stress tolerator under the C-S-R (Common: Scarce: Rare) model of primary strategies. *S. herbacea* has a wide circumpolar distribution in Europe, Asia and North America. Within Britain it is confined to upland sites in Scotland, Wales and the Lake District. (Meikle, 1984).

3.2.2 The sampling and collection of *S. herbacea* material.

Modern material.

Fresh *S. herbacea* leaves were collected from the Oetztaler Alpen, Tyrol, Austria in July 1994 and 1995.

Collection sites.

Collections of *S. herbacea* were kindly made by Dr. J.A. Pearson and are described in Tables 3.1 and 3.2.

Table 3.1 1994 collection sites of *S. herbacea.*

No	Altitude (m)	Site	Description	Nature of Collection
1	2100	Breitlehntal	East to West Hanging valley,	Flat ground random
2	2100	Breitlehntal	East to West Hanging valley,	Sloped ground random
3	2200	Timmelsjoch	Level ground meadow on alpine ridge	Random
4	2200	Rotmoostal	Glacial vorfeld, open glacial	Random
5	2500	Timmelsjoch	Level ground meadow on alpine ridge	Random
6	2500	Timmelsjoch	Level ground meadow on alpine ridge	Random
7	2500	Timmelsjoch	Level ground meadow on alpine ridge	Male Plants
8	2500	Timmelsjoch	Level ground meadow on alpine ridge	Female Plants
9	2670	Hohe Mut	High level alpine pasture	Random
10	2670	Hohe Mut	High level alpine pasture	Random
11	2800	Tiefenbach	Loose large boulder scree	East facing random
12	2800	Tiefenbach	Loose large boulder scree	South Facing random

Table 3.2 1995 collection sites of *S. herbacea.*

No.	Altitude (m)	Site	Description	Nature of Collection
1	2100	Breitlehntal	East to West Hanging valley,	Male Plants
2	2100	Breitlehntal	East to West Hanging valley,	Female Plants
3	2100	Breitlehntal	East to West Hanging valley,	Random
4	2500	Timmelsjoch	Level ground meadow on alpine ridge	Male Plants
5	2500	Timmelsjoch	Level ground meadow on alpine ridge	Female Plants
6	2500	Timmelsjoch	Level ground meadow on alpine ridge	Random
7	2500	Rettenbach	Rocky consolidated scree, open site	Random
8	2670	Hohe Mut	High level alpine pasture	Random
9	2670	Hohe Mut	High level alpine pasture	Male same plant sampled
10	2670	Hohe Mut	High level alpine pasture	Male same plant sampled
11	2800	Tiefenbach	Loose large boulder scree	Male same plant sampled
12	2800	Tiefenbach	Loose large boulder scree	Female same plant sampled
13	2800	Tiefenbach	Loose large boulder scree	Random

The sites were chosen to cover a wide altitudinal range, to represent a change in CO_2 concentration of 20 p.p.m.v (Dr. J. A. Pearson, personal communication) and to incorporate as many aspects as possible. Random samples were collected by throwing a quadrant (10cm by 10cm) over areas of *S. herbacea.* Ten mature leaves were then selected and placed in the sampling container and the process repeated. Leaves were then chosen at random from the sampling container for stomatal parameter analysis. Leaves from male and female plants were collected in the same way, but kept separate from each other. A number of leaves were also taken from an individual plant and these are referred to as 'same plant' collections.

Storage of *S. herbacea* leaf samples.

Leaves removed from *S. herbacea* plants were either stored in Formyl-Aceto-Alcohol (FAA) (50% 100ml Ethanol, 37-41% 6.5 ml Formalin, 2.5ml concentrated Glacial acetic acid) or pressed dried at the site. Whole plant specimens were removed carefully and transported.

Fossil material.

Collection site of fossil leaves of *S. herbacea.*

Fossil material was obtained from cores taken from the Morrone Birkwoods, a site in the Eastern Highlands of Scotland which extends over *ca.* 100ha and stretches between elevations of *ca.* 380m and *ca.* 600m. The sedimentary record spans the last *ca.* 12,500 years as dated by a series of radiocarbon determinations. The Morrone Birkwoods are known for their sub-Arctic vegetation and rich flora. Arctic-Alpine, and Northern Montane biogeographic elements are especially well represented. The macrofossil record indicates that the flora has had this general character, for some time. The pollen record further indicates that the sub-Arctic character of the vegetation has most likely persisted since earliest Holocene times (Huntley, 1994).

Coring locality.

The cores were kindly provided by Professor B. Huntley and Dr. J Allen. The coring locality was situated at National Grid Reference 37/131905; Longitude 3° 25' 51" W. latitude 56° 59' 53" N: elevation *ca.* 425m. The cores were taken from the deepest sediment area from small infilled basins within the woodland area.

Core sampling.

Sediments were sampled using a Wright-modified Livingstone piston corer (Wright, 1967). A fibrous sedge peat 30cm layer formed the uppermost surface. This was collected as a monolith before sampling with the corer. Sediment was collected to 430cm in depth. The core segments studied spanned 100-184 cm, 184-269cm, 269-343cm and 343-430cm. When the corer struck a boulder or bedrock coring was halted. Cores were later extruded in the laboratory, wrapped in plastic film and aluminium foil prior to storage at 4°C. When macrofossils were required the cores were unwrapped and the material present noted. The nature of the Morrone Birkwood cores used for this study is described in Diagrams 3a, 3b, 3c and 3d.

Plants macrofossils were extracted from both 5cm and 2cm half core cut sections. The sections were disaggregated in water. The resulting suspensions were wet-sieved using 3 sieves of mesh sizes 1mm, 400μm and 140μm. The residues collected in each were washed thoroughly. Residues were then examined under a Wild stereo-microscope. Leaf macrofossils of *S. herbacea* were counted, removed and stored in 50% aqueous glycerol.

3.2.3 Stomatal counts and guard cell measurements.

Leaf surface sampling.

Leaf surface replicas were used to determine stomatal characteristics. The region of the *S. herbacea* leaf studied was located midway between the tip and the base of the leaf, in order to avoid trichromes and large veins. Several methods were used to obtain a permanent record of the leaf epidermal features and obtain details of stomatal and epidermal cell counts as well as guard cell dimensions.

Diagram 3a. Description of material recorded in core sections taken from the Morrone Birkwood (100 - 184cm).

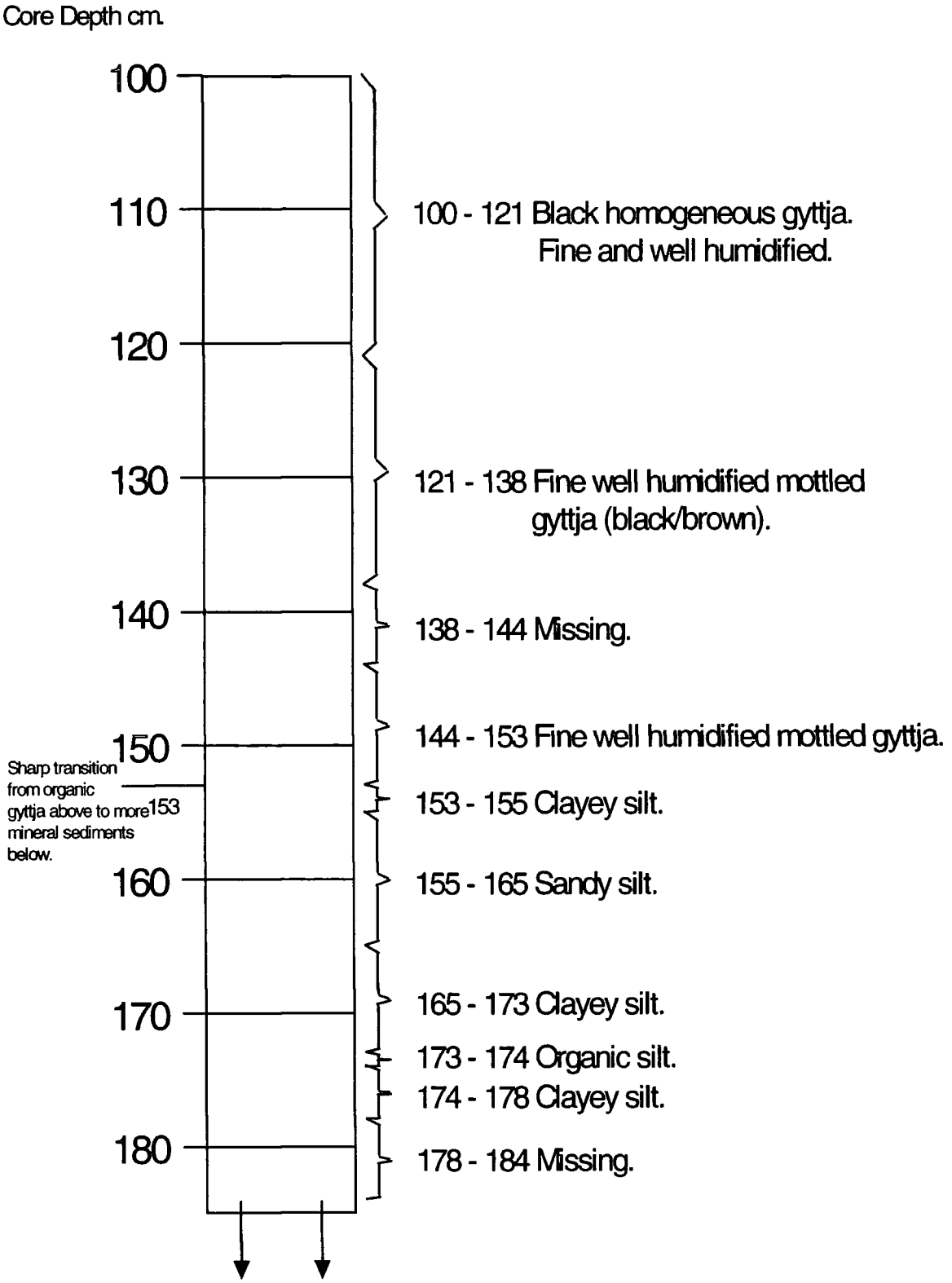


Diagram 3b. Description of material recorded in core sections taken from the Morrone Birkwood (184 - 269cm).

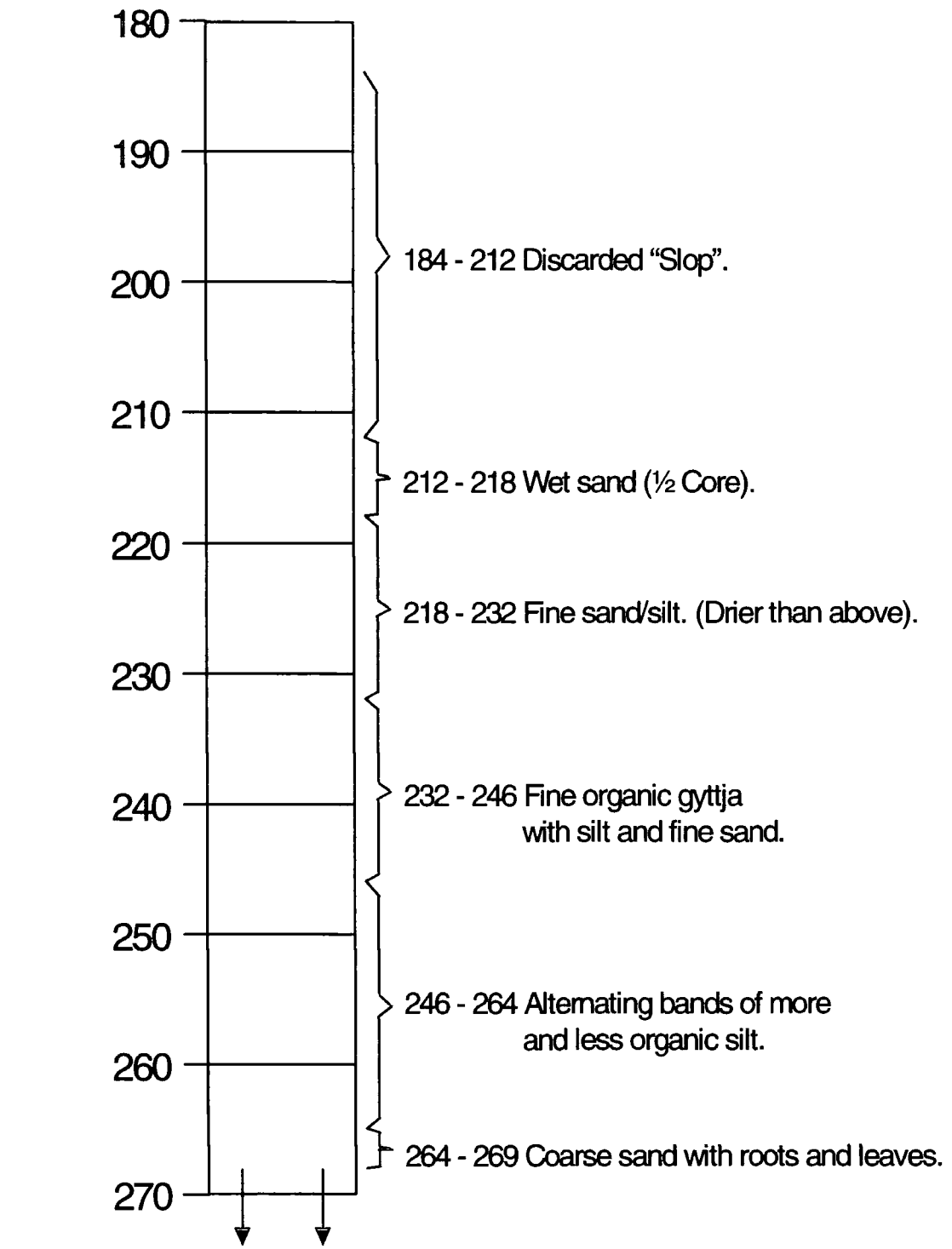


Diagram 3c. Description of material recorded in core sections taken from the Morrone Birkwood (269 - 343cm).

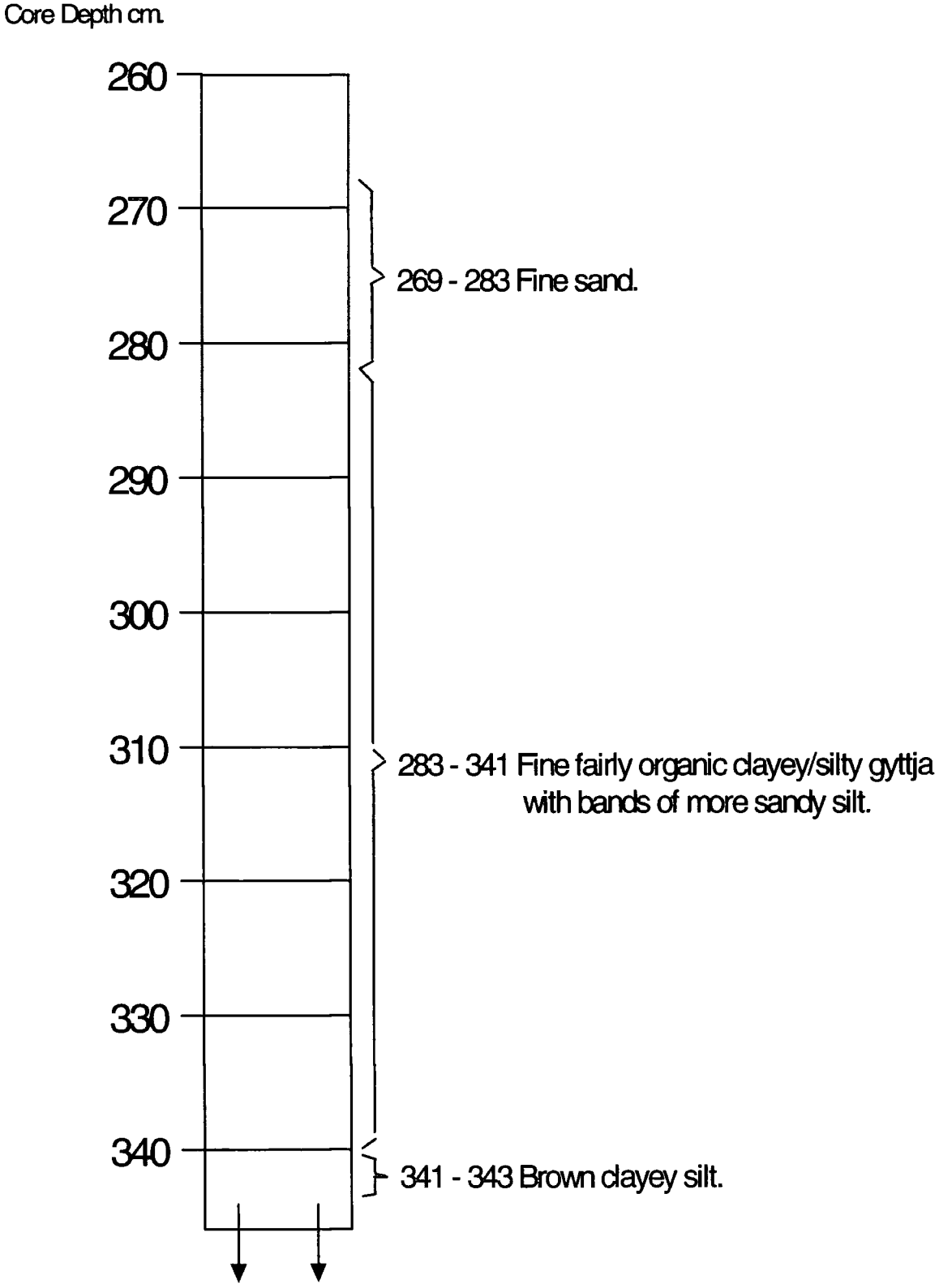
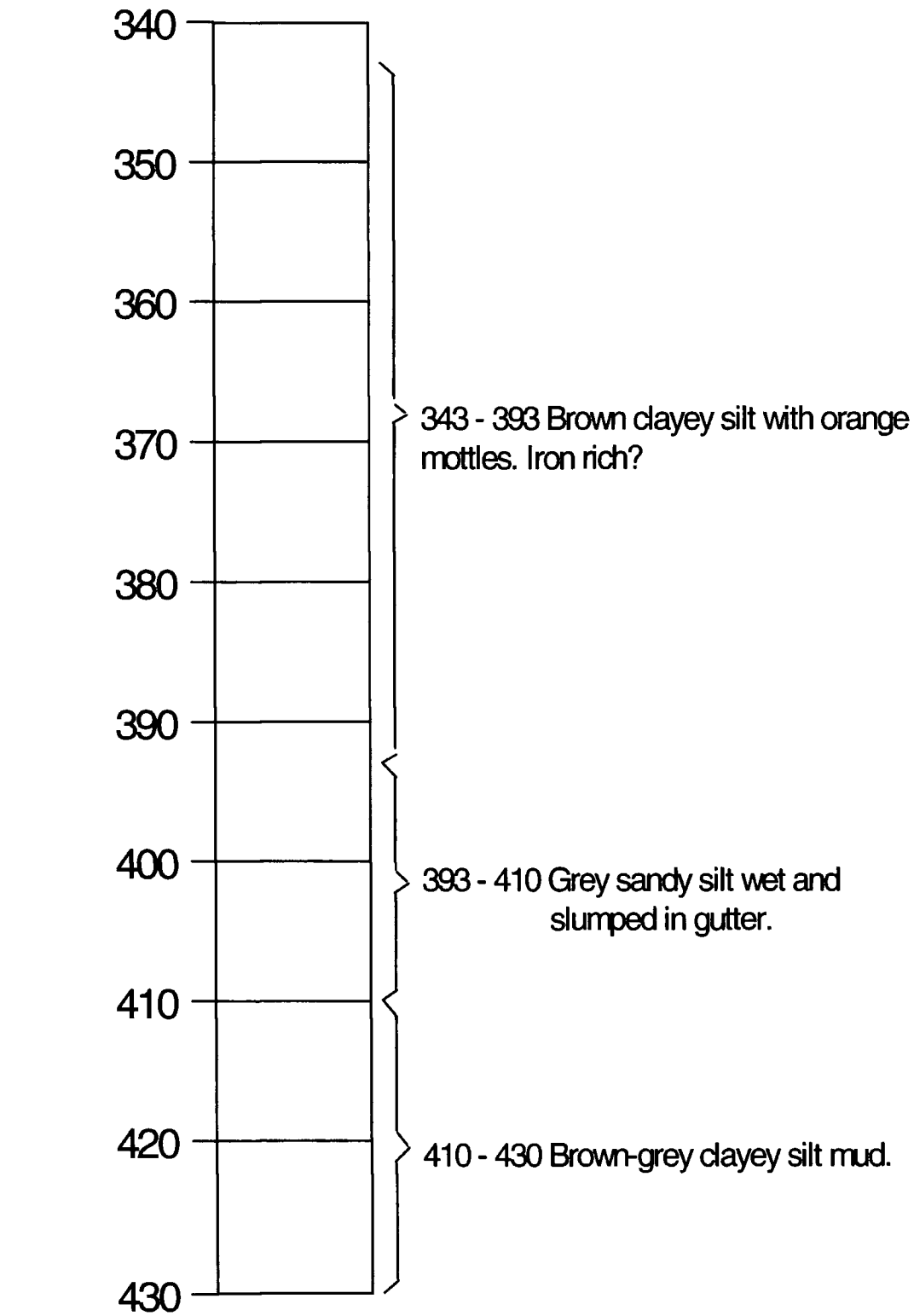


Diagram 3d. Description of material recorded in core sections taken from the Morrone Birkwood (343 - 430cm).



3.2.4 Recording of leaf epidermal features.

Micro-relief methods.

Four micro-relief methods were attempted on the leaf material. All replicas obtained by micro-relief techniques were placed on a microscope slide and covered with a coverslip for examination.

Cellulose acetate peel.

Casts of the leaf surface were prepared using colourless acetate, (clear nail varnish, Boots No. 17) (e.g. Hsiao & Fisher, 1975; Jones, 1983). A thin layer of nail varnish was painted on both the adaxial and abaxial surfaces of leaves for examination. The nail varnish was allowed to dry for between 20 and 30 minutes and then carefully peeled off with forceps.

Ethyl acetate.

An acetate replica of the leaf surface was obtained using small sections of clear acetate sheets. The leaf surface under investigation was flooded with absolute acetone and a section of acetate sheet applied. This was then compressed between two microscope slides (76 x 26 x 0.8-1.0 mm thick) and kept under compression to dry for approximately 20 minutes. It was then possible to peel away the leaf material from the acetate sheet leaving a negative replica of all the topographical features. The sheet replica was then studied using a microscope as previously described.

Acetate sheet.

A novel method was attempted using acetone and acetate sheeting. Sections of acetate sheet were added to an excess of absolute acetone until a gelatinous mixture was obtained. This was then painted across the leaf surface and allowed to dry for 30 minutes before the acetate impression was carefully peeled off.

Dental wax.

Weyers & Johansen, (1985), described the use of "Xantopren", a low viscosity dental impression material, which was applied to make estimates of stomatal aperture size. The application of Xantopren has the advantage of being fundamentally non-destructive, and the impressions can be stored indefinitely prior to examination. Xantopren or similar dental impression material is made up from base and activator in an appropriate proportion. It is essential that the components are well mixed at speed to avoid the mixture hardening before application. The dental wax used for this investigation was Xantopren Vl (Bayer Dental, Leverkusen, Germany). It was heated to melting point and mixed with a glass rod. The matrix was firstly spread across the leaf surface using a spatula. In addition a variation was attempted whereby the wax was gently heated and then the leaf carefully pressed into it in order to create a surface impression. Once the matrix had set, the leaf was removed. A layer of clear nail varnish was then applied, which resulted in the formation of a positive replica. Once the nail varnish had dried it was removed as before.

Epidermis/cuticular removal methods.

Three methods were used to remove the epidermal/cuticular layer for analysis.

Removal of the epidermis by Sellotape.

Leaves were gently placed on a strip of household Sellotape. The Sellotape and leaf were then compressed between two microscope slides. The Sellotape was then carefully peeled away from the leaf surface in an attempt to remove the epidermal layer.

Removal of the epidermis by the use of a razor blade.

Leaf material was softened, using a rehydrating mixture of water and glycerol (50:50). When the material was pliable, a razor blade was used to remove the epidermal layer. In order to achieve this, a small incision was firstly made to the leaf tip. Then using a binocular microscope and precision forceps, the epidermal layer was raised and removal attempted. In addition, the leaf surface was also "shaved" using a razor blade in an attempt to remove the upper layer.

Removal by maceration.

A solution of equal parts 20% hydrogen peroxide and glacial acetic acid was prepared. The leaf under examination was placed in this solution and gently heated in a water bath at approximately 60°C (Catling & Grayson, 1982). The leaf was removed when it was transparent in nature. Gentle and precise heating resulted in the epidermis and cuticle being separated away from the remaining leaf.

3.2.5 Microscopy methods.

Microscopy was used to directly examine the leaf surfaces for stomatal characteristics.

Fluorescence microscopy.

Macrofossil leaves were placed on a microscope slide. The material was then stained carefully with a drop of Auramine O dye (5% in water) and a coverslip applied. Slides were examined under a Nikon Diaphot-TMD inverted microscope with TMD-EF epiflourescence provided with an ultra-violet (UV) blue (excitation cassette) filtered mercury light. This method was used to clarify the positions of cell walls to make distinction of individual cells easier in fresh material, and to visualise stomata and obtain stomatal density results in leaf macrofossils.

Light microscopy.

Leaves were soaked in a 5% sodium hypochlorite solution for 20 minutes. The bleached leaves were then mounted on slides and examined as entire specimens under the light microscope. This was used as a method to view entire leaf surfaces.

Scanning electron microscopy

Leaves for examination under a scanning electron microscope (SEM) were air dried and attached to electron microscope stubs. These were then sputter coated with gold (Edwards, UK) to eliminate charging of the surface at an accelerating voltage of 15kV and a working distance of 20mm. Examination was carried out using a JEOL JSM848 SEM scanning electron microscope. For scanning electron micrographs, Ilford black and white professional ISO 125/22° films were used and developed on site, or the images

were downloaded directly and manipulated using Adobe Photoshop™ on an IBM ™ compatible computer.

Replica surface Examination.

The micro-relief replicas were placed on a microscope slide and covered with a cover slip. The replicas were then examined under the light microscope (C.Baker Light Microscope) to obtain the stomatal density, epidermal cell density and stomatal index for each leaf replica sampled. The number of stomata and the number of epidermal cells were counted (unless otherwise stated) from ten fields of view (area 0.091mm^2) from each of the individual leaf surface replicas (usually total 50 microscopic fields of view per sample) at x400 magnification. This method was used on the Austrian *S. herbacea.*

3.2.6 Cell density measurements, stomatal index and stomatal guard cell dimensions.

These measurements were carried out as described in Chapter 2.

Mapping of stomatal density across a leaf surface.

Entire leaf surface replicas of Austrian *S. herbacea* leaves were obtained using the cellulose acetate peel technique. These were placed on a microscope slide and a coverslip applied. The slides were examined under at x400 magnification. The microscope was connected to a television screen. Stomata were counted in each full screen view.

3.3 Results.

Analysis of stomatal parameters recorded from *S. herbacea* leaves collected at the Oetztaler Alpen, Tyrol Austria in July 1994 and 1995.

Error bars in all figures show standard error.

3.3.1 Variations in stomatal characteristics along an altitudinal gradient from 1994 collection sites.

Abaxial surface responses.

Fig. 3.1 and Fig. 3.2 illustrate the variation of stomatal characteristics recorded on the abaxial surface of *S. herbacea* leaves collected in 1994. There are no clear trends (i.e. an increase or decrease of a parameter) observed in any of the stomatal parameters with respect to an increasing altitudinal gradient. Stomatal density would appear to show a decrease with increasing altitude, however, there is considerable 'noise' within the data set. Stomatal density and stomatal index do not record parallel results. For example, Timmelsjoch 2500m male plants (site 7) have a mean stomatal density of $99.11 \pm 3.45 mm^{-2}$, and Timmelsjoch 2500m female plants (site 8) of $93.39 \pm 3.24 mm^{-2}$. The male plant site has an increased epidermal cell density than that of the female plant site, the corresponding stomatal index is further increased. Guard cell measurements do not reveal any consistent trends with changing altitudinal gradient. The responses of stomatal length, pore length and stomatal width do follow a similar pattern with respect to the sampling site, however, the recorded changes are not identical.

Adaxial surface responses.

Fig. 3.3 and Fig. 3.4 show the recorded stomatal characteristics for the adaxial surface. Once more there is no clear trend in any of the parameters in relation to altitudinal gradient. Stomatal and epidermal cell densities showed inconsistent responses with altitudinal change as did stomatal index.

Abaxial and adaxial surface responses.

With respect to all 1994 site recorded data it would appear that the patterns observed on the abaxial surface are not identical to those on the adaxial surface. For example the mean stomatal index was found to be greatest on the abaxial surface at Timmelsjoch meadow collection 2200m (13.54±0.65%) in comparison, stomatal index on the adaxial surface was found to greatest in male plants on Timmelsjoch 2500m (13.98±0.39%). Stomatal density was found to be consistently lower on the abaxial surface.

3.3.2 Variations in stomatal characteristics along an altitudinal gradient from 1995 collection sites.

Abaxial surface responses.

The response of stomatal characteristics on the abaxial surface to an increase in altitudinal gradient is illustrated in Fig. 3.5 and Fig. 3.6. Stomatal density does not reveal a clear trend with an increase in altitudinal gradient. For example the stomatal density of Breitlehntal female plants 2100m (site 2) was recorded as
126.43± 4.93mm^{-2} at 2100m the closest recorded site mean stomatal density to that was recorded at Tiefenbach 2800m on leaves taken from one female plant (site 12). Epidermal cell densities and stomatal index also were found to show variation which did not appear to be associated with altitudinal gradient and in addition were not parallel to those of stomatal density. Guard cell dimensions showed inconclusive patterns.

Adaxial surface responses.

Fig. 3.7 and Fig. 3.8 show the recorded stomatal characteristic responses of the adaxial surface with altitude. As noted previously, no apparent trend was revealed when sites were related to altitudinal gradient. In addition parallel site responses were not observed consistently with respect to stomatal densities, epidermal cell densities and stomatal indices. Guard cell dimensions do show a similar pattern with respect to the site of sampling, however, no consistent trend is observed with respect to an increase in altitudinal gradient.

Abaxial and adaxial surface responses.

When all the 1995 sites are considered, the abaxial surface response is found to differ from that of the adaxial surface with respect to all stomatal parameters under investigation. For example, stomatal density was found to increase from Rettenbach 2500m to Hohe Mut 2670m on the abaxial surface, however, the adaxial surface recorded at the same site and calculated from the same leaves was found to show a decrease in stomatal density. Stomatal density was consistently greater on the adaxial surface.

3.3.3 The difference in stomatal parameters between years.

It was not possible to obtain identical collections at each site point in each year. However, it is observed that there is considerable variation at sites of the same altitude when sampled a year apart. There is no consistent trend in with respect to cell densities and stomatal indices, guard cell dimensions are generally recorded to be greater in plants collected in 1994 in comparison to the 1995 collection.

3.3.4 The difference in stomatal parameters between male and female plants.

In 1994 one 'female only' plant collection was made at Timmelsjoch 2500m (site 8) and a corresponding 'male only' plant collection sampled (site 7). Three collections were made in 1995 comparing male and female plants, these were taken at Breitlehntal 2100m, Timmelsjoch 2500m and Tiefenbach 2800m. It was noted that at each site, irrespective of altitude, the 'female only' samples had consistently greater stomatal indices on the abaxial surface in comparison with 'male only' collections (see 3.3.11 for further statistical analysis).

3.3.5 Variations in stomatal parameters of 'pooled' sites along an altitudinal gradient in 1994.

Site data was pooled at each altitude to give a mean value at each elevation. The data was pooled to investigate if there were any significant trends when the variations observed at each collection site were incorporated within the mean value for that altitude.

The mean values of each stomatal parameter were plotted against altitude. Figs. 3.9-3.16 illustrate the results obtained. A trend line was applied using Microsoft EXCEL. The data was examined statistically using the correlation coefficient. The results of the statistical analyses for 1994 are presented in Table 3.3 and those for 1995 in Table 3.4. When a significant response is recorded it is relative to an increasing altitudinal gradient.

Table 3.3 Statistical analysis of pooled site data for 1994 (Figs 3.9, 3.10, 3.11 and 3.12). (There are 3 degrees of freedom in each case and $p<0.05 = r> 0.878$)

	Abaxial			Adaxial	
Parameter	**Significance**	**Correlation coefficient *r***	**Response**	**Significance**	**Correlation coefficient *r***
SD	p<0.05	0.951	Decrease	n.s	0.654
ECD	p<0.05	0.980	Decrease	n.s	0.0346
SI	n.s	0.806	----------	n.s	0.597
SL	p<0.05	0.912	Increase	n.s	0.094
PL	p<0.05	0.947	Increase	n.s	0.408
SW	n.s	0.656	----------	n.s	0.007

Table 3.4 Statistical analysis of pooled site data for 1995 (Figs. 3.13, 3.14,3.15 and 3.16). (There are 2 degrees of freedom and $p<0.05 = r> 0.95$)

	Abaxial		Adaxial	
Parameter	Significance	Correlation coefficient *r*	Significance	Correlation coefficient *r*
SD	n.s	0.206	n.s	0.704
ECD	n.s	0.094	n.s	0.073
SI	n.s	0.620	n.s	0.426
SL	n.s	0.01	n.s	0.693
PL	n.s	0.181	n.s	0.44
SW	n.s	0.017	n.s	0.788

3.3.6 Variations in stomatal parameters of 'pooled' sites along an altitudinal gradient observed in 1994.

Abaxial surface response.

The pooled data of the abaxial surface showed a significant decrease ($p<0.05$) in stomatal and epidermal densities in relation to an increasing altitudinal gradient (Fig 3.9). An insignificant change in stomatal index was recorded. Stomatal and pore lengths increased significantly ($p<0.05$) with respect to an increase in altitude (Fig 3.10). Stomatal width was found to have a non-significant relationship to altitudinal gradient.

Adaxial surface response.

Fig. 3.11 and Fig. 3.12 show the linear relationship of stomatal parameters of pooled site data with altitudinal gradient. A decrease of stomatal density was observed in respect to increasing altitude, however it was non-significant. The other parameters showed no correlation with altitudinal gradient.

The comparison of abaxial and adaxial surface responses.

The abaxial and adaxial surfaces do not show the same responses to altitudinal gradient. The abaxial surface showed statistically significantly decreased cell densities and

increased guard cell dimensions (with the exception of stomatal width) along the altitudinal gradient. There were no recorded significant changes on the adaxial surface.

3.3.7 Variations in stomatal parameters of 'pooled' sites along an altitudinal gradient in 1995.

Abaxial surface response.

No correlation was found between any of the stomatal parameters in relation to a changing altitudinal gradient.

Adaxial surface response.

A decrease of stomatal density was recorded with increasing altitude, however it was not statistically significant. Once again no correlation of stomatal parameters with altitude was recorded.

Comparison of 1994 and 1995 samples.

The response of the abaxial surface was found to vary depending on the sampling year. The 1994 data set revealed significant linear correlation of stomatal parameters, (cell densities showing a significant decrease and cell dimensions a significant increase) with increasing altitudinal gradient, however, no correlation was found in the 1995 data set. The stomatal parameters obtained for the adaxial surface did not reveal any correlation in either year.

3.3.8 Variation in stomatal parameters within altitudinal sites.

There were no clear trends recorded when stomatal parameters from all sites were analysed along an altitudinal gradient. The results revealed considerable 'noise' within the data sets when collections were made at the same altitude. Statistical analysis was carried out using one-way ANOVA (analysis of variance) to investigate variation in stomatal parameters within altitudinal sites.

3.3.9 1994 collection results.

2100m.

Two collections were made at 2100m from Breitlehntal. Leaves were collected at random from a flat ground sampling point and along an inclined ground. Table 3.5 shows statistical analysis of the data.

Table 3.5 One way ANOVA results calculated for stomatal parameters at 2100m from 1994. F crit = 3.94

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	p<0.05	100.36	n.s.	0.31
ECD	p<0.05	7.85	p<0.05	17.93
SI	p<0.05	56.35	p<0.05	16.31
SL	n.s	2.61	n.s	1.04
PL	p<0.05	12.01	n.s	0.47
SW	p<0.05	68.47	p<0.05	7.12

Significant differences in all stomatal parameters with the exception of stomatal length were recorded on the abaxial surface. Epidermal cell density, stomatal index and stomatal width showed significant variation on the adaxial surface.

2200m.

S. herbacea leaves were collected at random from two different sites at 2200m, Timmelsjoch and Rotmoostal. Table 3.6 shows statistical analysis of the data.

Table 3.6 One way ANOVA results calculated for stomatal parameters at 2200m from 1994. F crit = 3.94

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	p<0.05	8.17	n.s.	1.18
ECD	p<0.05	68.53	n.s	0.06
SI	p<0.05	65.48	n.s	1.71
SL	p<0.05	11.39	p<0.05	15.21
PL	p<0.05	28.10	p<0.05	12.19
SW	p<0.05	9.09	p<0.05	18.76

Statistically significant differences between all studied stomatal parameters were observed on the abaxial surface between sites at 2200m. Guard cell dimensions varied significantly on the adaxial surface but there was no significant variation in cell densities or stomatal index.

2500m

Four collections were made at 2500m from the Timmelsjoch site. Two random plant collections were made and male and female 'only' plants sampled. Table 3.7 shows statistical analysis of the data.

Table 3.7. One way ANOVA results calculated for stomatal parameters at 2500m from 1995. F crit =2.65

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	p<0.05	11.14	p<0.05	9.55
ECD	p<0.05	81.24	p<0.05	16.19
SI	p<0.05	33.32	p<0.05	30.47
SL	p<0.05	30.07	p<0.05	7.46
PL	p<0.05	30.52	p<0.05	7.97
SW	p<0.05	28.55	p<0.05	17.01

There are statistically significant recorded differences in all stomatal parameters on both leaf surfaces between the four sample collections.

2670m.

Two random collections of *S. herbacea* were made at the Hohe Mut site. Table 3.8 shows statistical analysis of the data.

Table 3.8 One way ANOVA results calculated for stomatal parameters at 2670m from 1994. F crit = 3.94

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	n.s	3.77	$p<0.05$	68.43
ECD	$p<0.05$	9.24	$p<0.05$	13.94
SI	n.s	0.0006	$p<0.05$	110.11
SL	n.s	0.83	n.s	2.12
PL	$p<0.05$	17.84	n.s	1.28
SW	$p<0.05$	7.43	n.s	0.09

The abaxial surface showed a significant difference in epidermal cell densities, pore length and stomatal width between the two random collections. There was significant variation on the adaxial surface of stomatal and epidermal cell densities and stomatal indices between collections.

2800m.

S. herbacea was collected from south and east facing slope aspects at Tiefenbach at an altitude of 2800m. Table 3.9 shows statistical analysis of the data.

Table 3.9 One way ANOVA results calculated for stomatal parameters at 2800m from 1994. F crit = 3.94

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	p<0.05	17.18	n.s	0.80
ECD	p<0.05	7.58	n.s	3.86
SI	p<0.05	23.61	n.s	0.11
SL	n.s	0.30	p<0.05	36.64
PL	n.s	1.21	p<0.05	31.96
SW	n.s	0.28	n.s	0.40

There was significant variation in stomatal and epidermal cell densities and stomatal indices recorded on abaxial surfaces of leaves collected from different slope aspects. This was not apparent on the adaxial surfaces where significant differences were found in stomatal and pore lengths.

3.3.10 1995 collection results.

2100m.

Three collections were made in 1995 from Breitlehntal at 2100m. Male and female 'only' plant collections were in addition to a random collection. Table 3.10 shows statistical analysis of the data.

Table 3.10 One way ANOVA results calculated for stomatal parameters at 2100m from 1995. F crit = 3.06

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	p<0.05	4.46	p<0.05	27.77
ECD	n.s	2.07	n.s	3.32
SI	n.s	0.29	p<0.05	15.39
SL	n.s	0.46	p<0.05	5.08
PL	n.s	1.10	n.s	2.04
SW	p<0.05	6.58	p<0.05	9.16

Significant variation was recorded in stomatal density and stomatal width on both surfaces between the sample collections. A significant difference was also observed on the adaxial surface with respect to stomatal index and stomatal length.

2500m.

Four collections were made at 2500m. These incorporated male, female and random collections from Timmelsjoch and a random collection made at Rettenbach. Table 3.11 shows statistical analysis of the data.

Table 3.11 One way ANOVA results calculated for stomatal parameters at 2500m from 1995. F crit=2.65

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	p<0.05	15.64	p<0.05	47.00
ECD	p<0.05	3.93	p<0.05	4.50
SI	p<0.05	19.51	p<0.05	75.67
SL	p<0.05	2.82	p<0.05	9.14
PL	p<0.05	5.13	p<0.05	9.23
SW	p<0.05	6.79	p<0.05	14.30

Significant variation was recorded in all stomatal parameters between the four collections.

2670m.

A random collection was made at Hohe Mut 2670m. In addition leaf collections from two single plants were made. Table 3.12 shows statistical analysis of the data.

Table 3.12 One way ANOVA results calculated for stomatal parameters at 2500m from 1995. F crit = 3.06.

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	n.s	1.32	n.s	2.99
ECD	p<0.05	29.49	p<0.05	36.86
SI	p<0.05	5.24	p<0.05	6.39
SL	p<0.05	6.72	p<0.05	29.61
PL	p<0.05	22.32	p<0.05	48.43
SW	n.s	3.00	p<0.05	13.45

Significant variation was recorded in all parameters with the exception of stomatal density and stomatal width on the abaxial surface, and stomatal density on the adaxial surface.

2800m.

Three collections were made from 2800m at Tiefenbach. Male and female plant 'only' collections were made in addition to a random collection. Table 3.13 shows statistical analysis of the data.

Table 3.13 One way ANOVA results calculated for stomatal parameters at 2800m from 1995. F crit = 3.06.

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	p<0.05	18.41	p<0.05	25.36
ECD	p<0.05	4.53	p<0.05	17.51
SI	p<0.05	15.86	p<0.05	57.13
SL	n.s	2.75	n.s	2.15
PL	n.s	0.85	n.s	2.68
SW	p<0.05	7.90	p<0.05	3.85

Both the abaxial and adaxial surface showed significant variation between the sample collections with respect to stomatal and epidermal cell densities, stomatal indices and stomatal width.

3.3.11 Comparison of 'male only' plant collections with 'female only' plant collections.

1994.

Male and female plant collections were made at Timmelsjoch 2500m Table 3.14 shows statistical analysis of the data.

Table 3.14 One way ANOVA results calculated for stomatal parameters between male and female collections at 2500m from 1995. F crit = 3.94.

	Abaxial		Adaxial	
Parameter	Significance	F Value	Significance	F Value
SD	n.s	1.46	p<0.05	6.7
ECD	p<0.05	275.32	p<0.05	17.62
SI	p<0.05	79.78	p<0.05	23.18
SL	p<0.05	78.63	p<0.05	23.49
PL	p<0.05	72.90	p<0.05	19.07
SW	p<0.05	74.02	p<0.05	38.27

There was significant variation observed in all parameters with the exception of stomatal density on the abaxial surface between the male and female collections. The responses were not identical on each surface, for example the female plants recorded a significant increase in stomatal index on the abaxial surface, yet demonstrated a significant decrease on the adaxial surface. Guard cell dimensions were consistently significantly greater on the abaxial surface and consistently reduced on the adaxial surface of the female plant collections.

1995.

Three collections of male and female plants were made in 1995. These were taken from Breitlehntal 2100m, Timmelsjoch 2500m and Tiefenbach 2800m. Table 3.15 shows statistical analysis of the data.

Table 3.15 One way ANOVA results calculated for stomatal parameters between male and female collections at 2100m, 2500m and 2800m from 1995. F crit = 3.94.

	2100m				2500m				2800m			
	Abaxial		Adaxial		Abaxial		Adaxial		Abaxial		Adaxial	
	Sig.	F Value	Sig.	F Value	Sig.	F Value	Sig.	F Value	Sig.	F Value	Sig.	F Value
SD	n.s	1.70	p<0.05	11.36	n.s	0.55	p<0.05	9.72	p<0.05	24.29	p<0.05	50.58
ECD	n.s	2.57	n.s	0.04	p<0.05	9.72	p<0.05	7.90	n.s	0.22	p<0.05	36.44
SI	n.s.	0.36	p<0.05	8.80	n.s.	0.02	p<0.05	18.79	p<0.05	23.05	p<0.05	100.91
SL	n.s	0.165	n.s	2.75	n.s.	0.34	p<0.05	12.58	n.s	1.11	n.s	2.59
PL	n.s	0.12	n.s	2.22	n.s	3.21	p<0.05	10.03	n.s.	0.55	n.s	0.02
SW	p<0.05	5.11	n.s	3.72	n.s	0.25	p<0.05	13.47	n.s	1.08	n.s	2.94

There is considerable recorded variation between male and female collections which is found to differ depending on the site of sampling.

Collections at 2100m showed little significant variation between male and female data sets with respect to both surfaces sampled. The abaxial surface showed that male plants exhibited a significantly higher stomatal width. Stomatal density and index were significantly reduced on the adaxial surfaces of male plants.

At Timmelsjoch 2500m the abaxial surface showed little variation in stomatal parameters, with only significantly greater epidermal cell densities recorded in female plants. The adaxial surface however, revealed significant variation in all the stomatal parameters recorded. Female plants had increased stomatal densities, decreased epidermal cell densities, increased stomatal index and the measured guard cell dimensions were significantly increased in comparison to the male plants.

Male and female plant collections at Tiefenbach 2800m showed a significant increase in stomatal density and stomatal index over female plants on the abaxial surface. These parameters on the adaxial surface also showed a significant increase in the female plants, a significant reduction in epidermal cell density was also recorded. There were no recorded differences in guard cell dimensions.

There appears to be no clear stomatal parameter response with respect to the differences recorded between male and female plants at different altitudinal sites in either of the years studied. The surface response does not appear to show consistent patterns of variation.

3.3.12 The relationship between stomatal density and stomatal length.

Mean stomatal density was plotted against mean stomatal length to investigate if a relationship existed between recorded stomatal density and stomatal length. The results for both leaf surfaces in 1994 and 1995 are illustrated in Fig. 3.17. The results were analysed statistically using the correlation coefficient. Table 3.16 shows the correlation coefficient value *r* and illustrates if they is a significant association recorded in *S. herbacea* leaves with respect to stomatal density and stomatal length.

Table 3.16. The statistical investigation of the relationship between stomatal density and stomatal length. There are 10 degrees of freedom in each case, $p<0.05 = r< 0.576$

1994				1995			
Abaxial		Adaxial		Abaxial		Adaxial	
r value	sig.	*r* value	sig.	*r* value	sig.	*r* value	sig.
0.557	n.s	0.408	n.s	0.329	n.s	0.069	n.s

A non-significant relationship was observed between stomatal density and stomatal length in both collection years.

3.3.13 Variation of stomatal density recorded over individual leaf surfaces.

Stomatal density was mapped across individual leaf surfaces as outlined in 3.2.6. The results illustrated are taken from four mapped leaves. Two leaves were taken from Timmelsjoch 2500m and two from Hohe Mut 2670m.

Fig. 3.18 shows there is considerable variation in stomatal density across a single leaf surface when considering leaf 1 abaxial surface, collected at Timmelsjoch 2500m. There appear to be isolated areas of increased stomatal density. The stomatal density appears to be decreased around the leaf edge and the mid vein area. Fig 3.19 illustrates the adaxial surface of the leaf. Although in agreement with the abaxial surface there is a further

reduction in stomatal density at the leaf edge and the mid vein area. The observed areas of increased stomatal density do not appear in identical positions to those recorded on the abaxial surface.

Fig. 3.20 and 3.21 show that a second leaf collected at the same site also shows variation in the distribution of stomatal density across both leaf surfaces. Once more there is a reduction in stomatal density recorded at most points on the leaf edge, however, there was no significant reduction observed in the mid vein region as in leaf 1.

Mapped stomatal densities of leaves collected at Hohe Mut 2670m are illustrated in Figs. 3.22, 3.23, 3.24 and 3.25. These maps show further variation in the areas of increased and decreased stomatal densities across leaf surfaces and on both leaves.

There is variation in stomatal densities across leaf surfaces. Areas of increased or reduced stomatal densities as observed on one leaf surface are not apparent on the associated opposite leaf surface or in other leaves collected at the same site. There would appear to be no clear trend as to the distribution of stomatal density across *S. herbacea* leaf surfaces.

3.3.14 Analysis of stomatal parameters recorded from *S. herbacea* macrofossil leaves collected from the Morrone Birkwood core.

The recovery and SEM analysis of core extracted *S. herbacea* leaves

S. herbacea leaves were removed from the Morrone Birkwood core from one meter onwards. The sample area was known to extend approximately 2,765 years, from 9,830±150yrs to 12,595±210yrs (Dr. J.Allen, personal communication). 255cm were recovered from the 4m core and 5cm core intervals were sieved and sampled. Intervals corresponded to 54yrs of deposition.

Leaves were sampled and recorded at core section intervals, the leaves preservation states were noted and outlined in Table 3.17.

Table 3.17. The preservation state of *S. herbacea* leaves recovered at core section intervals from the Morrone Birkwood core. The leaves are recorded as being whole, ¾ leaf, ½ leaf ¼ leaf or as a fragment (frag).

Depth (cm)	147-152	168-173	218-223	243-248	263-268	288-293	313-318	338-343	363-368	388-393	413-418
whole	0	1	1	1	3	3	10	4	0	0	0
¾	0	1	0	0	0	0	0	0	0	0	0
½	0	1	1	2	6	5	6	5	0	0	0
¼	0	0	0	0	0	1	0	0	0	0	0
Frag	0	0	0	2	2	1	0	0	0	0	1

The *S. herbacea* leaves were much reduced in size compared with the modern specimens, with an average leaf being approximately 12mm^2. The fossil leaves bore little resemblance to the modern day samples. In order to confirm identity specimens were sent to Dr R.D. Meikle and DNA analysis attempted to further confirm identify (see Chapter 5).

SEM analysis.

Analysis of the leaf surface by SEM illustrated the differences in leaf surface topography of fossil and modern specimens. Plate 3.1 illustrates the degraded and fragmented nature of the fossil leaf. In comparison to plate 3.2 which is that of a quarter section of modern *S. herbacea* observed under the same magnification. In the fossil leaf the margins are observed to be almost entire which, is rare (Meikle, 1984), unlike the crenulate-serrate margin of the modern specimen. Plates 3.3 and 3.4 demonstrate the lack of epidermal cell distinction on the fossil material in comparison to modern material, making the determination of stomatal index difficult. Guard cell dimensions on the fossil material were also difficult to determine due to the lack of definition as shown in comparison with a modern stoma (Plates 3.5 and 3.6).

3.3.15 Variations in stomatal density as recorded from four core sections.

All of the methods outlined in section 3.2.4 were attempted on the fossil material to obtain details of stomatal parameters. It was only possible to visualise stomata using fluorescence microscopy. Epidermal cell densities, stomatal index and guard cell dimensions could not be distinguished.

Leaves were selected for analysis from four core sections. 263-268, 288-293, 313-318 and 338-343cm. These sections were chosen as they possessed sufficient leaf material to sample both the abaxial and adaxial surface from three entire leaves. Each section spanned approximately 54 years of deposition and they were approximately 216 years apart from each other in succession. Stomatal counts were made from ten fields of view from each leaf surface.

Fig 3.26 illustrates the variation displayed between the fossil leaves sampled from each core site and the variation observed between core samples. Statistical analysis was then carried out to observe if there was significant difference between leaves within the same core section (see Table 3.18).

Table 3.18 One-way ANOVA results of the variation between leaves recorded within the same core section. F crit = 3.35

Surface	Abaxial				Adaxial			
Section	263-268	288-293	313-318	338-343	263-268	288-293	313-318	338-343
F value	0.415	0.125	2.22	1.75	2.13	0.298	1.91	0.266
Sig.	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

There was no significant variation recorded among leaves of the same core section.

The data was then pooled. Fig 3.27 illustrates the variation observed in stomatal density of the pooled data for the abaxial and adaxial surfaces. It can be observed that there is no consistent increase or decrease of stomatal density associated between leaves within the same core section.

Statistical analysis was carried out to investigate if there was a significant difference between the pooled core sections, and achieved using two comparisons. Firstly the variation observed across the four core sections on both surfaces, recorded as 'overall comparison'. This aimed to observe the overall variation spanning approximately 864 years. Secondly comparisons were made between adjacent cores which aimed to investigate any significant differences over the approximate 216 year period. One-way ANOVA results are shown in Table 3.19.

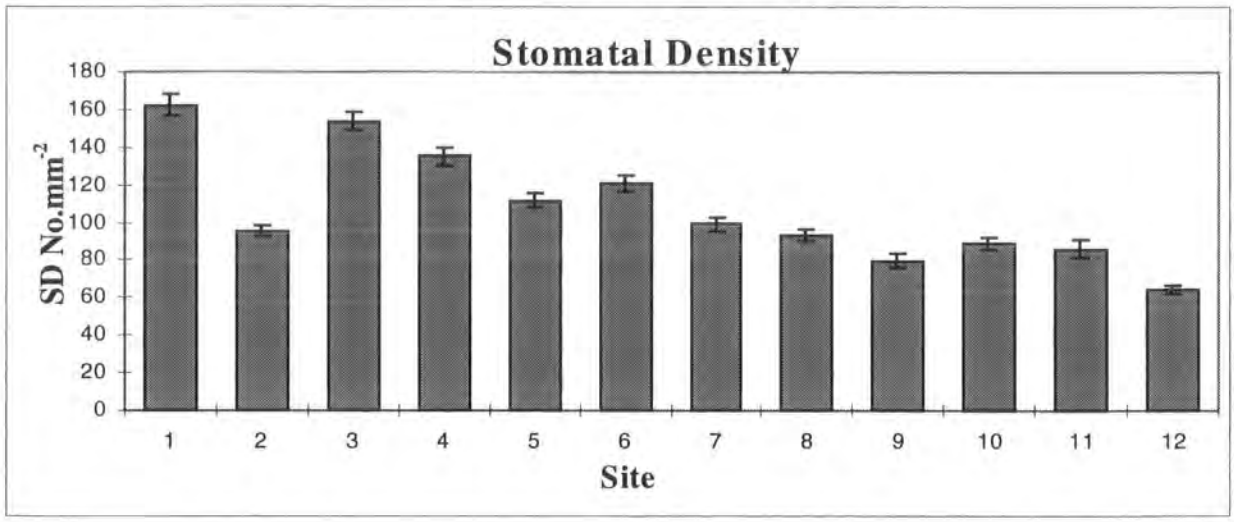
Table 3.19 One-way ANOVA results of the variation between pooled core sections. F crit = 2.68 for overall comparisons and F crit = 4.01 for comparison between adjacent core sections.

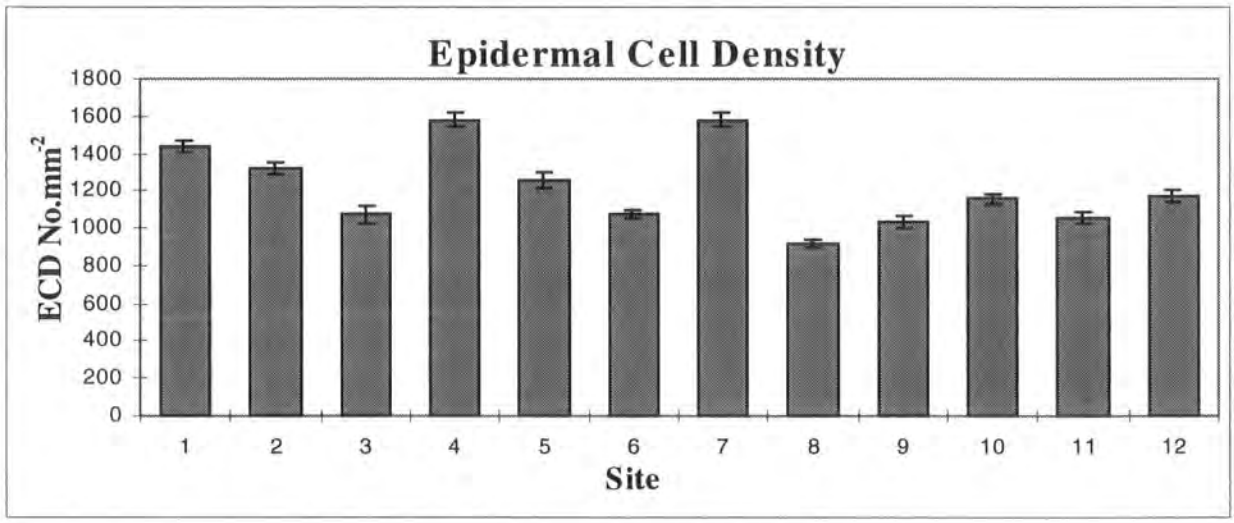
Surface	**Abaxial**				**Adaxial**			
Comparison	Overall	1&2	2&3	3&4	Overall	1&2	2&3	3&4
F crit	6.64	6.76	11.30	14.38	0.522	0.153	0.756	0.920
Sig.	$p<0.05$	$p<0.05$	$p<0.05$	$p<0.05$	n.s	n.s	n.s	n.s

There is significant variation observed among all the samples and between adjacent core sections on the abaxial surface. The adaxial surface showed no statistically significant variation.

Figure 3.1

The effect of increasing altitude on Stomatal Density, Epidermal Cell Density and Stomatal Index on the abaxial surface of *Salix herbacea* in 1994.





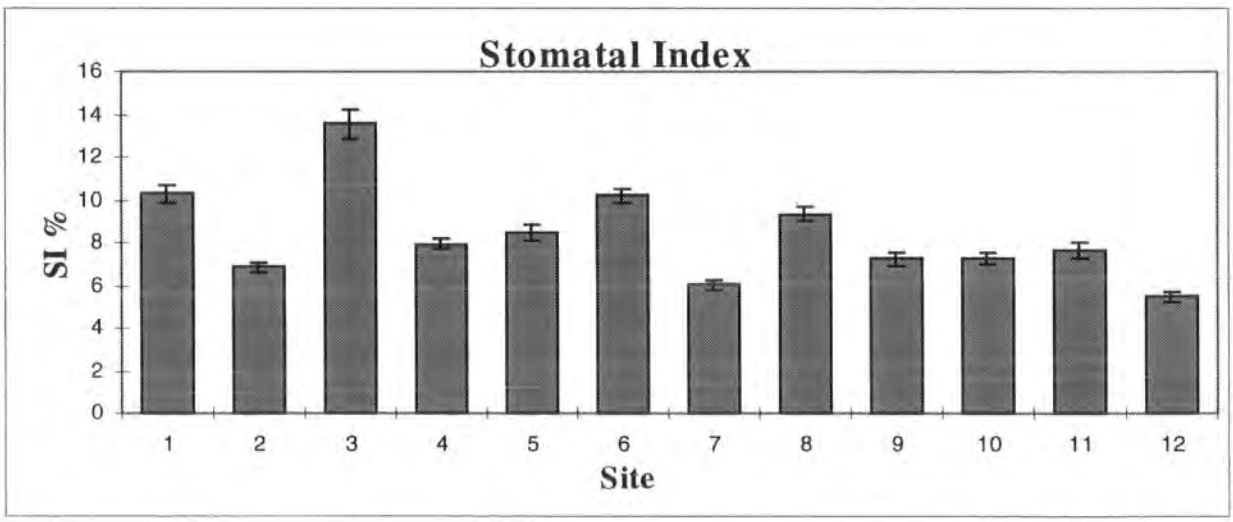


Figure 3.2

The effect of increasing altitude on Stomatal Length, Pore Length and Stomatal Width on the abaxial surface of *Salix herbacea* in 1994.

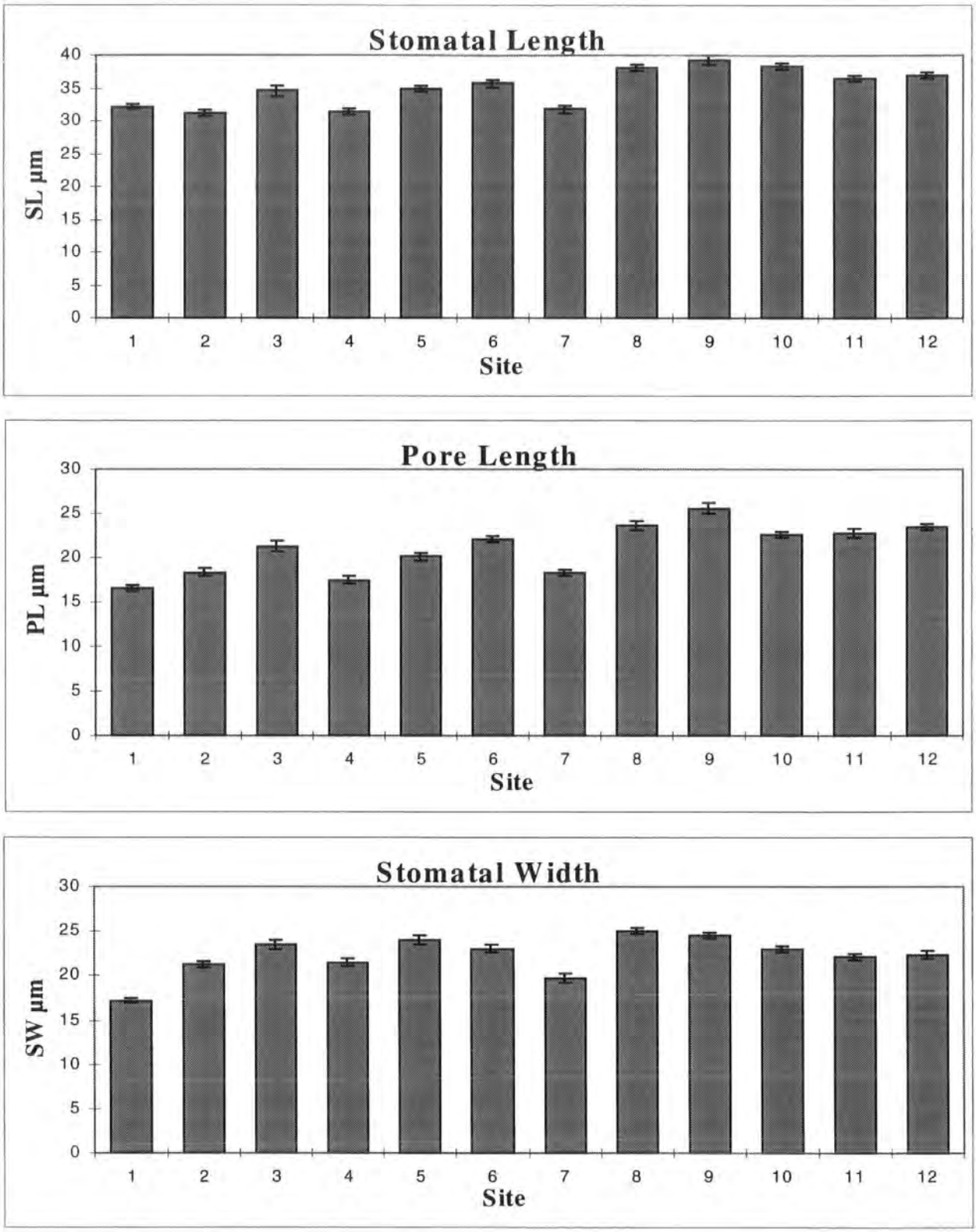
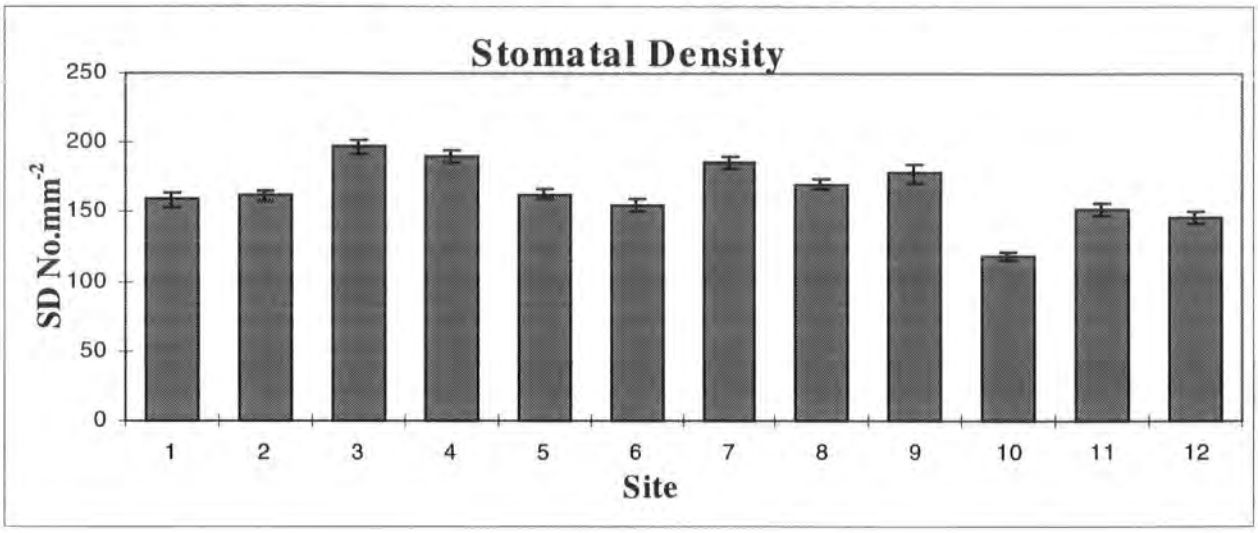
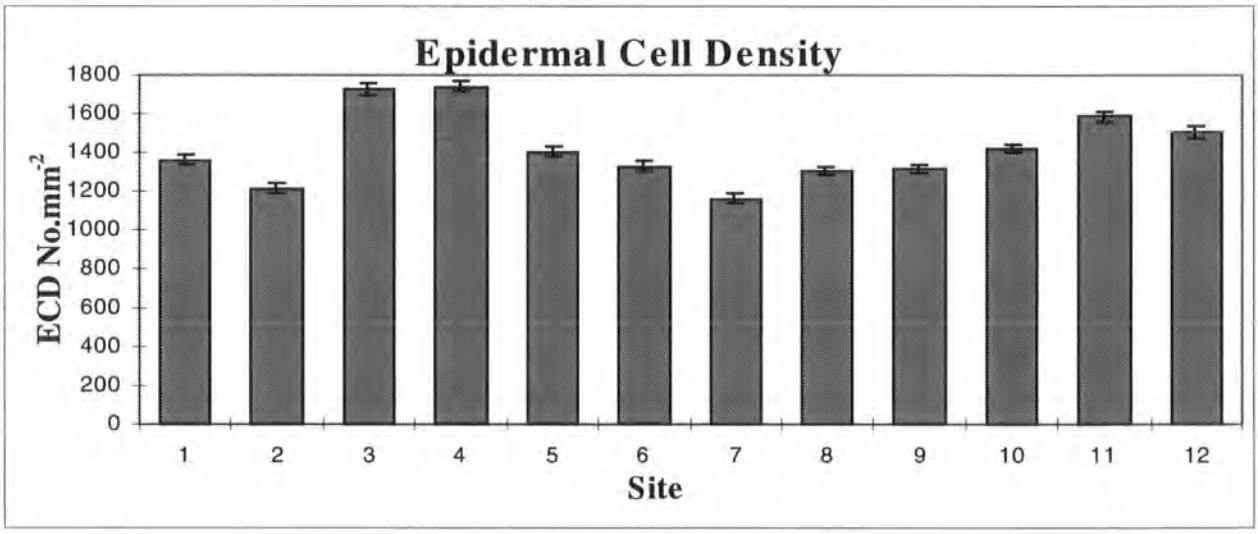


Figure 3.3

The effect of increasing altitude on Stomatal Density, Epidermal Cell Density and Stomatal Index on the adaxial surface of *Salix herbacea* in 1994.





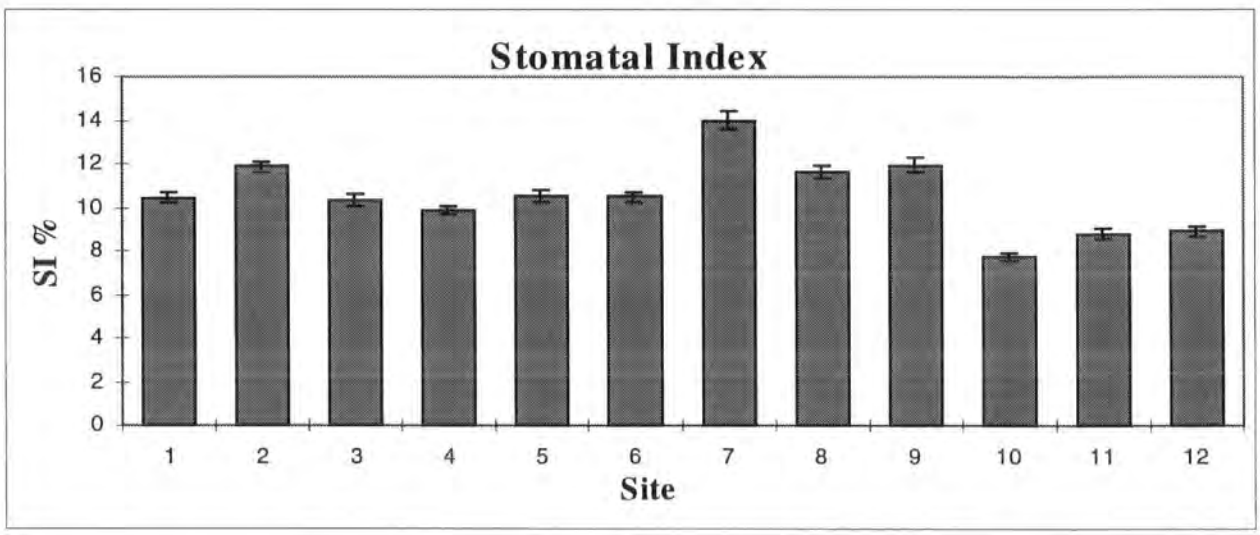


Figure 3.4

The effect of increasing altitude on Stomatal Length, Pore Length and Stomatal Width on the adaxial surface of *Salix herbacea* in 1994.

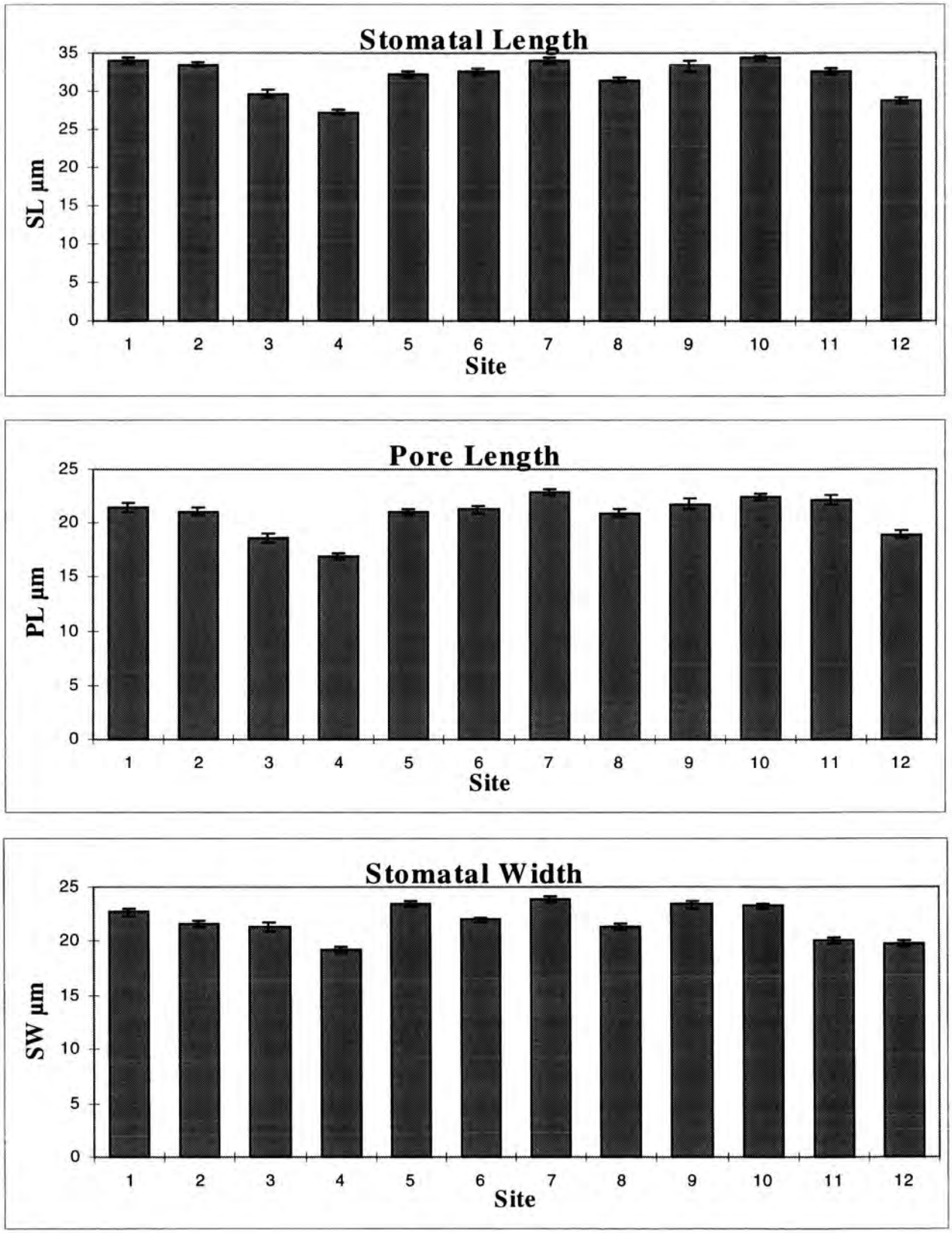
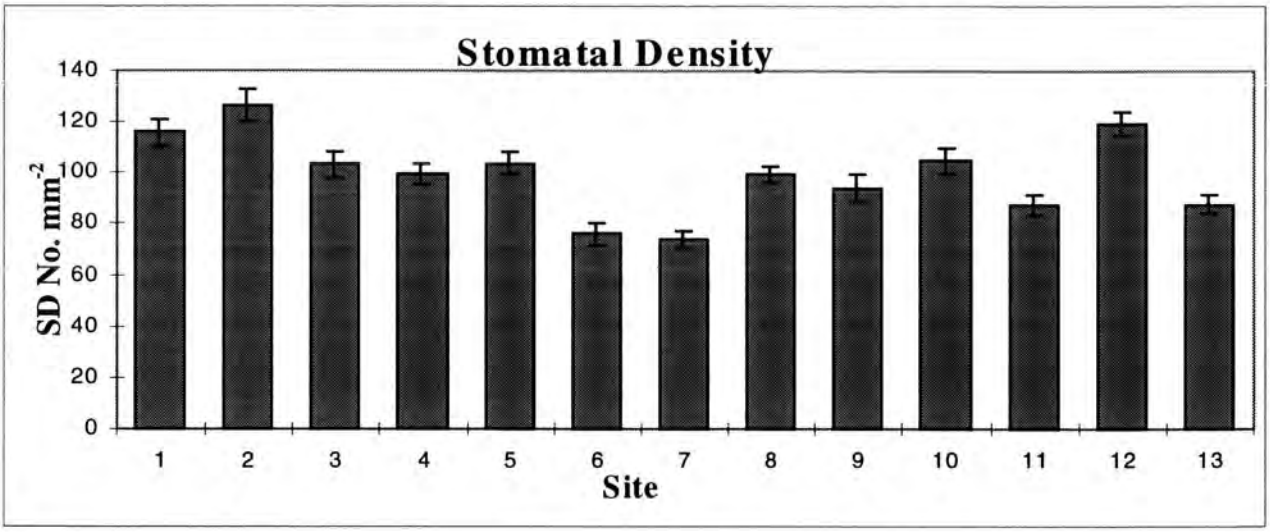
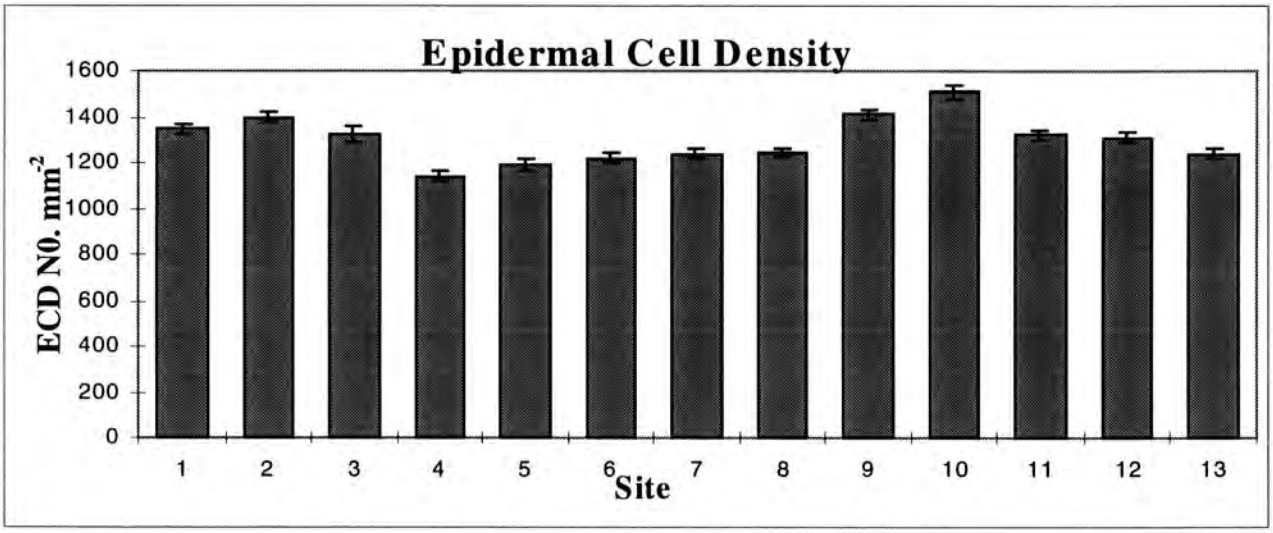


Figure 3.5

The effect of increasing altitude on Stomatal Density, Epidermal Cell Density and Stomatal Index on the abaxial surface of *Salix herbacea* in 1995.





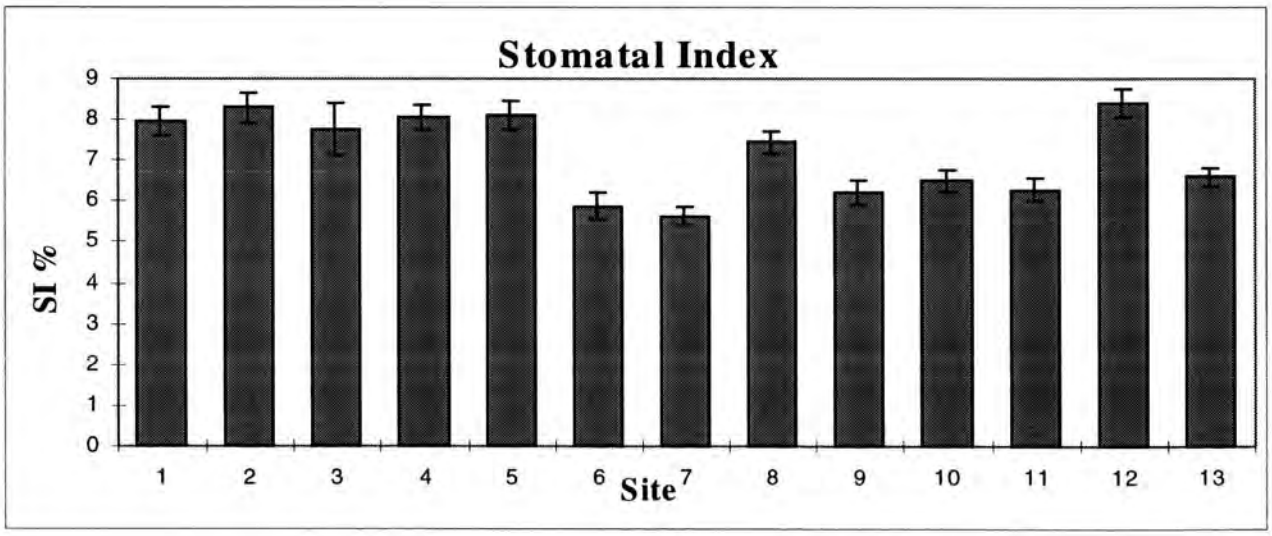


Figure 3.6

The effect of increasing altitude on Stomatal Length, Pore Length and Stomatal Width on the abaxial surface of *Salix herbacea* in 1995.

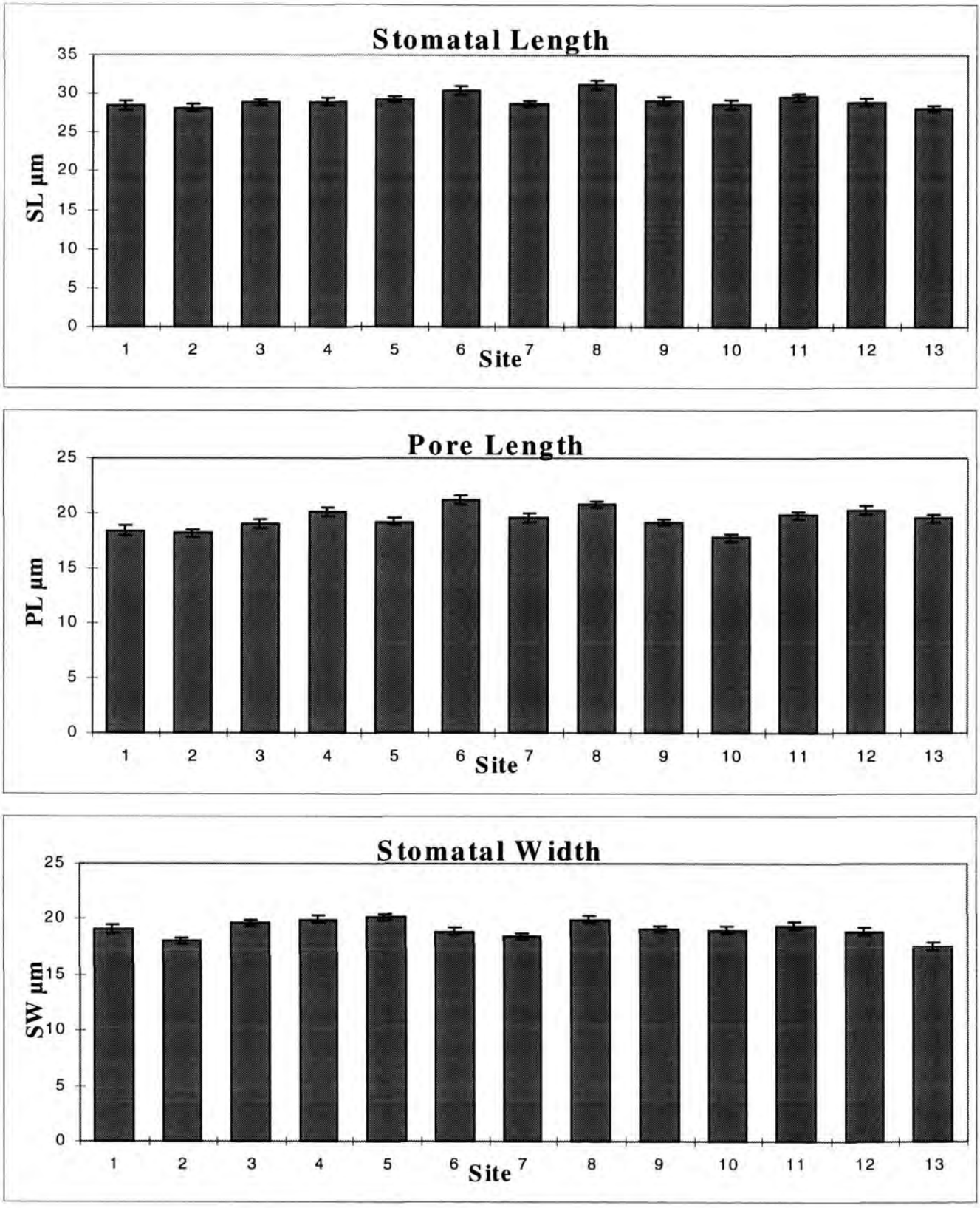
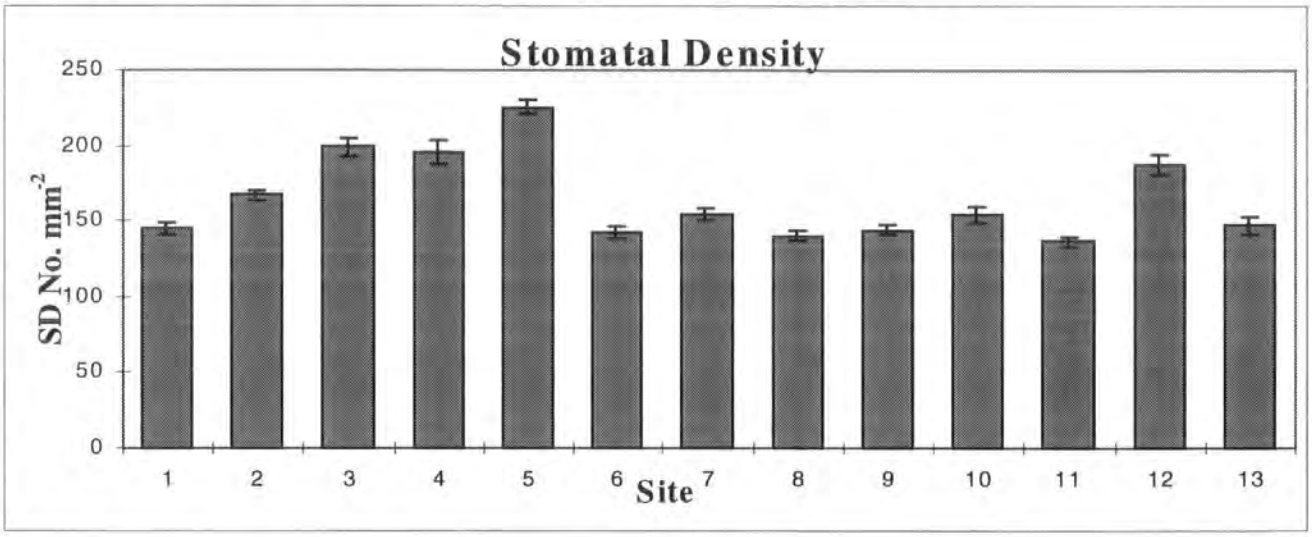
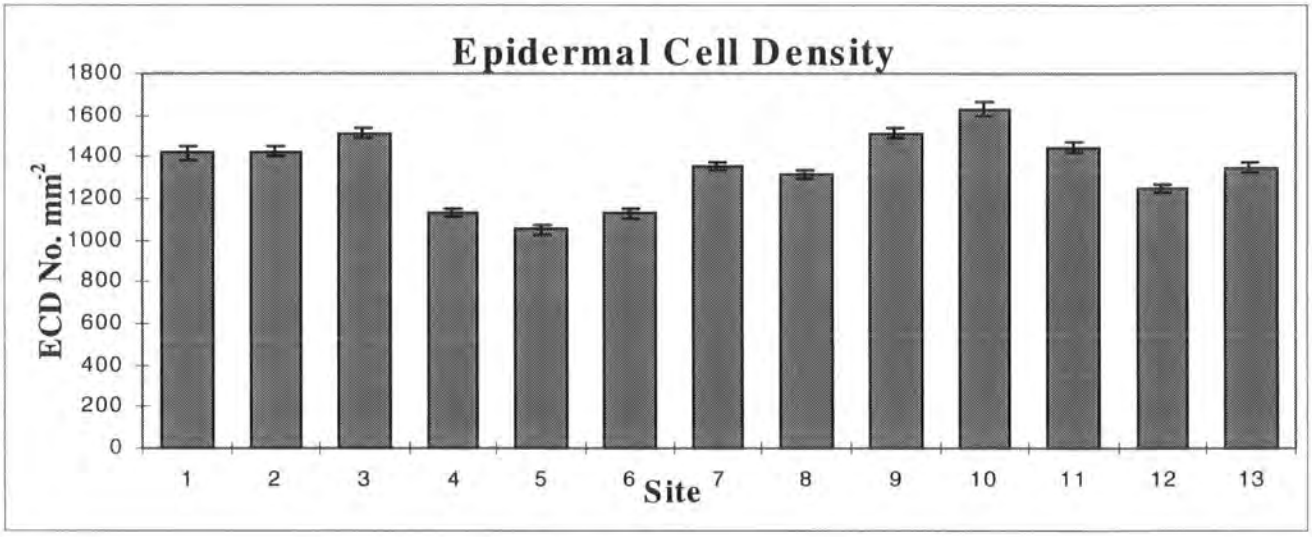


Figure 3.7

The effect of increasing altitude on Stomatal Density, Epidermal Cell Density and Stomatal Index on the adaxial surface of *Salix herbacea* in 1995.





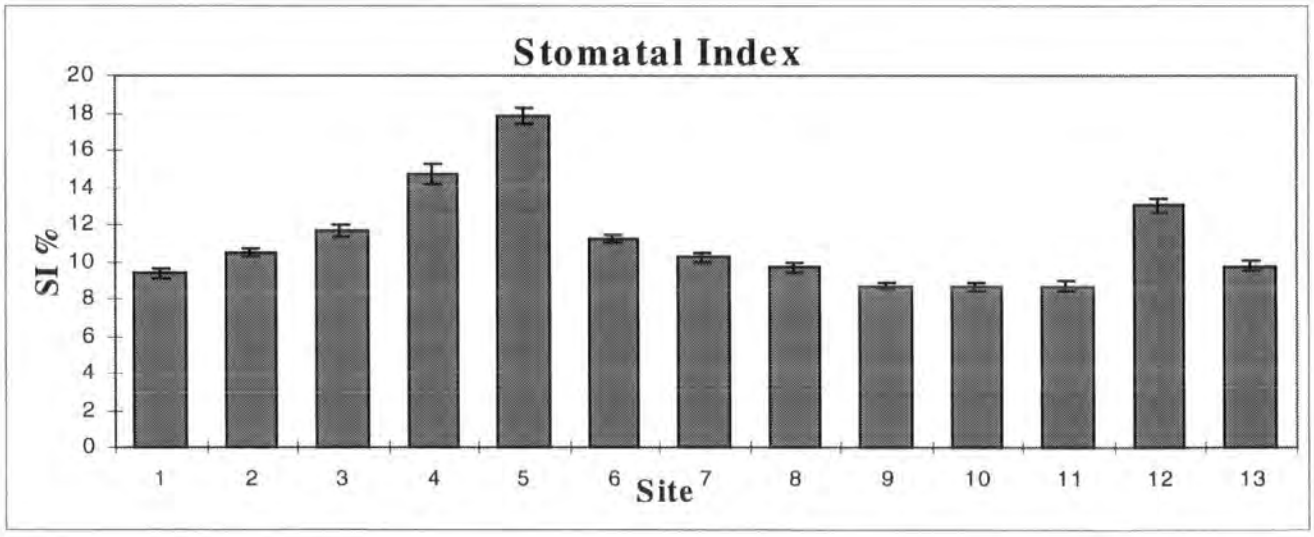


Figure 3.8

The effect of increasing altitude on Stomatal Length, Pore Length and Stomatal Width on the adaxial surface of *Salix herbacea* in 1995.

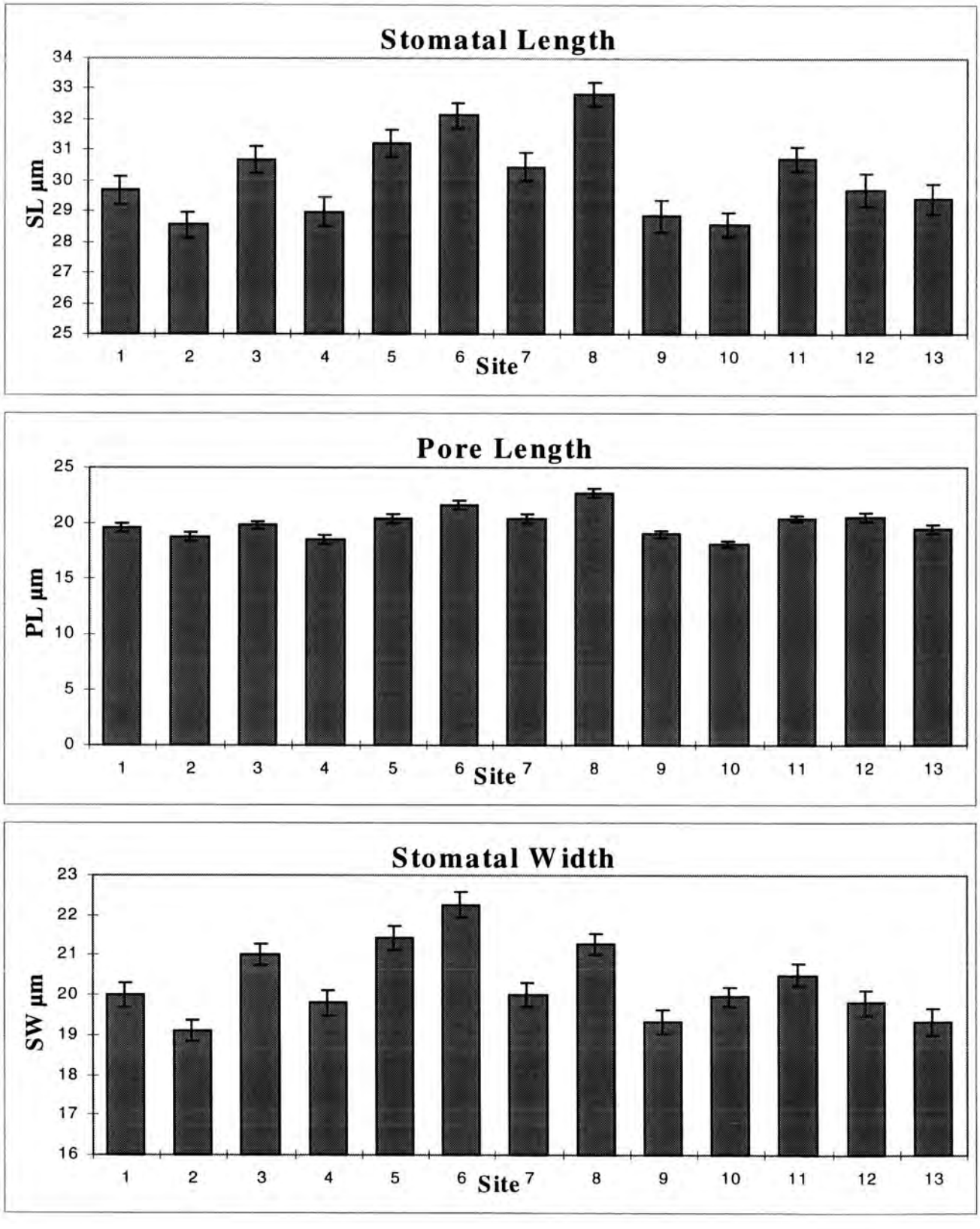
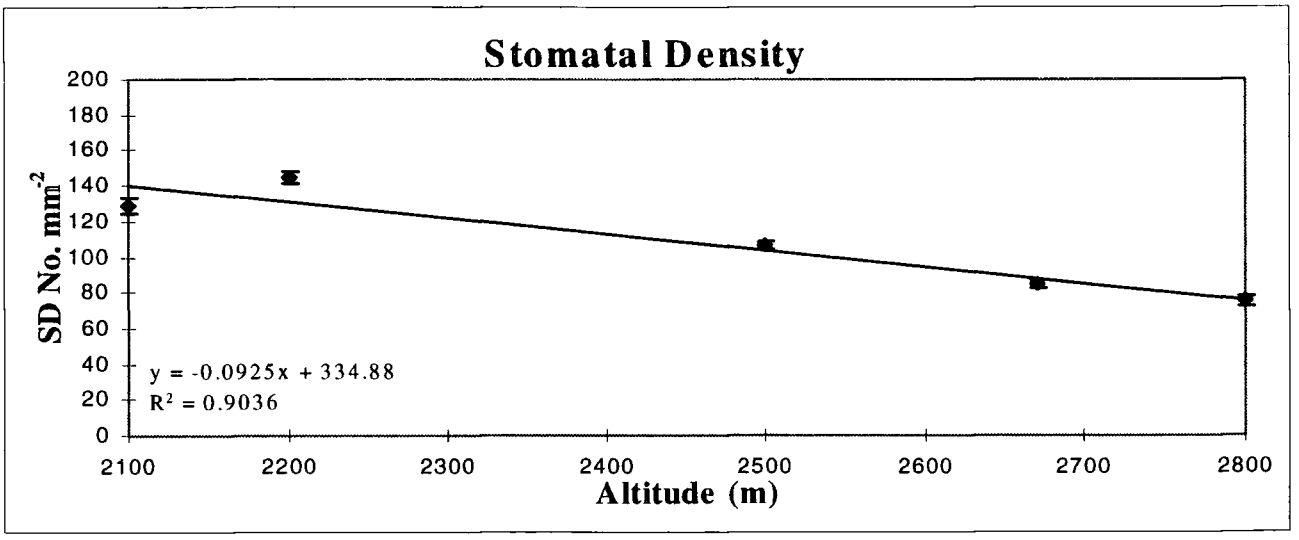
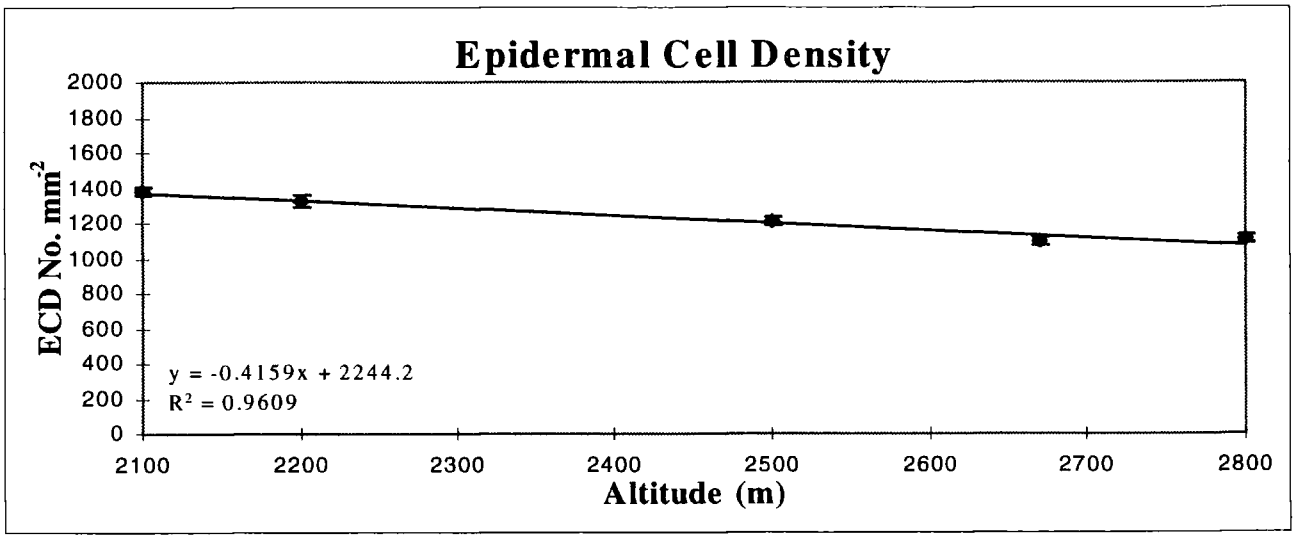


Figure 3.9

The effect of changing altitude on Stomatal Density, Epidermal Cell Density and Stomatal Index on the abaxial surface in 1994 (using pooled sampling sites).





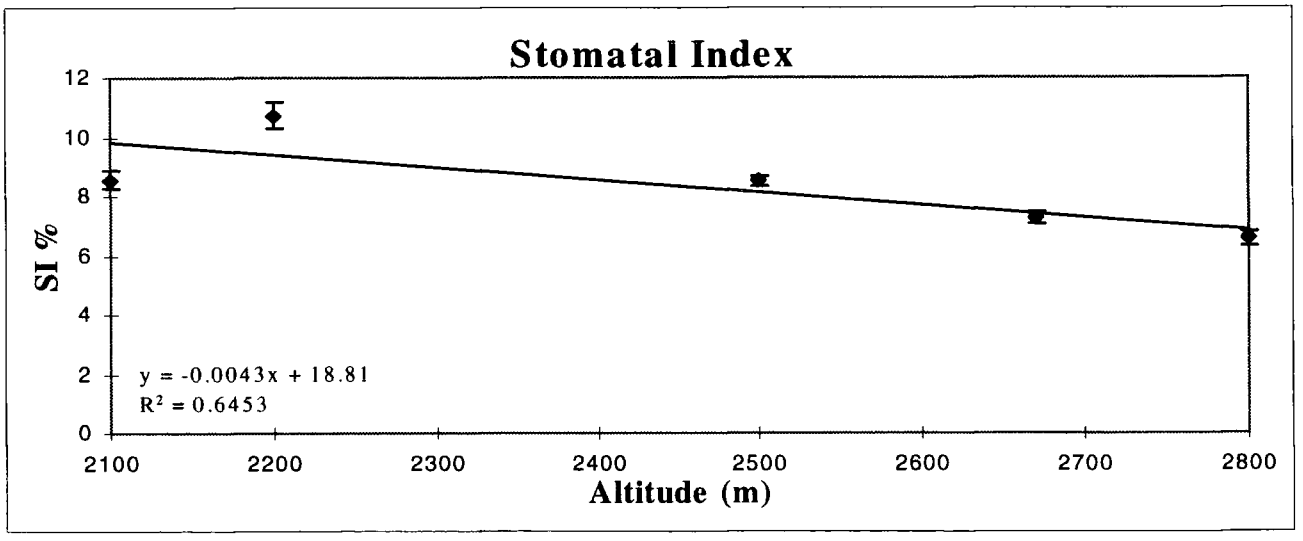
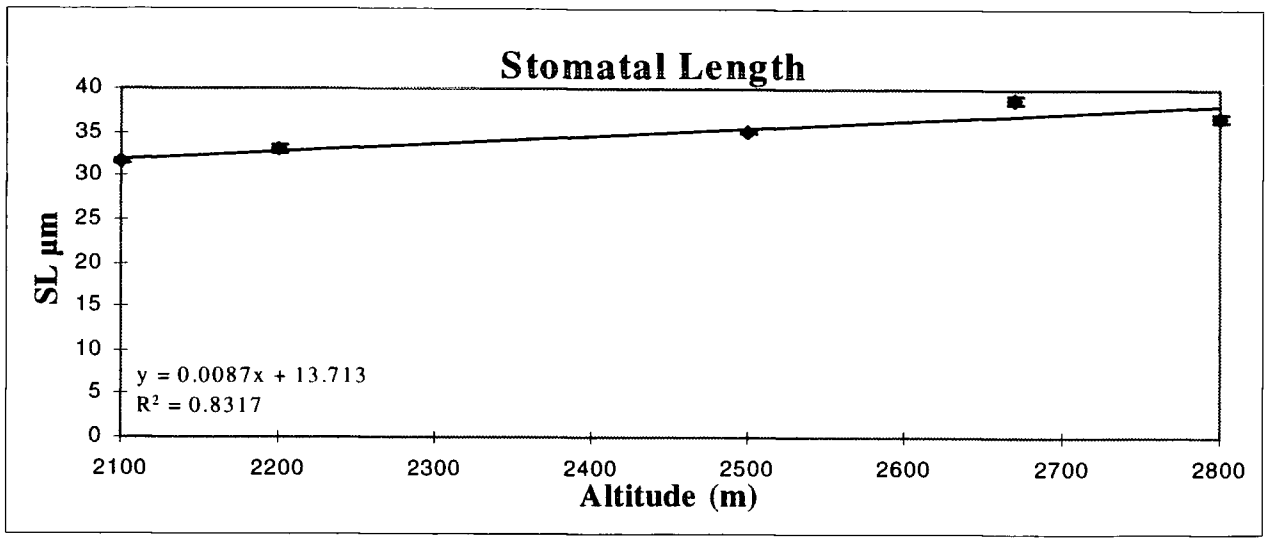
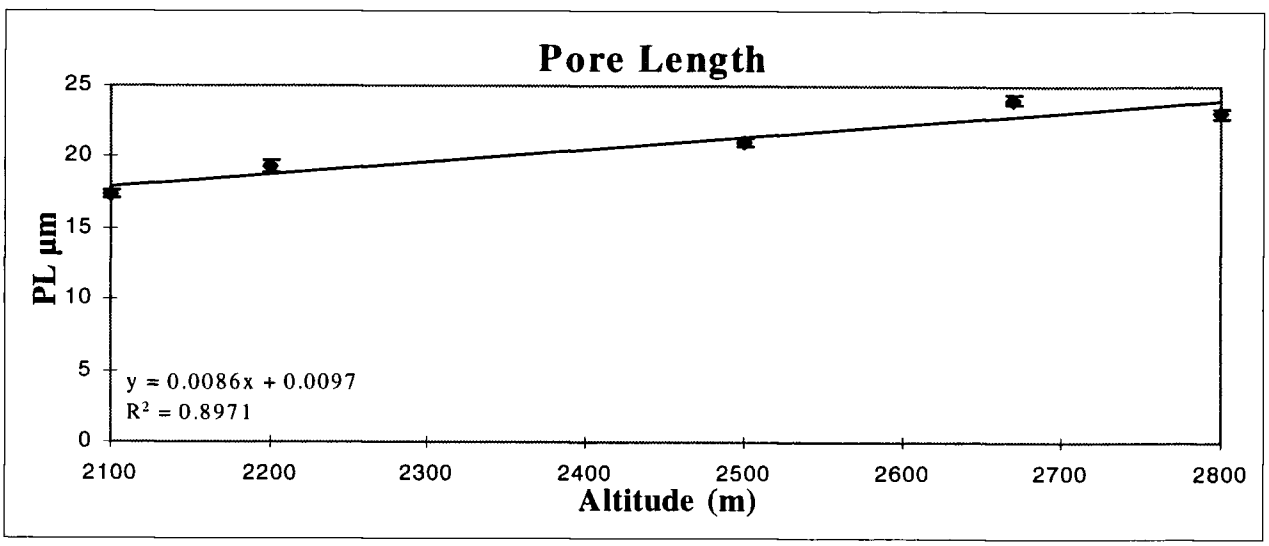


Figure 3.10

The effect of changing altitude on Stomatal Length, Pore Length and Stomatal Width on the abaxial surface in 1994 (using pooled sampling sites).





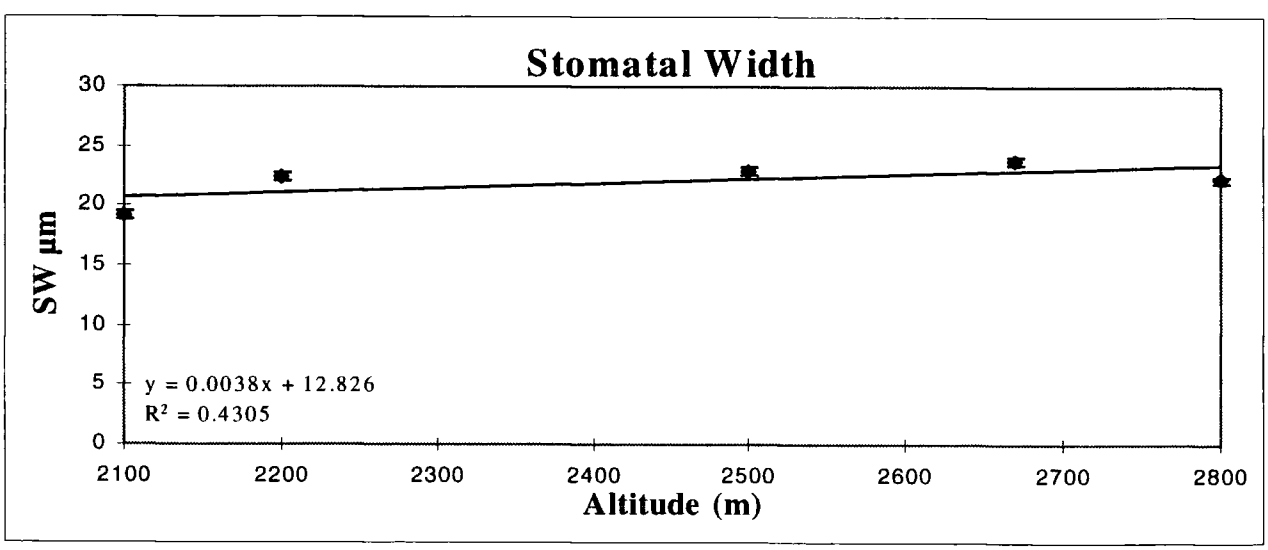
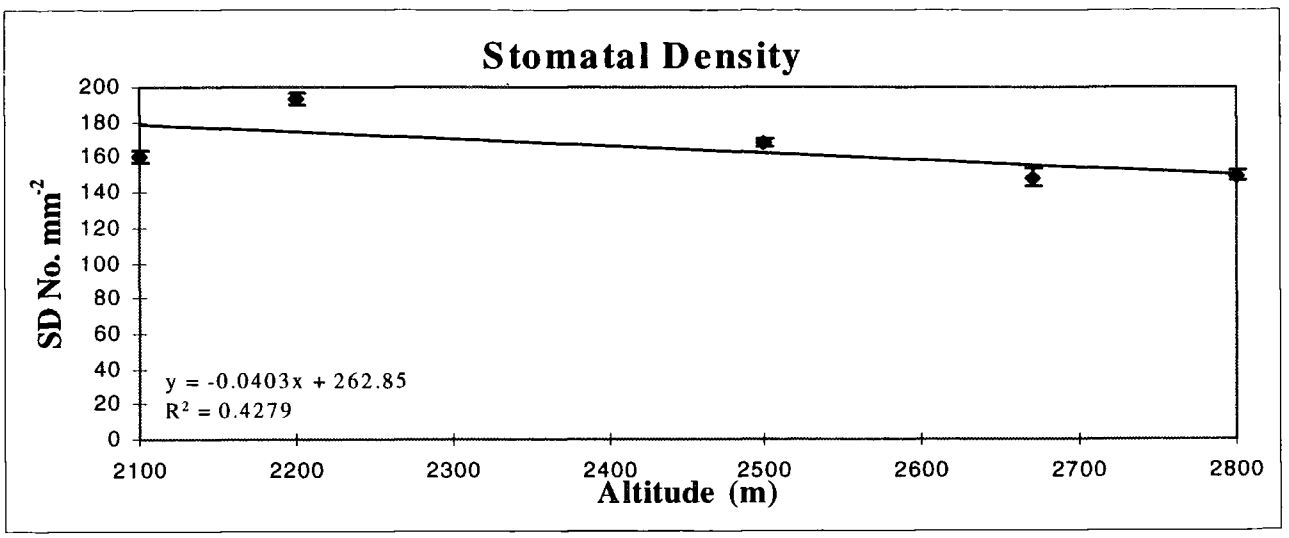
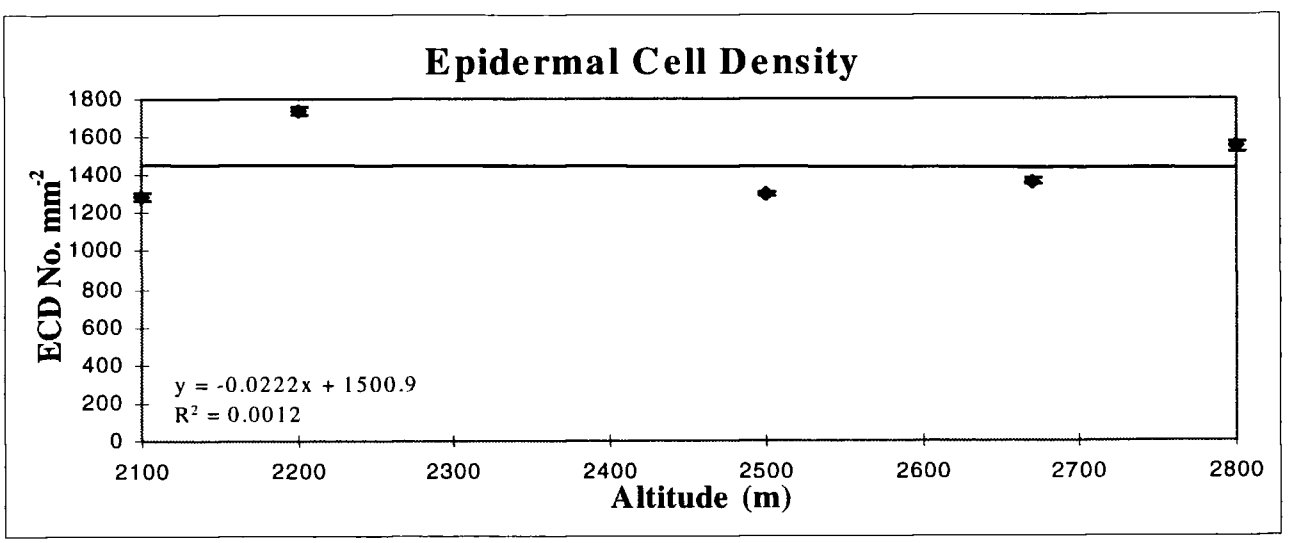


Figure 3.11

The effect of changing altitude on Stomatal Density, Epidermal Cell Density and Stomatal Index on the adaxial surface in 1994 (using pooled sampling sites).





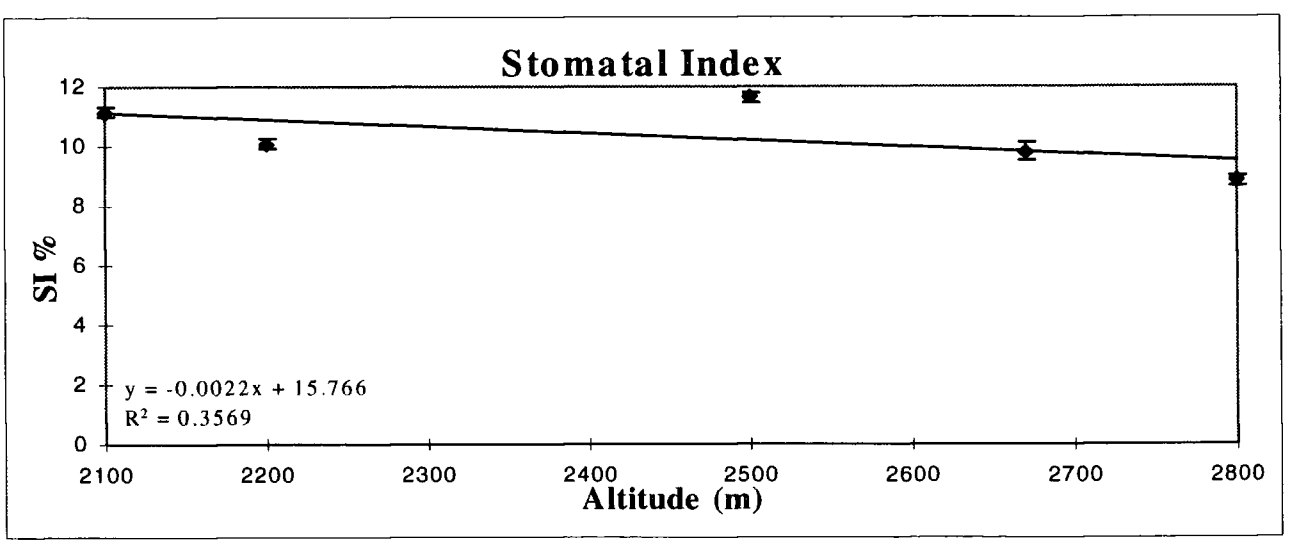
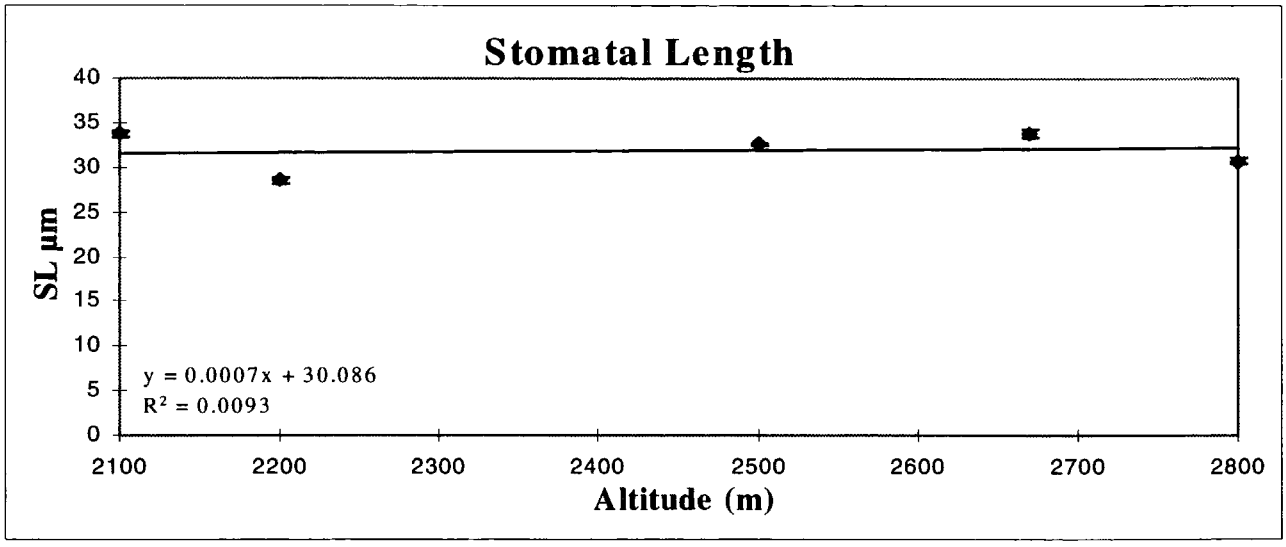
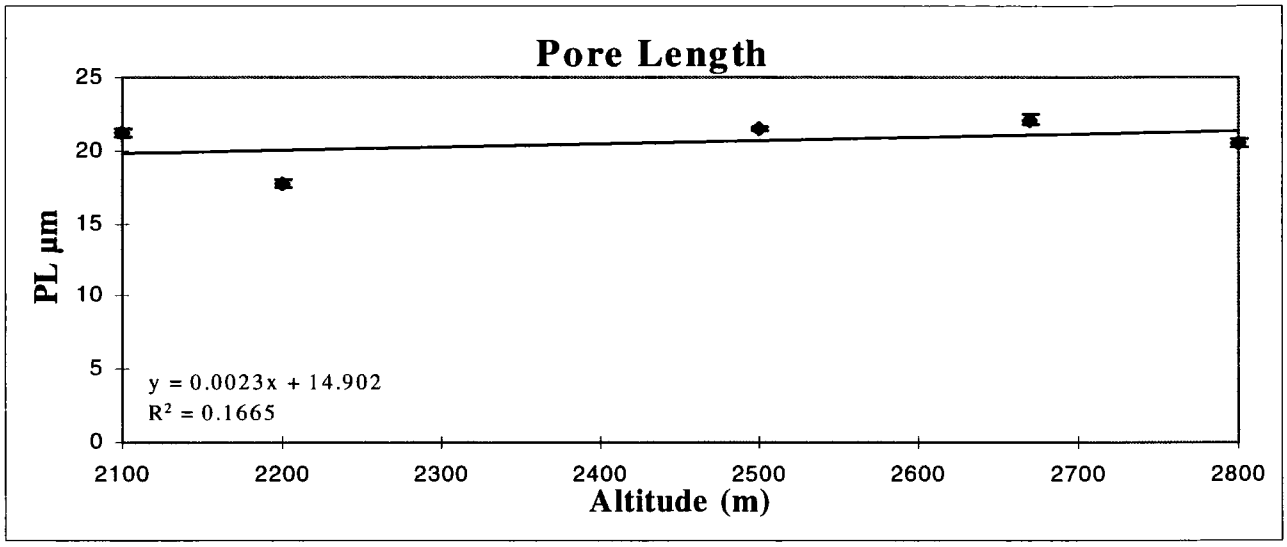


Figure 3.12

The effect of changing altitude on Stomatal Length, Pore Length and Stomatal Width on the adaxial surface in 1994 (using pooled sampling sites).





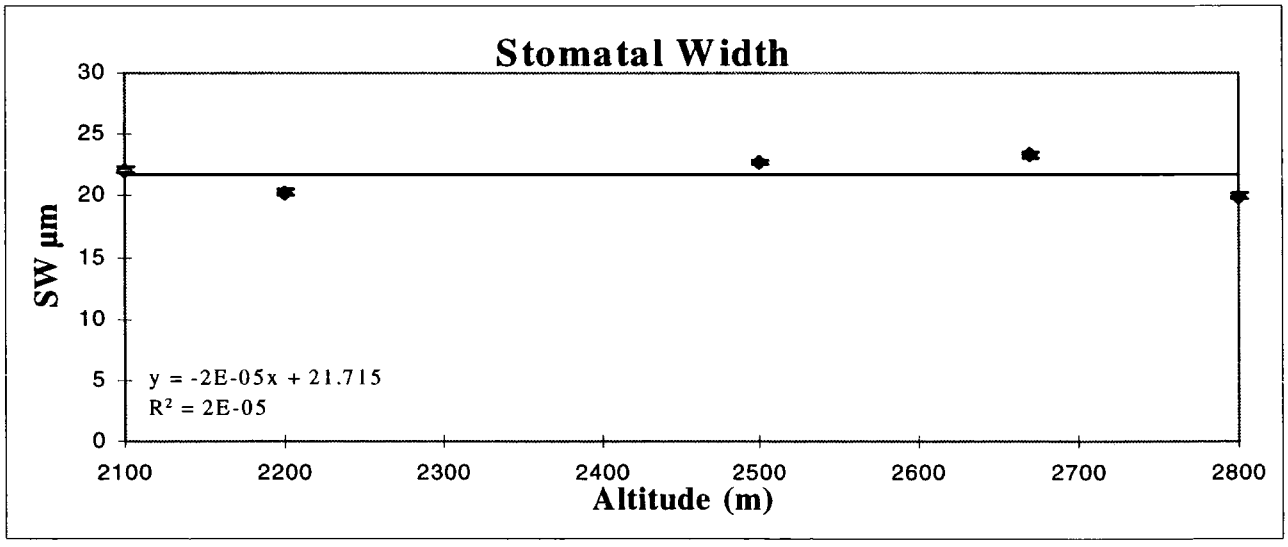
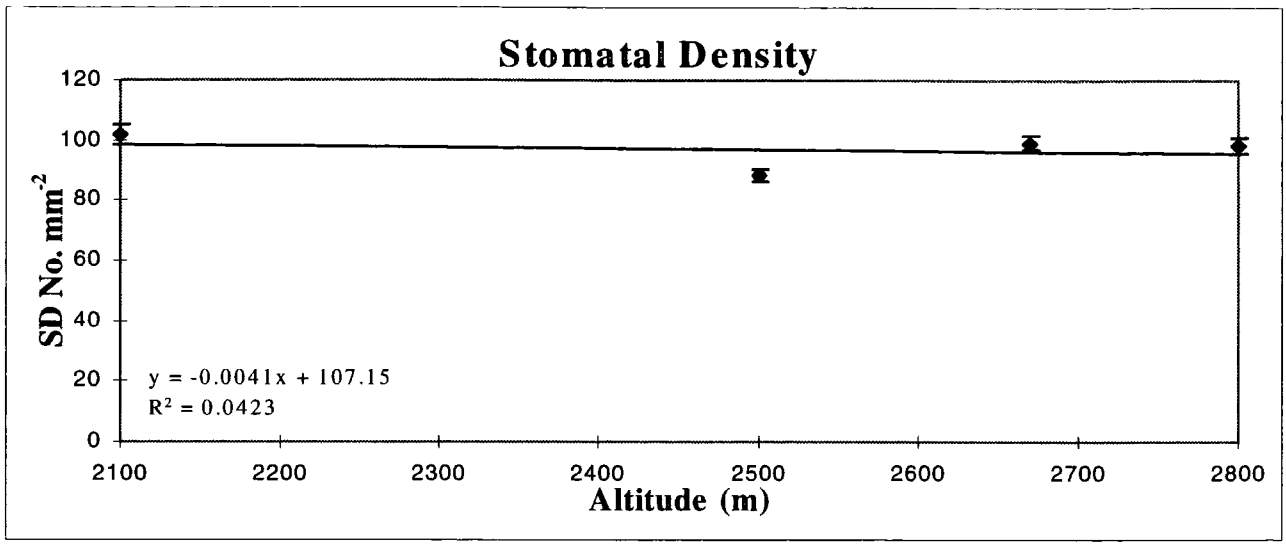
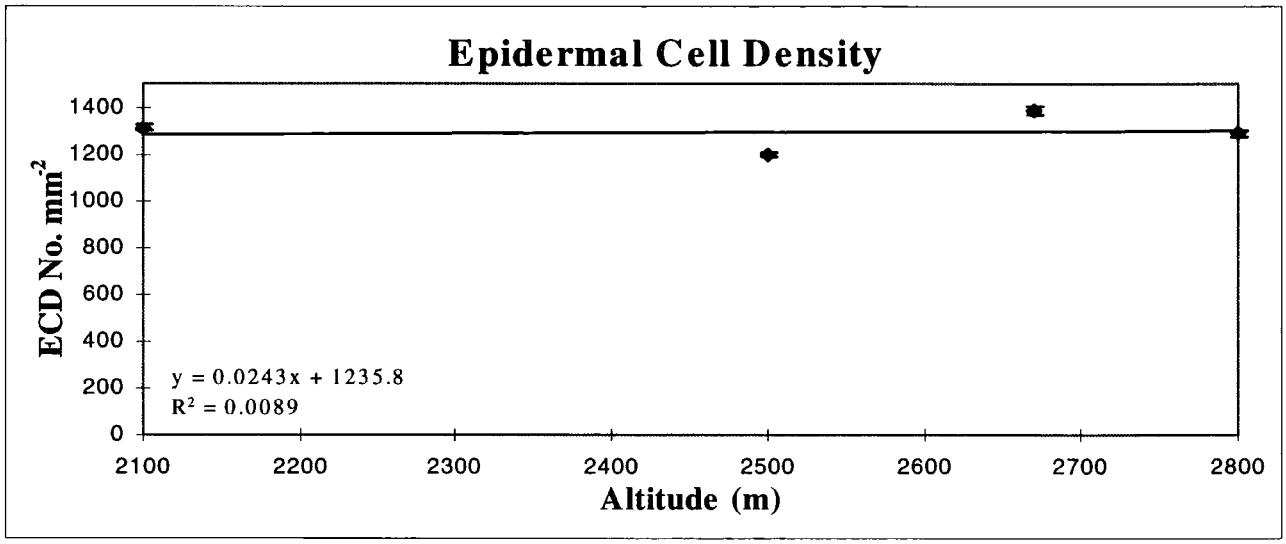


Figure 3.13

The effect of changing altitude on Stomatal Density, Epidermal Cell Density and Stomatal Index on the abaxial surface in 1995 (using pooled sampling sites).





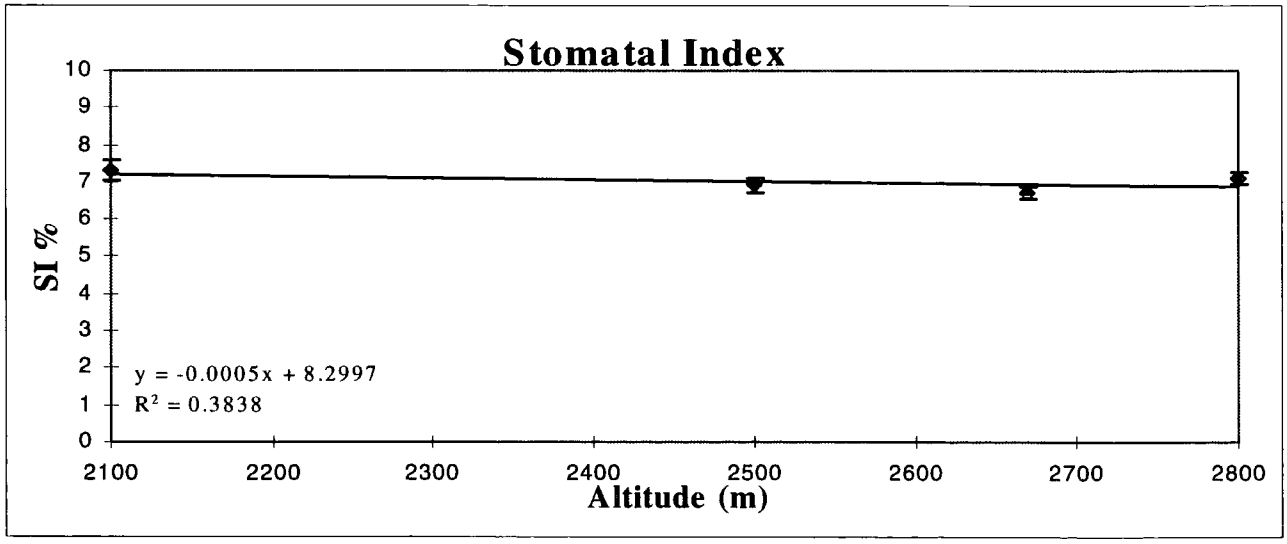
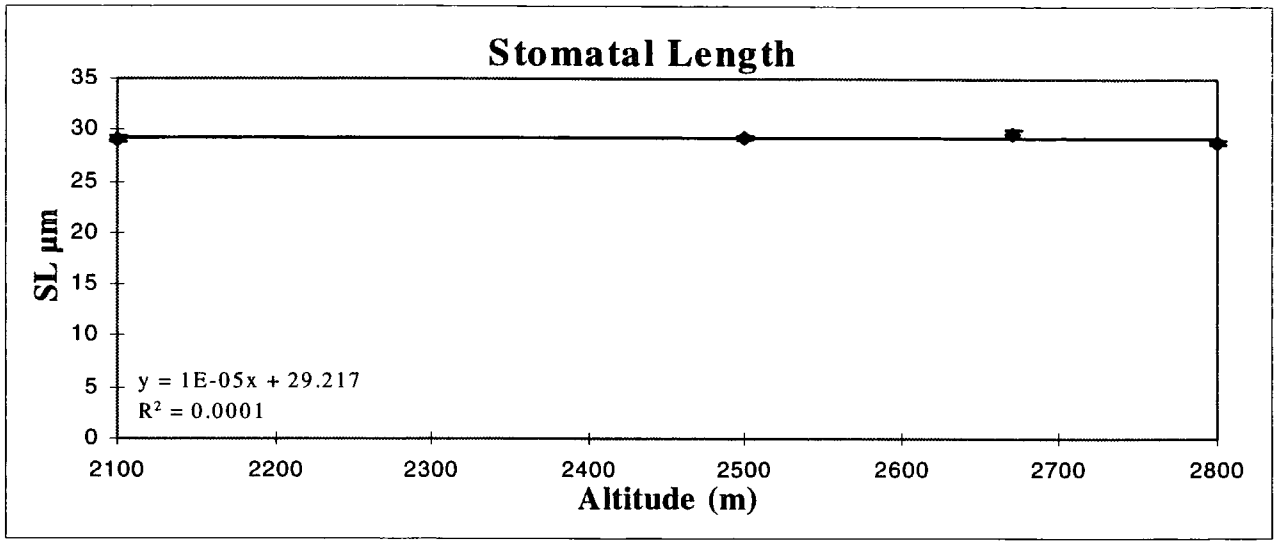
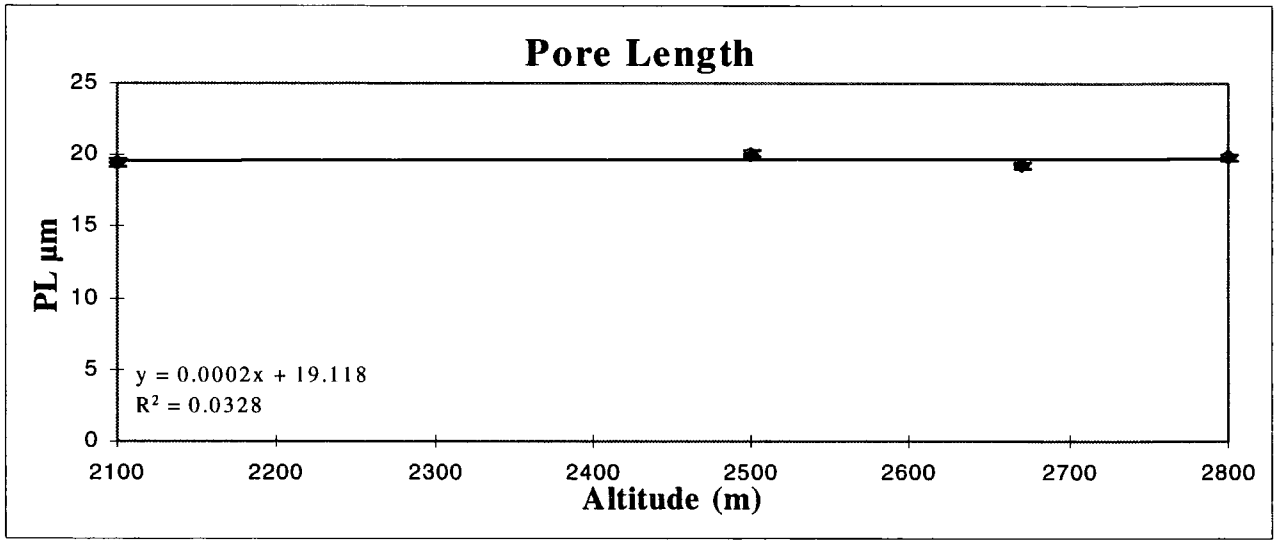


Figure 3.14

The effect of changing altitude on Stomatal Length, Pore Length and Stomatal Width on the abaxial surface in 1995 (using pooled sampling sites).





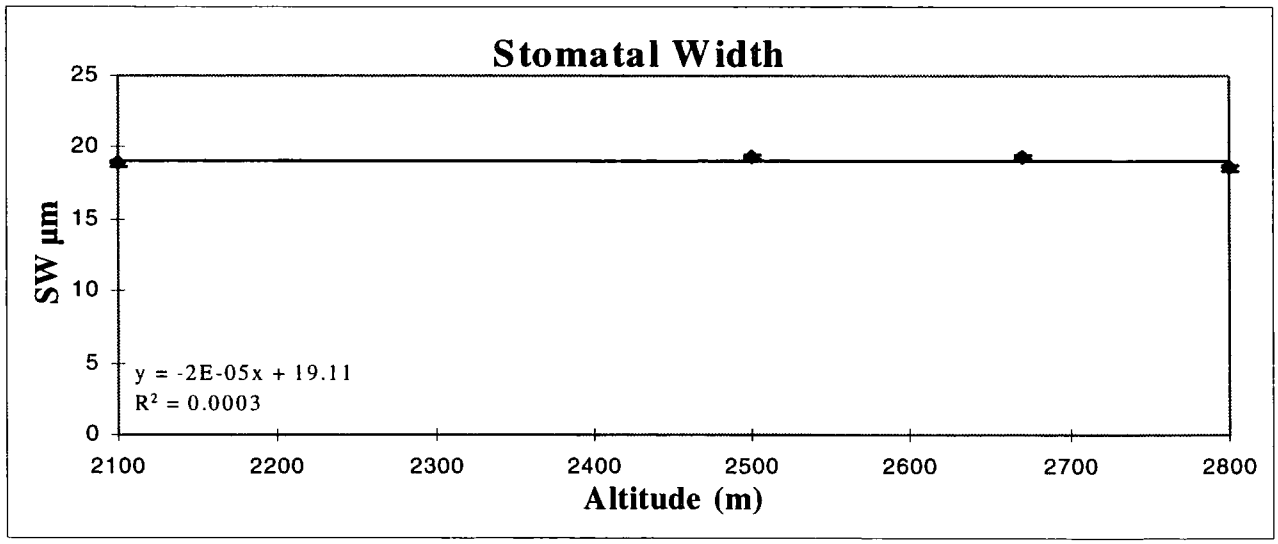
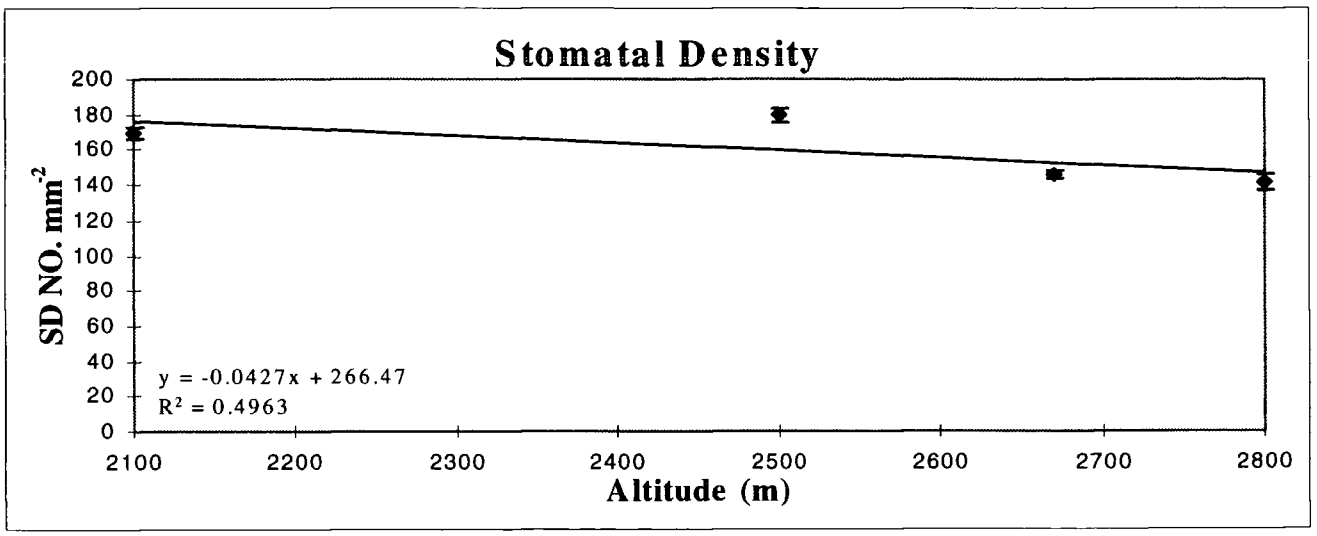
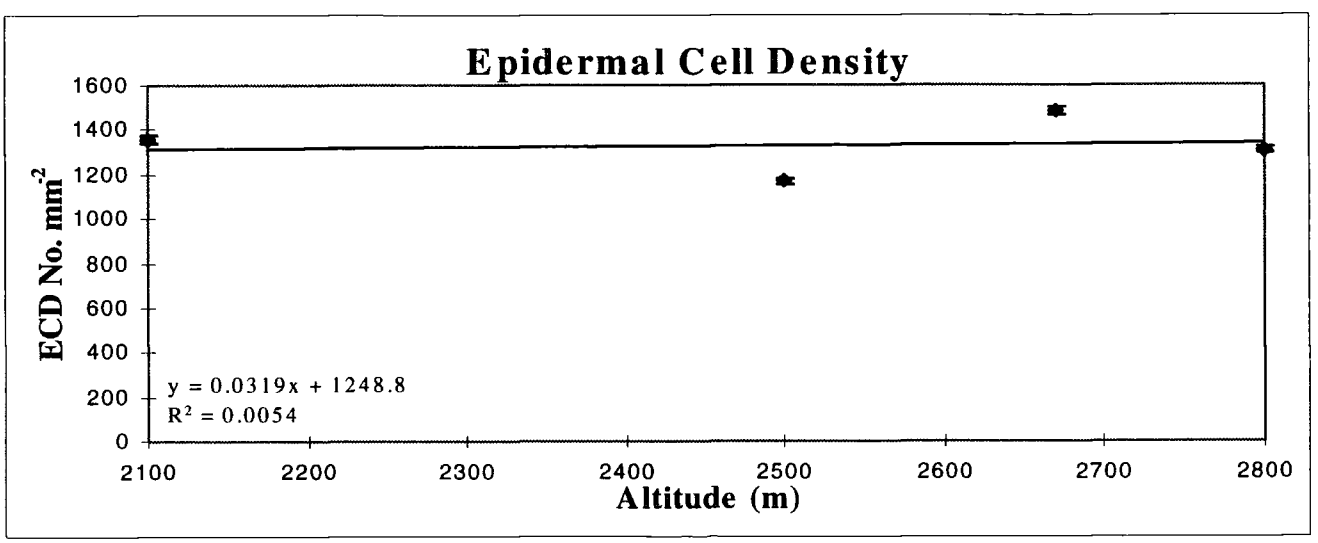


Figure 3.15

The effect of changing altitude on Stomatal Density, Epidermal Cell Density and Stomatal Index on the adaxial surface in 1995 (using pooled sampling sites).





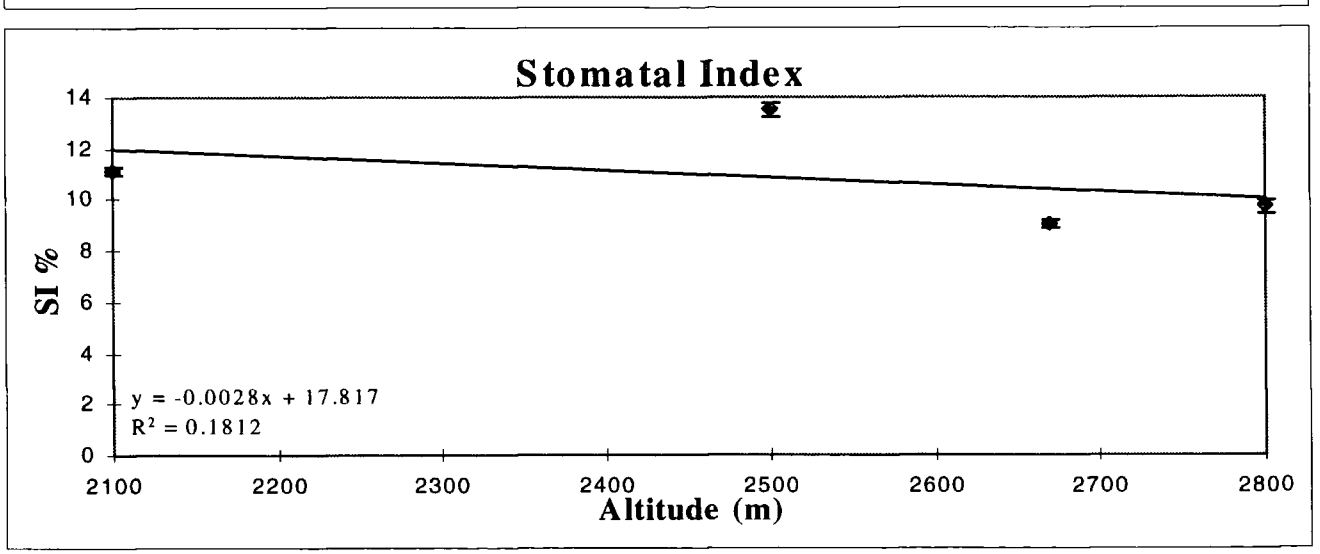


Figure 3.16

The effect of changing altitude on Stomatal Length, Pore Length and Stomatal Width on the adaxial surface in 1995 (using pooled sampling sites).

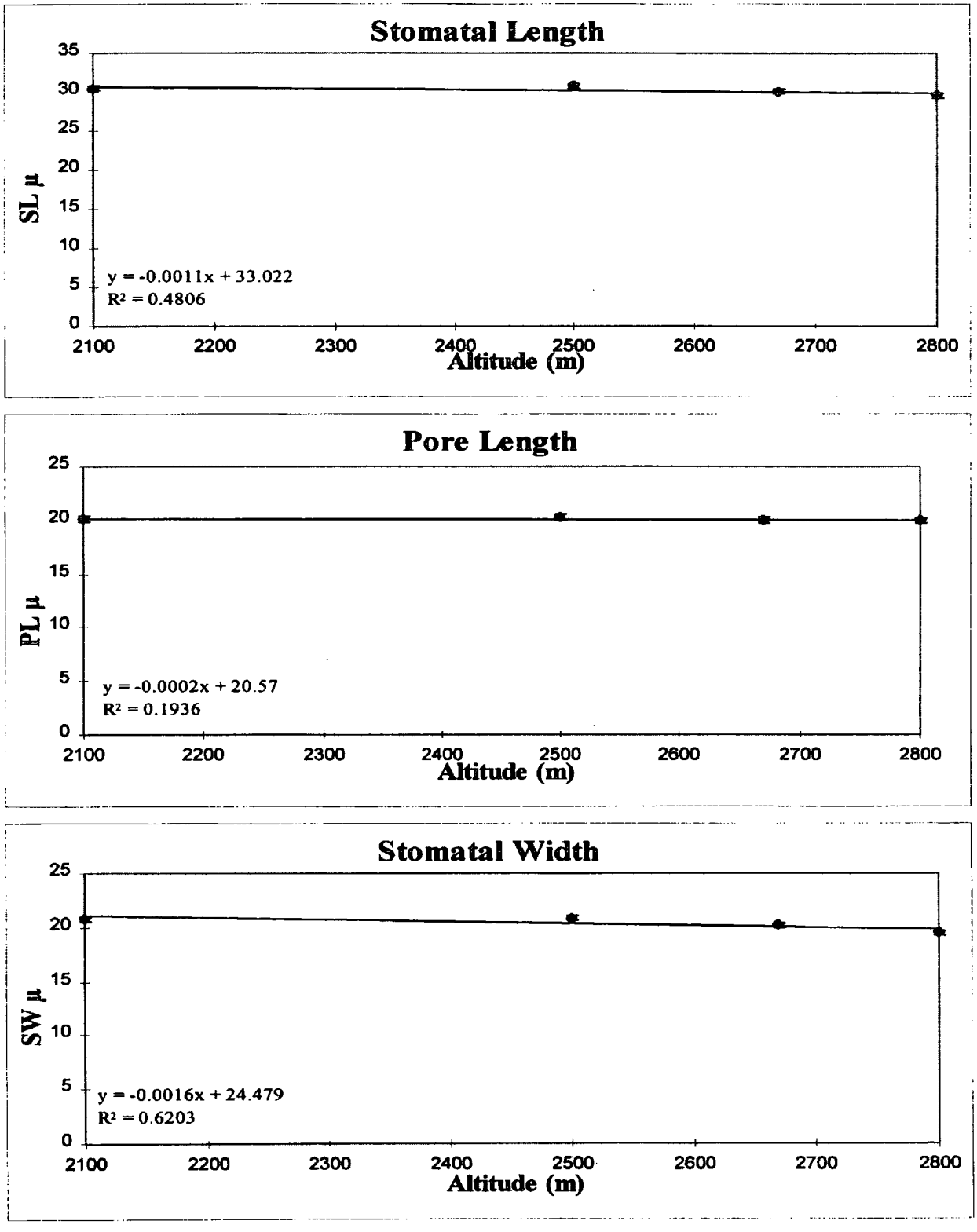
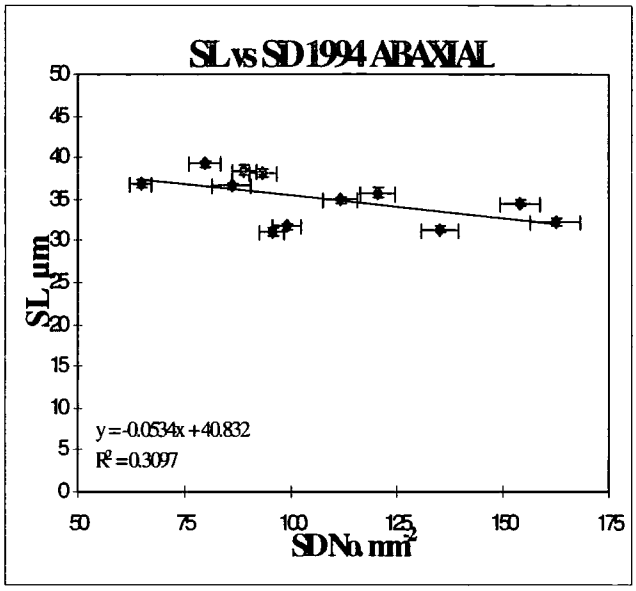
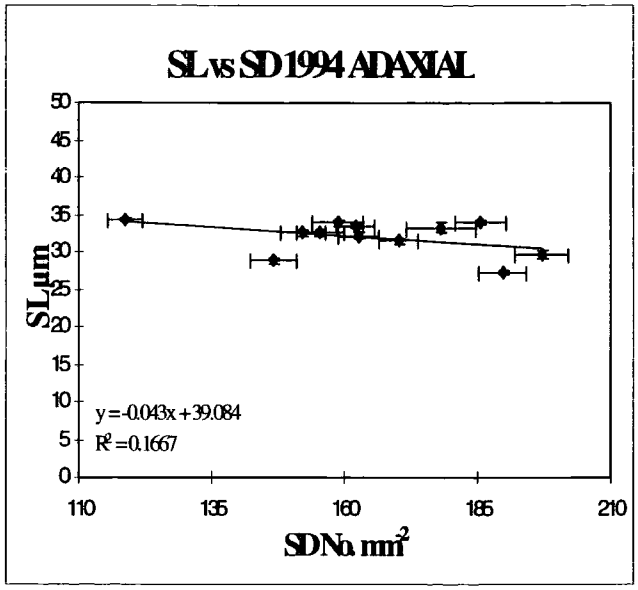
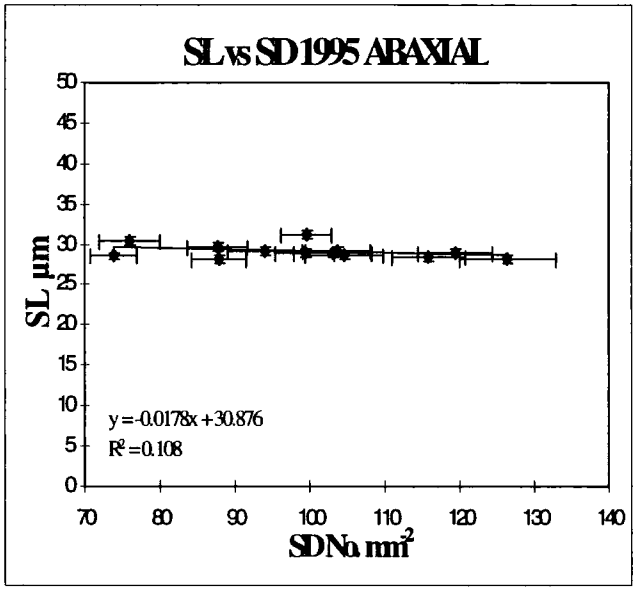


Figure 3.17. Relationship between stomatal length (SL) and stomatal density (SD) in *Salix herbacea*.







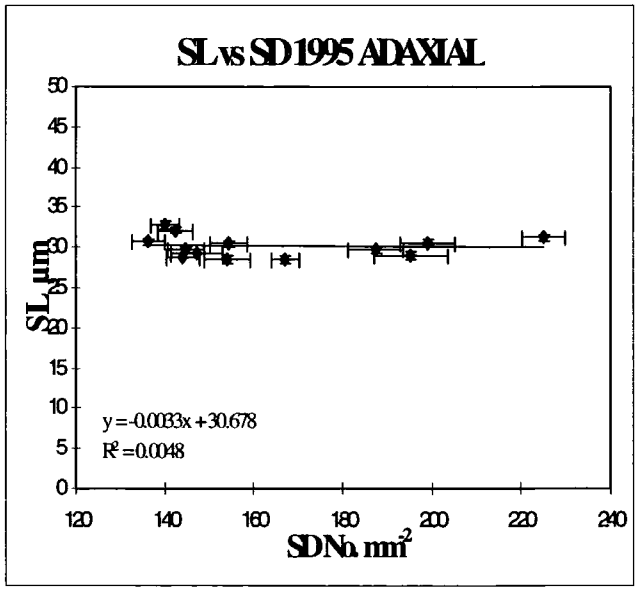


Figure 3.18.
Stomatal density mapped across the abaxial surface of leaf one collected from Timmelsjoch 2500m in 1995.

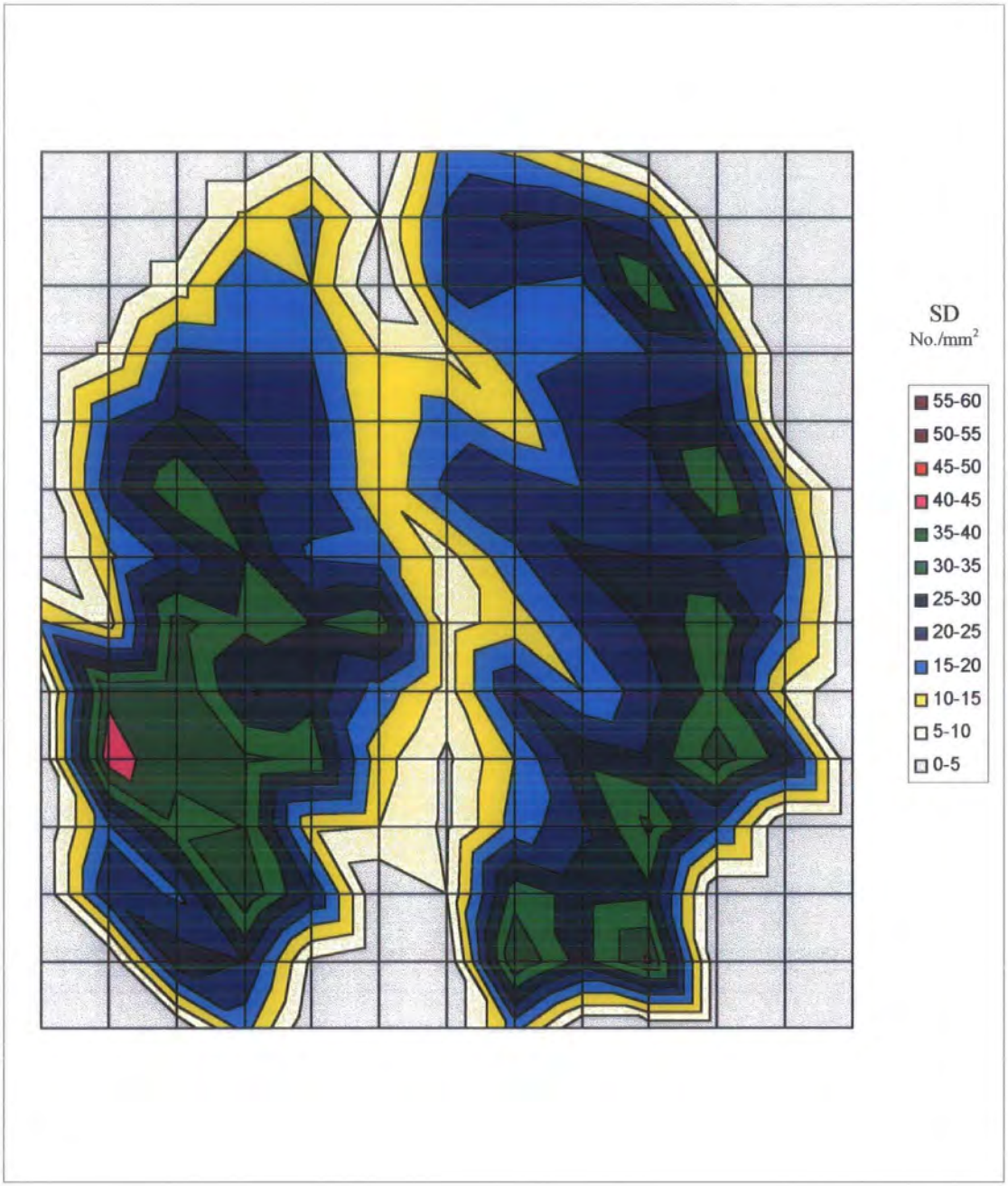


Figure 3.19.
Stomatal density mapped across adaxial surface of leaf one collected from Timmelsjoch 2500m in 1995.

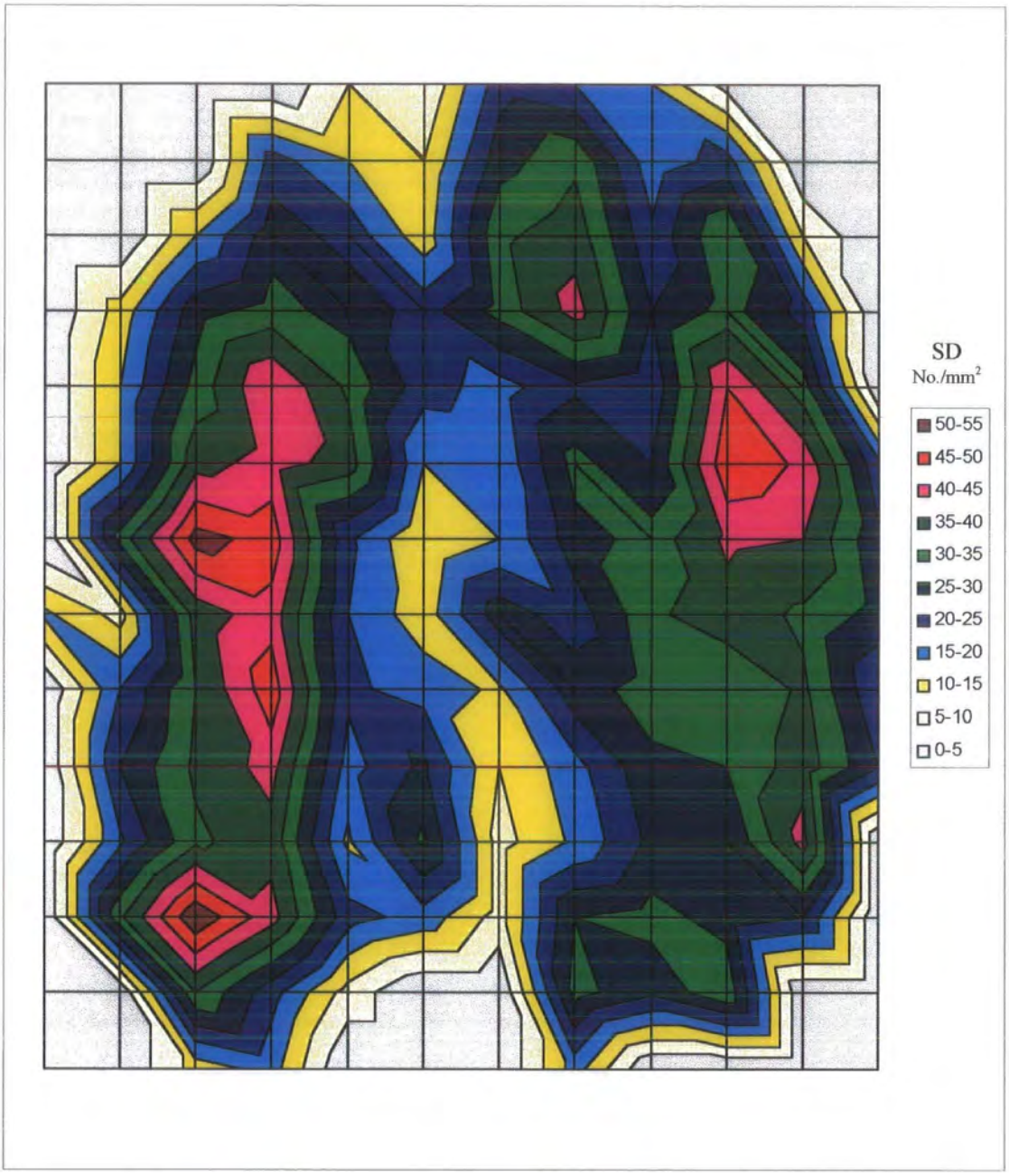


Figure 3.20.
Stomatal density mapped across the abaxial surface of leaf two collected from Timmelsjoch 2500m in 1995.

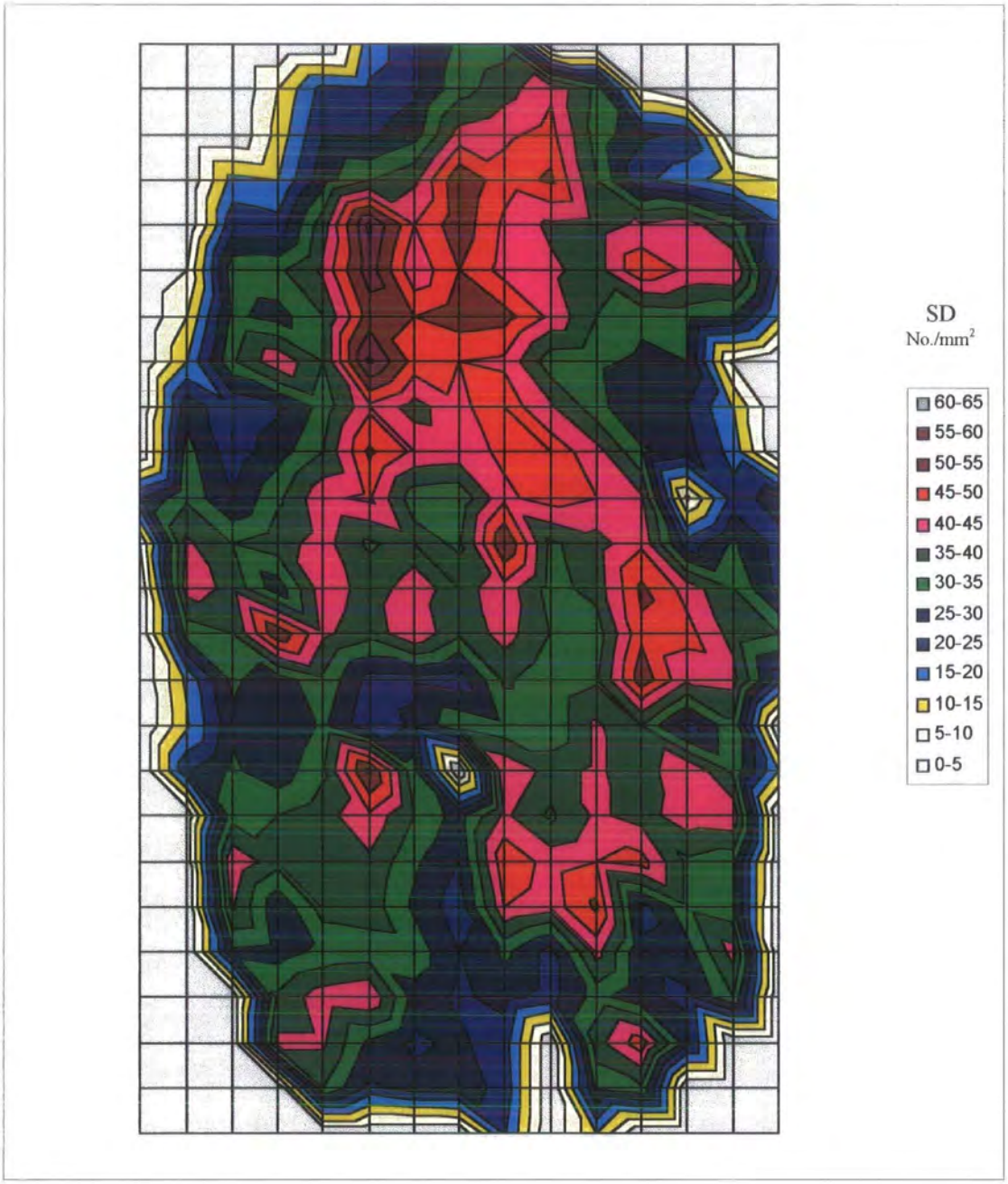


Figure 3.21.
Stomatal density mapped across the adaxial surface of leaf two collected from Timmelsjoch 2500m in 1995.

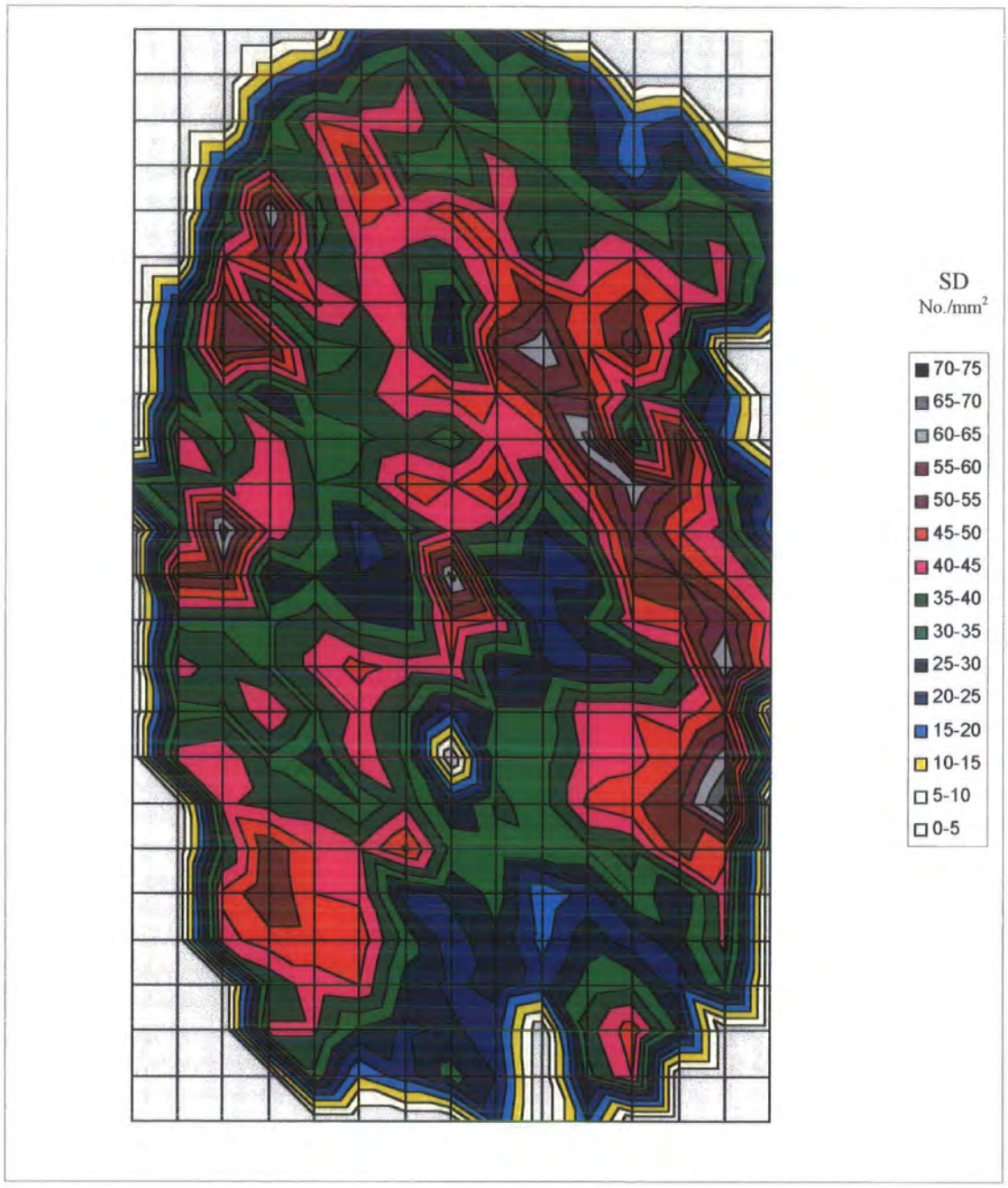


Figure 3.22
Stomatal density mapped across the abaxial surface of leaf one collected from Hohe Mut 2670m in 1995.

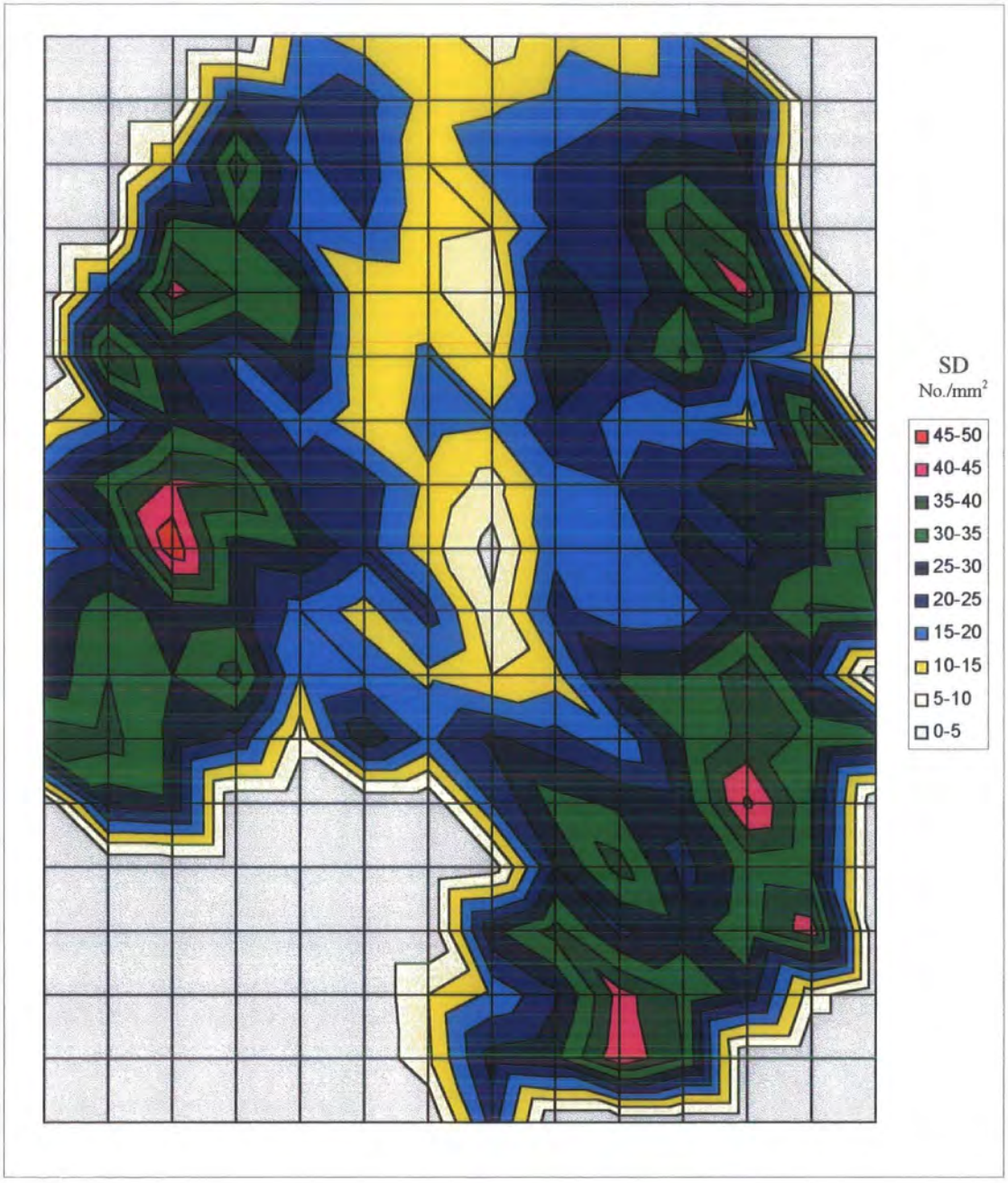


Figure 3.23.
Stomatal density mapped across the adaxial surface of leaf one collected from Hohe Mut 2670m in 1995

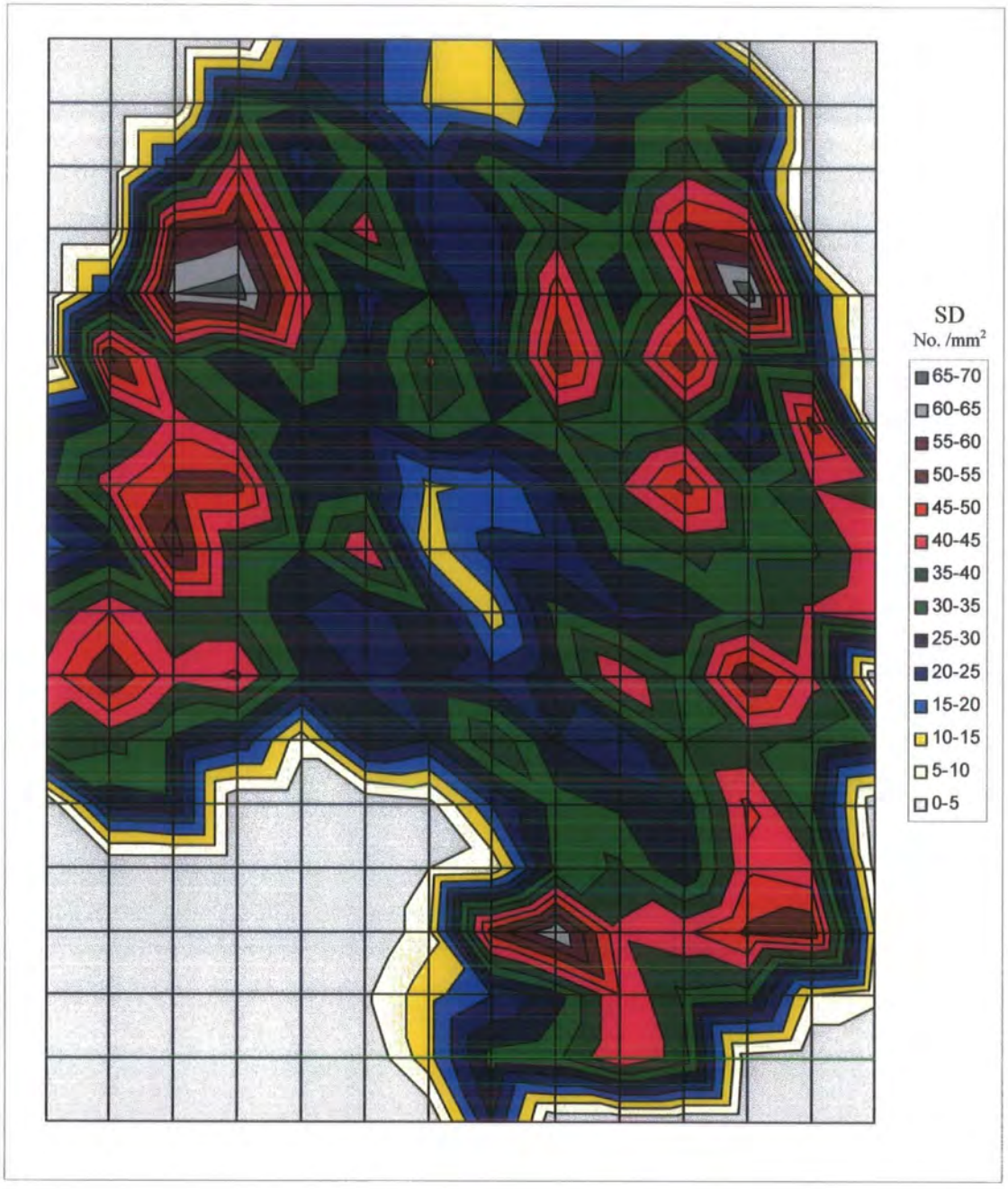


Figure 3.24.
Stomatal density mapped across the abaxial surface of leaf two collected from Hohe Mut 2670 in 1995

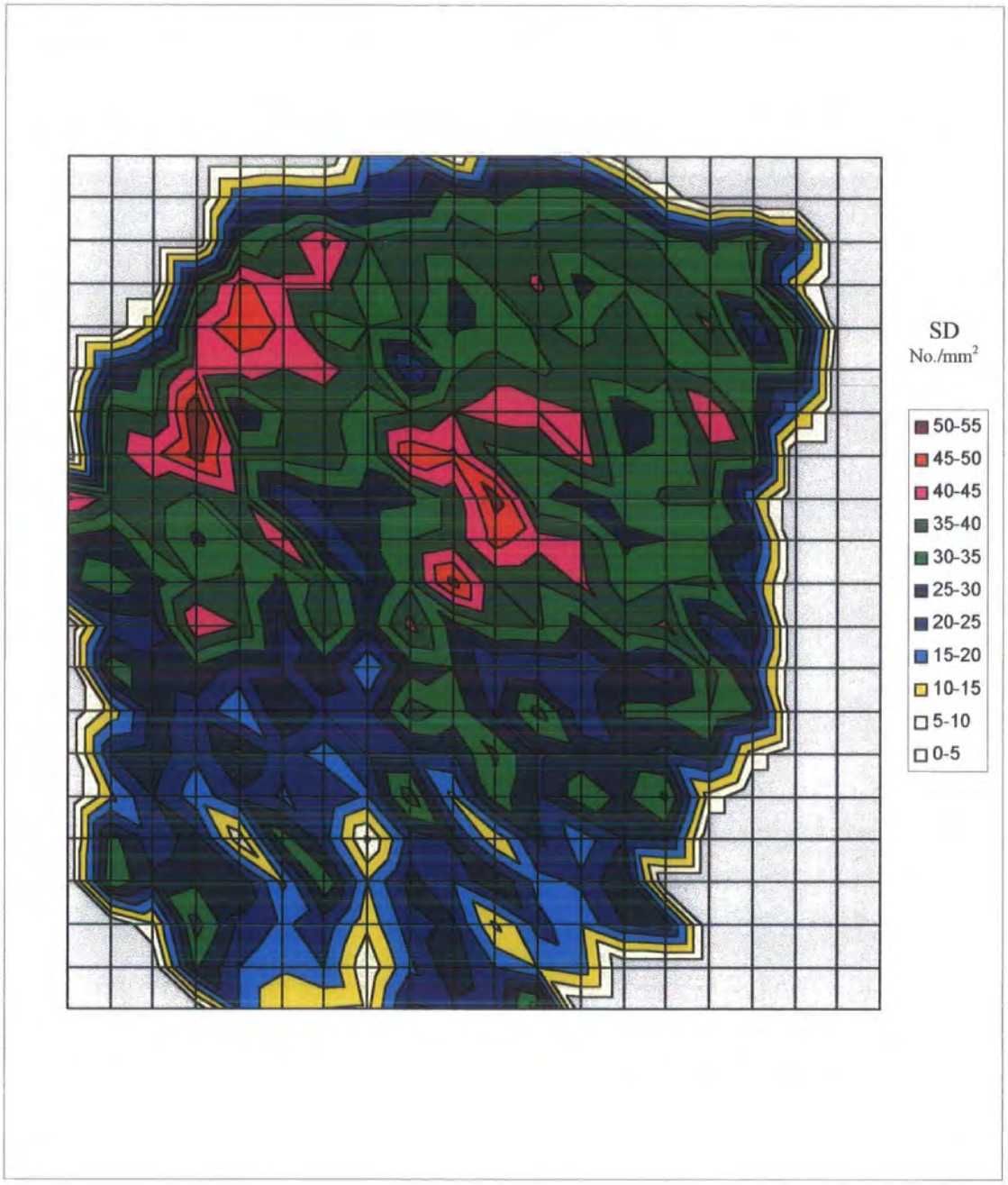


Figure 3.25.
Stomatal density mapped across the adaxial surface of leaf two collected from Hohe Mut 2670m in 1995.

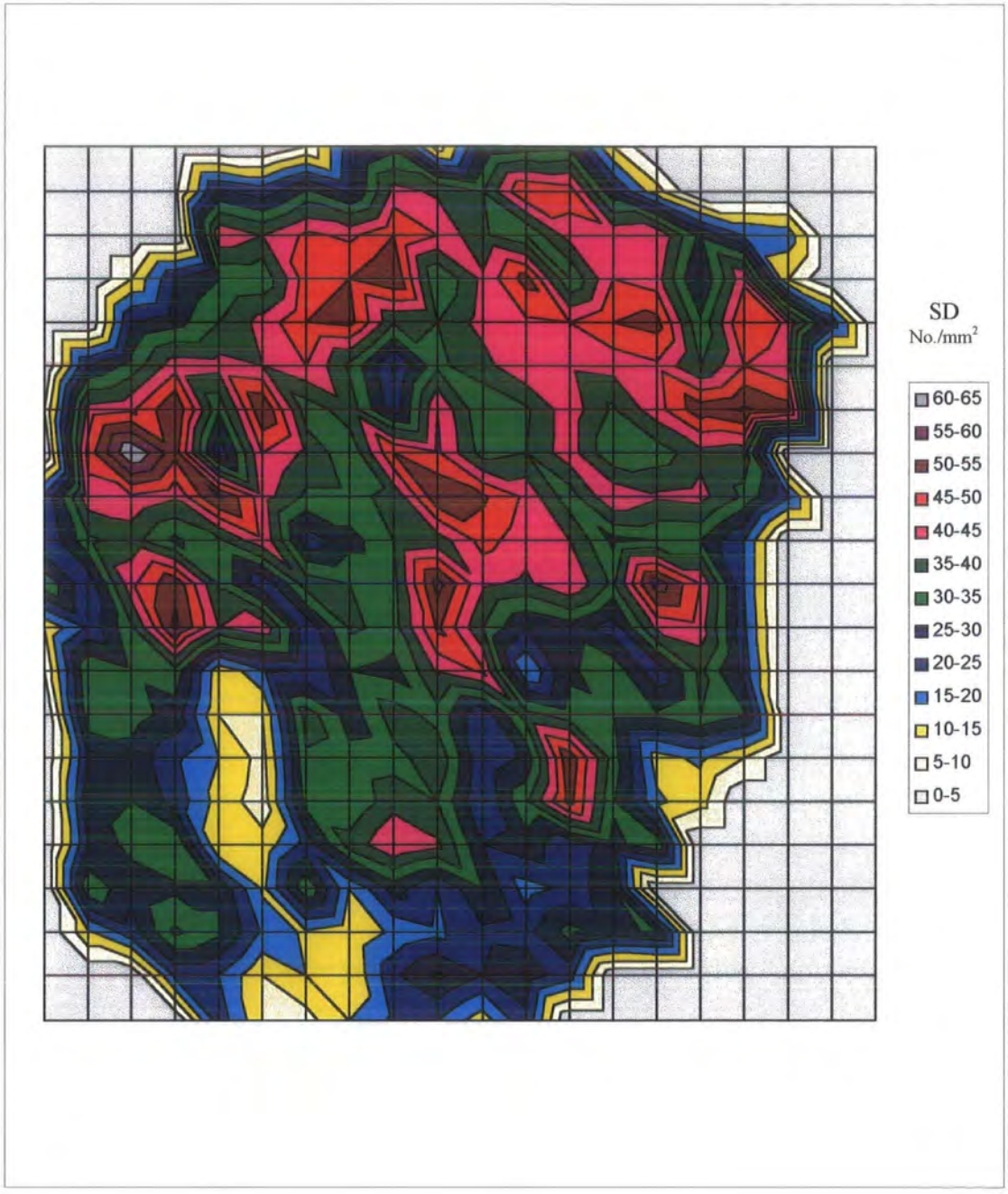
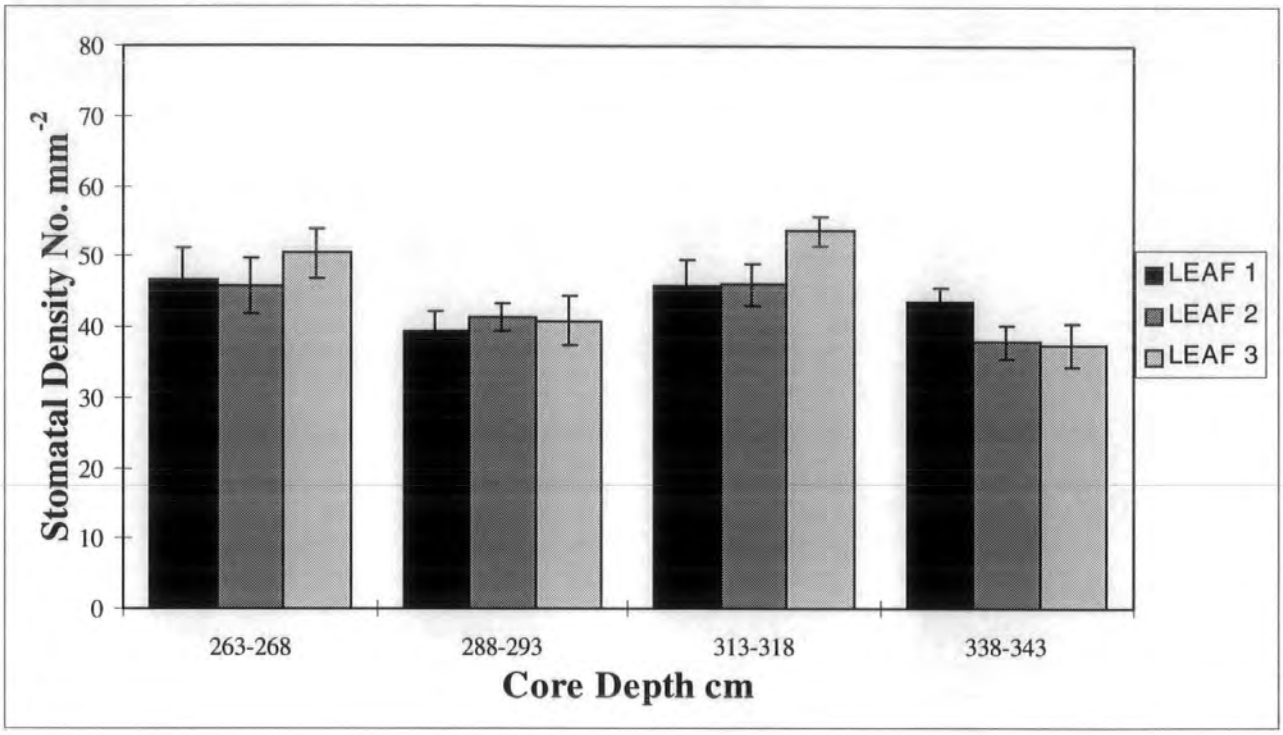


Figure 3.26. Variations in stomatal density of fossil leaves of *S. herbacea* taken from the Morrone Birkwood Core, illustrating individual leaf counts.

Abaxial surface



Adaxial surface

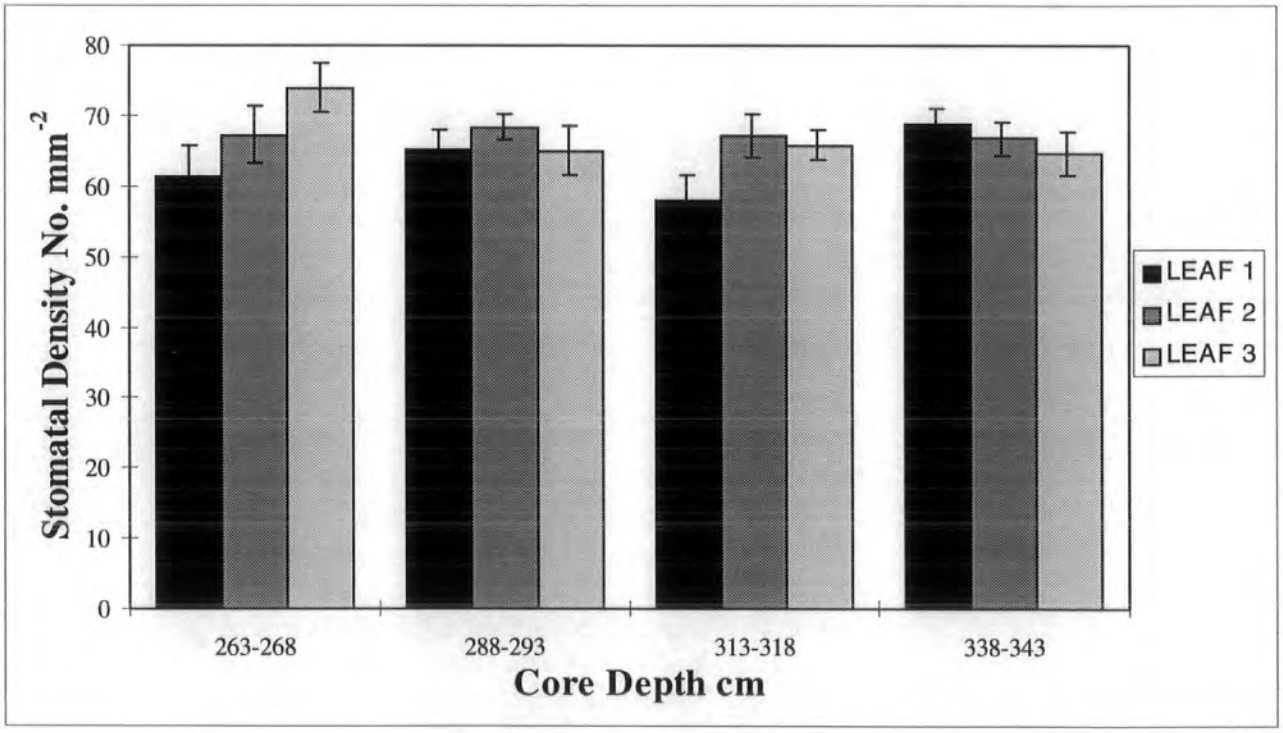
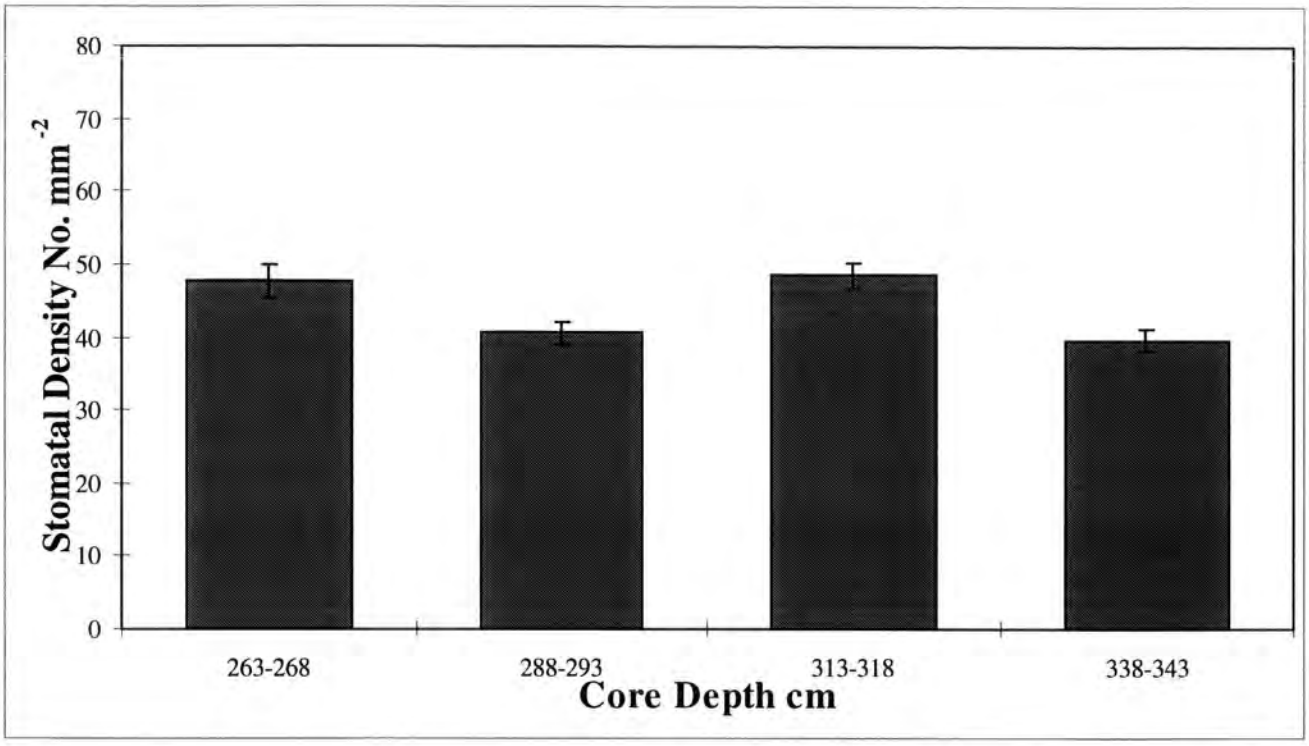


Figure 3.27. Variations in stomatal density of fossil leaves of *S. herbacea* taken from the Morrone Birkwood Core, illustrating pooled leaf counts.

Abaxial surface



Adaxial surface

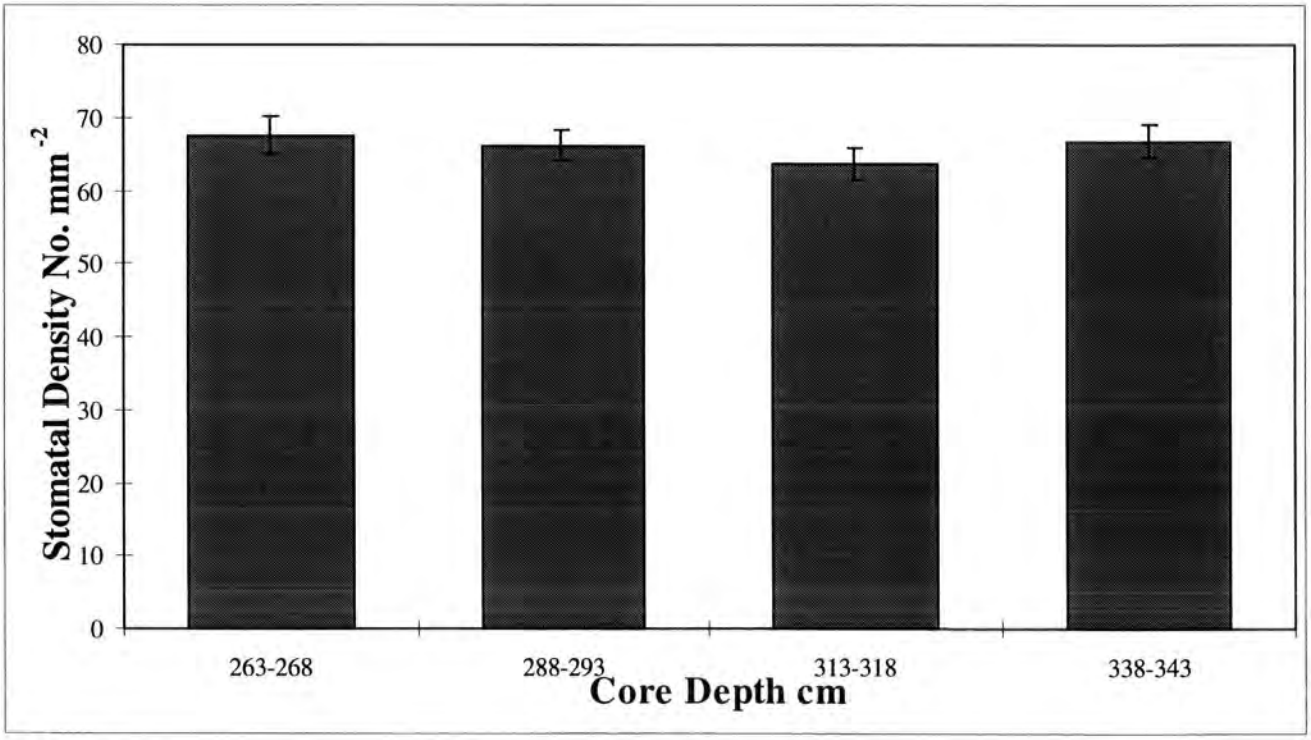
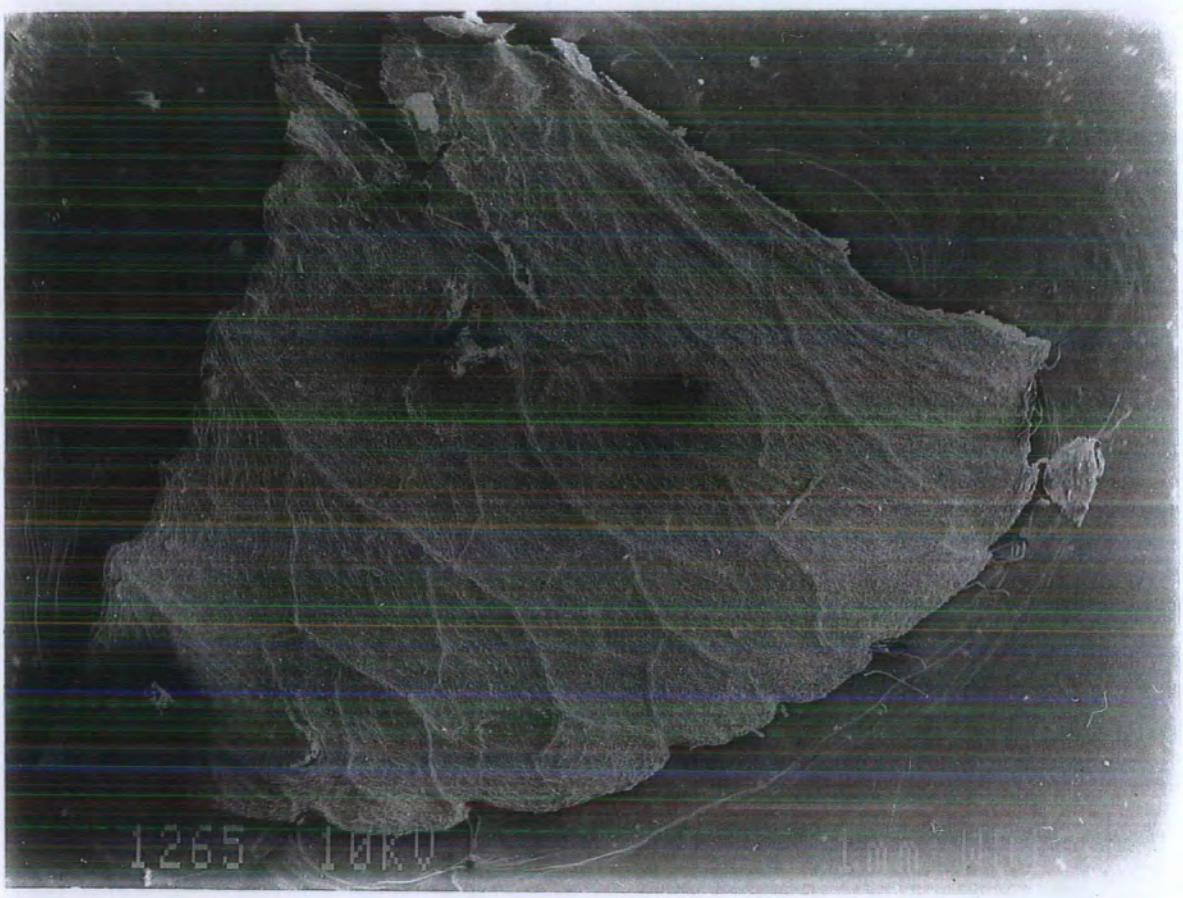


Plate 3.1 SEM photograph of fossil *S. herbacea* leaf.

Plate 3.2 SEM photograph of modern *S. herbacea* leaf.



Bar = 1 mm

Plate 3.3 SEM photograph of fossil *S. herbacea* leaf surface.

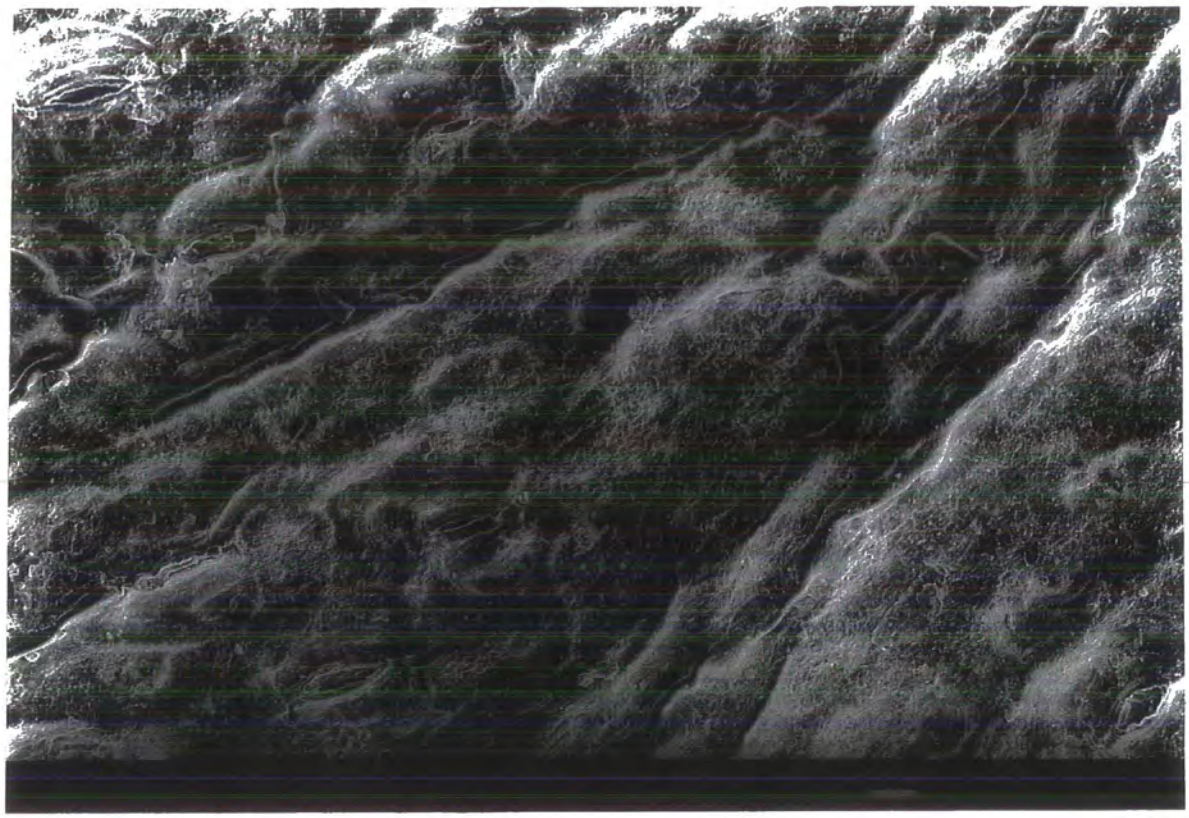


Plate 3.4 SEM photograph of modern *S. herbacea* leaf surface.

Bar = 10 μm

Plate 3.5 SEM photograph of fossil *S. herbacea* stoma.

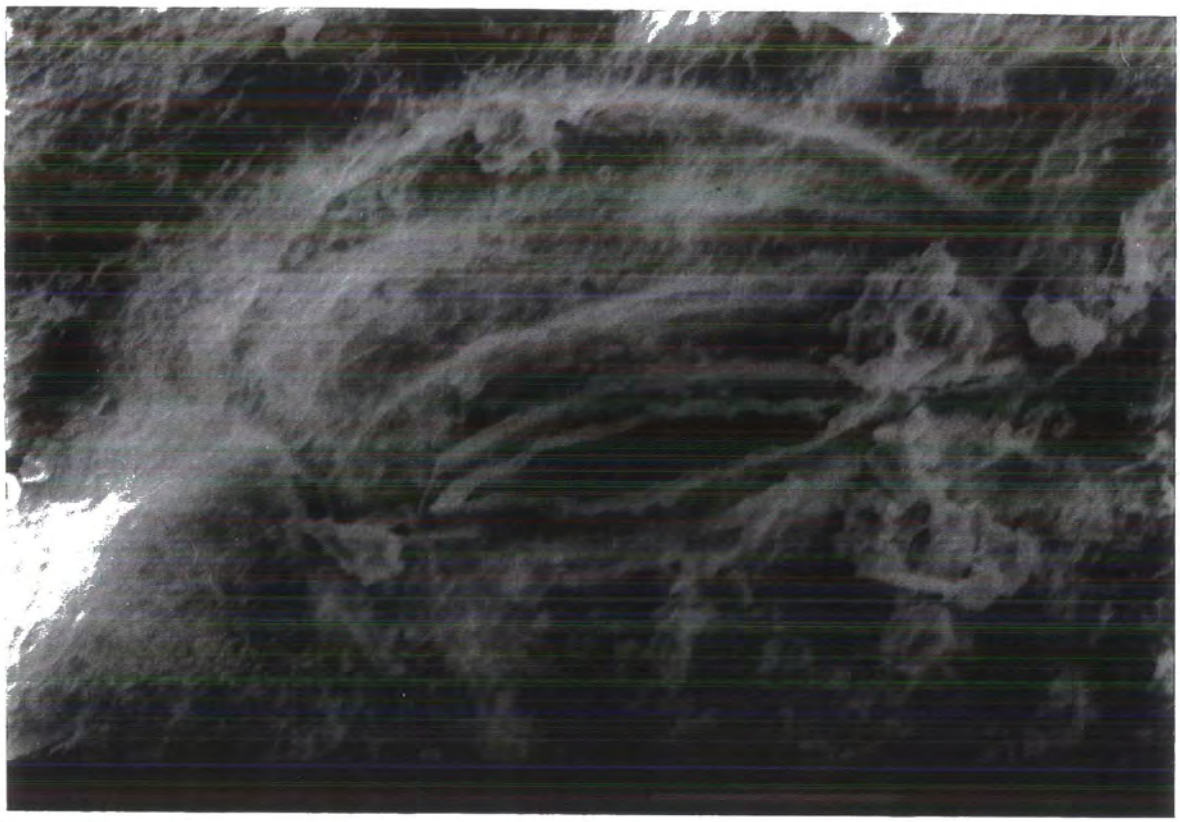
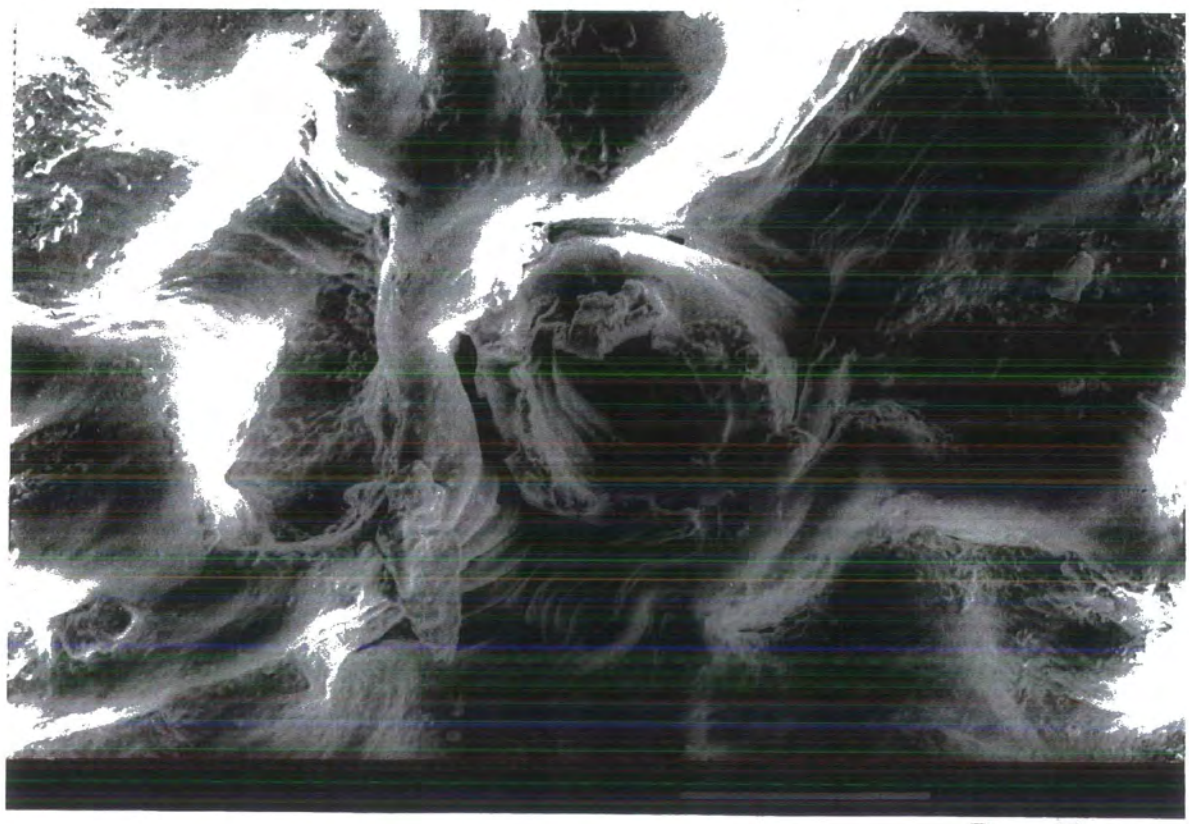


Plate 3.6 SEM photograph of modern *S. herbacea* leaf stoma.



Bar = 10 μm

3.4 Discussion.

3.4.1 Variations in stomatal parameters along an altitudinal gradient in Austria.

The study of *S. herbacea* leaves taken from plants growing along an altitudinal gradient aimed to investigate the effects of changing CO_2 partial pressures on stomatal parameters. Those leaves collected from plants at higher altitudes would have been formed under reduced CO_2 partial pressures (Gale, 1972), with a difference in CO_2 level of 20 p.p.m.v between 2100m and 2800m (Dr. J.A. Pearson, personal communication). The different collections at each altitudinal site were carried out to investigate the extent of variation of stomatal parameters that could not be attributed to changes in CO_2 partial pressures associated with altitudinal gradient.

Binns & Blunden, (1980), reported that the stomatal densities of leaves of *S. herbacea* recorded from a single plant growing in Ireland were in the range of 35-140mm^{-2} for the adaxial surfaces and more than 140mm^{-2} for the abaxial surfaces. The stomatal density values recorded in this study differ from those. The adaxial leaf surface was found to consistently record a higher stomatal density in comparison with the abaxial surface. The stomatal density range was also found to be higher on both surfaces than those recorded by Binns & Blunden, (1980). Most species with amphistomatous leaves have higher stomatal densities on the adaxial surface (Mott *et al.*, 1982). The observation that *S. herbacea* plants in this study possessed higher values on the adaxial surface is in agreement with the work of Beerling *et al.*, (1992).

3.4.2 Variations in stomatal characteristics along an altitudinal gradient as observed from all sampled sites.

The results revealed considerable variation in the response of the plants with regard to the stomatal parameters along the altitudinal gradient. There were no consistent trends observed on either leaf surface in association specifically with the gradient. This does not agree with the findings of Beerling *et al.*, (1992), who also investigated the stomatal densities of *S. herbacea* leaves along an altitudinal gradient in the Oetztaler Alpen in 1989. Using sampling sites at 2000m, 2200m (male and female) 2300m and 2670m

(north and south slope), and illustrating data in a similar manner they reported that leaves exhibited a reduction in abaxial stomatal densities with increasing altitude, whilst the adaxial surface densities remained constant. However, the number of leaves sampled, the counts recorded and the sites of sampling in this study were extended in comparison with those used by Beerling *et al.*, (1992). The increased sampling at each site and the collection technique used appeared to increase the amount of observed variation in stomatal densities and masked any effects which had previously been attributed to altitudinal gradient. This study further showed significant variation with stomatal characteristics within leaves (see 3.3.13). Woodward, (1986), also recorded a decrease in abaxial stomata with increasing altitude when studying *Vaccinium myrtillus* L. and a tendency towards an amphistomatous state. In amphistomatous species the adaxial surface tends to decline with increasing CO_2 concentration, whilst the abaxial surface remains relatively constant (Woodward, 1986).

Stomatal indices revealed no consistent trend and were found to show variation between sampling sites. Therefore, as stomatal index is essentially independent of leaf size once cells have differentiated these responses could not simply be attributed to the developmental stages of the leaves (Salisbury, 1928). It is therefore suggested that stomatal initiation is not being influenced in a consistent manner with altitudinal gradient.

The abaxial and adaxial leaf surface responses of *S. herbacea* leaves to the gradient were not always identical. Pearson *et al.*, (1995), investigated the response of adaxial and abaxial stomatal conductance in *Rumex obtusifolius* to growth at elevated concentrations of CO_2. They found the stomatal conductance of both the adaxial and abaxial surfaces were reduced by elevated concentrations of CO_2. The stomatal conductance showed a greater reduction on the adaxial surface than for the abaxial. However, there was a recorded absence of effects on stomatal density, indicating that the reductions were probably the result of changes in stomatal aperture. Although conductance experiments were not carried out during this investigation, stomatal densities were affected, so this would not appear to be the case.

Variation in stomatal parameters was also observed between male and female leaves. The female plants were recorded as having consistently higher stomatal indices on the abaxial surface. This response may be related to greater requirement for CO_2 assimilate by female plants compared with male plants for the production of flowers, fruit and seeds. Statistical analysis (see 3.3.11) of male versus female plants revealed that there was considerable variation observed in all stomatal parameters between leaf surface, site and time of sampling, suggesting that random sampling is important if sampling is not to be biased.

3.4.3 Variations in stomatal parameters of 'pooled' sites along an altitudinal gradient.

The pooled data sets did not reveal consistent trends in stomatal parameters associated with altitudinal gradient. The abaxial surface of 1994 collections revealed a significant decrease in stomatal and epidermal cell densities, thus it could be argued that the 1994 abaxial surface is responding to reducing partial pressure of CO_2 by showing a reduction in stomata. This result would be in agreement with Woodward, (1986), and Beerling *et al.*, (1992). However, a similar response was not recorded on the adaxial surface or in the 1995 collection. This inter-annual variation in pattern therefore casts doubt on any conclusions drawn from any one data set.

3.4.4 Variations in stomatal parameters within altitudinal sites.

The analyses of stomatal characteristics within altitudinal sites once more revealed much variation. There were significant differences between stomatal parameters of plants collected at the same altitude but of a different sex or geographical location. Even the collection methods used varied the stomatal parameters recorded. The leaf surface responses were also inconsistent. These results suggest that there are other factors effecting the response of the stomatal parameters that are unrelated to changes in partial pressure of CO_2 associated with altitude. In contrast to the conclusions drawn by Beerling *et al.*, (1992), this study found that the variations in stomatal density would appear to incorporate an extremely broad range of abiotic factors. In addition, the between plant variability due to genotypic or local environmental effects on *S. herbacea* is sufficient to

mask any clear trend response of stomatal density to changes in CO_2 levels. Thus there is a wide range of factors which may be causing these variations which may be attributed to the environment, to genetic composition or individual leaf variation which are now discussed.

3.4.5 The variation due to environmental factors affecting stomatal patterning.

Differences in light intensity, temperature, water availability, atmospheric humidity and other environmental factors besides the atmospheric CO_2 concentration could well account for the differences observed in stomatal parameters between the plants. There is no documented information available depicting levels or changes of such environmental factors at the Austrian sampling sites. Therefore it is difficult to attempt to attribute observed variations to an, or a combination of environmental factors.

3.4.6 Other environmental factors which influence the stomatal density of *S. herbacea.*

The influence of light intensity.

S. herbacea forms flattened mats and therefore would tend to avoid the influence of leaf shading on individual leaves. However, the light intensity within and between sites may have varied significantly, for example, between slope aspects. Light is probably more important in determining the course of stomatal behaviour than any other environmental component (Meidner & Mansfield, 1968). The differentiation of stomata in a leaf is a process specific to an individual leaf. Stomatal morphogenesis is under a genetic programme that can be affected by light during the critical period preceding unfolding of the blade, and hence this factor can influence stomatal parameters. Stomatal initiation is most active early in the development of the leaf and affects of on light initiation were greatest at this stage (Gay & Hurd, 1975). Gay and Hurd, (1975), found that light influenced the rate and duration of all stages of leaf expansion, and that the final stomatal densities achieved under natural conditions varied with leaf light history. The red to far red light ratio is thought to affect leaf growth and stomatal differentiation (Mitchell & Woodward, 1988). In addition, Wild & Wolf, (1980), recorded a higher stomatal density

on plants grown in full sunlight or high photon flux density (PFD) than plants grown in shade as was also reported by Givnish, (1988), and Osborn & Taylor, (1990).

Investigations have also revealed significant differences in stomatal indices in response to light within individual plants and between populations. Poole *et al.,* (1996), found that sun and shade leaves of *Alnus glutinosa* at the same site showed significant reduction in cell densities and stomatal index of shade leaves on the abaxial surface. The decrease in stomatal density was expected, due to an increase in surface area that is associated with the shading response, but the epidermal cell densities were not inversely proportional. Therefore, the overall decrease in stomatal indices in the shaded leaves were due to there being less reduction in epidermal cell density than would have been expected from the increase in leaf area.

Abaxial and adaxial surfaces are also known to respond differentially to light (Willmer & Fricker, 1996). This is thought to be due to differences in microclimate within the leaf and to inherent differences between the two epidermes. In most species the abaxial surface is found to be more sensitive than the adaxial surface as they are found to open at lower light levels and have wider open stomata at all levels compared with adaxial stomata.

As previously stated no information is available as to the prevalent light intensities at all the recorded sampling sites for *S. herbacea,* or between the plants sampled in this study. Therefore, it is impossible to accurately determine the effect of light on the development and initiation of stomata. However, it is possible to hypothesise that the variation observed in stomatal parameters during this study could be partly due to differences in light intensities.

The influence of water availability.

There are no data available describing water availability at each sampling site, or for each *S. herbacea* plant sampled. The altitudinal gradient at which *S. herbacea* was collected incorporated a wide range of soil types and slope aspects. It is highly likely that some

plant samples were taken from sites better supplied with water than others. For example, certain sites may have been susceptible to surface water run off such as the inclined slope sampled at Breitlehn, whereas environments such as the flatter meadow at Timmelsjoch may have retained water. Therefore it is important to consider the effect of water availability on stomatal parameters.

The responses of stomata to water stress must be regarded as fundamental to plant survival both from a functional and developmental perspective. Numerous studies have been carried out to investigate the effects of changing water regimes on stomatal patterns in a variety of plant species. The early studies of Gindel, (1969), on the stomata constellation in the leaves of cotton, maize and wheat plants reported differences associated with water availability as a function of soil moisture and environment. Rawson & Craven, (1980), studied *Helianthus annus* L., establishing that drought conditions led to an increase in stomatal density with an accompanied decrease in stomatal size and leaf area. Ciha & Brun, (1975), found similar effects in field grown Soybeans and differences in the water availability of soil water has been shown to affect leaf size in *Betula nana* (Beerling, 1993).

The difference in leaf size affects stomatal density by affecting leaf epidermal size. Plants growing under water limiting conditions will tend to have smaller epidermal cells, resulting in stomata being packed closer together and hence an increase in stomatal density. An opposite response is exhibited on leaves growing under conditions of high water availability, which are found to have lower stomatal densities (Tichá, 1982). The advantage of using stomatal index as a parameter is that if an environmental factor, such as restricted water availability, only affected epidermal cell expansion then its effects will largely disappear when stomatal index is calculated. In order to have an impact on stomatal index the treatment must affect either the proportion of guard cell initiates or differentiation from them. Changes in stomatal density in response to environmental factors are mediated through their having an indirect effect on epidermal cell expansion and size. Stomatal index, however, remains stable in response to these environmental parameters except under extreme experimental environments, where plants would

probably not survive in nature (McElwain & Chaloner, 1995). However, the results of El-Hashani, (1996), and Ferris, (1991), suggest that water availability does not just influence stomatal densities, since they found decreases in stomatal indices in response to a reduction in water availability. This suggests that water stress is affecting cell differentiation in young developing leaves and thus inhibiting stomatal initiation and contrasts with the suggestions of McElwain & Chaloner, (1995).

It could therefore be suggested that some of the variation observed in stomatal characteristics between and within sites could be due to variation in water availability. However, without accurate measurements of this factor it is impossible to determine as to what extent.

The influence of temperature and climate.

The stomatal parameters of *S. herbacea* in this investigation were assessed over two years. It was noted that *S. herbacea* leaf characteristics varied between study years. Beerling, (1993), found that the response of stomatal density to increases in CO_2 levels in *Betula nana* leaves showed considerable inter-annual variability. Variations in stomatal density observed in the present study could be due partially to annual variations in climate leading to differences in leaf expansion and overall leaf size. Beerling & Chaloner, (1993*a*), reported that *Quercus robur* leaves formed under warmer summer temperatures had reduced stomatal densities and indices from all parts, compared with their spring counterparts. It was suggested that for *Q. Robur,* temperature overrides the influence of irradiance intensity and small seasonal variations in CO_2 concentration in determining stomatal density. As temperature rises it is possible that the leaves are formed with fewer stomata, which is a possible adaptive response to reduce evapotranspirational water loss by lowering the stomatal conductance of the leaf. However, the work on *Q. robur* did not take into account that stomata would have been initiated in leaves at the bud stage in the previous season.

With respect to this study on *Salix,* no information was available describing the temperature or annual climatic conditions prevalent at each sample site. Therefore, in

addition to the environmental factors of light intensity and water availability variations observed in *S. herbacea* stomatal parameters may result from fluctuations in climate and temperature.

This study therefore could be improved by accurate measurements of other environmental factors known to affect stomatal parameters.

Environmental factors in combination.

A study undertaken by Körner *et al.*, (1989), found that stomatal densities of plants at higher altitude were in general higher than those from lowland regions, but they reported that the influence of temperature changes on stomatal density became increasingly difficult to determine. The authors suggested this was due to changes in temperature and atmospheric CO_2 concentration, which broadly fall with altitude, and irradiance which increases with altitude. Nevertheless they concluded that the recorded data showed that temperature is potentially a more important factor determining stomatal density than light intensity. However, with respect to the results obtained from *S. herbacea* the combination of environmental factors as discussed by Körner *et al.*, (1989), do not account for the variation found within the Austrian altitudinal sites. Therefore it would not appear that other factors and not simply the combination of factors outlined by Körner *et al.*, (1989), were entirely causal.

Other influential environmental factors.

The environmental factors discussed previously are the main potential influences occurring at the Austrian site. Additional factors of humidity and nutrient availability (Hsiao & Fisher, 1975), are also known to effect stomatal developmental behaviour and patterning.

The influence of genetic variation.

Stomatal density can vary greatly among different genotypes of the same species growing under identical conditions (Willmer & Fricker, 1996). *S. herbacea* is known to hybridise effectively (Meikle, 1984), and a slow growing species it being noted that propagation

from seeds is low (Pearson, personal communication). Therefore there is the potential for *S. herbacea* to develop in a clonal pattern. Genetic variation could therefore be responsible for aspects of the observed variation in stomatal parameters.

3.4.7 Variations among leaves on a single plant.

Stomatal differentiation generally continues until the leaf has reached between 10 to 50% of its final size (Tichá, 1982). The total number and density of stomata will increase during this period, then the density will decline as the epidermal cells continue to expand. Therefore, the age at which the leaf is sampled will have an effect on recorded stomatal parameters. 'Anatomical gradients' are apparent when leaves are mature. Tichá, (1982), reviewed data from many publications and concluded that the total number of stomata per leaf reaches a peak in leaves of middle insertion level; that there is a trend of increased stomatal frequency on both surfaces of fully expanded leaves with increasing height of insertion; and that cell sizes in general, tend to be smaller in leaves of higher insertion. The absolute values within these anatomical gradients are affected by plant water relation, irradiance level, temperature and other factors, but the essential character of trends is maintained (Weyers & Meidner, 1990). Thus it is important that sample leaves are fully developed and from the same insertion level, otherwise there are additional factors which may cause differences in recorded stomatal parameters among leaves on a single plant. In this study only mature leaves were collected, however, it is impossible to omit entirely the influence of leaf age. Given that *S. herbacea* forms flattened mats the effect of leaf insertion on stomata parameters is not as influential as in other species. Nevertheless, the effect of leaf insertion cannot be entirely discarded without carrying out extensive studies on *S. herbacea*, this was not possible within the scope of this investigation.

Variation across individual leaf surfaces.

The results of mapping stomatal density across individual leaf surfaces revealed further variation. It is known that stomatal frequency is not constant over the surface of a leaf (Tichá, 1982).

Research on both monocotyledons and dicotyledons have reported that stomata were found to be more dense at the margins and/or tips of the leaves (Salisbury, 1928; Miranda *et al.*, 1981; Smith *et al.*, 1989). Beerling & Chaloner, (1993*b*), suggested three possible reasons for the difference in patterning: the stomatal differentiation was uneven over the leaf surface resulting in different densities of stomata or both, (the differentiation hypothesis); conversely the leaf underwent uneven expansion after the cells had differentiated, resulting in uneven spacing of the stomata, (the expansion hypothesis); or that the spatial variation in both leaf expansion and stomatal differentiation contributed to the uneven spacing of stomata, (the mixed differentiation hypothesis). Any of these may account for some of the observed variation. In the case of *S. herbacea* leaves examined in this study, there appeared to be no consistent trends across the leaf surfaces when leaves were compared. Also, the abaxial surface recorded patterning was not consistent with that of the adaxial surface. Little is known about the factors which may cause such local variation in stomatal differentiation, especially as no consistent pattern has been observed (Poole *et al.*, 1996), but it may, for example, be related to the vein development site. The inconsistent patterning of stomata across individual *S. herbacea* leaf surfaces demonstrate clearly the need to take account of variation within leaves when comparing leaves between sites. There is the danger that if the sampling procedure does not take account of varying stomatal density within leaves, significant differences between sites could be masked or artificially increased. In this study whole leaves of *S. herbacea* were used, however, these results illustrate that without knowledge of the exact origin of an epidermal sample within a leaf it would be difficult to make definitive conclusions, especially when the use of fossil fragments is considered.

3.4.8 The relationship between stomatal density and stomatal length.

Stomatal characters are often related to each other (Jones, 1987), with stomatal density found to be inversely related to the size of guard cells in some species (Pallardy & Kozlowski, 1979; Ferris, 1991). This relationship causes stomatal aperture per unit surface to remain approximately constant despite the variability in stomatal density among different leaves and plants. Results obtained in this study do not reveal a significant relationship between these two parameters in *S. herbacea*. The work of

Gardener *et al.,* (1995), with hybrid poplars revealed that stomatal densities were found to be inversely correlated with guard cell length, following exposure to elevated CO_2. This illustrated that treatment influences on cell expansion also affect the stomatal complex. Miglietta & Raschi, (1993), reported findings which suggested that elevated CO_2 may have reduced the size of guard cells, leaving stomatal density and stomatal index unaltered. No such conclusions can be drawn from the data presented here.

Variation in guard cell dimensions

Stomatal length, pore length and stomatal width did not display any significant trend on either the adaxial or abaxial leaf surface with altering altitudinal gradient and once more illustrated inconsistent variation between and within sample sites. Pooled data obtained in 1994 showed a significant increase in stomatal length and pore length on the abaxial surface demonstrated with increasing altitude. This was not apparent in the 1995 data and therefore it cannot be concluded that altitude is affecting guard cell dimensions. The adaxial surface does not demonstrate any significant changes. If any of the guard cell dimensions were to illustrate a response to changing altitudinal gradient it would be expected to be that of stomatal length as it is not dependent on functional aspects of stomatal behaviour. There are contrasting reports as to the response of guard cell dimensions with respect to CO_2 concentrations. Radoglou & Jarvis, (1990), reported that elevated CO_2 concentrations on hybrid poplars leaves caused no effect on the stomatal pore length. However, Beerling & Woodward, (1995), reported an overall decrease in guard cell length with elevated CO_2 on variegated leaves, suggesting the pore length was smaller. Guard cell dimensions are known to show differences even when recorded under identical environmental conditions. The causes of general variability in stomatal size and aperture in such cases are expected to be due to a mixture of endogenous and exogenous factors that generate natural heterogeneity in biological systems. There can be considerable variation in stomatal dimension even within mature areas of the same leaf (Smith *et al.,* 1989). Smaller guard cells are usually found to be associated with higher stomatal densities (Willmer & Fricker, 1996), and therefore it may have been possible to speculate associated changes in stomatal density if accurate measurements of guard cells could be made and the species under investigation was known to demonstrate this

response. Such predictions were not valid in this study. The results obtained suggest that there is little value in measuring guard cell dimensions in order to relate them to environmental factors since variation with respect to other stomatal parameters and sampling sites is so great.

This study demonstrates clearly the need to appreciate the stomatal variation within leaves and between sampling sites when making comparisons and implicating causal factors. Although the study is classified as 'short term' it incorporates the response of *S. herbacea* in a natural environment where it is subjected to a wide range of environmental fluctuations. The *S. herbacea* plants are clearly established and have developed in the respective sites for a number of years, hence the differences in stomatal parameters observed can be concluded to be a true reflection of the intra-specific variation. Therefore, it is essential that before using stomatal parameters as a model to predict environmental change the variations described in this Chapter should be further assessed and taken into consideration.

3.4.9 Variations in stomatal density of *S. herbacea* leaf macrofossils.

Stomatal density analysis on *S. herbacea* fossil leaves taken from the Morrone Birkwood core revealed no overall trend associated with the age of the core section. The abaxial surface showed significant differences between each successive core section, however, the recorded changes in stomatal density varied between sections. There was a time interval of approximately 216 years between each section analysed and although the actual date of each section was difficult to qualify, those studied were known to correspond to a period of environmental change (Pearson, personal communication). The leaves were taken from the 'late-glacial' period which covers the interval 13,000 to 10,000 years BP, during which CO_2 concentration rose from 200 to 280 p.p.m.v (Neftel *et al.,* 1988). This study aimed to record long-term changes in stomatal density and extended the work of Beerling *et al.,* (1992), who had also studied the response of fossil leaves taken from 260 and 325cm depth from a Morrone Birkwood core. Beerling *et al.,* (1992), reported that the rising CO_2 of the past 11,500 years had been accompanied by an increase in stomatal density. In contrast, Beerling *et al.,* (1993*c*), later reported that

changes in climatic and atmospheric composition of a glacial-interglacial cycle *S. herbacea* showed a fall in stomatal density in relation to increases in atmospheric CO_2 over this time, as in agreement with the response observed in the dwarf-heathland species *Betula nana* (Beerling, 1993). The results presented here do not indicate similar responses. There was no clear trend in stomatal density associated with the time period studied. As it was not possible to determine stomatal index it is difficult to predict changes in stomatal initiation. In view of the variations found in the modern Austrian *S. herbacea*, it is possible that other factors influenced the stomatal density of the fossil leaves and are responsible for the observed variations.

In addition, it is known that about 1% of the organic matter biosynthesised in the biosphere enters the geosphere to become part of the sedimentary organic matter. Of this only a small amount is present as a morphologically well defined structure (de Leeuw, *et al.*, 1995). Therefore, in comparison with modern material, the total number of fossils which are available for study is relatively low (Poole, *et al.*, 1996). This was highlighted in this study, as it was only possible to sample three leaves from each core section in order to make statistically viable comparisons. It has been shown that even in modern material, stomatal density varied within an altitudinal site when ten leaves were analysed within a similar CO_2 concentration. This emphasises the problems associated with making accurate predictions from a few fossil leaves. Also, poor replication and inability to measure stomatal index in some ancient samples (Beerling & Chaloner, 1993*b*), have meant the understanding of the effects of predicted future concentrations of CO_2 on stomatal and epidermal cell development remain uncertain.

When using fossil data a number of assumptions have been made previously (e.g. Beerling *et al.*, 1992, 1993; Beerling & Chaloner, 1993 *a,b*). These include the similarity of growth conditions between leaves with factors such as nutrition, water availability, temperature and light availability remaining constant. Also fossils were considered similar in leaf aspect, insertion number, sex, and developmental state at the time of preservation. In addition if a fossil leaf fragment is obtained and analysed there is the problems of the influence of variations in stomatal parameters across the individual

leaf surface. Therefore, if it is accepted that variations in stomatal parameters are solely due to changes in CO_2, erroneous and/or biased conclusions can be easily drawn for palaeo and past vegetation, environments or climates based on this sort of fossil evidence alone (Poole *et al.*, 1996).

With the aim of further determining changes in atmospheric CO_2 levels through geological time, studies have also considered the use of extrapolating information from fossilised land plants and relating this to their nearest living relatives (van de Burgh *et al.*, 1993; McElwain & Chaloner, 1995). In light of this research and that of Poole *et al.*, (1996), this should be viewed with caution.

Previous studies on stomatal parameters of fossil and sub-fossil leaves rarely take into account either natural variation or that introduced by the bias of fossilisation and collection. This research demonstrates the importance of appreciating variations in stomatal parameters observed on modern material, before extrapolating data from fossil plants with the aim of determining past CO_2 concentrations. Thus, until the variability in extant material has been fully assessed conclusions drawn solely from counts of stomata from fossil material are of limited value and must be treated with caution.

Chapter 4.

The use of $\delta^{13}C$ as a tool to monitor environmental change.

4.1 Introduction.

The physiological studies of plant stomatal characteristics reported in Chapters 2 and 3 concluded there was a wide variation with respect to environmental change. To further establish whether plants responded to such a change a physiognomy approach was attempted. This involves looking at differences in element isotopes, in this case carbon, within plant material. Using changes (δ) observed in the ratio of carbon isotopes, hypotheses can be made as to whether the plant is responding to environmental change. Modern material will first be assessed and, depending on the conclusion, the study could be extended to fossil material.

4.1.1 The carbon isotope.

Carbon, along with many other elements, can occur in the environment as stable isotopes. Isotopes are chemically identical, but differ according to the number of neutrons present in the nucleus. Stable isotopes, as the name suggests, do not decay, and their relative proportions in the biogeochemical system remains fixed. They are, however, subjected to fractionation, especially during thermodynamic processes. Therefore, whilst the global proportions remain constant, the proportions of one isotopic form to another varies between the atmosphere and various sinks, such as plant material.

4.1.2 $\delta^{13}C$ values.

With respect to carbon, the lighter isotope ^{12}C constitutes the major proportion of the element and the heavier isotope, ^{13}C, accounts for a smaller component (Handley & Raven, 1992). Relative proportions of naturally occurring CO_2 are outlined in Table 4.1. Changes in the proportion of the heavy isotope of carbon ^{13}C in plant cellulose have been analysed in this study. Changes (δ or "delta") in the ^{13}C:^{12}C ratio in plant material is referred to a standard and expressed in parts per 1000 (see equation 1).

Table 4.1 The proportion of ^{13}C and $\delta^{13}C$ value ranges found in nature.

Heavy isotope	^{13}C
Mean fractional abundance	0.0111
1 δ as a fractional change of isotopic composition	1.1×10^{-5}
Usually observed ranges of δ values in nature	-40 to 0
Observed range as fractional change of isotopic composition	4.4×10^{-4}

(From: Handley & Raven, 1992).

The derivation and calculation of $\delta^{13}C$.

The isotopic fractionation of carbon is recorded by analysing plant cellulose ($C_6H_{10}O_5$). It is calculated as follows:

$$\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000 \qquad (1)$$

where R_{sample} and $R_{standard}$ are the ^{13}C:^{12}C ratios of the sample and of the universally accepted Pee Dee Belemnite (PDB) carbonate standard (Craig, 1957). As plants have a lower ^{13}C:^{12}C ratio than the standard, $\delta^{13}C$ is negative. The simplified theoretical relationship between gas exchange characteristics of C_3 plants and the isotopic composition of the leaf cellulose laid down during the development of the leaf is by:

$$\delta^{13}C = \delta^{13}Ca - a\,(b - a)\,p_i/p_a \qquad (2)$$

where $\delta^{13}C$ and $\delta^{13}Ca$ are the ^{13}C to ^{12}C ratios of the leaf and atmosphere respectively, and where a is the fractionation due to CO_2 diffusion in air through the stomatal pore (4.4‰), b is the net fractionation by Rubisco (27‰) and p_a and p_i are the ambient and intercellular partial pressures of CO_2 respectively (Farquhar *et al.*, 1982). Although a fractionation value for Rubisco of -27‰ is commonly used in modelling C_3 terrestrial photosynthesis, the actual values may be rather lower (Raven & Farquhar, 1990). $\delta^{13}C$ thus expresses the consequences of both biological processes and the source air isotope composition.

The present day proportion of $^{13}CO_2$ in total atmospheric CO_2 is roughly 1.1% (Larcher, 1995). Generally plants possess a below average $\delta\ ^{13}C$ value, due to plant carboxylases exhibiting a greater affinity for $^{12}CO_2$ than $^{13}CO_2$ (Larcher, 1995).

Within plant species $\delta\ ^{13}C$ values typically range from -40 to 0 per mil (‰). This broad variation is due to individual carboxylases of C_3, CAM and C_4 plants possessing slightly different affinities for either isotope. C_3 plants are found at the lower end of the range, CAM plants in mid ranges and C_4 plants close to zero.

$\delta^{13}C$ values are also affected by weather conditions, especially during drought, and also light irradiance received by the leaves, uptake of CO_2 from the air in the soil, low growth temperature, the decreasing partial pressure on mountains, and by ozone stress (Larcher, 1995).

4.1.3 The relationship between $\delta^{13}C$ and stomata.

Stomata are known to respond to environmental stimuli in order to maintain water balance within the plant. Factors upsetting this balance would lead to a change in stomatal conductance. $\delta^{13}C$ is principally determined by the relative magnitudes of stomatal and mesophyll conductance, and thus the intercellular partial pressure of CO_2 (p_i) during photosynthesis (Farquhar *et al.*, 1982). When mesophyll conductance is high (or stomatal conductance is low) p_i will be low and $\delta^{13}C$ will be high. Thus, as a consequence, changes in environmental conditions may be reflected in isotopic studies. For example, a decrease in stomatal aperture caused by production of abscisic acid (ABA), in turn induced by water stress, results in an increase in the efficiency of water use, a decrease in the discrimination against ^{13}C thereby resulting in less negative $\delta^{13}C$ values. Such a scenario will cause variation in $\delta^{13}C$ values in plants from the same species growing under different environmental conditions.

Stomatal density and size are determined at the time of leaf production (Chapter 1). The examination of stomatal characteristics as described in chapter 3 has been implicated for use in analysing past environments (van de Burgh *et al.,* 1993; McElwain & Chaloner, 1995). Previous work has also suggested that a relationship may exist between $\delta^{13}C$ and environmental change therefore it may be possible to use $\delta^{13}C$ values in association with the stomatal density record to offer the possibility of determining the comparative timing of changes (Beerling *et al.,* 1993). In addition, it has also been suggested that there is a

relationship between stomatal density and $\delta^{13}C$ (Beerling *et al.,* 1993). However, whether this is a causal relationship remains open to question, since it takes no account of physiological variations which impact on $\delta^{13}C$ and cannot be measured after the plant has died (Jones & Sutherland, 1991).

When considering both stomatal densities and $\delta^{13}C$ values obtained from plant leaves collected from a variety of sites it may be possible to qualitatively determine the importance of physiology and physiognomy on carbon isotope discrimination in the plants analysed as Malone *et al.,* (1993). In addition, as Chapter 3 has outlined considerable variation between and within sites, which were known to be under the influence of a CO_2 partial pressure gradient, it may provide an alternative perspective from which to assess the influence of environmental factors.

4.1.4 Variation in $\delta^{13}C$ associated with temporal changes.

The isotopic composition of atmospheric CO_2 varies naturally according to the trends in global biological productivity over glacial - interglacial periods (Coplen *et al.*, 1994; Leavitt & Danzer, 1992; Meyers & Horie, 1993; White, *et al.*, 1994), and also annual time scales (Farquhar & Lloyd, 1993; Farquhar *et al.*, 1993).

CO_2 is released back into the atmosphere following human activity such as the burning of fossil fuels and biomass destruction. This has caused a net increase in atmospheric CO_2 concentration, as well as changes in isotopic composition, since plants contain relatively smaller quantities of ^{13}C (discussed previously). In fact, CO_2 derived from fossil fuel combustion and biomass destruction result in ^{13}C values of $\approx$ -25‰, compared with $\approx$-7‰ for atmospheric CO_2 (Friedli *et al.,* 1986). Therefore, increased usage of fossil fuels over the past few decades will have decreased the proportion of ^{13}C in the atmosphere, which in turn will decrease the proportion of ^{13}C incorporated in modern plant material. A significant correlation between overall $\delta^{13}C$ and corresponding atmospheric CO_2 concentration during the last three centuries has been reported by Peñuelas & Azcón-Bieoto, (1992). $\delta^{13}C$ significantly decreased in the 1980's after four decades of accelerating atmospheric CO_2 concentration by approximately 40μmolmol^{-1} (Friedli *et*

al., 1986). In addition, meteorological data obtained from the collection area revealed that the 1980's were in fact drier than the 1940's and therefore the $\delta^{13}C$ values obtained from C_3 plants in the area may have been further affected by dry conditions as well as increased atmospheric CO_2 concentrations.

Beerling *et al.,* (1993), investigated the short- (200 years) and long-term (> 140 000 years) effects of changing CO_2 concentrations on plant $\delta^{13}C$ values. The study of *S. herbacea* herbarium leaves showed decreased $\delta^{13}C$ values in response to the CO_2 increases of the last 200 years. This was once again attributed to the decrease in atmospheric ^{13}C as a result of fossil fuel burning and deforestation. The examination of quaternary leaf macrofossils suggested that the higher leaf $\delta^{13}C$ value observed reflected the palaeotemperature under which the leaves formed; low temperatures being associated with high $\delta^{13}C$. Short term studies on herbarium material collected from southern Sweden extending back 200 years, have been used to examine the theory that these documented increases in the atmospheric CO_2 concentration over the past two centuries, (Friedli *et al.*, 1986), have affected the $\delta^{13}C$ values obtained from cellulose present in an arctic alpine species. From this work it would appear theoretical that fossil leaves could provide potential evidence for atmospheric CO_2 change from the stomatal density record and palaeotemperature change from the leaf $\delta^{13}C$ content.

4.1.5 Changes in $\delta^{13}C$ associated with spatial (altitudinal) gradients.

Montane environments provide sites where climate and vegetation change rapidly over short distances, with respect to altitude and slope aspects. The partial pressures of CO_2 are known to be progressively reduced with increasing altitude (Gale, 1972). Previously there have been several reports showing the influence of altitude variations on $\delta^{13}C$ (Körner *et al.,* 1988; 1991).

In this study the plant material used for $\delta^{13}C$ analysis, *S. herbacea,* had been collected along an altitudinal gradient in order to assess the response of stomatal characteristics with respect to changes in carbon dioxide partial pressure (see Chapter 3). Beerling *et al.,* (1993), had previously investigated the response of three carbon isotope related

parameters ($\delta^{13}C$, discrimination and p_i/p_a ratio) determined from *S. herbacea* leaves along an altitudinal gradient in Austria. The collection sites used were similar to the those in this study. The results of Beerling *et al.*, (1993), revealed a non-significant decrease of leaf $\delta^{13}C$ with increasing altitude. These results support the view that local trends in leaf $\delta^{13}C$ may follow quite different patterns to those observed globally (Friend *et al.*, 1989). Global trends along mountainsides have been reported to show an increase in $\delta^{13}C$ of plant leaves with altitude (despite a wide scatter) in response to a decrease in the partial pressure of CO_2 and possibly oxygen and temperature (Körner *et al.*, 1988; 1991). The results of Beerling *et al.*, (1993), also contrast with the earlier work of Körner & Diemer, (1987), who concluded that p_i/p_a decreased with altitude, therefore in accordance with equation (2) $\delta^{13}C$ should increase with altitude (assuming no change in $\delta^{13}C_a$).

It would appear that local climatic factors can influence $\delta^{13}C$ values in response to changes in CO_2 partial pressure. Observations made on plant material from the Scottish Highlands, reported after two consecutive years of monitoring $\delta^{13}C$; a decrease and no response in $\delta^{13}C$ composition, respectively with altitude. These investigations were carried out in a small geographic range and concentrated on changes within species (Friend *et al.*, 1989). The leaf $\delta^{13}C$ values for both *Nardus stricta* L. and *Vaccinium myrtillus* L., between 200 and 1100m were found to increase (though not significantly) with altitude in 1986 but were found to decrease in 1987. It is suggested that the change in trend between years is related to marked differences in annual climate. $\delta^{13}C$ values appeared to be influenced as much by climate as by the ambient partial pressure of CO_2. Therefore, when studying the effect of altitudinal CO_2 changes on leaf $\delta^{13}C$ values the association is not straightforward. It is complicated by the fact that the investigations necessarily include the effects of climate such as precipitation, dewfall and incidence of freezing (Körner *et al.*, 1988; 1991; Friend & Woodward, 1990; Morecroft *et al.*, 1992), which can influence the $\delta^{13}C$ values of leaves. Morecroft & Woodward, (1990), found that the general increase in $\delta^{13}C$ observed with altitude (Körner *et al.*, 1988), may result from plastic responses of plants to environmental changes with altitude. Freezing and low temperature both cause changes consistent with the global altitudinal trend demonstrated

by Körner *et al.*, (1988). Drought, which may be caused by dry air or low soil water content, increases $\delta^{13}C$ because of reduced stomatal conductance, as described by Farquhar *et al.*, (1989). In addition, it has been observed that leaf nitrogen concentration increases with increasing altitude; this has also been linked to an increase in carboxylation efficiency and carbon isotope ratio ($\delta^{13}C$) (Morecroft *et al.*, 1992). The factors influencing altitudinal trends in carbon isotope ratio of plants as outlined by Morecroft *et al.*, (1992), are presented in Table 4.2.

Table 4.2 Factors influencing altitudinal trends in carbon isotope ratio of plants

Promoting an increase in $\delta^{13}C$ with increasing altitude	**Promoting a decrease in $\delta^{13}C$ with increasing altitude**
Decreasing stomatal and boundary layer conductance with falling temperature	Increasing stomatal and boundary layer conductance with falling atmospheric pressure
Decreasing stomatal conductance with increased incidence of frozen soil	Increasing boundary layer conductance with increasing wind speed
Increasing carboxylation efficiency through higher nitrogen concentration and/or lower specific leaf area (SLA)	Decreasing mesophyll conductance with low reaction rates at low temperature
Decrease in stature with altitude	Lower dark respiration because of lower temperatures
Higher internal resistance because of thicker leaves	Decrease in leaf area index with altitude

Environmental factors such as light, temperature, salinity and drought will also have effects *via* changes in intercellular $p(CO_2)$.

4.1.6 The use of $\delta^{13}C$ values in this study.

The analysis of the stable carbon isotope was carried out in this investigation on *S. herbacea* material collected along an altitudinal gradient in the Oetztaler Alpen, Tyrol Austria (see 3.2.1) The partial pressure of CO_2 is known to decrease with increasing altitude (Gale, 1972). The analysis aimed to investigate any relationship between the isotopic discrimination and stomatal parameters along an altitudinal gradient.

Firstly modern *S. herbacea* was examined and the results analysed. This would provide a basis to determine the feasibility of using $\delta^{13}C$ values to monitor environmental change before extending the study to fossil material.

4.2 Materials and Methods

4.2.1 Analysis of $\delta^{13}C$.

Material used for analysis.

Fresh *S. herbacea* material was collected in 1994 from the Oetztaler Alpen, Austria during July. Leaves were collected from single plants located at different altitudinal sites (see Table 4.3 for site descriptions). Plants and leaves for analysis were selected at random. Collections were made at either close proximity sites, (i.e. the next random plant sampled was less than 2m from the previous), or at sites approximately 10m apart.

Table 4.3 Altitudinal site collection points for $\delta^{13}C$ analysis.

Altitude (m)	Site	Collections
2100	Breitlehntal	2 close proximity (less than 2m apart)
2200	Timmelsjoch	1 collection
2200	Rotmoostal	3 close proximity (less than 2m apart)
2200	Timmelsjoch	2 close proximity (less than 2m apart) female plants 2 sets of close proximity male plants 10m apart from each other (A&B)
2670	Hohe Mut	1 collection (A) 2 close proximity (less than 2m apart) (B) which are ≅ 10m from (A)
2800	Tiefenbach	1 East 1 South

The leaves were stored in formyl-aceto-alcohol solution (FAA).

Stable isotope determinations were made on each set of leaves collected. α-cellulose was isolated from the *S. herbacea* leaves and used to produce CO_2 required for $\delta^{13}C$ analysis.

4.2.2 The isolation and preparation of α-cellulose from *S. herbacea* leaves for $\delta^{13}C$ analysis.

The method used to isolate the α-cellulose was that described by Twiddy, (1996), after Sternberg, (1989).

Ten *S. herbacea* leaves were oven dried at 60°C for 48 hours. The dried leaves were ground in a pestle and mortar and boiled in distilled water for 2 hours.

The temperature was reduced to 70°C. 0.5ml of concentrated acetic acid and 1g of mercuric chloride was added. This was repeated every 15 minutes until the leaves were bleached. The material was then washed in distilled water, before being immersed in 17% (w/v) sodium hydroxide for 45 minutes. It was further washed in distilled water before a 10 minute immersion in 10% (v/v) acetic acid and finally washed in distilled water prior to drying at 50°C.

4.2.3 Production and isolation of CO_2 from α-cellulose for $\delta^{13}C$ analysis.

Following the isolation of α-cellulose from *S. herbacea* leaves, CO_2 was produced and isolated from the α-cellulose ready for $\delta^{13}C$ analysis. This work was kindly carried out at the Department of Biology, University of Miami, by E. Twiddy (see Twiddy, 1996). The following protocol was followed:

9mm outside diameter quartz glass necked cuvettes were heated for 2 hours at 600°C. 4-6mg of *S. herbacea* α-cellulose and an excess (c. 1g) of pre-heated copper filings were added. Each cuvette is pumped to vacuum ($<10^{-3}$ mbar) and sealed. The sealed cuvettes were heated to 850°C for 2 hours in a muffle furnace, after which they were slowly cooled back to room temperature. When the samples had cooled, the vessels were scored with a glass cutter and placed in a cracker on a vacuum line. The line was pumped out and then isolated from the pump. A dewar flask containing a slush of dry ice/acetone was placed over the first trap on the line this was necessary for the removal of water vapour. A second trap contained liquid nitrogen into which the CO_2 gas produced would condense. The vessel was then broken. Any trace impurities (such as nitrogen), which registered on the vacuum gauge were pumped away. Liquid nitrogen was placed around the collection vessel and was taken away from the second trap. Once all the CO_2 had re-condensed, the collection vessel was isolated and, depending upon design, was either physically removed or sealed using an oxyacetylene torch. The isotopic composition of the CO_2 released was analysed in a triple beam stable- isotope ratio mass spectrometer (VG Isogas Sira) (see also Sofer, 1980, Heaton, 1990a,b, 1991; cf. Murnick & Peer, 1994). During all analyses standards of known isotopic composition were run alongside

the samples. Three standards were used: One graphite standard (National Bureau of Standards #21), and two carbonate standards from Beta Analytic Inc. (as Twiddy, 1996).

4.3 Results.

4.3.1 The effects of altitude on the carbon isotope composition of *S. herbacea* leaves.

The number of samples analysed was limited due to time and financial constraints. Therefore, it was not possible to achieve $\delta^{13}C$ values from all the samples collected in the Oetztaler Alpen. The results presented are from a representative collection. In each case the reported value was a mean of five determinations made by the stable isotope-ratio mass spectrometer at that sample site.

Unless otherwise stated leaves were collected randomly. Figure 4.1 shows the wide scatter in $\delta^{13}C$ values in respect to the altitudinal gradient.
Substantial variations in $\delta^{13}C$ values were observed even when plants were sampled at the same altitude.

2100m. Site sampled: Breitlehntal.

Two separate α- cellulose preparations were analysed at this site. Leaf samples were collected from two separate *S. herbacea* plants within close proximity (1&2) (less than two metres apart). The minimum value recorded was -24.93‰ and the maxima of -23.62‰. The recorded variation at this site is 1.31‰.

2200m. Sites sampled: Timmelsjoch and Rotmoostal.

The $\delta^{13}C$ value obtained from material collected from the Timmelsjoch site was different to that obtained from the Rotmoostal region. A range of 2.43‰ was recorded. The triplicate plants sampled within close proximity at Rotmoostal did not correlate closely.

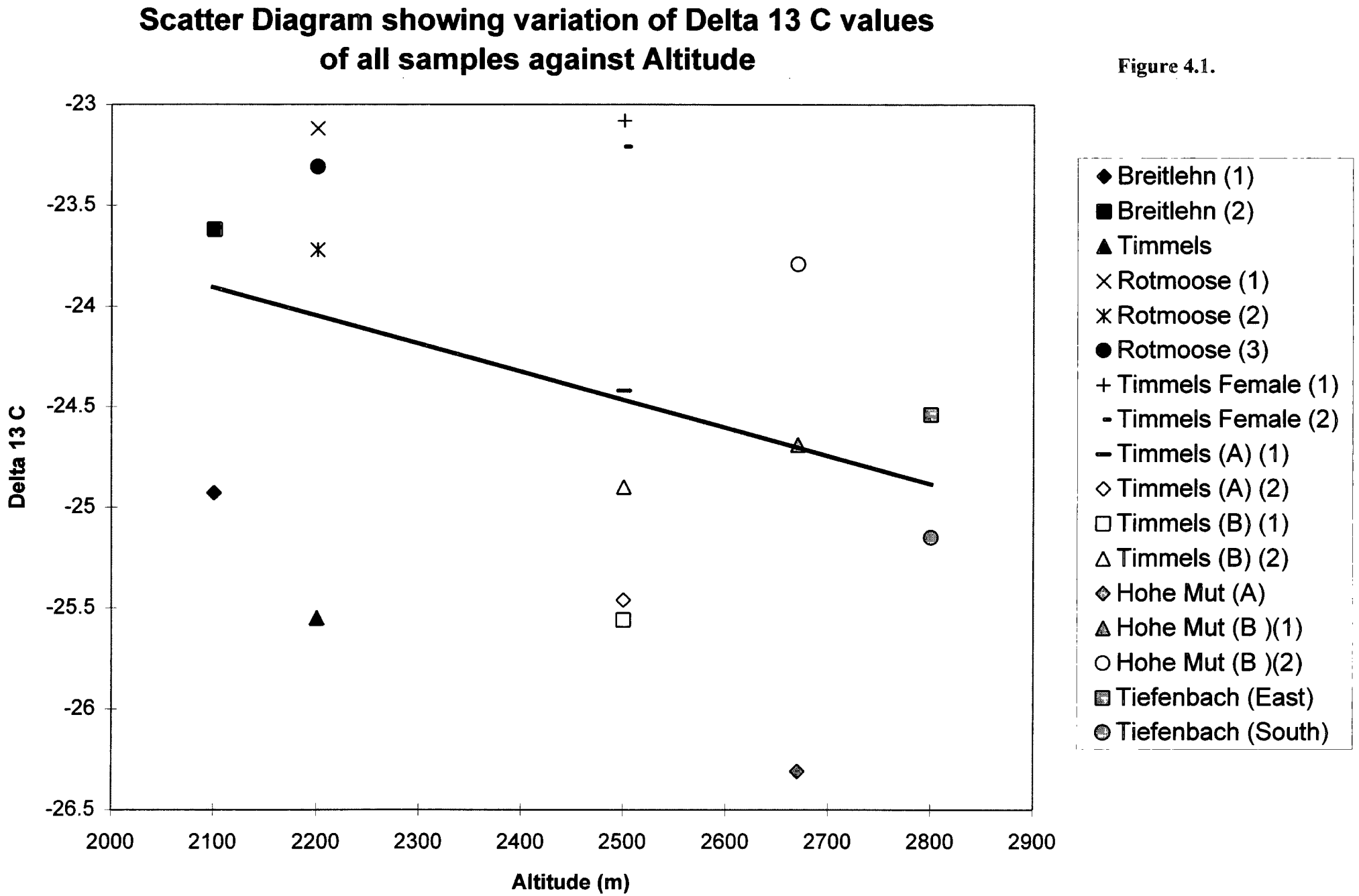

Scatter Diagram showing variation of Delta 13 C values of all samples against Altitude
Delta 13 C
-23
-23.5
-24
-24.5
-25
-25.5
-26
-26.5
2000
2100
2200
2300
2400
2500
2600
2700
2800
2900
Altitude (m)
Breitlehn (1)
Breitlehn (2)
Timmels
Rotmoose (1)
Rotmoose (2)
Rotmoose (3)
Timmels Female (1)
Timmels Female (2)
Timmels (A) (1)
Timmels (A) (2)
Timmels (B) (1)
Timmels (B) (2)
Hohe Mut (A)
Hohe Mut (B)(1)
Hohe Mut (B)(2)
Tiefenbach (East)
Tiefenbach (South)

Figure 4.1.

2500m. Site sampled: Timmelsjoch.

Six samples were analysed at this altitude. Female plants were collected located in close proximity (less than 2m apart) (1&2), and two sets of male plants (A & B) were collected from sites 10m apart. A variation of 2.48‰ was recorded over the entire data range. The altitudinal mean value of all the samples recorded as -24.44‰ with an associated standard error of 0.442‰. This suggested wide scatter within the data. Although the female plants sampled showed similar values, the random collections did not.

2670m. Site sampled: Hohe Mut.

Three plants were sampled here. Sample (A) was collected from a distance approximately 10m from that of site (B). Two close proximity samples were taken at (B). Once more an extensive data range was recorded (2.52‰).

2800m. Site sampled: Tiefenbach.

Two samples were collected from East and South Facing slopes. The $\delta^{13}C$ values differed by 0.61‰. This variation, although prominent, was not as extensive as plants collected in a close proximity from other slopes, for example Breitlehn (1.31‰).

Statistical analysis.

A linear trend line was fitted using EXCEL. This fit revealed a decline in $\delta^{13}C$ with increasing altitude. The r^2 value associated with this line was 0.1053. This is not a significant decrease at the p= 0.01 level. The correlation coefficient was calculated over the altitudinal range and found to be -0.326. This value is not significant at the p=0.05 level.

4.4 Discussion

4.4.1 Oetztaler Alpen site results.

S. herbacea leaf $\delta^{13}C$ values in this study were found to show wide variation with respect to altitudinal gradient. No significant trend was observed with increasing altitude and a non-significant correlation coefficient was recorded.

Beerling *et al.,* (1993), had reported a non-significant decline in $\delta^{13}C$ with altitude when studying *S. herbacea* from the same area collected in 1989. The material in this investigation was collected in 1993, and a further non-significant decrease observed. However, the extent of the decline was much less, in this case with r^2=10.53% compared with that of 52.7% as recorded by Beerling *et al.,* (1993).

The data obtained in this study revealed great variation. Plants sampled at the same altitude and from sites within close proximity to each other were found to show considerable variation at all locations, except that of the female plants sampled from the Timmelsjoch 2500m. As all these plants had been collected within a 2m radius of each other, it would be expected that if altitude was the only factor effecting the $\delta^{13}C$ values, there would be no significant difference in the values observed. In addition plants collected at the same altitude, but from different sites revealed considerable variation. Differences in leaf $\delta^{13}C$ values were observed between random and female plants collected at 2500m in the Timmelsjoch, and between east and south facing plants collected at Tiefenbach 2800m. It is therefore suggested that further variation could be attributable to the sex of the *S. herbacea* plant studied and the aspect of the sampling site.

The recorded *S. herbacea* leaf $\delta^{13}C$ values did not correlate closely with those obtained by Beerling *et al.,* (1993). The recorded leaf $\delta^{13}C$ values ranged from -23.08‰ to -26.31‰ (a difference of 3.23‰) compared with that of approximately -25.6‰ to -26.9‰ (a difference of 1.3‰) obtained by Beerling *et al.,* (1993). In addition, the values recorded in 1993 varied to those recorded in 1989. It would appear that as the methods in

these two studies were similar a temporal factor may also be effecting the recorded leaf $\delta^{13}C$ values.

Although the number of samples analysed in this study were limited it was possible to obtain a data set greater than that of Beerling *et al.,* (1993), thus the extent of the value ranges could be attributed to increased sampling. The shortcoming of using field collected *S. herbacea* is that there is no accurate record available as to the climatic conditions prevailing during the growth of these plants. Therefore, it is impossible to present a causal factor or factors which are attributable to the recorded variation in leaf $\delta^{13}C$ values in respect to both the differences observed in one sampling year, within and between sampling sites and collection year. Inter-seasonal variations in $\delta^{13}C$ have been recorded in tree rings of oak by Switsur *et al.,* (1994), and in grassland material (Smedley *et al.*, 1991), but as these leaves in this study were all sampled at a similar time, inter seasonal variation cannot account for the wide scatter observed.

Variations in the carbon isotopes present in plant cellulose are thought to be due to changes in the relationship between p_i and p_a, which may be caused by species-specific physiological variations, by changes in p_a, or changes in p_i as a result of the response of stomatal conductance to stress. In this study, since the plants are of the same species and the samples were taken at a fixed time, p_a should remain constant, thus the observed changes in $\delta^{13}C$ values are assumed to be the result of variations in p_i.

The results presented here suggest that the isotopic content of leaves taken from an altitudinal gradient do not necessarily follow global trends (Körner *et al.,* 1988), and contrasts with the observations of Körner & Diemer, (1987). The occurrence of local trends in leaf $\delta^{13}C$ have been reported (Beerling *et al.,* 1993). Studies have shown other factors changing with altitude, such as the climate (Friend *et al.,* 1989), low temperatures and increased incidence of freezing temperatures (Morecroft & Woodward, 1990), and those outlined by Morecroft *et al.,* (1992), can all substantially change leaf $\delta^{13}C$. It would appear that the results reported here for *S. herbacea* are in agreement with this analysis, in that the value of $\delta^{13}C$ would appear to be affected by other environmental factors which cause the great variation and range of values obtained.

However, it is somewhat surprising that there is still considerable recorded variation when plants are sampled within close proximity. It would be assumed that the climatic, partial pressure and environmental influences in such a close area would be similar. If specimens of the same species from different sites and ages are to be used for the basis of environmental reconstruction's then genotypic factors should also be considered. Therefore, it could be hypothesised that factors such as leaf morphology as affected by irradiance, and plant position, developmental age and genotypic variation are therefore also influencing isotopic discrimination.

4.4.2 $\delta^{13}C$ and stomatal parameters.

Stomatal parameters of *S. herbacea* were described in Chapter 3, and found to show variation between and within altitudinal sites which in turn was attributed to a range of environmental factors. The $\delta^{13}C$ values were also found to show variation, and although it was not viable to statistically assess the significance of variation between and within sites, it is clear that a number of environmental factors could be influencing the values. The variation did not appear to correlate with that of stomatal parameters, which may have been due to the extent of the variation and therefore it is not possible to imply that the changes observed are in association with stomatal parameters. Since stomatal parameters exhibited variation it is not surprising $\delta^{13}C$ values showed a similar trend.

4.4.3 Implications for extending the use of $\delta^{13}C$ as a tool to monitor environmental change.

The wide scattering of $\delta^{13}C$ values recorded from modern *S. herbacea* leaves suggest the reliability of using $\delta^{13}C$ as a tool to analyse environmental change in *S. herbacea* fossil material is questionable. Although a relatively small sample size was used in this investigation, the number of *S. herbacea* macrofossils available for a comparable analysis are very limited, therefore it is very difficult to obtain statistically viable data sets. Also, if the recordings achieved from fossil material were to reveal a significant trend related to the time of sampling, it would not be possible to specify a sole environmental factor that may have caused the change in isotopic discrimination to vary as outlined by studying modern fresh material. In addition the degradation of cellulose is not fully understood. If

any intra-molecular differences were to occur during degradation it may be possible that these could account for some of the observed variation as suggested by Twiddy, (1996).

Cellulose is also thought to be relatively stable and resilient through time, but $\delta^{13}C$ may undergo a degree of post-depositional alteration (Meyers, 1994). This degradation process is not fully understood. If the cellulose molecule is not preserved in pristine form, it could be that these intra-molecular differences, cause as yet unaccounted, variations between sub fossil and contemporary $\delta^{13}C$ values from cellulose.

The value of $\delta^{13}C$ in C_4 species has been shown to be less susceptible to variation than in C_3 species (Larcher, 1995). Consequently the remains of C_4 plants could be a far better source of information on past changes in atmospheric CO_2 composition than material from C_3 plants (Marino & McElroy, 1991). However, fossil material available for C_4 plants is limited.

Therefore, in conclusion it would appear that the analysis of the stable carbon isotope ratios on plant material as a tool with which to study environmental change may be limited by the complexity of environmental factors that are able to influence $\delta^{13}C$ values.

Chapter 5.

Using molecular biology techniques to identify *Salix herbacea* leaf samples.

5.1 Introduction.

In order to relate any changes in stomatal characteristics to environmental conditions it is essential that the leaf material studied is correctly identified. This is especially critical when fossil samples are being used. Leaves identified as *S. herbacea* were extracted and recorded from the Morrone Birkwood core as described in 3.2.2. R.D. Meikle (personal communication) further confirmed identification of the species. However, close inspection using SEM (see 3.2.5) and light microscopy revealed some physiological differences between fresh leaves sampled from Austria and those extracted from the Morrone Birkwood core (see 3.3.14).

In order to further confirm the identity of leaves present in the Morrone Birkwood core a molecular biological approach was attempted based on DNA characteristics that are unique to each species. Thus DNA sequences could provide a tool to help identify the species without having to rely completely on morphological features. Hence leaf identity could be confirmed if DNA could be extracted, analysed and identified.

This study aimed to determine specific regions of DNA sequence within the *S. herbacea* genome. Since no DNA sequence was available for *S. herbacea* in the plant DNA databases it was important to firstly obtain "reference" sequence from fresh leaf specimens of 'confirmed' *S. herbacea* collected from The Botanical Gardens Edinburgh. If successful, this could then be compared with DNA sequence from fresh Austrian *Salix* samples and show whether the analysis of DNA sequences was in agreement with visual identification. Field preservation methods could then be assessed to provide information on the most suitable way to preserve fresh material for DNA analysis. The study would then be extended to investigate whether DNA sequence could be obtained from older samples. The extraction of DNA from herbarium specimens would illustrate the feasibility of extracting 'ancient *Salix* DNA', and would also provide a way of checking the viability of the extraction procedure for use on fossil material. Finally, the aim was to extract 'ancient DNA' from leaf macrofossils of different ages throughout the core to aid

in the confirmation of these samples. This would be especially useful when considering fossil leaf fragments. As the study of plant fossil ancient DNA is poorly studied this investigation additionally aimed to further understanding within the field.

5.1.1 Ancient DNA

Ancient DNA (aDNA) refers to nucleic acids present in preserved biological material. DNA and RNA can be extracted in trace amounts from fossils, subfossil remains, biological sources and museum specimens. The term aDNA thus incorporates any bulk or trace of DNA from a dead organism or parts of it, as well as extracorporeally encountered DNA of a living organism (Herrmann & Hummel, 1992). DNA that has undergone diagenetic or autolytic processes or that has been fixed in anyway after the death of the organism therefore can be considered to be ancient. Evidence of the presence of nucleic material in old plant tissues was originally demonstrated at the beginning of the 20th Century using staining methods (Herrmann & Hummel, 1992). The introduction of molecular cloning techniques led to rapid advances in the study of aDNA.

The presence of aDNA in an animal tissue source was first demonstrated successfully by Higuchi *et al.*, (1984). DNA was extracted and cloned from dried muscle taken from museum specimens of salt preserved skin of a quagga. This extinct zebra-like animal had died approximately 140 years previously. Pääbo later reported the molecular cloning of aDNA from much older material, that of Egyptian mummies, which had been radiocarbon dated to 2430±120 years BP (Pääbo, 1985). aDNA has also been extracted from plant material. Rogers & Bendich, (1985), extracted aDNA from herbarium specimens dated 22-118 years old and from mummified seeds and embryos ranging from 500 to greater than 44,600 years old.

The observation that aDNA was present in material of this age generated considerable controversy, as the stability and thus authenticity of ancient nucleic acids was doubted, especially since little progress followed these initial successes. It has been established that when an organism dies, its tissues quickly decompose and the DNA is heavily modified. Cell death exposes nucleic acids to a variety of damaging agents which lead to

chemical modification and thus, without preservation, DNA fragmentation occurs resulting in average DNA fragments of only a few hundred base pairs (Doran *et al.,* 1986; Pääbo, 1989). This fragmentation is thought to occur rapidly, since DNA of similar base pair length were observed in material obtained from a 13,000 year-old ground sloth of Southern Chile and a 4 year-old piece of dried pork (Pääbo, 1989). It is generally considered that less than 1% of the DNA molecules extracted from museum specimens or archaeological finds can be expected to be undamaged (Herrmann & Hummel, 1992).
Chances of obtaining aDNA are increased when the samples are present in preserving conditions. The preservation of material can be enhanced by certain environmental conditions, in particular the absence of water and microbial activity. Therefore xeric conditions offer ideal sites and samples recovered from such areas often contain aDNA. However, it has been reported that aDNA has been recovered from hydrated sites. Brain tissue was excavated from an 800 year-old human and found to be well preserved. The relatively high humidity, mineralised, anaerobic and non-acidic water saturated environment of the recovery site resulted in inhibition of bacterial and fungal growth, and ensuing degradation (Doran *et al.,* 1986).

The age at which aDNA could be extracted was extended with the examination of specimens encapsulated in amber (formed from plant resin). Insect specimens trapped in amber would have been encased rapidly. The sugar and terpenes in amber inhibit the growth of bacteria and act as a tissue preservative. Two reports simultaneously claimed the successful extraction and amplification of tiny remnants of DNA from both a 30 million year-old termite and a stingless bee fossilised in amber (DeSalle *et al.,* 1992; Cano *et al.,* 1992). These were followed by the account of the amplification and sequencing of segments of DNA from a 120-135 million year-old nemonychid weevil found in Lebanese amber (Cano *et al.,* 1993). However, it has not been possible to repeat the results of these reports. There is scepticism about the survival of DNA over extensive periods of time such as millions of years. Lindahl, (1993), argues that it is feasible to obtain useful DNA sequences from material tens of thousands of years old but due to degradation, it was not possible to recover sequence from older material.

In this study, only samples found in preserving conditions that permitted limited degradation and contained abundant DNA were of use for DNA analysis.

5.1.2 Amplification of aDNA.

A major problem of working with aDNA is that it is very low in abundance. A major breakthrough came following the introduction of molecular biology techniques, and in particular that of the polymerise chain reaction (PCR) which is discussed in depth later. PCR is a technique that enables amplification of large quantities of DNA from extremely small starting quantities of DNA. Using PCR technology, mitochondrial DNA sequences were obtained from a 7000 year-old human brain tissues excavated from Little Salt Spring in Florida (Pääbo *et al.*, 1988). This site preserved material well due to the anaerobic and neutral conditions prevailing in the waters of Florida peat bogs. The limits of the field were further extended with reports of DNA extraction from a 13,000 year-old specimen of an extinct ground sloth, and then a 40,000 year-old woolly mammoth preserved in Siberian permafrost (Pääbo, 1989). Originally only soft tissues were used in the search for aDNA until the presence of aDNA was demonstrated in human bones, the oldest dated to 545 BP (Hagelberg *et al.*, 1989).

The advent of PCR also allowed advances to be made with the amplification of aDNA from plant tissues. Very short nucleic acid fragments from 3300 year-old seeds of cress (*Lepidium sativum* L.) had been isolated by Rollo, (1985), but using PCR, Rollo *et al.*, (1989), went on to report the first clear evidence of aDNA in plant remains. These were in the form of endogenous DNA in maize kernels, radiocarbon-dated 980±95 ^{14}C years from a Huari archaeological site.

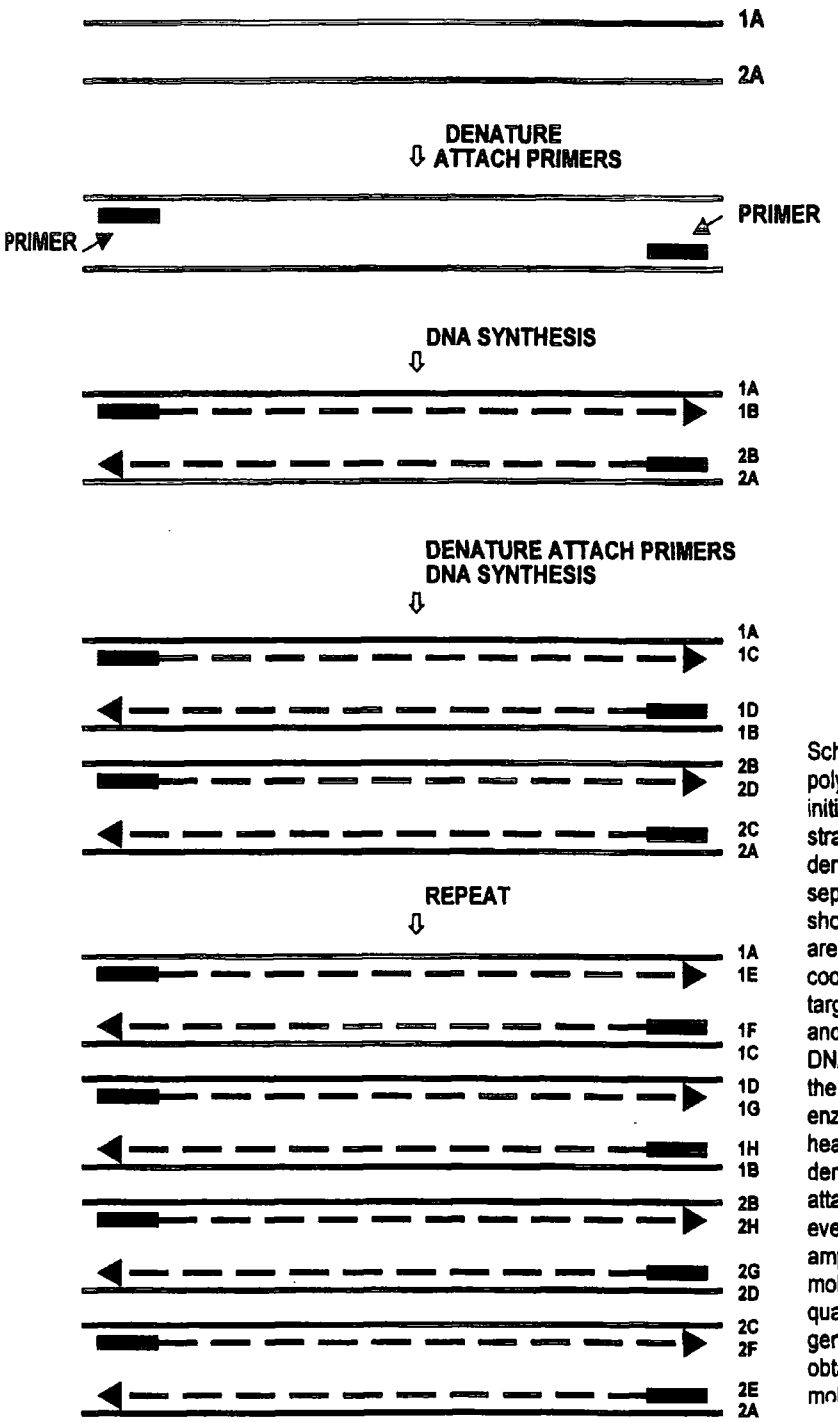
5.1.3 The Polymerase Chain Reaction.

The polymerase chain reaction (PCR) was invented at Cetus by Kary Mullis and others in 1985 (Mullis *et al.*, 1986; Mullis & Faloona, 1987). It was described as a "test tube cloning technique of extraordinary sensitivity and power" (Mullis *et al.*, 1986). Initial success in obtaining aDNA sequence was achieved by researchers using cloning techniques directly on extracted aDNA (Higuchi *et al.*, 1984; Pääbo, 1985). However,

due to the relatively low abundance of aDNA, cloning is often difficult to achieve. Successful DNA cloning requires the extraction of a significant amount of DNA from the material under investigation. Ancient samples are subject to damage and degradation and therefore it is highly likely that if DNA has been preserved it is only present in minute quantities and is fragmented. This can result in extremely low cloning efficiencies (Pääbo *et al.*, 1989).

PCR allows the *in vitro* amplification of specific regions of DNA from small amounts of genetic material, or in theory, a single DNA molecule. The process begins by denaturing the DNA sequence of interest (template) by heating to 94°C, resulting in the dissociation of the double helix. The region to be amplified is specified using two short synthetic oligonucleotide primers. These are chemically synthesised fragments of DNA, which are homologous to regions flanking the target DNA sequence. As the temperature of the reaction is dropped these primers are able to hybridise to opposite strands. The presence of a DNA polymerase and the four deoxyribonucleotide triphosphates in the reaction mixture allow DNA synthesis in the 5'-3' direction. The DNA polymerase is able to bind at the primer:template complex and extends the annealed primers at the exposed 3 prime (3') hydroxyl group end by adding complementary nucleotides. This extension is carried out typically at 72°C. The initial few amplification cycles are know as the screening phase, where the desired DNA fragment is selected by specific primer binding. The amplification stage of the subsequent cycles increases the copy number of the selected fragment exponentially (Saiki *et al.*, 1988). This process is summarised in Fig. 5a.

The first DNA polymerase enzyme to be used in PCR was the Klenow fragment of *Escherichia coli* DNA polymerase I (Saiki *et al.*, 1985; Mullis & Faloona, 1987). This polymerase was however, inactivated by the high temperature required by denaturation and therefore fresh enzyme had to be added at the onset of each cycle (Erlich, 1989). Amplification reactions of small segments of DNA (200 bp) generally were successful but results with larger templates were disappointing. Yields were often poor, and products were frequently heterogeneous in size (Scharf *et al.*, 1986).



Schematic representation of the polymerase chain reaction. To initiate the reaction a double stranded DNA molecule is denatured (i.e. the strands are separated) by heating. Two short oligonucleotdes ('primers') are added and the mixture cooled. The primers attach to target sites on the DNA strands and act as starting points for the DNA synthesis, catalysed by the DNA polymermerase enzyme. Additional cycles of heating and cooling in repeated denaturation – primer attachment – DNA synthesis events, leading to exponential amplification of the original molecule. In this way microgram quantities of DNA (sufficient for genetic analysis) can be obtained from a single staring molecule.

Figure 5A The principles of the Polymerase Chain Reaction (PCR)

A thermostable DNA polymerase, isolated from *Thermus aquaticus* (*Taq*) alleviated these shortcomings. *T. aquaticus* strain YT7, a thermophile, eubacterial micro-organism was isolated from a hot spring in the Yellowstone National Park as first described by Brock & Freeze, (1969). *Taq* DNA polymerase has a relatively high temperature optimum (T_{opt}) for DNA synthesis (Gelfand, 1989). Although *Taq* DNA polymerase has a very limited ability to synthesise DNA above 90°C, the enzyme is relatively stable and is not denatured irreversibly by repeated exposure to the high temperatures required for strand denaturation. Annealing and extension of oligonucleotide primers can be carried out at elevated temperatures, which greatly reduced non-specific primer binding, and produced homologous products. However, there are still some disadvantages of using *Taq* polymerase for PCR. Firstly it lacks 3'-5' exonuclease activity or "proof-reading" activity, which means incorrect nucleotides may be incorporated during DNA synthesis. If mis-matches are formed early on in the amplification process, these too will increase exponentially with each subsequent PCR cycle. Therefore, it is important to appreciate that minor discrepancies between DNA sequences obtained using PCR may not be true differences, but result from PCR errors. In recent times a range of DNA polymerases have been isolated or engineered, some of which have a proof reading activity eg. *Pfu* DNA polymerase and thus a lower error rate. However, these enzymes are relatively expensive, and are only used when accurate amplification is critical.

PCR Design and Optimisation.

Since PCR involves a number of complex interactions it is important to optimise the many variables. It is highly unlikely that there will ever be one set of amplification conditions that will prove optimal in all situations (Erlich, 1989).

Primer Design.

The most crucial PCR parameter in all cases is the primers. Primers need to be designed to anneal to the template DNA with high specificity so that only the synthesis of desired products proceeds. If non-specific primer binding occurs, non-specific amplifications will result. Non-specific amplifications are intensified if they occur during the early PCR cycles when very little template DNA is available, since every amplification cycle will

exponentially increase non-desired products. Primer design should incorporate the following parameters: They should have random base distribution and composition with a Guanine and Cytosine content of 50-60 %. A Guanine/Cytosine nucleotide "GC clamp" should be situated at the 3' end. This ensures strong annealing to the template on subsequent cycles due to presence of three hydrogen bonds between the Guanine and the Cytosine and hence reduces non-specific primer hybridisation, there should be no self complementarity and an absence of secondary structures. Primers are designed to be ideally 18-25 base pairs in length. Primer pairs should have similar melting points (annealing temperatures) to allow simultaneous melting as determined by Tm.

The approximate melting points of the primers are calculated by: $Tm = 4(G+C) + 2(A+T)$. Actual primers used for PCR, designed using these considerations are discussed later.

PCR Buffering conditions.

Each *Taq* polymerase is supplied with an optimal reaction buffer. Variation of the components of this reaction buffer can affect amplification success, therefore it is important to optimise conditions as discussed by Innis *et al.*, (1990).

Altering the magnesium (Mg^{2+}) ion concentration in a reaction can affect amplification specificity and yield. *Taq* polymerase requires Mg^{2+} concentrations normally of 1.5 mM $MgCl_2$ for optimum performance. A range of Mg^{2+} concentrations is usually used, with deoxynucleotide triphosphates (dNTP'S) added in excess. Mg^{2+} chelating agents can be present after some DNA extractions that decrease the overall Mg^{2+} concentration present in the reaction mixture, therefore it is necessary to increase Mg^{2+} concentrations. By applying a range of Mg^{2+} PCR conditions can be optimised.

Taq polymerase has an optimum concentration of between 1 and 4 units per 100 µl. If the enzyme concentration is increased above this level this can lead to greater production of non-specific PCR products.

Thermal Cycling .

As discussed, PCR requires thermal cycling of the reaction mixtures. Three temperatures are needed to accomplish the cycle. After the initial denaturation step, the temperature is lowered to allow the primers to anneal. This temperature is dependent on the specific characteristics of the primers (Tm), and is altered accordingly. If non-specific PCR products are formed during the reaction it is possible to increase the annealing temperature and thus the stringency of the primer annealing. The temperature is then raised for extension, the period of which is dependent on the size of the amplified product. As a rule of thumb extension is considered to proceed at a rate of 1kb/min. The number of cycles required to achieve successful amplification is dependent on the number of copies of target DNA present in the original sample. With modern specimens 30 cycles are usually sufficient, with ancient samples the number of cycles is typically increased to 40.

Other PCR considerations.

During the initial DNA denaturing step it is possible for primers to bind in a non-specific manner due to the low stringency binding conditions present at lower temperatures. *Taq* polymerase is known to possess activity at room temperature and can extend the non-specifically bound primers. During subsequent cycling the primers will bind as normal and the presence of the undesired products increase exponentially. It is possible to minimise this occurrence by using a method known as 'hot start PCR' as described by Bassam & Caetanoanolles, (1993). Hot start PCR prevents DNA synthesis until the temperature has reached 72°C and thus encourages specific primer annealing. This is achieved either by adding the *Taq* polymerase once the reaction temperature is above 72 °C or by separating the primers initially from the template by a layer of wax which then melts to allow mixing at 94°C.

Contamination.

Theoretically the PCR can amplify from as little as one molecule of DNA and hence minute amounts of contaminating DNA from other modern sources can also be amplified. It is especially important to eliminate contamination when universal primers are used due

to the fact that these primers are designed to conserved DNA sequences, and can therefore amplify DNA from a number of species. Modern contaminants are likely to be amplified in preference to ancient target molecules as the degree of degradation is probably much reduced. In order to minimise the risk of the amplification of a contaminant a number of precautionary measures can be adopted. Primers can be designed which are specific to the target organism, or targeted towards unique characteristic features, such as the chloroplast genome in plants and hence the amplification of human, bacterial and fungal matter which may become incorporated with the specimen during preservation is avoided.

A major problem when working with aDNA is that PCR amplified products from modern material could contaminate and "seed" subsequent amplifications in other samples. In order to minimise this risk, modern and ancient analyses were carried out in separate laboratories which were situated in different buildings. All solutions and reagents were prepared separately and kept isolated. Laboratory coats and gloves were always worn, these items were also contained in separate laboratories dependent on the material being analysed. Filtered tips were used to prevent any carry over contamination from pipettes during PCR and other experimental stages. In order to detect false positive results, blank controls were always run, these enabled the authenticity of any detected DNA from ancient and modern samples to be assessed. Reactions were always repeated to confirm the reproducibility of results obtained.

Amplification of damaged DNA in ancient materials may result in non-specific products which are caused by an effect known as 'Jumping PCR' (Pääbo *et al.*, 1990). Jumping PCR occurs when amplification starts from shorter fragments of the DNA instead of from intact template molecules spanning the entire interprimer sequence. The primers will be extended during the first PCR cycle up to the point where lesions or ends of fragments cause the polymerase to stop. In subsequent cycles, these extended primers can anneal to other template molecules and further extend. As cycling continues primer length is able to increase until 3' ends overlap. This overlapping produces a full-length double stranded molecule which is able to act as template for the conventional chain reaction (Pääbo *et al.*, 1989).

Despite these shortcomings PCR technology has led to great advancements in the field of aDNA. If it is applied carefully and controls incorporated PCR is the most effective and rapid method available for producing multiple copies of intact DNA which can be sequenced and analysed for phylogenetic studies.

5.1.4 Primer Choice.

Choosing a plant DNA sequence for investigation

It is very important to choose carefully the target DNA sequence before attempting to recover genetic information from ancient remains. The state of preservation of the DNA in the available sample must be taken into consideration. As discussed previously DNA is subject to degradation so a DNA sequence of realistic preserved size should be chosen. The choice of primers enables the size of the target DNA region to be determined. Ideal target sequences are present in multiple copies within the genome and therefore increase the chance of DNA recovery. In order to design specific primers for use with PCR it is necessary to have sequence knowledge of the sample under investigation. Since there was no known sequence data available for *Salix* it was necessary to use "universal primers". Universal primers have been extensively used when sequence information is not known. They are designed to anneal to conserved DNA sequence regions. The shortcoming associated with their use is that of an increased probability of amplifying any contaminating DNA.

When considering extraction and amplification of aDNA in plant material, three genomes can be targeted, mitochondrial, chloroplastic and nuclear.

Targeting Mitochondrial DNA.

Amplification of aDNA from humans and animals usually target mitochondrial DNA. Mitochondrial DNA (mtDNA) is present in several hundred copies per cell and is transmitted to the progeny uniparentally. Within mtDNA lie tracts such as the D-loop region which exhibit considerable intraspecific divergence and are flanked by conserved regions for primer design. It is due to the presence of such properties that mtDNA has been successfully used for analysing ancient human and animal remains (Higuchi *et al.*, 1987). Analysis of mtDNA is not as revealing in plants as such considerations apply only

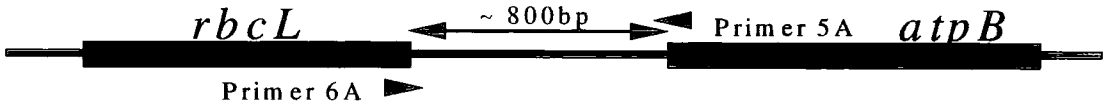
in part. Even though plant mtDNA is present in high copy number per cell and often inherited uniparentally, it has been found to undergo an extraordinary evolutionary pattern. The sequence evolution of mtDNA is slow with a rate of plant mtDNA point mutation that can be 100 times slower than that in animal mtDNA (Palmer & Herbon, 1988). Rollo *et al.,* (1989), reported that plant mtDNA was not expected to reveal appreciable polymorphism when evolutionary related organisms are compared.
Despite the reported successes of the use of mtDNA in the animal field very little has been reported on plant mtDNA sequences. It is therefore difficult to design primers suitable for use with plant material. For these reasons mitochondrial DNA was not targeted in this investigation.

Targeting Plastid DNA

Targeting the chloroplastic genome for aDNA analysis has been used (Golenberg *et al.,* 1990). The chloroplast (cp) genome of flowering plants consists of circular DNA molecules between 120 and 217 kilobases in size. Chloroplast genomes occur in thousands of copies in leaves, the chloroplasts themselves being differentially well preserved in comparison with other subcellular structures (Niklas *et al.*, 1985). Therefore, there is the increased likelihood of the presence of preserved DNA. The evolution of chloroplast genes is slow, allowing for intra- and inter- famial phylogenetic comparisons. Early comparative sequencing studies uncovered the conservative nature of cpDNA evolution (Zurawski *et al.,* 1984). This led to the development of sets of synthetic primers that correspond to relatively conserved stretches of cpDNA genes. The gene *rbc*L codes for the large subunit of ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco) and an extensive data set of *rbc*L sequences is available.

The material available for DNA analysis in this study was leaf matter. Subject to degradation it would therefore be appropriate to target the chloroplastic genome for PCR amplification.

Primers used for targeting Plastid DNA.



Primers were designed to amplify a region that was expected to be polymorphic (see above) (Al-Janabi *et al.*, 1994). An intergenic region between *rbcL* and *atpB*, approximately 800 bp of DNA was targeted.

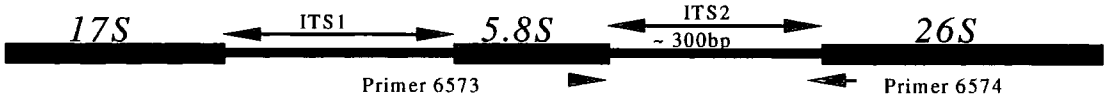
Primer 5A 5' GGAAAAGTGATATCCAGCAC 3'
Primer 6A 5' TTGAGTTGTTGTTATGGTAA 3'

Targeting Nuclear DNA

The overall rate of nuclear DNA evolution is about twice that of cpDNA (Doebley *et al.*, 1984; Zimmer *et al.*, 1988). All organisms encode ribosomal RNA genes (rDNA), the protein products of which make up approximately half of the mass of the ribosomes of both eukaryotes and prokaryotes. rDNA are arranged in tandem and are present in thousands of copies throughout the typical plant genome and can comprise of up to 10% of the total plant DNA (Hemleden, 1988).

rDNA is used for a broad range of phylogenetic studies due to the fact that different components of the rDNA show variation in their degree of conservation (Hamby & Zimmer, 1992). As the three ribosomal gene subunits 17S, 5.8S and 26S are very conservative throughout organisms with regard to both their length and nucleotide sequence these can be used for phylogenetic analyses at broad levels.

Internal transcribed spacer regions separate each gene sequence. The internal transcribed spacer regions (ITS) ITS1 and ITS2 lie in-between the 17S and 5.8S and 5.8S and 26S subunits respectively.



Primer 6573 and 6574 are shown above.

These ITS regions are more divergent in their nucleotide sequences and have been found to be quite variable even among closely related species. As these variable regions are flanked by the conserved subunits, it is possible to amplify these regions using primers designed to anneal to the subunits near to the ITS borders. ITS sequences have been used to gain phylogenetic information, and used to study evolution for example in the Compositae (Baldwin, 1992).

The ITS2 region was targeted in this study. There were several reasons for the choice. The ITS2 region is highly repeated and therefore demonstrates a high DNA copy number. The sequence flanking the conserved region is known and therefore primers can be designed to amplify this region. It is also known to allow differentiation to the species level and shows the greatest degree of variation. The target length would also be in the region of 300 base pairs, a site length which would be appropriate for use with preserved fossil material which is subject to the constraints of DNA degradation.

Primers used for targeting nuclear DNA.

The primers used in this study were modified from those of Sun *et al.*, (1994), and Hsiao, (1993). The top-strand primer had a GC:AT ratio of 14:5 and an approximate Tm of 66°C. This new primer was designated 6573.

6573 5' GTCTGCCTGGGCGTCACGC 3'

Sun *et al.*, (1994) used a primer situated 100 base pairs into the 26S gene. However, for this ancient DNA work in which the ITS2 region only will be considered a different primer was required. Therefore, the bottom-strand primer designated ITS4, which is complementary to the 26S rDNA near the ITS2 border used, was adapted from those designed by White *et al.*, (1990). This primer was renamed 6574 for these experiments.
6574 5' TCCTCCGCTTATTGATATGC 3'

6574 is a 20mer which contains a GC:AT ratio of 9:11, and requires an approximate Tm of 58°C.

5.1.5 Obtaining "Template" DNA from *Salix* for use in PCR.

Fresh Material

DNA is most readily extracted and amplified from fresh specimens. The collection of field material, however, often requires the sample to be preserved in some way before laboratory analysis. It is this preservation step which presents problems for the extraction and amplification of DNA which has high molecular weight and purity. In the past a variety of techniques have been employed to preserve specimens for herbarium, cytological and anatomical investigations. However, little was known about their effects on the integrity of DNA until the work of Doyle & Dickson, (1987), and Pyle & Adams, (1989).

The research of Doyle & Dickson, (1987), using leaf samples removed from *Solanum,* reported the effects of several methods on DNA preservation. These included the chemical treatments, formyl-aceto-alcohol (FAA) (70% ethanol : formalin : glacial acetic acid, 18:1:1), Carnoy's solution (ethanol:acetic acid, 3:1). 70 % ethanol, chloroform:ethanol (4:3) and brine (10 % NaCl), and also the effect of drying at 42 °C overnight and then pressing at room temperature. They found that all the chemical treatments resulted in DNA degradation. Although storage in chloroform:ethanol (4:3) for one week allowed the recovery of high molecular weight DNA, after this time rapid break down occurred. Of all the methods employed drying proved to be the most effective means of preservation. Further methods were attempted by Pyle & Adams, (1989). 27 treatments were carried out on leaves of spinach (*Spinacia oleracea* L) which were then examined to determine any effects on the quality and quantity of DNA. One of the methods used formaldehyde (7.4 %). Scott & Timmis, (1984), had previously achieved DNA restriction bands of 2-8 kbp following the use of 0.5 % formaldehyde in 0.01 M HEPES as a fixative. These bands appeared smaller after fixing for 1-2 hours, but were still apparent. The work of Pyle & Adams did not reveal the presence of such DNA restriction bands. In fact, of the chemical treatments, with the exception of EDTA, none were successful in preserving spinach leaf DNA after 24 hours. EDTA did not yield high molecular weight DNA after 7 days of storage.

These findings suggest that the exposure time and concentration of the fixation appears to relate to the speed of degradation. Only the extraction from fresh, frozen and dried leaves yielded good quality high molecular weight genomic DNA (30-50 kbp). The way in which the leaf was dried was also found to be important. Drying by microwaving for 3 minutes at 700 watts was found to cause DNA degradation. As spinach is a relatively easy species from which to obtain DNA, Pyle & Adams, (1989), also examined more difficult species such as oak, juniper and magnolia. These species are known to contain high tannin and other secondary compounds. High molecular weight DNA was obtained from fresh leaves of these species but it had undergone some degradation. Drying the leaves resulted in variable DNA yields that appeared to be species dependent. Such variation is likely to be attributable to the complexity of phenolics/tannins as well as polysaccharides either before or during extraction (Coradin & Giannasi, 1980; Doyle & Dickson, 1987; Doyle & Doyle, 1987). From the conclusions drawn from these studies it would appear that for field collection if it is not possible to examine fresh specimens then drying is the best preservative method. Freezing in liquid nitrogen is another alternative but this method requires additional field equipment that may not always be feasible. A rapid drying procedure is important for quality DNA preservation (Savolainen *et al.*, 1995). Prolonged drying periods induce extreme water stress, nutrient shortage and wounding, which in turn result in the production of phenolic compounds and free radicals. These can effect the success of DNA extraction.

Herbarium Material.

Herbarium collections offer enormous potential for the extraction and amplification of DNA. Encompassing an immense collection of taxa, herbarium material provides a means of studying molecular phylogenetics without having to resort to collecting living material of some rare species or travelling extensive distances. It can act as a reference with which to compare modern living field specimens, provide a sample of populations for studies of changing genetic structure and be used to settle taxonomic disputes. The routine use of chemical constituents preserved in dried herbarium specimens as taxonomic markers extends back at least 100 years most notably in its use in the systematics of lichens (Hale, 1983).

While herbarium and other dried material were regularly used for secondary metabolite studies, little attention was given to the possibility of the preservation of macromolecules of systematic influence (Giannasi, 1992). Advances in the development of restriction endonucleases (Crawford, 1990), in the late 1970's and early 80's coupled with Southern blotting analysis allowed the visualisation of DNA. Also, the advent of plasmid sub-cloning procedures and DNA sequencing (Ritland & Clegg, 1987), resulted in the isolation of and sequencing of, specific genes. Initially Rogers & Bendich, (1985), were successful in obtaining DNA from fresh, herbarium and mummified plant tissues. The aim of their study was to develop an isolation method suitable for use with milligram amounts of plant tissue. Herbarium specimens ranging from 22-118 years old were used. This work revealed on average DNA of 400 base pairs in length, however, sizes up to 20-30 kbp were found in all of the samples. They concluded that the extent of DNA degradation of the herbarium specimens appeared to be related to the condition of the leaf rather than the year it was dried. Leaf condition is dependent on how, and at what developmental stage the leaf was dried. It is possible to extract enough DNA from herbarium specimens to directly visualise DNA presence in electrophoretic gels, or indirect visualisation by hybridisation to labelled probe DNA. However, this approach is not desirable as it requires large quantities of herbarium material. Use of PCR, which enables the amplification of DNA preserved in minute template quantities in small amounts of leaf tissue, (ca 0.1 g or less), greatly extended the possibility of using herbarium material for DNA extraction. This technique greatly reduces the need to irreparably destroy large quantities of herbarium material. 30 mg of herbarium leaves (ca $1cm^2$) can be used in detergent minipreps of DNA (Liston *et al.,* 1992). However, extraction and amplification is not always "straight forward". As with freshly preserved material it would appear that the technique employed to preserve leaves as herbarium specimens can determine the success of recovering DNA. 17 herbarium specimens representing a variety of different conditions of preservation collected from 2-151 years were examined (Savolainen *et al.*, 1992). 4 DNA extraction methods were used, CTAB, DTAB, guanidine thiocyanate and silica and a further CTAB extraction protocol of Doyle & Dickson, (1990), as modified by Taberlet *et al.,* (1991). Several attempts were made to remove PCR inhibitory activities detected in the herbarium DNA extracts. Such

procedures were not successful with all samples. DNA templates could also be of insufficient quantity for amplification. Hence, PCR amplification was not always successful. As a result, out of the 17 DNA extractions in which inhibitory activities had been removed and PCR reactions optimised, only 8 were found to contain amplifiable cpDNA. 900 base pairs DNA fragments were amplified in 6 samples, and in the two remaining only 369 bp. There appeared to be no apparent correlation between the age of herbarium sample and the success of DNA amplification. There are numerous factors that appear to be related to the conservation of amplifiable DNA.

Fossil Material

aDNA from fossils is of limited availability and of variable quality and presents problems in assessing whether the samples are truly contemporaneous. However studies on fossil material demonstrate a wide range of applications. Fossil DNA may open the possibility for co-ordinated studies of macro- and microevolutionary patterns, reconstruction of relationships between contemporous taxa by inferring ancestral states and distances between them and in addition providing a tool to decouple the identification of species from morphology (Golenberg, 1992).

Considerable research had been carried out reporting the chemical analysis of organic constituents of fossilised plants and compression material, but little had been done relating to the possibility of finding preserved DNA. As suggested by Eglington & Logan, (1991), nucleic acids are one of the molecules most susceptible to destruction over time. To have aDNA leaf fossils would ideally be rapidly and directly deposited in order to minimise physical damage, and reduce external and internal induced degradation. Preservation in anoxic conditions may also reduce oxidative damage.

Plant fossils have been found to contain extractable DNA in a variety of locations. Rapid tissue dehydration had been considered to be necessary for DNA preservation, as determined by Pääbo, (1989). However, the successful extraction of DNA from a number of fossil tissues that have been hydrated throughout preservation has been reported.

These include plant matter such as the *Magnolia* (Golenberg *et al.,* 1990), and *Taxodium* (Soltis *et al.,* 1992). However further work of Sidow *et al.,* (1991), revealed that the high molecular mass DNA that can be detected in some extracts from Clarkia fossils is mostly, if not exclusively of bacterial origin. Neutral conditions may also be considered more suitable for DNA preservation than acidic or alkaline ones. The field of plant fossil aDNA extraction has been poorly studied. There are few examples of plant fossil aDNA extraction and those such as Golenberg *et al.,* (1990), and Soltis *et al.,* (1992), have not been repeated.

In spite of the factors known to effect DNA preservation, it is obviously not always possible to recover the required fossil material under ideal conditions. However, by rapidly processing fossil material, reducing exposure to oxygen, light, water and external debris would aim to enhance the chance of DNA recovery.

The high profile research that has been outlined in this introduction has received considerable attention. It is possible to apply aDNA technology as a tool with which to authenticate the identity of material. This is successful if the technology is carefully controlled to omit contamination. Therefore, if it is possible to isolate DNA from the various *Salix* samples the aims of this research are realistic.

5.2 Materials and methods

5.2.1 Chemicals and preparation kits.

All inorganic chemicals were of AnalaR quality and purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. unless otherwise specified.

All organic chemicals and enzymes were from Sigma Chemicals Plc., Poole, Dorset, U.K. unless otherwise specified.

Lab M Nutrient Broth (no. 2), Lab M Nutrient Agar, Amersham Ltd., Bury, U.K.

Restriction endonucleases, corresponding buffers, *Taq*, T4 DNA ligase, Klenow enzyme, X-gal and wild type λ DNA were IGI Fermentas.

Agarose was from BRL, Gaithersburg, U.S.A..

Metaphor agarose was from FMC BioProducts.

Polaroid film was from Polaroid (UK) Ltd., St. Albans, Hertfordshire, U.K..

Laboratory sealing film was from Whatman International Ltd., Maidstone, U.K..

Primers were from Perkin Elmer Ltd.

QIAGEN kits were from QIAGEN Ltd., Boundary Court, Gatwick Road, Crawley, West Sussex.

5.2.2 Sources of *S. herbacea* leaf material.

Norwegian leaves.

S. herbacea leaves were kindly collected by Dr. J. Allen at 1230m from Norway and pressed dried.

Austrian leaves.

Fresh *S. herbacea* leaves were collected from the Oeztaler Alpen, Austria. The fresh leaves used in this study were collected in July 1995 from Timmelsjoch 2500m.

S. herbacea leaves stored in FAA were also taken from Timmelsjoch 2500m in July 1995.

Royal Botanical Garden Edinburgh leaves.

R. McBeath kindly supplied fresh leaves from the Botanical Gardens Edinburgh.
These leaves were catalogued 19721425 *S. herbacea* L. Taken from a 1972 cutting. Source Brownlie, J.S.N collected directly from wild; origin known United Kingdom; Scotland: Perthshire: Glen Isla, Caenlochan.

Herbarium leaves.

The Natural History Museum London kindly supplied herbarium leaf material. The samples used in this investigation were catalogued as Flora of the British Isles. Herb. Musei Britannici, *S. herbacea* L. Origin Perth: Sow of Atholl. 11.9.32 Collected by G. Taylor and described by" the flat suborbicular leaves show the glossy upper surface, more glossy under surface and raised venation on both sides. This is the common form".

Fossil leaves.

Macrofossil leaves were extracted and stored from the Morrone Birkwood core. Leaves for these studies were taken from the core section 313-318, from which the greatest numbers of macrofossils were recovered.

5.2.3 DNA techniques.

Aseptic conditions.

Aseptic conditions were used whenever feasible. Plasticware, glassware and other equipment were sterilised by autoclaving prior to use (15psi, 121°C, 20 minutes). Unless otherwise stated solutions were prepared according to Sambrook *et al.,* (1989), and

autoclaved or filter-sterilised through a 0.22μm nitrocellulose filter (Sartorius) into a sterile container.

Ancient DNA techniques.

Sterile gloves, laboratory coats and face masks were worn throughout experiments. The extraction of aDNA was carried out in a separate laboratory to that of modern DNA. This laboratory contained a laminar flow cabinet, a separate stock of all solutions and equipment including a PCR block. The extraction of aDNA and the set up of PCR reactions were carried out in a sterile laminar flow cabinet. In addition filtered tips were used throughout aDNA extraction and amplification.

DNA extraction technique for leaves.

The CTAB (Hexadecyl-trimethyl-ammonium bromide) modified extraction protocol (Rogers & Bendich, 1985), was used to extract DNA from plant tissue. Centrifugation, unless otherwise stated, was carried out at 13,500g

Sampled leaves were placed in a pre-chilled pestle and mortar and ground under liquid nitrogen to a fine powder. This was followed by the addition of 1ml of CTAB extraction buffer (2% CTAB, 100mM Tris HCl pH8.0, 1.4M NaCl, 20mM EDTA), which had been pre-warmed to 60°C. was added. The extract was pipetted into a 1.5ml Eppendorf tube and incubated at 60°C for 30 minutes.

The large debris was removed by centrifugation at 13,5000g for 3 minutes. The supernatant was transferred to a fresh tube and two volumes of chloroform : isoamyl alcohol (24:1) was added. The sample was vortex mixed for ten seconds and centrifuged for 2 minutes, and the upper aqueous layer was pipetted off into in a fresh Eppendorf tube. The phase-separation was then repeated.

The DNA was then precipitated by the addition of 2 volumes of precipitation buffer (1% CTAB, 50mM Tris-HCl pH8.0 and 10mM EDTA) to the aqueous layer and left at 4°C for 1 hour. The precipitated nucleic acids were pelleted by centrifugation for 10 minutes after which the supernatant was removed. The pellet was washed with 1ml of 70%

ethanol and centrifuged again. The pellet was air dried at room temperature, and resuspended in 50µl of TE buffer (10mM Tris-HCl pH8.0, 1mM EDTA).
DNA samples were stored at -20°C. A control extraction ('the extraction blank') was always carried out where no leaf material was used in the extraction procedure. This was to monitor for any contamination, and if any was seen the results were discarded.

Agarose gel electrophoresis.

DNA samples were separated using agarose gel electrophoresis.

TAE agarose gels.

Agarose gels were used according to the predicted sizes of the DNA to be analysed. Lower percentage, (0.7%) gels were used to separate linear DNA of the range 800bp to 10kb, whereas higher percentage (2.0%) gels were used to separate PCR products of the range 100bp to 2kb.

Agarose was added to 1 x TAE (50 x stock = 242g Tris, 100ml EDTA pH8.0, 57.1ml glacial acetic acid per litre (Sambrook *et al.,* 1989),) according to the percentage gel required and dissolved using a microwave oven. Upon cooling the solution to approximately 60°C, ethidium bromide (EtBr) (10mg.ml^{-1}) was added to a final concentration of 0.2µg.ml^{-1}. EtBr interacts with the DNA helix and allows visualisation under UV light. The gel was then poured into gel trays of either 100 x 80 mm (volume 70ml) or 77 x 55 mm (volume 50ml) in size to which combs had been added and left to set. Gels were run in an electrophoresis tank (Pharmacia GNA-100) containing running buffer (1 x TAE, containing EtBr 0.2µg.ml^{-1}).

TBE metaphor agarose gels.

A 2% metaphor agarose gel accurately resolves PCR products in the range 100bp to 1kb. TBE percentage gels were made as for TAE gels, but replacing TBE for TAE (10 x TBE stock = 216g Tris, 110g boric acid, 18.6g EDTA). Once set, it was placed at 4°C for 30 minutes. Samples were loaded onto the gel which was then submerged in TBE running buffer (1xTBE containing 0.2µg.ml^{-1} EtBr).

In order to visualise faint DNA bands it is possible to omit the addition of EtBr in the gel and running buffer mixtures, due to the fact that EtBr can cause bright staining across the gel. The post-electrophoresis staining step was achieved by placing the gel in a fresh tray containing 100ml of distilled water and 0.2μg.ml^{-1} EtBr for 20 minutes. The gel was then rinsed with distilled water and then placed in fresh distilled water for at least one hour.

Size markers

In order to estimate the size of DNA bands, size markers were run on each electrophoresis gel.

Lamda *Pst*I markers (λ*Pst*) are used on 0.7% agarose gels.
λDNA was digested with *Pst*I and produces a series of fragments of the following sizes in kilobases:
14.05, 11.49, 5.07, 4.75, 4.51, 2.84, [2.56, 2.46, 2.44] 2.14, 1.99, 1.70, 1.16, 1.09, 0.81, 0.52, [0.47, 0.45], 0.34.
The sizes enclosed in square brackets are generally seen as a single band as they migrate together on an agarose gel. Small fragments are also produced but rarely seen.

100bp ladder (MBI Fermentas (0.5mg/ml)) was used on 2% and 3% TAE and TBE agarose gels.
This ladder produced a series of fragments 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 and 80 base pairs in length.

Loading samples.

Loading buffer was added to each sample to ensure that DNA remained in the wells. 6 x Gel Loading Buffer was used (Sambook *et al.,* 1989), (0.25% bromophenol blue, 0.25% cyanol FF and 40% sucrose in distilled water and had been filter sterilised and stored at 4°C). The DNA samples (and size markers) were loaded and electrophoresis carried out at 5-10V.cm^{-1} for the required amount of time.

Gel band isolation.

The DNA band was visualised and excised from an agarose gel using a sterile scalpel, and placed in a sterile Eppendorf tube.

Purification of DNA from an extracted gel band.

Two methods of purification of the extracted DNA band from agarose were used.

a) The QIAGEN Quick Gel Extraction technique was used.
The protocol used was according to the manufacturer's instructions.

b) Silica fines (protocol, a gift from Prof. N.J. Robinson).

Preparation of the silica fines.

250ml of silica 325 mesh powder was resuspended in distilled water to give a total volume of 500ml. The suspension was stirred for 1 hour and left to settle for a further hour. The suspension was then centrifuged at 5000g in a Beckman J2-HS centrifuge using a JA 14 rotor. The pellet was resuspended in 150ml of distilled water until the pH was greater then 5.5. Silica fines were stored at 4°C as 50% slurry in sterile distilled water.

Silica fines protocol

Two volumes of sodium iodide solution (90.8g NaI, 1.5g Na_2CO_3) dissolved in distilled water and saturated with 0.5g $Na_2 SO_3$, stored at 4 °C in a light proof bottle) were added to the DNA solution (minimum 200µl volume). This was heated at 70°C and mixed occasionally until the agarose fragment had completely melted. The tube was then left at room temperature to cool for 5 minutes. 5µl of silica fines were then added to the DNA and mixed continuously for 10 minutes to allow the DNA to bind to the silica fines. This was followed by centrifugation at 13,500g for 15 seconds. The supernatant was removed and the fines pellet washed with 1 ml of 70 % ethanol. After drying the silica fine pellet under vacuum it was resuspended in 50µl of TE buffer (10mM Tris-HCl pH 7.5, 1 mM EDTA), vortexed and left for 10 minutes at 37°C with occasional shaking to elute bound

DNA. After microcentrifuging for 15 seconds the supernatant containing the DNA was collected and saved. The fines pellet was then resuspended once more in TE buffer and the latter step repeated.
The DNA was stored at -20°C.

5.2.4 Amplification using PCR.

PCR reactions were set up in a laminar flow cabinet. Reactions using modern leaf DNA extractions were set up in different laminar flow cabinets and in separate laboratories to those using ancient material in order to minimise the risk of cross contamination. The extraction blank that had been prepared at the same time was also used as a template in PCR reactions to reveal any contamination. In addition a further blank was set up at this stage. This blank involved the addition of sterile water to a PCR reaction that would further monitor contamination

The amplification reactions usually took place in a reaction volume of 25µl.
In a typical reaction mixture being as follows:

REAGENT	STOCK CONCENTRATION	FINAL CONCENTRATION
dNTP's	2.5mM	0.2mm (each)
Magnesium ions	25mM	1-4mM
Taq polymerase	4-5units/µl	1 unit
10 x buffer contents	10 x	1 x
Primers	Varied	10 pmol (each)

The DNA extract template was added to the above reaction. When using DNA from modern specimens, 500ng was used as a template. A dilution range was established to optimise the quantity of aDNA required. Inhibitors that are often found to be present in the extraction could seriously effect PCR success, and result in the absence of PCR products. By diluting the extracts and thus altering the concentration of template DNA added it is possible to alleviate such problems. The dilution series usually consisted of 100% extraction, that is undiluted extract, 10% extraction (1µl DNA extract with 9µl milli-Q water) and 1% extraction (1µl DNA extract with 99µl milli-Q water).

Amplification reactions were set up in PCR Eppendorf tubes. In order to prevent evaporation of the reaction mixture, modern DNA specimens required the addition of a layer of mineral oil before they were placed in a Perkin Elmer Thermal Cycler. As a Perkin Elmer 2400 Thermal Cycler was used for ancient reactions which had the additional facility of a heated lid, it was not necessary to add mineral oil to these samples.

When 'Hot start PCR' (Bassam & Caetanoanolles, 1993), was carried out it was necessary to separate the reaction mixture. This ensured that synthesis did not occur prior to the first annealing cycle and thus reduces the risk of non-specific primer annealing. This was achieved by adding a volume of 12µl of dNTP's, 10 x buffer, Taq and one primer, and then a tablet of wax. The wax was then melted and solidified and thus formed a barrier that separated the 13µl volume of template and second primer layer.
A typical PCR involving rDNA primers 6573 and 6574 is shown in Table 5.1.

Table 5.1 The PCR reaction mixture set up using 'Hot Start PCR' The wax layer initially separates the reaction mixture. All given volumes are in µl.

Reaction	H_2O	Buffer	dNTP	Mg^{2+}	*Taq*	6573	W	H_2O	6574	Temp*
Blank	4.35	2.5	2	2	0.2	0.95	A	12.15	0.85	0
Ex Blank	4.35	2.5	2	2	0.2	0.95	X	11.15	0.85	1
1 µl DNA	4.35	2.5	2	2	0.2	0.95	W	11.15	0.85	1
$^1/_{10}$ µl DNA	4.35	2.5	2	2	0.2	0.95	A	11.15	0.85	1
$^1/_{100}$ µl DNA	4.35	2.5	2	2	0.2	0.95	X	11.15	0.85	1

(* temp= template, 6573 and 6574 are rDNA primers)

In some cases a "Band Stab" was carried out to supply template (Bjourson & Cooper, 1992). This involved stabbing a sterile needle into the DNA band on the agarose and the corresponding site on the blank lane and swirling these in the PCR reaction mixture.

The annealing temperature was dependent on the DNA sequence of the primers was calculated. The extension time of approximately 1Kb/minute was determined by the size of the expected products. For rDNA primers the PCR cycles were:

94 °C for 5 minutes
94 °C for 1 minute]
60 °C for 30 seconds] Cycles of x 25 to x 40 (depending on material)
72 °C for 15 seconds]
72 °C for 5 minutes
(94°C denature, 60°C anneal and 72°C extension)

Filling in 5'/3' -recessed termini "Blunt Ending".

A characteristic of many thermostable DNA polymerases, including *Taq* polymerase, is that they add an extra nucleotide, typically adenine, to each 3' end of synthesised DNA strand. This base addition interferes with blunt-end cloning. It is possible to fill in this base overhang (blunt end) of the DNA, and thus remove the extra nucleotide by incubating the reaction for 30 minutes at 37°C with T4 DNA polymerase, or the Klenow fragment of DNA polymerase. In practice 50µl of gel extracted DNA was "blunted ended" by adding 1µl of T_4 DNA polymerase (or Klenow DNA polymerase), 5µl of dNTP's (a solution containing all 4 dNTP's each at 1mM) and 14µl of T_4 DNA polymerase buffer (5x) (or Klenow buffer 10x).

Purification of PCR products.

PCR products were purified using the QIAGEN PCR Purification Kit according to the manufacturer's instructions and the concentration of DNA was assessed on a gel relative to that of the vector before ligation.

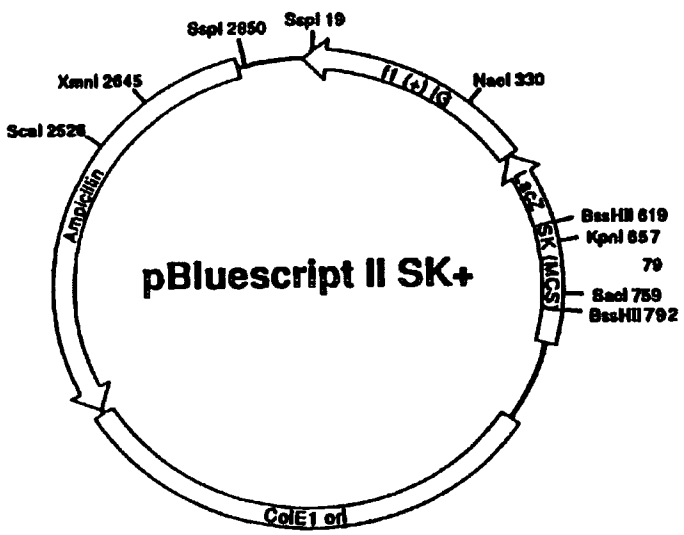
5.2.5 DNA cloning.

Cloning of PCR products into Bluescript SK+ vector

Ligation of DNA into Bluescript SK+.

The "blunt ended" DNA was ligated into Bluescript II SK+ plasmid that had been cut with the restriction endonuclease *Sma*I.

Bluescript SK+



Reverse Sequencing Primer
5'AACAGCTATGACCATG3'

Sac I *Sac* II
5'GGAAACAGCTATGACCATGATTACGCCAAGCGCGCAAT TAACCC TCACT AAAGGGAACAAAAGCTGGAGCT CCACCGCGG
3'CCT T TGTCTATACTGGTACTAATGCGGT TCGCGCG TTAATTGGGAGTGAT T TCCC TT GTTT TCGACCTCGAGGTGGCGCC

Not I *Xba* I *Spe* I *Bam*H I ***Sma I*** *Pst* I *Eco*R I *Eco*R V *Hind* III *Sal* I *Xho* I
TGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGG
ACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAAGCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCC

Apa I *Kpn* I
GGGG GCCCGGTACCCAATT CGCC CTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAA3' +
CC CC CGGGCCATGGGTTAAGCGGGATATCACTCAGCATAATGCGCGCGAGTGACCGGCAGCAAAATGTT5' -
3'TGACCGGCAGCAAAATG5'
M13 - 20 Forward Sequencing Primer

The fragments of insert and vector DNA were mixed together at a ratio of 20:1 (insert:vector) with a maximum of 300ng DNA in a total volume of 16µl. 2µl of 10 x T4 DNA ligase buffer (MBI Fermentas) was then added to along with 2µl (1 unit) of T4 DNA ligase to make a total volume of 20µl. The reaction was then incubated at 16°C overnight.

Bluescript SK+ is a cloning vector that contains two selectable marker genes. The first, an antibiotic (ampicillin) resistance gene allows selection of transformed bacteria on ampicillin containing media. The second, the *LacZ* gene (β-galactosidase) allows blue / white colour selection of bacterial colonies. A multiple cloning site (MCS) exists within the *LacZ* gene. Under normal conditions, β-galactosidase, an enzyme that hydrolyses lactose and other galactosides into glucose and galactose is expressed. The addition of the galactoside Xgal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) to the selection plates enables transformed bacteria to be screened for insertion of DNA into the MCS. Xgal is hydrolysed by β-galactosidase therefore if an active β-galactosidase is expressed a blue indolyl derivative is formed within the colony. This indicates no DNA has been inserted into the MCS. If the *LacZ* gene has been inactivated by the insertion of DNA into the MCS, the Xgal cannot be hydrolysed and white colonies are formed.

*Sma*I cuts at a target site within the MCS in the *LacZ* gene. The ligation depends on the ability of DNA ligase catalysing the formation of a phosphodiester bond between 5' phosphate and 3' hydroxyl termini in blunt ended duplex DNA. Once the DNA of interest has been successfully ligated into the vector, competent bacteria (*Eschericia coli* DH5α) were transformed with the plasmid (Hanahan, 1983).

Preparation of competent cells.

5ml of LM. Broth (2g made up to 1 litre with distilled water gave a final concentrations of $10g.l^{-1}$ beef extract, $10g.l^{-1}$ balanced peptone no.1, $5g.l^{-1}$ NaCl, pH7.5 ± 0.2.) was inoculated and incubated overnight at 37 °C. This culture was subcultured 1 in 100 into fresh LM Broth. These cells were grown at 200rpm shaken to an optical density at 550nm (OD_{550nm}) of 0.3-0.35. Cultured cells were chilled on ice for 5 minutes before being centrifuged down in pre-chilled centrifuge tubes at 4000g, 4°C for 7 minutes. The supernatants were poured off and the cell pellets resuspended in 2/5 volumes of TF_{bI} (30mM potassium acetate, 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM manganese chloride and 15% glycerol. The solution was adjusted to pH 5.8 with 0.2M acetic acid and filter sterilised). The tubes were held on ice for 5 minutes and centrifuged as before. The cells were resuspended in 1/25 volume of TF_{bII} (10mM MOPS, (3-(*N*-morpholino) propane sulphonic acid) 75mM $CaCl_2$, 10mM $RbCl_2$ and 15% glycerol. The

solution was adjusted to pH6.5 with KOH and filter sterilised) and left on ice for 15 minutes. The stock was then aliquoted into pre-chilled Eppendorfs, frozen in liquid nitrogen and stored at -80°C.

Transformation of the vector DNA and insert. "Ligation Reaction".

The transformation procedure used was a modified version of that described by Hanahan, (1983). DH5α cells were thawed and then placed on ice for 10 minutes. The DNA was added (up to 2/5 volume cells/250ng/200µl cells) and the tube held on ice for at least 15 minutes (up to 45 minutes). The cells were then heat shocked at 42 °C for 90 seconds and held on ice for 2-3 minutes. 800µl of room temperature LM Broth was added. The tube was then incubated at 37°C for 60 minutes on a shaker.

All solutions and bacterial cultures were spread onto the agar plates using a glass spreader that had been sterilised in 70% ethanol. Pre-warmed LB-agar plates (containing 100µgml^{-1}ampicillin) containing 40µl of Xgal (20mg/ml). The plates were incubated at 37°C for 60 minutes. 100µl aliquots of transformed DNA in broth was spread on one plate and labelled 1/10. The remaining cell suspension was then centrifuged down. The supernatant was removed until 100µl including pellet remained. The pellet was resuspended and applied to a second plate and labelled 9/10.
Plates were incubated at 37°C overnight.

A selection of transformants was removed with either a flamed loop or a sterile cocktail stick. These were then inoculated in 5 ml of L-Broth that contained 10 µl of ampicillin (25mg/ml). The culture was shaken overnight at 37°C.

Plasmid minipreps.

The standard protocol of the QIAGEN QIA-prep-spin Plasmid kit was performed to recover plasmid DNA from DH5α cells. Samples were eluted in 50µl of water.

Restriction endonuclease digestion.

In order to ensure that a correct size insert is present, endonuclease restriction enzymes were used to cut out the insert from the vector. *Bam*HI and *Eco*RV sites were used unless otherwise stated. Digests were set up as follows. 11µl water, 5µl of DNA, 2µl of enzyme buffer, 1µl *Eco*RV and 1µl of *Bam*HI. This was incubated at 37°C for 2 hours. Digestions were then run on an agarose gel for assessment.

5.2.6 Sequencing of DNA.

DNA sequencing requires an approximate minimum of 500ng of DNA in 10µl of sample. In order to assess DNA concentration prior to sequencing 1µl of uncut DNA in a volume of 10µl is run on a 0.7% TAE agarose gel against 1µl of the DNA concentration marker pGEM (200ng/µl). If the concentration is assessed to be sufficient, sequencing proceeds. In the case of direct sequencing, i.e. before cloning, the primers used in the sequencing reactions are the same as those for the PCR. Cloned samples were sequenced using Universal M13 forward and reverse primers that flank the MCS and therefore it was possible to sequence through the insert to the other side.

DNA was analysed using the Applied Biosystems 373A DNA sequencer. Fluorescent labelled Univesal M13 primers from the Applied Biosystems PRISM™ Ready Reaction Dye Primer Cycle Sequencing Kit were used according to manufacture's instructions.

5.2.7 Computer analysis of DNA.

Preliminary DNA analysis was carried out using DNA Strider™ and the University Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Software Package at BBSRC , Daresbury, UK. The DNA sequences were compared with GenBank and EMBL databases using the FASTA program. Alignments were performed using the gap program which enabled comparison of two sequences, and the Clustal V program which was used for multiple sequence comparison.

5.3 Results.

5.3.1 PCR using chloroplastic *rbcL* primers.

PCR of DNA from press dried *S. herbacea* leaves taken from Norway.

PCR was attempted using the chloroplastic *rbcL* primers on dried Norwegian *S. herbacea* leaves, to assess their suitability for use on leaf samples.

DNA was extracted from four press-dried leaves using the CTAB extraction method.

A 10µl aliquot of the DNA extract obtained was analysed using a 0.7% TAE agarose gel. A λ/*Pst* I marker ladder was run on the gel to size the DNA, and an extraction blank (containing no leaf material).

A genomic DNA band and ribosomal (rRNA) bands were observed as expected in the extract as shown in Fig.5.1. The absence of bands in the extraction blank indicated that there was no visible DNA contamination from other sources during the extraction procedure.

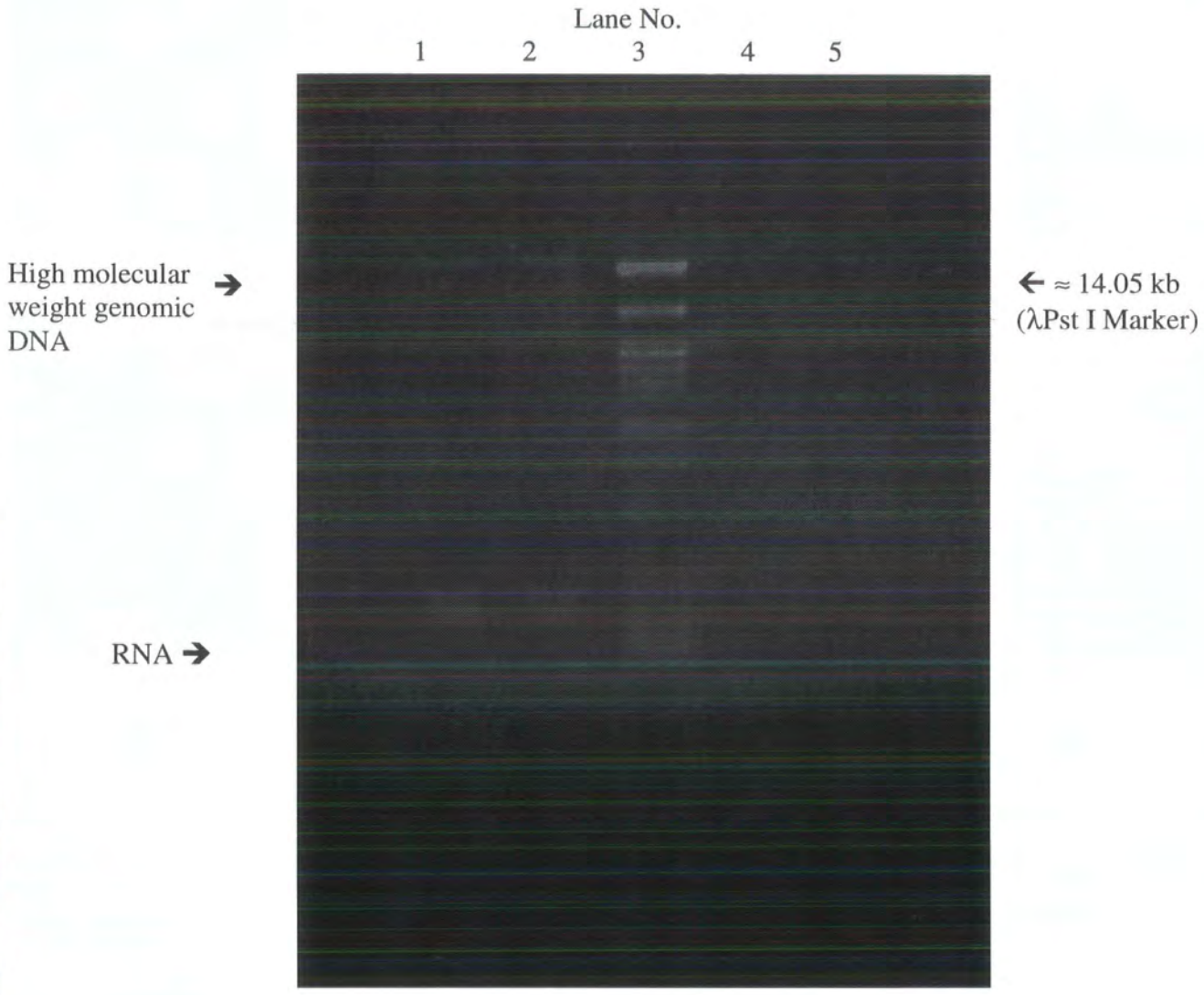
The genomic DNA band was excised and purified using the silica fines protocol for use in PCR. A section was also excised and purified from an identical area on the extraction blank lane as a negative control in the PCR experiments. The purified DNA was checked by running triplicate aliquots on a TBE metaphor gel. Band stabs were made using the method described in materials and methods.

The PCR conditions used were as follows: 94°C for 5 min; (94°C for 1 min, 60°C for 1 min, 72°C for 1 min) x35 cycles; 72°C for 5 min.

Figure 5.2 shows the single band PCR products obtained using a Mg^{2+} range and visualised on an agarose gel. The extraction blank control and the PCR negative controls produced no visible products, indicating the absence of contamination during the extraction, amplification and preparation of the DNA used for PCR. The experiment was repeated and these results found to be reproducible.

To allow DNA sequencing of the PCR products they were cloned into the pBluescript SK+ plasmid at the *Sma*I site and transformed into DH5α using the method described in materials and methods. Plasmid DNA was recovered from 'white' colonies using the QIAGEN QIA-prep-spin DNA miniprep kit.

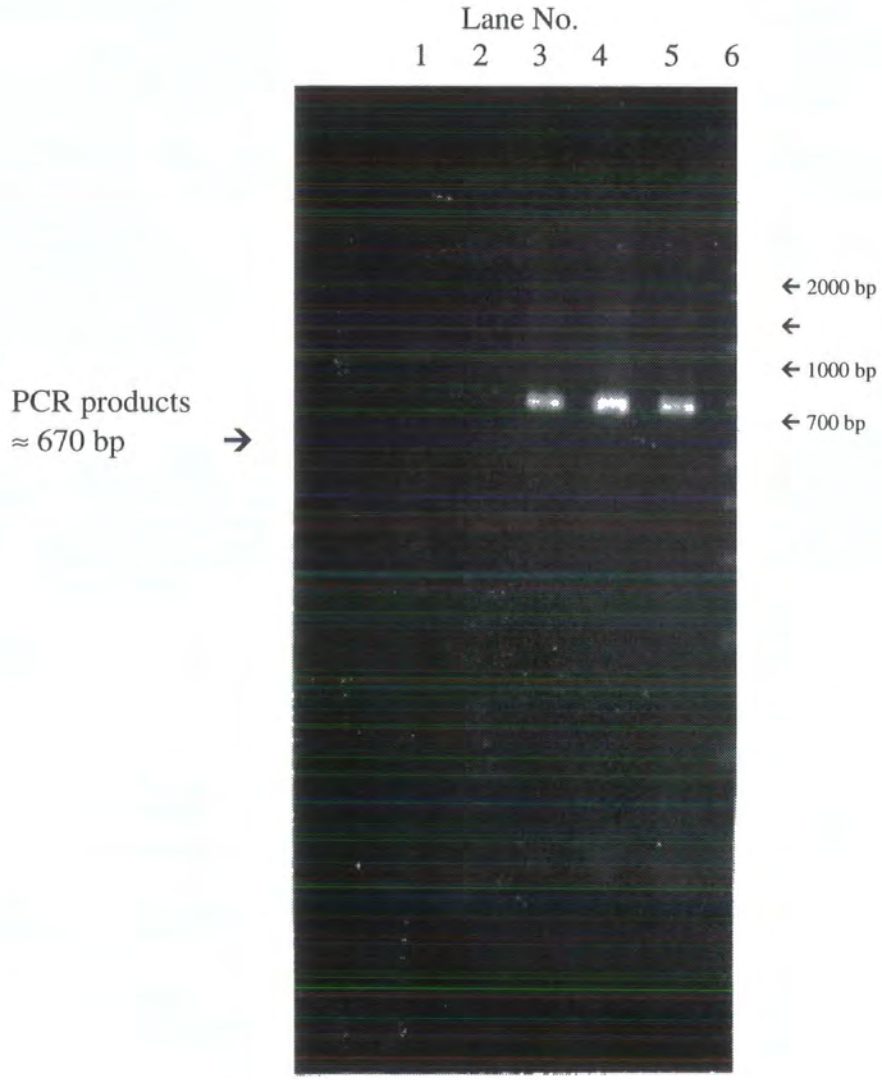
Figure 5.1 **Image of agarose gel showing genomic DNA bands and rRNA bands observed in the DNA extraction of *Salix herbacea* collected in Norway**



Lane 1	Extracted DNA Norway (1)
Lane 2	Extracted DNA Norway (2)
Lane 3	λPst I Marker
Lane 4	Extraction Blank (1)
Lane 5	Extraction Blank (2)

Figure 5.2

Image of agarose gel showing PCR products observed using *rbcl* 5A and 6A primers on extracted DNA from *Salix herbacea* collected in Norway.



Lane 1	Extraction Blank
Lane 2	PCR Blank
Lane 3	DNA Mg 1
Lane 4	DNA Mg 2
Lane 5	DNA Mg 3
Lane 6	Marker 100 bp Ladder

To ensure that the plasmid DNA recovered contained a cloned insert of the correct size it was cut with the endonuclease restriction enzyme EcoRI (see pBluescript SK+ map Page 172). Cutting with EcoR1 causes the plasmid DNA to linearise, and hence it can be sized against known markers. A plasmid containing an insert will therefore appear larger when analysed on an agarose gel than one containing no insert.
The plasmid DNA obtained was quantified using know DNA standards and was sent for DNA sequence analysis.

The use of the *rbcL* primers amplified a region of 661 base pairs. This sequence was compared with known sequences on the GenBank plant DNA database using the SEQNET program FASTA. The 661 base pair sequence was found to be most closely matched to *Zea mays* chloroplast coupling factor complex (CF-1) beta & epsilon (*Z. mays*) (accession No. J01421; Krebbers *et al.,* 1982). The sequence was found to be a contaminant as it was closely matched to a cereal, and cereal analysis was also undertaken in the laboratory. As DNA is known to degrade considerably over time and fragment the target site that was in excess of 600bp is therefore not an ideal target for amplifying DNA from fossil material. It was aimed to use internal primers to amplify a region of 300bp if these external *rbcL* primers had been successful. However, from this result it would appear that apart from targeting a site that is too big for aDNA work the primers were also too universal. In addition, at the time of this work there were problems with laboratory water systems that had led to the amplification of water algae DNA. Therefore a smaller region of approximately 300bp was targeted using the rDNA primers.

5.3.2 The use of ribosomal (rDNA) primers.

PCR of press dried *S. herbacea* leaves taken from Norway.

Dried *S. herbacea* leaves were used to determine whether the rRNA primers were compatible with this species.

DNA was extracted from press dried leaves using the CTAB method. Extracted DNA was purified using electrophoresis and the silica fines method described previously. This DNA was then subjected to the PCR.

The PCR conditions used were as follows: 94°C for 5 min; (94°C for 1 min, 60°C for 30 s, 72°C for 15 s) x 35 cycles; 72°C for 5 min.

A 10µl aliquot of each PCR reaction (total 25µl) were loaded onto a 2.0% TBE metaphor agarose gel and sized using the λ/*Pst* I ladder.
A single band product of the expected size was observed (≈ 280bp) and its authenticity checked by direct sequencing.

The GenBank plant DNA sequence database was searched as for *Sh*rbcL.
The DNA sequence obtained, named Nor.*Sh* was found to be most closely related to that of *Populus deltoides* (*Popdel,* accession No. X64764, D'Ovidio, 1992). An identity of 87.2% over a 218 base pair region was observed (see Diagram 5a). *P. deltoides* is also a member of the Salicaceae family.

PCR using the rDNA primers had amplified a DNA region from *S. herbacea* that was closely matched to a member of the Salicaceae family and hence these primers were used to aid in the identification of *Salix* from other sources.

PCR of *S. herbacea* leaves obtained from Edinburgh Botanical Gardens.

Leaf samples were obtained from Edinburgh Botanical Gardens and DNA obtained as normal using the CTAB extraction protocol and purified.

Since the presence of inhibitors in the extractions was unknown, a dilution series that aimed to balance the presence of inhibitors with the concentration of DNA present was performed. The extracted DNA was aliquoted into 100% extraction, 10% extraction (1/10 diluted with water) and 1% extraction (diluted with water 1/100).
A range of magnesium concentrations was set up to optimise PCR conditions.
10µl of each PCR reaction was run out on a TBE metaphor gel and sized using the 100 base pair size ladder to size products. Single product PCR bands of the correct size were repeatedly observed in undiluted DNA extracts amplified under conditions of 1mM and 2mM magnesium

Diagram 5a.
The alignment of Norwegian *Salix herbacea* sequence to *Populus deltoides.*

The extracted Norwegian *Salix herbacea* DNA (Nor.*Sh)* was found to be most closely matched to *Populus deltoides (Popdel).* 87.2% identity in 218 bp overlap. The alignment was performed using the SEQNET program FASTA.

```
                                                10        20        30
Nor.Sh                                    TTCTGCCTGGGCGTCACGCATCGTCG-CCAC
                                          |||||||||| |||||||||||||| || |
Popdel TGCGCCCGAGGCCTCCTGGTCGAGGGCACGTCTGCCTGGGTGTCACGCATCGTCGCCCCC
      400       410       420       430       440       450

               40        50        60        70        80        90
Nor.Sh CACTCCCCTCGGCTTACGAGGGCGGGGGCGGATACTGGTCTCCCGCGCGCTCCCGCCCGT
        | ||||||||||||| ||||||||||||||||||||||||||||||||||||||||| ||
Popdel CGCTCCCCTCGGCTCACGAGGGCGGGGGCGGATACTGGTCTCCCGCGCGCTCCCGCTCGC
      460       470       480       490       500       510

             100       110       120       130       140       150
Nor.Sh GGTTGGCCTAAAATCGAGTCCTCGGCGACGGTCGCCACGACTAGCGGTGGTTGAGAGACC
        || ||||| ||||||||||||||||||||||||||||||| ||||||||||||||||||
Popdel GGCTGGCCCAAAATCGAGTCCTCGGCGACGGTCGCCACGACGAGCGGTGGTTGAGAGACC
      520       530       540       550       560       570

             160       170       180       190       200       210
Nor.Sh CTCGGACTCGGTCGTGCGCGTGCTTGTCGCCCCCGGGACCTCCCGGACCCCCGAGCATTG
       ||||||| | ||||||||   |   |||| |||||||| || | |||||| || |||| |
Popdel CTCGGACACTGTCGTGCG---GGCCGTCG-CCCCGGGATCTTCTGGACCCTCG-GCATCG
      580       590           600       610       620       630

Nor.Sh GCTTTCAA
        | |||| |
Popdel ACCTTCTAGGATGCCTCGTTGCGACCCCAGGTCAGGCGGGACTACCCGCTGAGTTTAAGC
           640       650       660       670       680       690
```

concentrations (Fig 5.3), suggesting these were the optimal PCR conditions. Where products of the correct size were seen, the PCR reactions were pooled and ran on a TBE metaphor agarose gel (Fig.5.4). The single band was extracted and purified using the QIAGEN Gel Quick Gel Extraction kit. 10µl of the purified DNA was quantified and sent for direct sequencing.

The 266bp sequence obtained revealed greatest similarity to *P. deltoides* (87.2% identity in 266 base pair overlap) demonstrating that the amplified DNA from *S. herbacea* obtained from the Edinburgh Botanical gardens was most closely related to that of *P. deltoides.*

The PCR product was then cloned into the pBluescript SK+ plasmid at the *Sma*I site and transformed into DH5α as before. The QIAGEN Mini-prep protocol was followed to recover plasmid DNA and the plasmids containing the required insert authenticated by digesting with *Eco*RV and *Bam*HI restriction enzymes (pBluescript diagram) to excise the insert. Released inserts were assessed firstly for size on a TBE gel as shown in Fig. 5.5 and their concentration established using a DNA sample (pGEM) of known concentration.

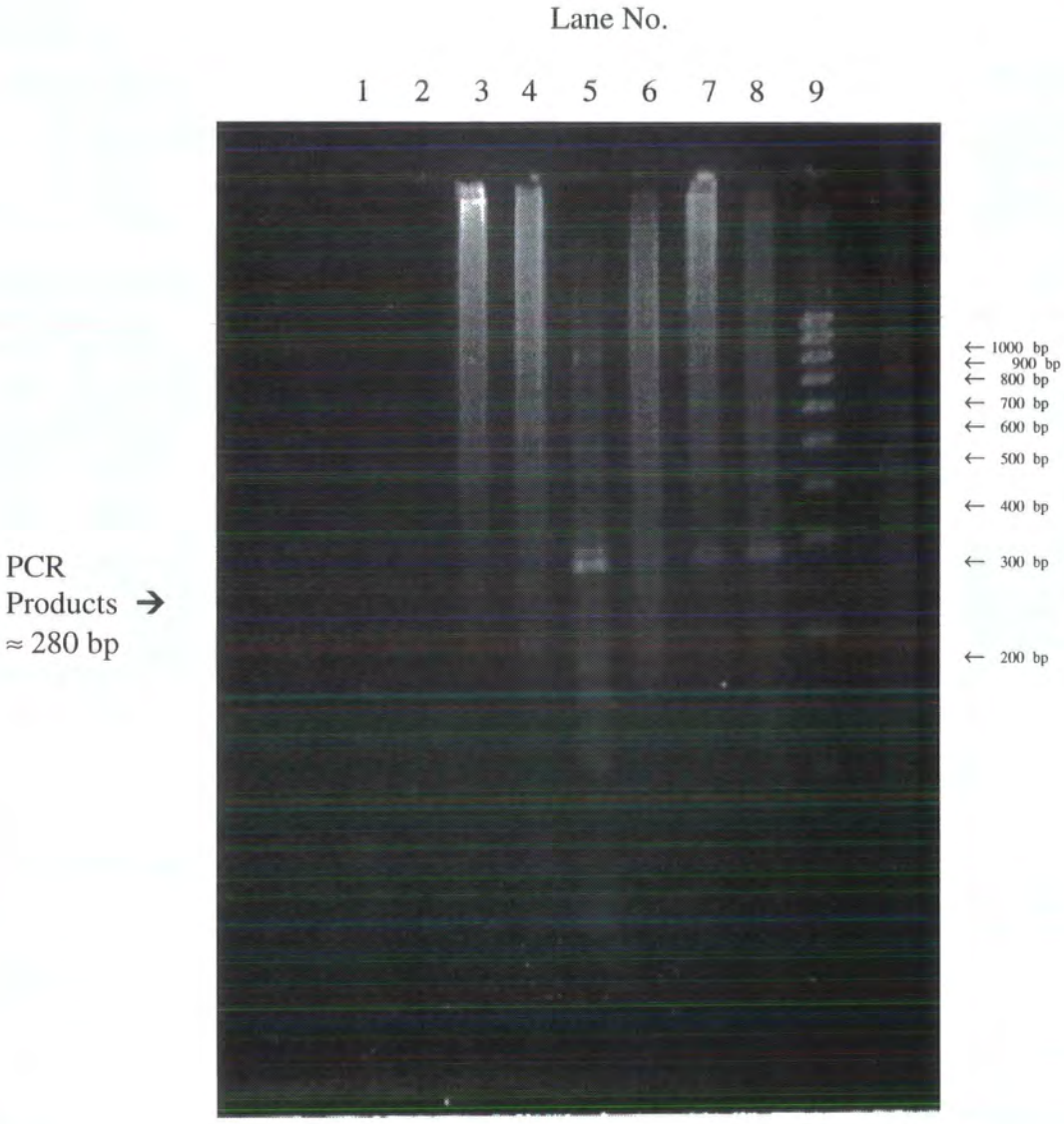
The DNA from three of the plasmid preps was then sequenced using Reverse primers. The DNA sequences obtained were entitled EBG1, EBG2 and EBG3.

The multiple sequence alignment (Diagram 5b) shows that, with the exception of one base pair difference in one clone (EBG3), the sequences were identical, which has probably arisen due to a PCR error (see discussion) The cloned sequences also show approximately 90.5% identity to *P. deltoides.* rDNA Edinburgh Botanical Gardens sequences will now be used as reference *S. herbacea* sequences when analysing field and fossil samples.

The next stage was to isolate rDNA from *S. herbacea* material collected for stomatal analysis.

Figure 5.3

Image of agarose gel showing the PCR products observed using rDNA primers on extracted DNA from *Salix herbacea* from the Botanical Gardens Edinburgh



Lane 1	Extraction Blank
Lane 2	PCR Blank
Lane 3	1/100 DNA Mg 2
Lane 4	1/10 DNA Mg 2
Lane 5	1 DNA Mg 2
Lane 6	1/100 DNA Mg 1
Lane 7	1/10 DNA Mg 1
Lane 8	1 DNA Mg 1
Lane 9	100 bp Ladder

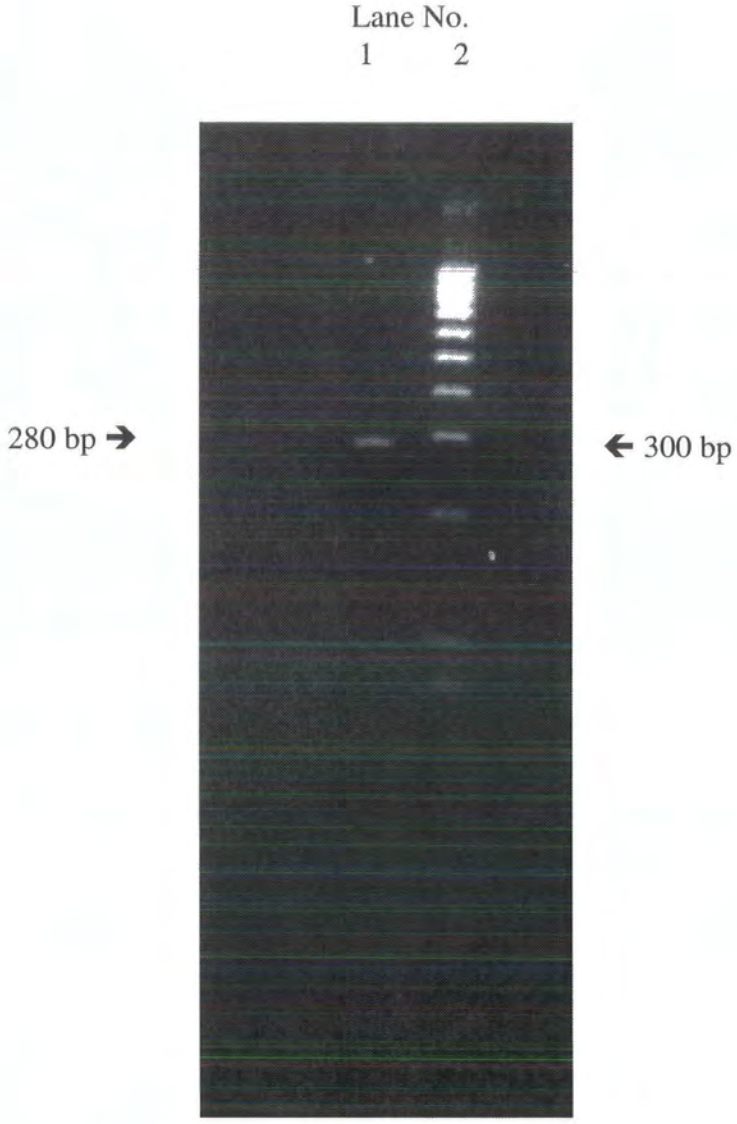
Figure 5.4

Image of an agarose gel showing the single band of DNA for gel extraction and purification

Lane 1	Marker 100 bp ladder
Lane 2	Combined PCR products

Figure 5.5

Image of agarose gel showing blunt-ended PCR product obtained using *Salix herbacea* from the Botanical Gardens, Edinburgh



Lane 1 Blunt-ended PCR Product
Lane 2 Marker 100 bp Ladder

Diagram 5b.
The multiple alignment of DNA sequences obtained as triplicates from *Salix herbacea* leaves from Edinburgh Botanical Gardens.

The multiple alignments of the triplicate sequences obtained from *Salix herbacea* leaves from Edinburgh Botanical Gardens are shown (EBG1, EBG2 and EBG3). Asterisks indicate identities, and base pair changes are denoted by dots. The alignment of the three sequences obtained was performed using the SEQNET program CLUSTAL V.

```
EBG1            GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAGGGCGGGGGCG
EBG2            GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAGGGCGGGGGCG
EBG3            GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAGGGCGGGGGCG
                ************************************************************

EBG1            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTCCTCGGCGACG
EBG2            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTCCTCGGCGACG
EBG3            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTCCTCGGCGACG
                ************************************************************

EBG1            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGCGCGTGCTTGTCGC
EBG2            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGCGCGTGCTTGTCGC
EBG3            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGCGCGTGCTTGTCGC
                ************************************************************

EBG1            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCA
EBG2            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCA
EBG3            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTTCAAGGATGCTCTCGTTGCGACCCCA
                ********************************** ************************

EBG1            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
EBG2            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAG--
EBG3            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
                *********************************************
```

PCR from *S. herbacea* collected from Oetztaler Alpen Austria.

Fresh *S. herbacea* leaves.

S. herbacea specimens were collected and transported with roots from the Oetztaler Alpen to the laboratory and fresh leaves harvested.

DNA was extracted from fresh leaves using the CTAB extraction protocol.
10µl of extract was run on a 0.7% TAE agarose gel against the size marker λ/Pst I. A band of high molecular weight genomic DNA was once again observed as shown in Fig 5.6. RNA and carbohydrate were also observed. The smearing was attributed to an excess of DNA and RNA present in the extract. The genomic DNA was band isolated and purified as previously. The PCR amplifications were optimised by reducing the number of cycles to 30.
Upon obtaining repeatable PCR single band products of the correct size, these were purified and cloned into pBluescript as before.
Duplicate sequences were carried out on plasmid DNA recovered from transformed colonies. These were named AUS1 and AUS2 and were shown to be 100% identical with each other. The sequences showed greatest identity to *P. deltoides* on the GenBank plant DNA data base search (90.1% identity in 284 base pair overlap). The sequence (AUS1) was also compared with the reference *Salix* sequence (EBG1) obtained from the Edinburgh Botanical Gardens (see Diagram 5c). The two sequences AUS1 & EBG1 contained 4 base pair differences over a region of 289 bp.

***S. herbacea* leaves stored in FAA.**

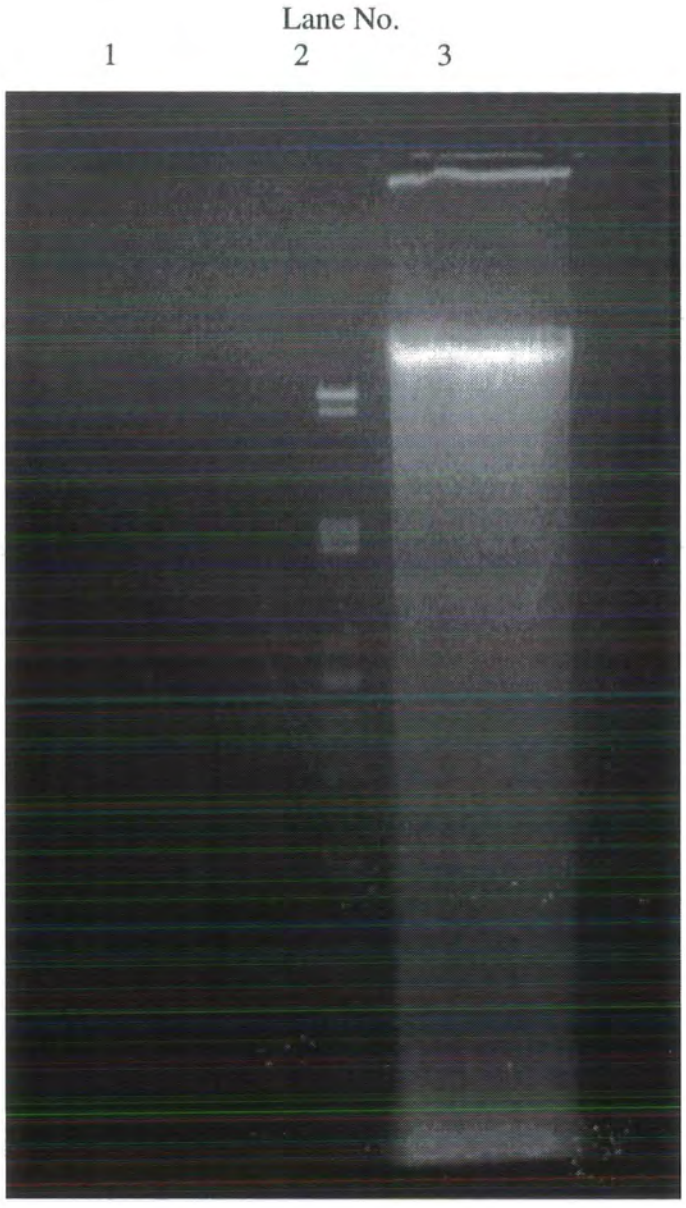
In order to analyse stomatal characteristics of *S. herbacea* material was collected in the field and stored in FAA.
An attempt was made to extract DNA from FAA stored leaves.

Leaves were soaked for 1 hour in Tris 10 mM pH 8.0 to remove some of the FAA from the leaves. The buffer was then changed and leaves left to soak overnight.

The CTAB DNA extraction protocol was followed.

Figure 5.6

Image of agarose gel showing extracted Austrian *Salix herbacea* DNA band prior to gel purification.



Lane 1 Extraction Blank
Lane 2 λ*Pst* I Marker
Lane 3 Extracted Austrian DNA

Diagram 5c.
The alignment of Edinburgh Botanical Gardens *Salix herbacea* sequence 1 to fresh Oetztaler Alpen, Austria *Salix herbacea* sequence 1.

The reference Edinburgh Botanical Gardens *S. herbacea* sequence 1 (EBG1) showed a 285bp gap alignment with that of fresh Oetztaler, Austria *S. herbacea* sequence 1 (AUS1) over a sequence length of 289bp as shown by the omission of a base match line. The alignment was performed using the SEQNET GCG GAP alignment program.

```
EBG1    1 GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAG 50
          |||||||||||||||||||||||| ||||||||| |||||||||||||
AUS1    1 GTCTGCCTGGGCGTCACGCATCGTCCCCCCCACTCTCCTCGGCTCACGAG 50

EBG1   51 GGCGGGGGCGGATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTA 100
          ||||||||||||||||||||||||||||||||||||||||||||||||||
AUS1   51 GGCGGGGGCGGATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTA 100

EBG1  101 AAATCGAGTCCTCGGCGACGGTCGCCACGACAAGCGGTGGTTGAGAGACC 150
          |||||||| ||||||||||||||||||||||||||||||||||||||||
AUS1  101 AAATCGAGTTCTCGGCGACGGTCGCCACGACAAGCGGTGGTTGAGAGACC 150

EBG1  151 CTCGGACACGGTCGTGCGCGTGCTTGTCGCCCCCGGGACCTCCCGGACCC 200
          |||||||||||||||| ||||||||||||||||||||||||||||||||
AUS1  151 CTCGGACACGGTCGTGTGCGTGCTTGTCGCCCCCGGGACCTCCCGGACCC 200

EBG1  201 CCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCAGGTCAGGCGG 250
          ||||||||||||||||||||||||||||||||||||||||||||||||||
AUS1  201 CCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCAGGTCAGGCGG 250

EBG1  251 GACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
          |||||||||||||||||||||||||||||||||||||
AUS1  251 GACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA 289
```

A visible DNA pellet was not observed following precipitation at 4°C for 1 hour and therefore the precipitation period was extended overnight. Still no pellet was visible at this stage, and therefore the extracts were not subjected to gel electrophoresis analysis. As PCR only requires a few molecules of template DNA for successful amplification, PCR reactions were still attempted using these extracts.

Although PCR conditions were altered, including number of cycles, magnesium concentrations and extract dilutions, PCR products were not obtained (discussed later).

5.3.3 PCR from ancient *S. herbacea*.

Herbarium *S herbacea* leaves.

A herbarium specimen of *S. herbacea* was obtained from The Natural History Museum.

The CTAB DNA extraction protocol was followed.

A faint pellet was visible after incubation at 4°C for 1 hour. The extraction was left overnight to precipitate further. This resulted in a visible pellet that was then washed and dried. The crude extraction mixture was diluted as before.

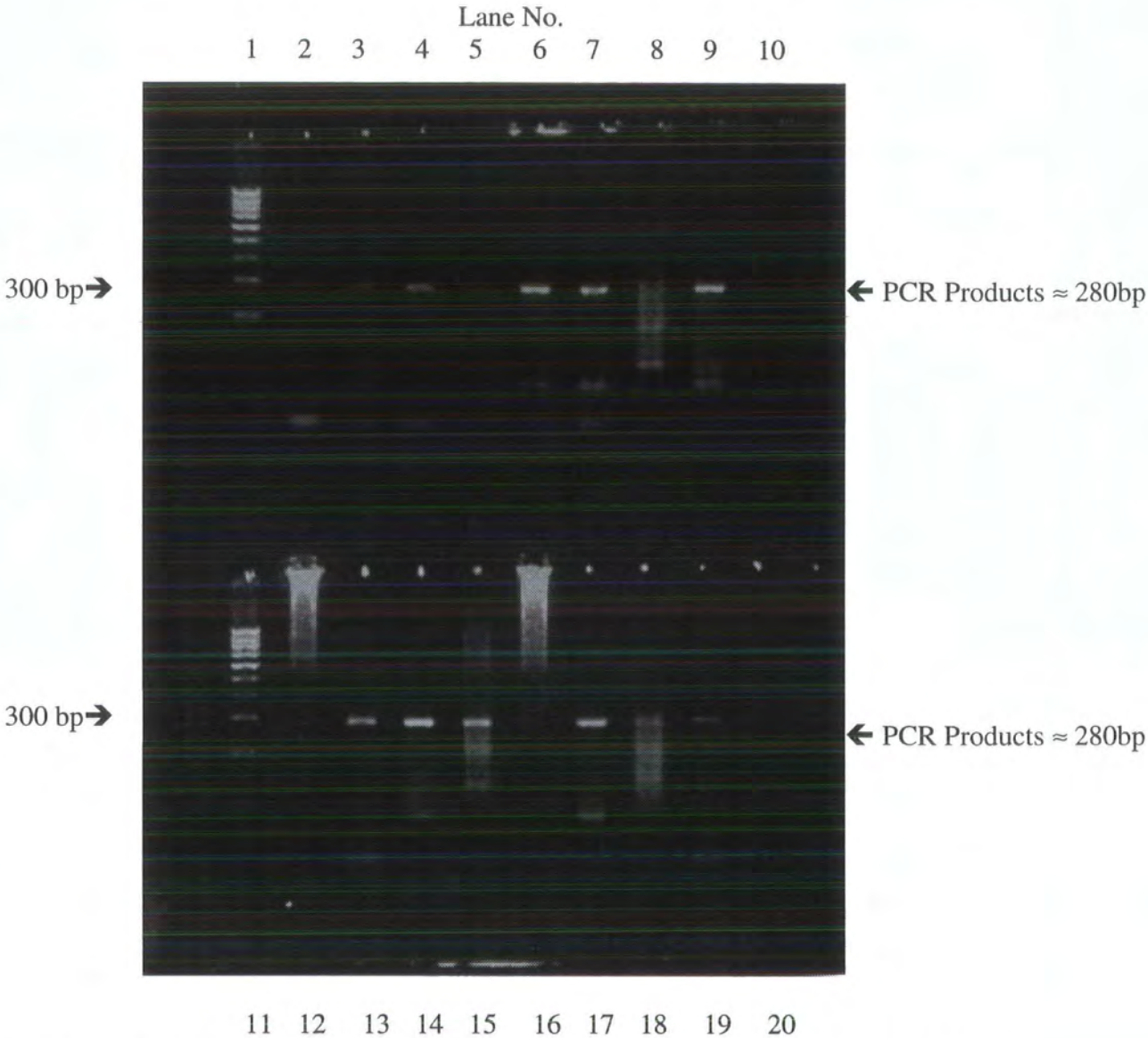
Single band PCR products were clearly observed when PCR conditions were optimised as shown in Fig 5.7.

In order to obtain PCR products from ancient DNA samples the number of amplification cycles was increased from 35 to 40. The amplified PCR products were found to be reproducible and were pooled before they were analysed using a 2% metaphor agarose gel.

The pooled products were purified from the gel and cloned as before. Plasmid DNA obtained from transformed bacterial colonies was assessed using restriction enzymes and found to be of the expected size and the cloned inserts sequenced. Two sequences obtained from the ancient herbarium specimens were named HER1 and HER2. HER1 matched HER2 in 288bp over a sequence length of 289bp. When in comparison with reference EBG1, HER1 showed one base pair difference whereas HER2 matched identically (see Diagrams 5d and 5e). The base pair change observed in HER1 could be due to a PCR error.

Figure 5.7

Image of agarose gel showing the PCR products observed using rDNA primers on extracted DNA from *Salix herbacea* from herbarium specimens



Lane 1	Marker 100 bp ladder	Lane 11	Marker 100 bp ladder
Lane 2	1/100 DNA Mg 1	Lane 12	1 DNA Mg 3
Lane 3	1/10 DNA Mg 11	Lane 13	1 DNA Mg 3 repeat
Lane 4	1 DNA Mg 1	Lane 14	1 DNA Mg 4
Lane 5	1/100 DNA Mg 2	Lane 15	1/10 DNA Mg 4
Lane 6	1/10 DNA Mg 2	Lane 16	1/100 DNA Mg 4
Lane 7	1 DNA Mg 2	Lane 17	1 DNA Mg 5
Lane 8	1/100 DNA Mg 3	Lane 18	1/10 DNA Mg 5
Lane 9	1/10 DNA Mg 3	Lane 19	1/100 DNA Mg 5
Lane 10	PCR Blank	Lane 20	Extraction Blank

Diagram 5d.
The alignment of duplicate herbarium *Salix herbacea* sequences.

The duplicate *S. herbacea* sequences were obtained from 'ancient' herbarium specimens. Herbarium sequence 1 (HER1) matches herbarium sequence 2 (HER2) on 288bp over 289bp. The alignment was performed using the SEQNET GCG GAP alignment program.

```
HER1    1 TCCTCCGCTTATTGATATGCTTAAACTCAGCGGGTAGTCCCGCCTGACCT 50
          ||||||||||||||||||||||||||||||||||||||||||||||||||
HER2  289 TCCTCCGCTTATTGATATGCTTAAACTCAGCGGGTAGTCCCGCCTGACCT 240

HER1   51 GGGGTCGCAACGAGAGCATCCTTGAAAGCCAATGCTCGGGGGTCCGGGAG 100
          ||||||||||||||||||||||| |||||||||||||||||||||||||
HER2  239 GGGGTCGCAACGAGAGCATCCTTGTAAGCCAATGCTCGGGGGTCCGGGAG 190

HER1  101 GTCCCGGGGGCGACAAGCACGCGCACGACCGTGTCCGAGGGTCTCTCAAC 150
          ||||||||||||||||||||||||||||||||||||||||||||||||||
HER2  189 GTCCCGGGGGCGACAAGCACGCGCACGACCGTGTCCGAGGGTCTCTCAAC 140

HER1  151 CACCGCTTGTCGTGGCGACCGTCGCCGAGGACTCGATTTTAGGCCAACCA 200
          ||||||||||||||||||||||||||||||||||||||||||||||||||
HER2  139 CACCGCTTGTCGTGGCGACCGTCGCCGAGGACTCGATTTTAGGCCAACCA 90

HER1  201 CGGGCGGGAGCGCGCGGGAGACCAGTATCCGCCCCCGCCCTCGTGAGCCG 250
          ||||||||||||||||||||||||||||||||||||||||||||||||||
HER2   89 CGGGCGGGAGCGCGCGGGAGACCAGTATCCGCCCCCGCCCTCGTGAGCCG 40

HER1  251 AGGGGAGTGGGGGCGACGATGCGTGACGCCCAGGCAGAC 289
          |||||||||||||||||||||||||||||||||||||||
HER2   39 AGGGGAGTGGGGGCGACGATGCGTGACGCCCAGGCAGAC 1
```

Diagram 5e.
The alignment of Edinburgh Botanical Gardens sequence 1 to herbarium *Salix herbacea.*

The reference Edinburgh Botanical Gardens *S. herbacea* sequence 1 (EBG1) showed a 288bp gap alignment with that of herbarium *S. herbacea* sequence 1 (HER1) over a sequence length of 289bp as shown by the omission of abase match line. The alignment was performed using the SEQNET GCG GAP alignment program.

```
EBG1        1 GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAG 50
              ||||||||||||||||||||||||||||||||||||||||||||||||||
HER1        1 GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAG 50

EBG1   51 GGCGGGGGCGGATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTA 100
          ||||||||||||||||||||||||||||||||||||||||||||||||||
HER1   51 GGCGGGGGCGGATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTA 100

EBG1  101 AAATCGAGTCCTCGGCGACGGTCGCCACGACAAGCGGTGGTTGAGAGACC 150
          ||||||||||||||||||||||||||||||||||||||||||||||||||
HER1  101 AAATCGAGTCCTCGGCGACGGTCGCCACGACAAGCGGTGGTTGAGAGACC 150

EBG1  151 CTCGGACACGGTCGTGCGCGTGCTTGTCGCCCCCGGGACCTCCCGGACCC 200
          ||||||||||||||||||||||||||||||||||||||||||||||||||
HER1  151 CTCGGACACGGTCGTGCGCGTGCTTGTCGCCCCCGGGACCTCCCGGACCC 200

EBG1  201 CCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCAGGTCAGGCGG 250
          |||||||||||||| ||||||||||||||||||||||||||||||||||
HER1  201 CCGAGCATTGGCTTTCAAGGATGCTCTCGTTGCGACCCCAGGTCAGGCGG 250

EBG1  251 GACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA 289
          |||||||||||||||||||||||||||||||||||||||
HER1  251 GACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA 289
```

The extracted aDNA of known *S. herbacea* was thus shown to match that of modern DNA from known *S. herbacea*. As the use of rDNA primers had been successful in amplifying DNA from ancient samples an attempt was made to extract and amplify aDNA from delicate leaf macrofossil material.

PCR from *S. herbacea* leaves extracted from Morrone Birkwood core.

S. herbacea leaf specimens were extracted from sample 338-343cm. The leaves were washed by soaking for 1 hour in Tris 10mM pH 8.0 after which fresh buffer was added and the leaves left overnight to soak. The CTAB DNA extraction protocol was followed and a dilution series made as previously.

Attempts were made to optimise PCR buffering conditions, and the number of amplification cycles increased to 50.

PCR products were observed but were not reproducible. These observed products were cloned and sequenced. The sequence data revealed that the sequences obtained were not analogous to either *P. deltiodes* or reference *S. herbacea.*

The extraction and PCR was repeated. No PCR products were observed.

5.3.4 Multiple Sequence Alignments.

Diagram 5f shows the multiple sequence alignment of all sequences obtained which had been successfully blunt ended and cloned. The multiple sequence alignment was performed using the SEQNET program CLUSTAL V. The 7 sequences only show base pair changes in 5 occasions.

Diagram 5f.
The multiple alignment of all sequences obtained which had been successfully cloned. The multiple sequence alignment was performed using the SEQNET program CLUSTAL V. The 7 sequences only show base pair changes in 5 occasions.

```
BGE1            GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAGGGCGGGGGCG
BGE2            GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAGGGCGGGGGCG
BGE3            GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAGGGCGGGGGCG
AUS1            GTCTGCCTGGGCGTCACGCATCGTCCCCCCCCACTCTCCTCGGCTCACGAGGGCGGGGGCG
AUS2            GTCTGCCTGGGCGTCACGCATCGTCCCCCCCCACTCTCCTCGGCTCACGAGGGCGGGGGCG
HER1            GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAGGGCGGGGGCG
HER2            GTCTGCCTGGGCGTCACGCATCGTCGCCCCCACTCCCCTCGGCTCACGAGGGCGGGGGCG
                ************************* ********* ************************

BGE1            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTCCTCGGCGACG
BGE2            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTCCTCGGCGACG
BGE3            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTCCTCGGCGACG
AUS1            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTTCTCGGCGACG
AUS2            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTTCTCGGCGACG
HER1            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTCCTCGGCGACG
HER2            GATACTGGTCTCCCGCGCGCTCCCGCCCGTGGTTGGCCTAAAATCGAGTCCTCGGCGACG
                ************************************************** **********

BGE1            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGCGCGTGCTTGTCGC
BGE2            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGCGCGTGCTTGTCGC
BGE3            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGCGCGTGCTTGTCGC
AUS1            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGTGCGTGCTTGTCGC
AUS2            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGTGCGTGCTTGTCGC
HER1            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGCGCGTGCTTGTCGC
HER2            GTCGCCACGACAAGCGGTGGTTGAGAGACCCTCGGACACGGTCGTGCGCGTGCTTGTCGC
                ********************************************** **************

BGE1            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCA
BGE2            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCA
BGE3            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTTCAAGGATGCTCTCGTTGCGACCCCA
AUS1            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCA
AUS2            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCA
HER1            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTTCAAGGATGCTCTCGTTGCGACCCCA
HER2            CCCCGGGACCTCCCGGACCCCCGAGCATTGGCTTACAAGGATGCTCTCGTTGCGACCCCA
                ********************************** ************************

BGE1            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
BGE2            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAG--
BGE3            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
AUS1            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
AUS2            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
HER1            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGA
HER2            GGTCAGGCGGGACTACCCGCTGAGTTTAAGCATATCAATAAGCGGAAGGA
                *******************************************
```

5.4 Discussion.

DNA sequences were successfully obtained from both modern and ancient herbarium *S. herbacea* samples using nuclear ribosomal DNA primers. Overall all the cloned sequences match each other closely. The similarity is much greater than to any other plant sequence available on the GenBank plant DNA database.

S. herbacea leaves which were collected from Edinburgh Botanical Gardens were used as reference material. Sequences were successfully obtained from these leaves and the GenBank plant database found them to be most closely matched to that of *P. deltoides. P. deltoides is* a member of the Salicaceae family and is closely related to *S. herbacea.* It is therefore highly probable that the extracted DNA is *S. herbacea* and will be called such.

The reference material was then used to validate the identity of leaf material collected in the field. The extraction, amplification and sequencing of DNA from fresh leaf material collected in the Oetztaler Alpen Austria revealed sequences that closely matched those of the reference. Although 4 consistent base pair changes were observed these were not significant. The mismatches could be due to experimental error that may have been caused by the misreading of *Taq* polymerase during amplification, or they may have occurred during cloning or sequencing. The mismatches could also be real changes found in the Austrian material. However, as the target sequence is non-coding, and therefore, polymorphisms can occur, these are intra-genic changes and they do not effect either the identification of the material or the suitability of this material for stomatal parameter studies. Thus it can be concluded that the field material is that of *S. herbacea.*

As DNA is most readily extracted and amplified from fresh specimens the *S. herbacea* collected from Edinburgh Botanical Gardens and the Oetztaler Alpen Austria should present little problem in obtaining sequence data, as was found. The sequence data achieved from the extraction of DNA from ancient herbarium material revealed at most one base pair mismatch to that of modern *S. herbacea* from Edinburgh Botanical Gardens. This result outlined that the method of extraction and amplification was suitable

for use on ancient specimens. In addition, from the good sequence data achieved it would appear that the technique employed to preserve these herbarium leaves allowed the conservation of DNA. The DNA templates were of sufficient quality for amplification.

The work of Savolainen *et al.,* (1995), outlined several methods which were used in an attempt to remove PCR inhibitory activities detected in herbarium DNA extracts. In this case it was possible to remove any inhibitors present in the extractions by diluting with water. It was necessary to increase the PCR cycle number to 40 in order to achieve visible PCR products. The sequence lengths achieved using the rDNA primers were comparable to those obtained using fresh material Therefore, the herbarium sample does not appear to have been subjected to significant DNA degradation in this region.

The chemical preservation of field material had been found to present problems for the extraction and amplification of DNA (Doyle & Dickson, 1987; Pyle & Adams, 1989). *S. herbacea* leaves from the Oetztaler Alpen Austria had been preserved in FAA Storage in FAA had been used as it was an effective way for preserving leaf material for stomatal analysis. It was necessary to store the leaf samples in FAA for a considerable period of time before the extraction of DNA. The extraction and amplification of DNA in these preserved samples was not successful, as was found by Doyle & Dickson, (1987) and Pyle & Adams, (1989). The leaves had been soaked and washed in an attempt to remove FAA from the leaves but this was not sufficient to enable DNA extraction. Ranges of PCR reactions were applied to optimise PCR conditions, none of these resulted in products. Samples were also diluted prior to PCR. Dilution aims to reduce any inhibitory reactions by reducing the absolute content of the inhibitor and thus its inactivating effect on the polymerase. However, since the DNA content is reduced proportionately, if there are very few templates this may be counterproductive (Herrmann & Hummel, 1992). Possible modifications include the addition of bovine serum albumin (BSA) and 'blotto' to the amplification reaction. BSA acts by having a positive effect on enzyme activity, whereas blotto sequesters contaminants. However, the addition of these reagents with complex molecular structure may introduce an additional source of contamination that must be monitored. The lack of PCR products could be attributed to a variety of factors. The preservation of material in FAA can induce the hardening of leaf material due to the

presence of formaldehyde (Jackson *et al.*, 1991). In addition it is known that formaldehyde breaks the purine-pyrimindine hydrogen bonds resulting in rapid and irreversible denaturation of double stranded DNA. When samples are stored for any length of time in FAA, leaves are often observed to lose coloration as a result of the chlorophyll leaching into the preservative. In addition if DNA is rapidly degraded it may form insoluble secondary metabolites. It is also possible that DNA may also be lost by leaching into the FAA solution, as suggested by Jackson *et al.*, (1991). In addition it has been reported that even if DNA can be extracted further problems arise during PCR. These may be due to structural changes or crosslinkage of the DNA induced by formaldehyde which can influence PCR performance or limit the size of amplification products (Karlsen *et al.*, 1994; Greer *et al.*, 1990). Therefore, in conclusion the reason for the failure of DNA extraction and amplification may be either due to the lack of presence of sufficient DNA templates in the leaf material prior to amplification, or the effect FAA can have on PCR.

Recently a protocol has been reported suitable for the recovery of DNA fragments at least 450bp in length from formaldehyde-fixed specimens (Vachot & Monnerot, 1996). They illustrated that the extent of DNA degradation in museum specimens can vary significantly from one sample to another, this may be attributable to several facts. It is not known in museum specimens how fresh the sample was before fixation, what type of fixative was employed, the concentration used and for how long and at what temperature the material was fixed. Although the study was devoted to amphibian specimens this work highlighted the importance of improving fixation conditions which may be used in the field and also maximise the potential for molecular studies on fixed material in the future.

In order to extract and amplify DNA from material collected in the field it would appear that storage in FAA is far from ideal. Alternative techniques such as drying. This has been reported to preserve high-grade DNA (Doyle & Dickinson, 1987; Pyle & Adams, 1989; Thomson & Henry, 1993; Harris, 1993). Drying should be rapid in order to prevent tissue injury that may result in the production of injuries and subsequent responses that

may influence DNA extraction and/or amplification. In the case of field material this could be implicated by immediately pressing leaves between papers and changing papers regularly to avoid any surplus moisture developing which could effect DNA. A method of reducing the detrimental effect of the senescence process by rapidly desiccating field material using silica gel is reported by Chase & Hills, (1991).

Apart from desiccating material an alternative preservation technique involves freezing leaves in liquid nitrogen, however this method requires additional field equipment that may not always be feasible.

The ITS2 region shows considerable length conservation throughout species. The ITS2 region of Sorghum was reported to be 217 base pairs (Hsiao, 1993). This length has been shown to be small enough to allow successful amplification of aDNA, and as the length is conserved, aDNA amplification of other species should be viable.

As modern, fresh and ancient herbarium *S. herbacea* specimens had resulted in the successful amplification of the ITS2 region of approximately 300 base pairs it is known that the extraction and amplification methods were successful, therefore this was attempted on fossil material. However, the success would be dependent on the preservation state of the DNA under examination. In this study it was not possible to extract and amplify ancient DNA from leaf macrofossils extracted from the Morrone Birkwood core. There are several reasons as to why this was not possible.

Firstly there are the reasons associated with the DNA preservation in the fossil material prior to core extraction.

There is no available knowledge as to the original preservation conditions of these leaf macrofossils. That is what conditions were prevailing at the time when the leaves would have become abscised from the branch or the *S. herbacea* plant preserved as a whole. Rapid tissue dehydration at this stage could have enhanced the preservation of DNA, due to susceptibility of the molecule to hydrolysis and also may be expected to reduce the activity of endogenous proteases and nucleases. This preservation condition would have been rather unlikely considering the area from which these leaf macrofossils were

recovered. Thus the degradation of DNA may have occurred before the leaf became incorporated in the core. Nevertheless, there are controversial reports of the extraction and amplification of aDNA from fossil plant tissues which have been preserved fully hydrated (Golenberg *et al.,* 1990; Soltis *et al.*, 1992).

Neutral conditions may also be desirable for DNA preservation. DNA can become depurinated under acidic conditions, and alkaline environments can cleave DNA at apurinic or apyrimidinic sites (Ausubel *et al.,* 1991). There is also the additional problem of the denaturation of double-stranded DNA, which removes the stability provided by the hydrogen bonds between the two strands when DNA is preserved under alkaline conditions. It may be that acidic conditions may initially inhibit microbial activity and, further, that humic acids can chelate metal ions, thereby contributing to prolonged preservation as suggested by Eglinton & Logan, (1991). The leaf macrofossils obtained from the Morrone Birkwood core are thought to be subjected to preservation conditions of pH 6.6 (Huntley, 1979*a*). This pH should not significantly effect the degradation of DNA

Physical damage to the fossil material is also another concern when considering preservation extent. If deposition occurred rapidly and was directed, physical damage would be minimised. Rapid deposition would in addition reduce externally and internally induced degradation. Externally induced degradation is due to microbial attack, and internally induced includes senescence, necrosis and wounding reactions (Golenberg, 1992). Rapid sedimentation will also reduce exposure of the tissue to biotic degradation (Eglinton & Logan, 1991). Examination of leaf surface structure using SEM had revealed leaf structure in some instances to be relatively intact in view of the time period of preservation. Therefore, it would not appear that the leaf macrofossils were subjected to external physical damage, but it is not possible to comment on the extent of internally induced degradation.

Anoxic conditions are also known to reduce oxidative damage to DNA. Although the compact core may be considered to be a relatively anoxic environment, the oxidative conditions to which the leaves were subjected prior to becoming compacted in the

core are unknown.

Secondly, there are several reasons as to why the field extraction of the fossils and the preliminary processing and storage of the cores may have resulted in degradation of any preserved DNA.

The cores were taken using a Wright-modified Livingstone piston corer (Wright, 1967). This was driven into the sediment up until a depth of 430cm or until a boulder was struck. The corer was removed from the ground, and transported back to the laboratory. The cores were later extruded in the laboratory, wrapped in plastic film and aluminium foil and stored at 4°C. None of these methods were ideal for DNA preservation. It would have been preferable to reduce the exposure of the macrofossil leaves to desiccation, oxygen, light and external debris as outlined by Golenberg, (1992). The cores had been stored for 2 years prior to the extraction of the *S. herbacea* leaf macrofossils. The cores were not dried and therefore any moisture present remained throughout this storage period and thus could have enhanced the degradation of any preserved DNA present at this time. In addition, storage at 4°C is not ideal, it would have been better if it had been possible to freeze the entire core to prevent further degradation.

The extraction of the plant macrofossils from the cores involved cutting the core in order to sample sections and then disaggregating these in water. The wet sieving technique employed, and the subsequent storage in 50% glycerol would have not aided in the preservation of aDNA.

At all the outlined stages, there is the risk of the introduction of contaminants. PCR products had been initially observed which were found to be unrelated to *S. herbacea*. This contamination could have been associated with the leaf macrofossil in the core segment prior to excavation and may have contained DNA that was amplified in preference to any remaining leaf macrofossil DNA. However, given the nature of the field extraction of the fossils and the preliminary processing and storage of the cores the contaminant may also have been introduced at any one of these stages

In light of the above discussion, for future attempts to extract and amplify aDNA from core material sampled in the field, a much faster processing method should be employed. Once the cores have been removed, specimens should be immediately recorded and then the extractions carried out. As suggested by Golenberg, (1992), the macrofossil leaves should be ground with a pestle and mortar at the earliest possibility. The ground material should then be transferred into a 1.5ml microfuge tube filling no more than one third of the tube in order to allow for the addition of extraction buffer and chloroform/isoamyl alcohol. After the extraction buffer have been added, and the grindate exposed evenly to the buffer the tubes can be kept on ice or stored in a refrigerator at this stage until the extraction can be completed in the laboratory. It has been reported that samples prepared in this manner have been stored several weeks before final extraction without any noticeable problems.

Future work.

As it has been reported that nuclear DNA, and in particular ribosomal gene sequences, in plants appear to evolve at relatively rapid rates compared with other DNA, eg. Chloroplastic DNA (Zimmer *et al.,* 1988; Doebley *et al.,* 1984), this seemed a good target for PCR. In addition, the conserved coding regions of the rDNA repeat units have been shown through restriction site analysis, to have no measurable variation in length between species (Jorgensen & Cluster, 1988). Therefore as both length and sequence are highly conserved, rDNA can be used as a tool to compare variation between species using similar primers. This study aimed to use the targeted rDNA region to confirm species identification. As discussed, this was successful up to the examination of the leaf macrofossils. Future work on such material should include the further use of chloroplastic primers. Although rbcL primers were initially used on modern *S. herbacea* material and it was possible to amplify regions of over 600 bp, this region was too extensive to use in respect to fossil specimens. Primers should be designed to target shorter regions within the chloroplastic genome suitable for use with ancient material.

Chapter 6.

Summary and Conclusions

The aim of this study was to examine the feasibility of using plant characteristics as a tool to monitor environmental change. Plants have an absolute requirement for CO_2; which enters the plant through specialised cells known as stomata. Thus, because of their role, stomatal function is critically important for plant survival. They are known to change both function and density in response to changes within their surrounding environment. Variations in atmospheric CO_2 levels are known to have occurred throughout history. A reliance on fossil fuels for energy in recent times has resulted in rises in the concentration of atmospheric CO_2, this trend is predicted to continue in the immediate future. If a definite pattern of association between the effect of changing CO_2 levels on stomatal characteristics can be determined, then it is hoped that a model to predict what effect, if any, future atmospheric CO_2 changes may have on plant viability may be created. Historic atmospheric CO_2 levels are currently obtained from ice core records, which due to their nature, often offer an incomplete record. This study was extended to look at fossilised plant material to try and calculate palaeo-CO_2 levels and hence add to the information available from the ice core record.

Previous studies have indicated that stomata are able to respond to environmental change and suggest that atmospheric CO_2 levels are linked closely with leaf stomatal density (Beerling & Chaloner, 1993*c*; Beerling *et al.* 1993), i.e. a rise in CO_2 level correlated to a stomatal density decrease. Such findings led to the extension of studies to investigate the long-term response of stomatal parameters to environmental change by considering fossil material (Beerling & Chaloner, 1993*b*). From the outlined correlation between CO_2 concentration and stomatal density it was postulated that stomatal characteristics recordings could be used as potential indicators of past CO_2 concentration. However, examination of the methods described indicates that these studies were based on limited data sets and did not take into account a number of variables, and as such cannot be considered "universal".

Work described in this thesis extended previous studies to determine accurately whether stomatal characteristics are an effective means of predicting both future plant responses to increased levels of atmospheric CO_2 and as indicators for palaeoclimates. In order to achieve this it was considered essential to assess stomatal responses in the natural environment, as opposed to an artificially controlled one. By extensive sampling, a data set was generated to determine whether CO_2 changes could be correlated directly to stomatal variations, or whether other environmental factors, known to possess the ability to affect stomata, and not fully considered before, have a greater influencing potential. If a correlation between CO_2 levels and stomatal characteristics is still apparent after sampling extensively both within and between extant plant species in the natural environment, then stomatal characteristics could indeed have the potential to be used as a tool to monitor both palaeo-CO_2 levels and model plant responses following future global environmental change.

The work presented in this thesis firstly describes the stomatal characteristics of three plant species within a geographical area that permitted the short-term investigation of plant responses to a relatively small (20 p.p.m.v) increase in CO_2 concentrations in a natural environment. To create a model to monitor environmental change using plant stomatal characteristics, it is essential that any resulting correlation be applicable across the plant kingdom. The study of *Ranunculus ficaria, Sambucus nigra* and *Hedera helix* revealed non-consistent stomatal responses to an increased CO_2 level between the species. Although a decrease in stomatal density and stomatal index was sometimes recorded in association with a naturally increased CO_2 gradient, the response was not universal either within or between the species studied. Stomatal parameter responses were seen to vary depending on leaf surface, within, as well as between sampling sites and the time at which samples were taken. This variation in response was thought to be under the influence of other factors and could not be solely attributed to the CO_2 gradient. The variation was of significant magnitude to make it impossible to relate changes simply to CO_2 levels. Therefore, it is apparent that a general relationship between the response of stomatal parameters to CO_2 changes could not be obtained. It is important that responses within individual species are understood thoroughly so that species specific responses may also be considered before any response correlation maybe applied. The results presented in this thesis

showed that it is considerably more difficult to predict plant responses to small, but relatively important increases in CO_2 levels, than previously thought. By increasing the sample size of previous studies, it is shown that a number of additional variables should be considered when sampling within the natural environment.

In order to determine whether a clear correlation between stomatal characteristics and CO_2 levels existed, the study using *R. ficaria* was extended using greatly elevated CO_2 levels in an artificial environment (340 p.p.m.v). Plants grown under elevated CO_2 regimes in controlled environmental chambers have been reported to decrease leaf stomatal density (Thomas & Harvey, 1983; Woodward, 1987). *R. ficaria* was also found to record significant reduction in both stomatal density and stomatal index in association with elevated CO_2 levels. In addition *R. ficaria* was also found to demonstrate response regulation if established plants were removed from the elevated CO_2 regime and returned to ambient CO_2 levels. These findings clarified that a correlation does indeed exist between stomatal characteristics and CO_2 when levels are greatly increased and other environmental factors that may affect stomatal parameters are strictly controlled. This further highlighted the importance of considering other environmental factors when using plant stomatal characteristics as a tool to monitor environmental change, since plant responses to relatively small increases in CO_2 in the natural environment, where other environmental factors are able to directly influence stomatal parameters, differ significantly from those observed in artificially controlled experiments. Caution should be applied when using results obtained from plants grown in artificially enhanced CO_2 regimes to predict global stomatal responses in the natural environment.

Due to the variation observed in these initial experiments it was deemed important to undertake a more detailed study of plant responses in their natural environment, to try and determine what other environmental factors may be exerting an effect on stomatal parameters. This was achieved by studying the response of stomatal characteristics to small increases in CO_2 concentration of plants grown along a natural altitudinal CO_2 gradient. As altitude increases the partial pressure of CO_2 is known to decrease. *Salix herbacea* was used as it is known to be established across altitudinal ranges and, for studying palaeoclimates, leaves are also known to be preserved in the fossil record.

Thus, this material would provide the potential of assessing the effects of differing CO_2 concentrations in both past and future studies.

Contrary to the work of Beerling *et al.* (1992), no consistent relationship was found between stomatal characteristics and CO_2 concentration in association with changing CO_2 levels in extant material. All measured parameters indicated great variation, which could be attributed to factors such as the sample site, collection method, plant sex, leaf surface and sampling year. Considerable variation was also found when sampling at the same altitude where the effects of CO_2 concentration would not be apparent. Stomatal patterning across individual leaf surfaces was also found to be inconsistent.

The frequency, manner and extent of sampling have been greatly extended in this investigation compared to previous studies. This revealed a much greater intra-specific variation than had been previously thought, which appeared to mask any effect that maybe attributed to the changing CO_2 level. This variation in extant material cannot be ignored and must be appreciated when studying fossil material. Research into fossil material also showed a non-consistent trend between stomatal density and palaeo-CO_2 levels as predicted by core section sampling of fossil remains. These investigations carried out on *S. herbacea* have shown the importance of studying extensively in the natural environment to fully appreciate the effect of other environmental factors on stomatal characteristics. Without an understanding of these effects on extant and fossil material, it is wrong to use stomatal characteristics to predict palaeo-CO_2 levels and model future effects.

To confirm the variation observed when directly studying stomatal parameters and their response to known CO_2 changes in their natural environment, another approach, using $\delta^{13}C$ analysis, was performed. Beerling *et al.* (1993) suggested that a relationship existed between $\delta^{13}C$ and environmental change, and also between $\delta^{13}C$ and stomatal density. Once again great variation was apparent in the $\delta^{13}C$ values obtained which could not be attributed to varying CO_2 levels. These findings, coupled with the previous experiments, suggested that other environmental factors were able

to significantly alter stomatal parameters and $\delta^{13}C$ values, and these factors should be further examined before predictions maybe made.

This work revealed that different plant species recorded different changes toward changing CO_2 concentrations. When attempting to obtain stomatal information from fossilised leaf remains it became apparent that leaf identification became crucial. To date, reliance has been made on 'expert' identification of leaves from the fossil record. Whilst this process is useful, it can sometimes be time consuming, inconvenient and inaccurate. It was therefore decided to utilise a novel approach of identification based on DNA characteristics. This study looked at variations in conserved genes within *S. herbacea* in fossil samples that had been expertly identified, field samples and herbarium extant species. These experiments did prove that DNA maybe used for identification purposes, since field samples of *S. herbacea* were correctly assessed, however characterisation of fossil samples remained problematical, due to the degradation of the DNA over time. DNA has previously been isolated successfully from fossil samples and it is thought that with some improvement to the method, or access to better preserved material, it may well be possible to use such a method to assist fossil identification.

Chapter 7.

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