The physiology of Dionysa aretiodes during micropropagation

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THE PHYSIOLOGY OF *DIONYSIA ARETIODES* DURING MICROPROPAGATION

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Declaration

The work described in this thesis has been completed by the undersigned at the Department of Biological Sciences of the University of Durham and has not been previously submitted for any other diploma or degree.

Signed

Date 15/8/94

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S. J. DANIELS

ABSTRACT

Dionysias are high alpine plants which are restricted in the wild and scarce in cultivation. Fourteen species have been cultured *in vitro*, but weaning has proved difficult, due to water status difficulties with the plant material. Physiological aspects of this problem have been investigated.

Acetate peels from adaxial leaf surfaces showed that control of stomatal density was lost during *in vitro* culture. Studies of the juxtaposition of glands and stomata showed that, apart from stomata of *pot grown (de novo)* material, clustering occurred for both structures. Glands appeared to progressively inhibit adjacent stomatal development, from *de novo* material where no inhibition occurred, to cultures subject to water stress where inhibition was pronounced. Experiments to determine the affect of stomatal density on weaning showed that only plants with very low stomatal densities survived to root. Stomatal apertures, determined by floating leaves in water or ABA solutions, fixing and removing epidermal strips, were found to be similar, independent of treatment.

Epidermal strips were removed and stained with sodium cobaltinitrite and ammonium polysulphide solutions to determine potassium accumulation in the guard cells. Low levels of potassium were found for all treatments. Higher accumulations were found in flaccid material. Cation accumulations in leaf material were analysed by atomic emission and atomic absorption for sample solutions prepared by dry combustion. The K:Na ratio was found to drop in *in vitro* cultures, but could be ameliorated by increasing the pH and/or calcium concentration of the media. No amelioration of stomatal operation was found.

Samples of farina from eight species, including *in vitro* and *de novo* material, were collected by washing leaves in acetone. Differences in farinal composition, revealed by TLC, were found between species, suggesting the possibility of a chemotaxonomic classification. Some quantitative and qualitative differences were found between *in vitro* and de novo material. It would appear that some biochemical changes occur during *in vitro* culture.

It is postulated that the morphological adaptions shown by *Dionysia aretiodes* regulate water loss and account for the apparent lack of stomatal functionality. Physiological differences occur when plant material is cultured *in vitro*, but no effective treatments to improve weaning were found. The survival and rooting of propagules from culture appears linked to water loss as a result of stomatal density and developmental epidermal patterns.

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LIST OF ABBREVIATIONS

ABA	Abscisic Acid.
ADP	Adenosine 5'-Diphosphate.
ATP	Adenosine 5'-Triphosphate.
BAP	6-Benzylaminopurine.
CI	Confidence Interval.
DW	Distilled Water.
FAA	Formalin-Aceto-Alcohol.
IBA	Indole-3-Butyric Acid.
IP_3	Inositol-1, 4, 5-Trisphosphate.
JI	John Innes Compost.
MAFF	Ministry of Agriculture, Fisheries and Food.
rf	Relative Flow.
STDEV	Standard Deviation
TLC	Thin Layer Chromatography.

INTRODUCTION

1.1 Dionysias

Dionysias are high alpine plants belonging to the family Primulaceae; the genus consists of forty one known species, twenty of which are currently in cultivation. Most species are native to Afganistan and Iran; *Dionysia mira* occurs in Oman, while *D. hedgei* also occurs on the border of Afganistan and the former Soviet Union. Although of great horticultural merit, many members of the genus have proved difficult to propagate vegetatively, several species only exist in very small numbers in cultivation and therefore remain vulnerable. Propagation by seed is also problematic as many species, derived from single introductions, are effectively rendered sterile. It is unlikely that further collections will be made in the foreseeable future due to the political and social situations in these countries. Populations are known to be limited in the wild, and this in itself is a cause for concern, particularly as it is not possible to verify current plant numbers.

Dionysias are difficult to grow in cultivation with only five species, *D. aretiodes, D. curviflora, D. involucrata, D. mira, D. tapetiodes, that* may be considered easier to cultivate. In the wild Dionysias grow in shaded or partially shaded cliffs, were they are sheltered from the hot dry

conditions of the summer, and snows of winter. The cool, moist climate of Europe makes Dionysias very difficult to grow as they are particularly sensitive to a moist atmosphere during the autumn and winter. Propagation by cuttings has proved successful for amenable species where a 70% take may be expected, but for rarer species such as *D. afganica* and *D. microphylla*, 5% would be an optimistic figure (Grey-Wilson, 1988).

Although it has proved possible to multiply thirteen species *in vitro*, during the course of this research (Table 1), it has proved difficult to wean this material off to normal alpine house conditions. Some limited success has been possible with *D. aretiodes*, *D. lamingtonii*, *D. freitagii*, however this has been associated with very large losses.

D. aretiodes, one of the easiest members of the group to cultivate, has been used to investigate physiological changes which might occur during micropropagation, and attempt to develop systems for improved weaning percentages.

Taxonomic Treatment

The genus may be divided into three sections, section Anacamptophyllum, section Dionysia, and the section Dionysiastrum (Table 1). Species vary from the large woody yellow flowered plants of Anacamptophyllum subsection

Scaposae, to the low growing rosette forming, purple flowered plants of Dionysiastrum, subsections Afghanicae and Microphyllae.

The origins of the genus *Dionysia* remain unclear, *Dionysia* and *Hottonia* may have evolved from *Primula* after heteostyly had been developed, or *Dionysia* may have evolved from a forerunner of the Primula section *Armeria*. *If* the latter is correct then the small rosette forming, pink flowered species, represent the most primitive types while the tall, large leaved, yellow flowered Dionysias evolved to resemble Primula section Sphondylia (Richards, 1993). Wendeblo (1961, in Grey-Wilson, 1989) suggested that the yellow flowered Dionysias were primitive and that cushion forming pink flowered species were more advanced.

Table 1. The Taxonomic Treatment of the Genus Dionysia

Section ANACAMPTOPHYLLUM

Subsection SCAPOSAE

1. D. mira
2. **D. bornmuelleri**
3. **D. teucrioides**
4. D. balsamea
5. D. paradoxa
6. D. lacei
7. D. saponacea
8. **D. hissarica**

Subsection REVOLUTAE

9. **D. aretioides**
10. D. leucotricha
11. **D. revoluta**
12. **D. archibaldii**
13. D. rhaptodes
14. D. oreodoxa
15. D. esfandiarii

Section DIONYSIA

Subsection CAESPITOSAE

16. D. lurorum
17. D. caespitosa
18. D. gaubae
19. D. diapensiifolia
20. D. odora
21. D. termeana

Subsection BRYOMORPHAE

22. D. sawyeri
23. D. haussknechtii
24. **D. lamingtonii**
25. D. michauxii
26. D. curviflora
27. **D. janthina**
28. D. iranshahrii
29. D. bryoides
30. D. zagrica
31. D. sarvestanica
32. **D. denticulata**
33. **D. tapetodes**
34. D. kossinskyi

Subsection HETEROTRICHAE

35. D. lindbergii

Section DIONYSIASTRUM

Subsection INVOLUCRATAE

36. D. involucrata
37. D. hedgei
38. **D. freitagii**
39. **D. viscidula**

Subsection AFGHANICAE

40. **D. afghanica**

Subsection MICROPHYLLAE

41. **D. microphylla**

Plants in bold indicate species cultured during the research project.

1.2 Micropropagation

Micropropagation has been used commercially to propagate an increased range of horticultural nursery stock and glasshouse material; this has included plants which could not otherwise have been propagated in the required numbers. Despite the successful micropropagation of many plant species there are still problems associated with the technique, including the transition from *in vitro* culture to the glasshouse environment.

Plants cultured *in vitro* must develop ways to prevent water loss, following their transfer from the water saturated environment in culture, to the lower humidity of the glasshouse. This transition may require a thickening of the epicuticular wax and changes in stomatal function (Brainerd and Fuchigami, 1982). The major source of water loss from these propagules appears to be through the stomata.

The stomata of cultured plants fail to respond, or respond to a smaller extent, to darkness, ABA, and higher than ambient carbon dioxide levels (Koshuchowa et al, 1990; Brainerd and Fuchigami, 1982; Santamaria 1993). A higher proportion of abnormal circular stomata, rather than the more normal elliptical stomata, may also formed in culture (Blanke and Belcher, 1989; Wetzstein and Sommer, 1993 [in Blake and Belcher, 1989]). Failure of stomatal closure may be due to the rigidity of the guard cell wall (Ziv et al, 1987 in Tort

and Coudret, 1993) and/or stomatal deformation (Blancke and Belcher, 1989).

Investigation of rose stomata from culture, or the glasshouse environment, showed no ultrastructural differences, except for the size of the vacuole. *In vitro* plants showed a vacuole occupying 40% of the guard cell area in both light and dark, whereas glasshouse plants showed 40% and 25%, in light and dark, respectively (Tort and Coudret, 1993). It was thought that this difference was, in main, due to guard cell wall rigidity. Rose stomata raised *in vitro* were found to remain nonfunctional after transfer to a normal environment.

Koshuchowa et al (1990) found that the degree of loss of function shown by stomata of birch depended upon the environmental conditions, with a smaller degree of loss under photoperiodic lighting and small temperature gradients. A decrease in humidity and an increase in illumination (Maene and Debergh, 1987 in Tort and Coudret, 1993) have also been suggested to increase functionality. Stomata of young birch leaves were found always to be functional but lost the ability to close after 4-8 days in culture, with the guard cell walls becoming rigid and inelastic. A high level of potassium was found in the guard cells of birch, but not in roses (Sallanon et al, 1993; in Koshuchowa et al ,1990).

Hardening of *in vitro* plants prior to acclimatization (weaning) in a glasshouse would seem to be essential for the

survival of a number of plant species. The patterning of stomata over the leaf surface, pore length and the functionality of stomata, may have an important effect on water loss from the leaf surface. This is particularly important in the weaning of micropropagules, where the rate of water loss may have a great impact on the rate of establishment.

1.3 Leaf Stomatal Patterns

Stomatal density varies amongst species from 20-2000 pores mm^{-2}, but more commonly between 40-350 mm^{-2} (Weyers and Meidner, 1990). The total number of stomata per leaf may be estimated by multiplying the stomatal density by the leaf area. However it has been shown that stomatal density is not constant over the leaf surface and that differences occur between leaves from the same plant (Ticha, 1982). It was found that leaves of middle insertion showed the largest total number of stomata per leaf and that the frequency of stomata in fully expanded leaves increased with higher levels of insertion. On individual leaves the highest stomatal densities were recorded at the leaf tip and showed a declined to the lowest numbers at the leaf base. Differences were found on the edge, the centre, and near the midrib of leaves, but these were not usually significant. Variation in stomatal characteristics, including stomatal density, appear to be a function of the genotype within the species for *Populus*

(Pallardy and Kozlowski, 1979), for *Sorghum* (Liang et al, 1975), and in barley (Mishin et al,. 1972).

Stomatal density, when correlated with stomatal aperture, gives a measure of the gas diffusion potential. This appears true for *Populus*, (Pallardy and Kozlowsky, 1979). Liang et al (1975) found a negative and significant correlation in *Sorghum*, but this was not found by Muchow and Sinclair (1989) where epidermal conductance was unrelated to stomatal pore length, which varied with genotype. Epidermal conductance increased with increasing stomatal density. *Commelina communis* shows differing patterns of stomatal density and stomatal aperture; stomata were not clustered, although the stomata were confined to interveinal areas of the leaf. Higher densities were found at the leaf tips and margins (Smith et al, 1989). Stomatal density in *Glycine max* cultivars was shown to vary due to the level of insertion, light intensity and water stress (Buttery et al, 1993). Irrigation caused a reduction in stomatal density due to leaf expansion. Ticha (1982) found that stomata differentiation continued until the leaf reached 10-50% of its final size. Peak values were found at a leaf area expansion of 10% in *Lycopersicum esculentum*, 15% in *Brassica hirta*, 25% in *Citrus* sp, and 60% in pepper.

1.4 Guard Cell Function

Stomatal Opening

Guard cells accumulate K^+ in order to open. Proton pumping hyperpolarises the membrane causing K^+ to enter through the inward channel, gated by voltage and potassium concentration. Potassium may also enter through a stretch activated channel. Energy for proton pumping is provided by the consumption of ATP, this is stimulated by light and auxins. Auxins also increase the loss of anions which depolarises the membrane, and may lead to stomatal closure (Kearns and Assmann, 1993). Uptake of chloride ions by proton co-transport (Weyers and Meidner, 1990) or stretch activated port, balances the uptake of potassium ions, as does the release of malate from the chloroplasts (Cosgrove and Hedrich, 1991), (Figure 1).

Stomatal closure

Potassium is lost through the outward channel modulated by voltage and extracellular potassium ions. Loss of anions through the anion channel also stimulates the outward potassium channel and causes a depolarisation of the membrane. The entry of chloride ions stimulates the outward potassium channel and anion channel. ABA, IP_3 and calcium ions have been found to inhibit the inward potassium channel in some species. IP_3 has also been found to accelerate the

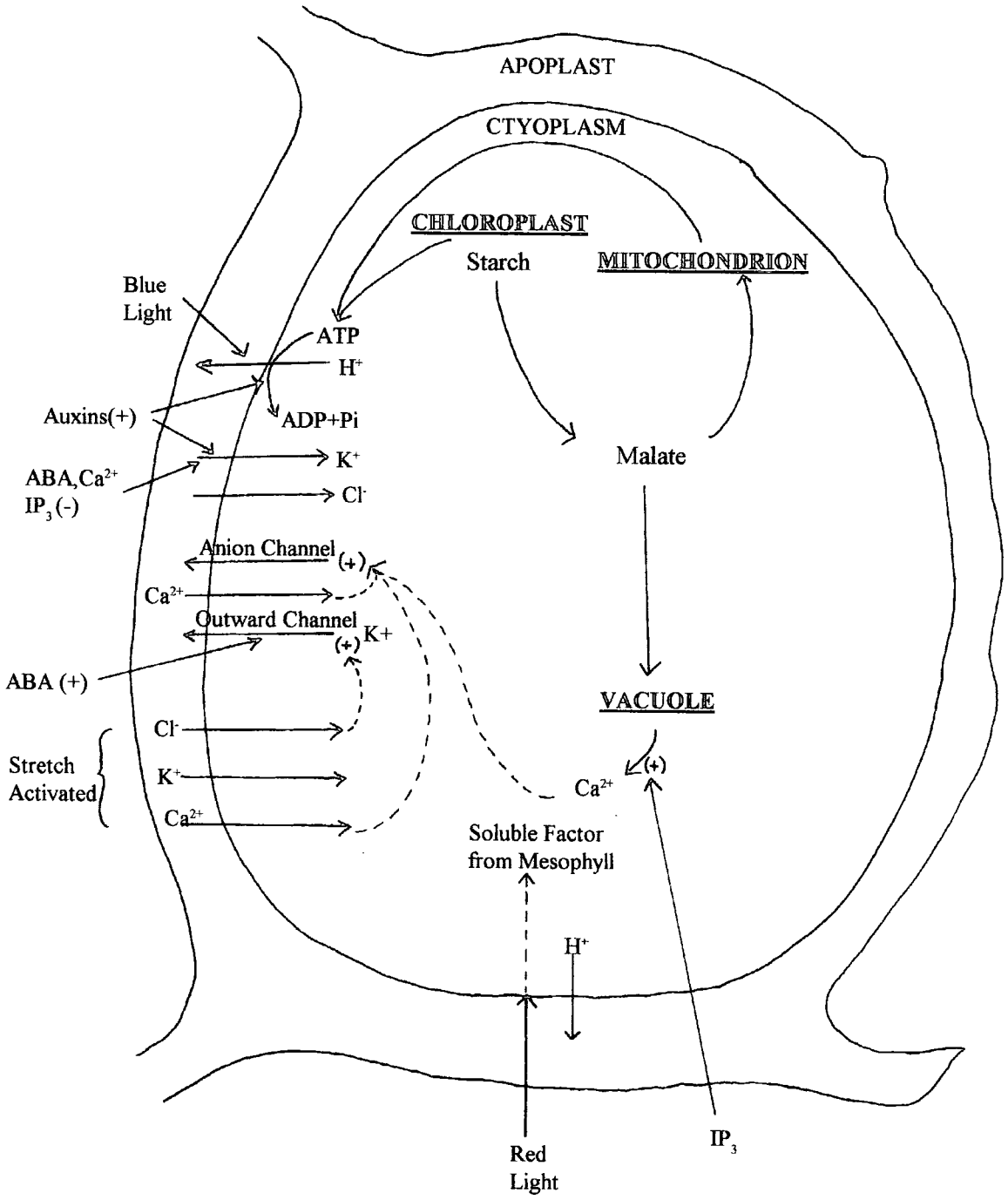


Fig 1 Illustration of the Processes Underlying Guard Cell Turgour as a Function of Stomatal Operation

Based on the ideas of Weyers and Meidner (1990), Cosgrove and Hedrich (1991),Kearns and Assam (1993), and Lee and Bowling (1993)

release of stored calcium in the guard cell (Kearns and Assmann, 1993).

1.4.1 Cations

It has been proposed that the lack of a stomatal closure mechanism in *vitro* cultures may be due to an altered K:Na ratio (Brainerd and Fuchigami, 1982). Wardle et al (1981) found that the K:Na ratio dropped further when *in vitro* material was subject to NaCl stress when compared to plants grown on vermiculite subject to the same treatment. A difference between cultures derived from seed or curd explants was reported; seedlings in vermiculite contained large amounts of calcium, a lesser amount in plants derived from curd and a negligible quantity in seed cultures. Differences were also found in the K:Na ratio in the two *in vitro* cultures. These results should be interpreted with care as the explants were exposed to liquid cultures, the leaf material was in contact with the media for most of the experiment and absorption through the leaves might effect selectivity. The description of seed cultures where 'cotyledonary leaves [were] much thicker and more brittle than those produced by seedlings grown in vermiculite' would also seem to suggest that one culture at least was vitrified and therefore likely to behave abnormally.

In whole plants exposed to NaCl stress sodium competes for uptake with calcium (Martinez and Lauchli, 1993) and/or affects intracellular levels of calcium. Sodium increases membrane porosity, and results in membrane depolarisation (Hawkins and Lewis, 1993). High salt levels reduces K/Na selectivity but can be ameliorated by the addition of calcium (Martinezand Lauchli, 1993). Nitrate ions and calcium were found to enhance the potassium concentration in wheat (Hawkins and Lewis, 1993).

Osborne et al (1993) working with the marginal plant *Gunnera tinctoria* observed that stomata were unable to close due to high concentrations of potassium in the epidermis. Cultivation on a potassium free substrate resulted in greater stomatal apertures in the light and stomatal closure in the dark. Responses were not solely due to potassium loading but also attributed to the K:Na ratio.

1.4.2 The effect of ABA

The closure mechanism of stomata is complex. ABA has been found to cause a rapid efflux of potassium through the outward channel and inhibit the inward channel; part of this initiation process may be calcium dependent, however it appears that the initial rapid stimulation of potassium efflux is calcium independent (MacRobbie, 1990).

Water stress causes an increase in ABA levels in the bulk-leaf tissue or in the transpiration stream several times greater than normal endogenous levels. This change is often accompanied by stomatal closure. Apoplastic ABA may increase due to a release of ABA from the symplast of the mesophyll cells when leaves become stressed (Tardieu et al, 1993). Differences between xylem ABA, or that produced in situ may occur. ABA from the transpiration stream may be removed faster than intracellular pools which may be shielded from cytoplasmic degradation by the chloroplastic envelope (Gowing et al, 1993). Cannon (1990, in Trejo et al, 1993) showed that mesophyll tissues modified the composition of the transpiration stream before reaching the sites of ABA action on the guard cells.

Environmental conditions, temperature, CO_2 concentrations and the nutritional state of the plant influenced the apparent sensitivity of the stomata to ABA. ABA in the transpiration stream due to soil drying was also found to have a highly variable effect on stomata (Trejo et al, 1993).

Gowing et al (1993) found that the rate of stomatal closure was proportional to the log of the concentration of ABA applied through the petiole, stomatal closure beginning after six to eight minutes. However it has been shown that the sensitivity to ABA is highly dependent on the method of feeding.

Trejo et al (1993) found that 1uM of ABA was sufficient to cause stomata on incubated epidermal strips to close, stomata from leaf pieces remained open, whilst leaves fed through the midrib showed an immediate response. The epidermal strips were found to accumulate a large amount of ABA, where as leaf pieces accumulated only small amounts of hormone (fifty percent of which was located in the cells of the mesophyll), the substantial uptake being offset by its rapid metabolism. The epidermal cells and guard cells were found to accumulate ABA in the floated epidermal strips, a linear relationship was found between the ABA concentration in the epidermis and stomatal response. Leaf mesophyll tissue appeared to have an controlling affect of ABA in the leaf.

A lack of response to ABA both in whole plants and with *in vitro* cultures has been shown for a number of species including *Gunnera tinctoria* (Osborne et al 1993), *Malus domestica in vitro* (Brainerd and Fuchigami, 1981).

1.5 Farina

Dionysias in common with other members of the genus *Primula* produce farina, a complex and enigmatic mixture of substances, from glandular hairs on the leaf surface.

Muller (1915 in Wollenweber and Schnepf, 1970) showed that the farina of *Primula pulverulenta* was mainly composed of

flavone ($C_{15}H_{10}O_2$), mixed with a small quantity of additional substances. Blasdale (1947) found that for twenty one species of *Primula* studied, at least seventy five percent of the farina was composed of flavone. Blasdale believed farina to be of taxonomic significance for defining sections of the genus *Primula*, but thought that farina served no useful purpose and was therefore excreted as a waste product.

Flavonoid patterns correlated with species and varieties has been observed in *Cheilanthes* and *Notholaena*. *Primula*, however, produces a genus-specific flavonoid pattern (Wollenweber, 1977). Wollenweber also speculated that as many flavonoid producing plants appeared to be from semi-arid regions there might be a correlation between flavonoid production and an ecological, or physiological, function.

Blasdale (1947) found differences in the structures of farinal glands from four species of *Primula* and also postulated that farina diffused through the wall of the gland. Wollenweber and Schnepf (1970) found that farina was secreted through the gland wall in *P. vulgaris* and was not stored in a subcuticular space as were some oils. Flavonoids of *Pityrogramma chrysoconia* were found to penetrated the cuticle and to crystalise on its the surface (Schnepf and Klasova, 1972). Smooth surfaced, tubular endoplasmic reticulum was found to be a prominent feature of the cytoplasm of glands in *P. vulgaris* (Wollenweber and Schnepf, 1970).

Limited work on *Dionysia* farina has been carried out; but has often been restricted by a lack of material (Wollenweber, 1977). Harborne (1968) produced a systematic study of the components of *Primula* farina; quercetin, kaemferol, gossypetin, and 3',4'-dihydroxyflavone were also found in the farina of *Dionysia aretiodes*

1.6 Aims

The aim of this research project was to investigate the physiology of Dionysias, particularly, *Dionysia aretiodes 'Phyllis Carter'*, during micropropagation. In particular the following objectives were pursued:

To determine the reasons for weaning failures from *in vitro* culture; to ascertain the effect of stomatal patterning and density on this process.

To investigate the activity of stomata under different conditions; to ameliorate stomatal activity and to improve water relations of propagules prior to weaning.

To examine the production and nature of farina under different environmental treatments, with particular reference to water loss and weaning.

2 METHODS AND STATISTICAL ANALYSIS

2.1 Plant Material

Material of *Dionysia aretiodes 'Phyllis Carter'* was derived from plants grown in an alpine house, at Houghall College, Durham (Plate 2). Plants were grown in 7" clay pots, with a compost consisting of:

1 part JI No 2
1 part sand
1 part chick grit
1 part perlite
1 part vermiculite

Pots were plunged in a sand bench, which was watered freely during the summer but kept on the dry side during the winter months. Additional water was given to the pots during the summer if the foliage showed a loss of turgidity. Plants weaned from culture (Plate 3) were potted into aquatic containers that could easily be transferred to larger pots without the need for root disturbance. Dionysias have been shown to be very sensitive to root disturbance and potting can often cause the death of the plant. The material for this, and the other species cultured, was kindly provided by Mr. E. Watson of Newcastle.

Cultures of *Dionysia aretiodes 'Phyllis Carter'* were initiated on 13/3/90 and had been subcultured 17 times at the conclusion of the project (Plate 1).

2.2 Initiation of Cultures.

Seven young shoots, 10mm in length and slightly etiolated, were removed from the centre of the stock plant. The explants were washed in four changes of sterile distilled water to reduce contamination levels; the first washing containing 0.01% Teepol. The explants were transferred to a 10% bleach solution (Chlorex) containing 0.01% Teepol for fifteen minutes followed by three washes of sterile distiled water.

Explants were initiated on half strength Murashige and Skoog media (Murashige and Skoog, 1962), and placed in a Baird and Tatlock cooled incubator with sixteen hour photoperiod, at 535 lux using Sylvania Grow-Lux F20W/Gro tubes, at a temperature of 23°C. *Dionysia aretiodes* was further subcultured on a medium containing no growth regulators, as was *D. lamingtonii*(Plates 4 and 5). Other species were found to benefit from the presence of 2 mg/l BAP and 0.1 mg/l IBA (Table 2).

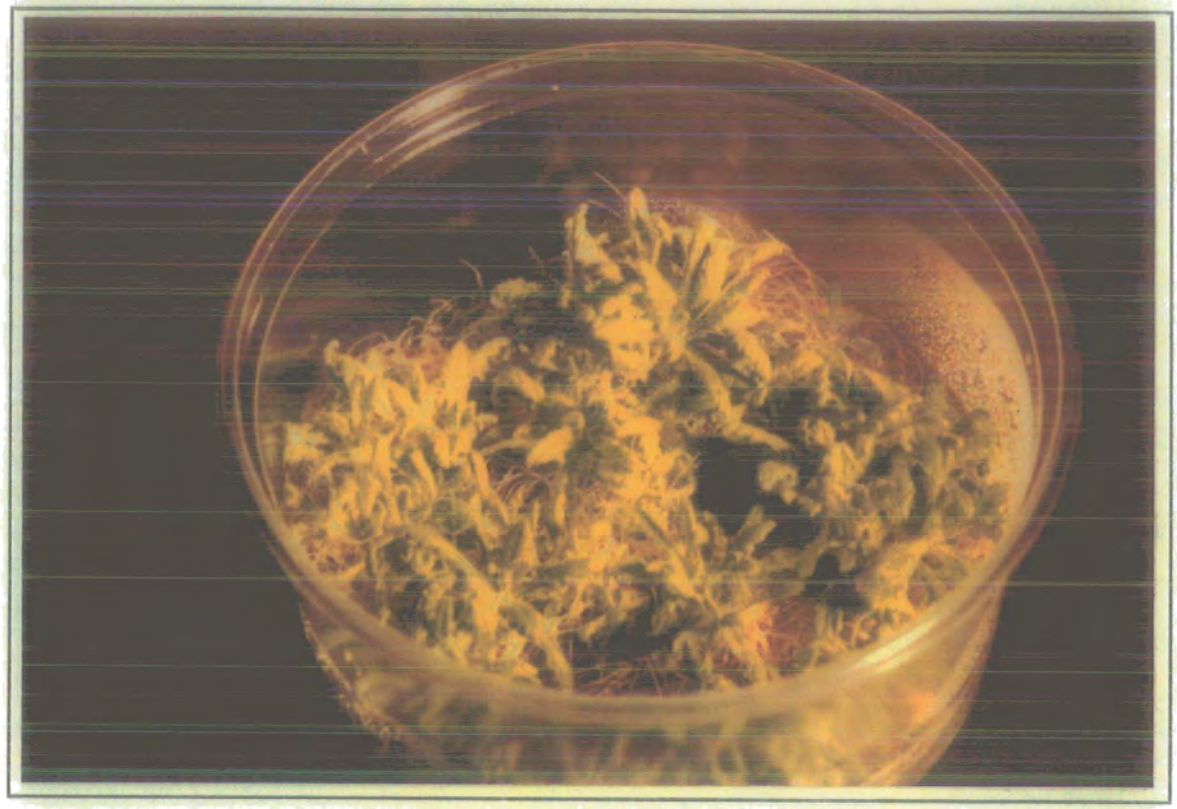


Plate 1 Culture of *Dionysia aretiodes 'Phyllis Carter'*

Plate 2 *Dionysia aretiodes 'Phyllis Carter'*

Plate 3 *Dionysia aretiodes 'Phyllis Carter'* after Weaning from *in vitro* Culture

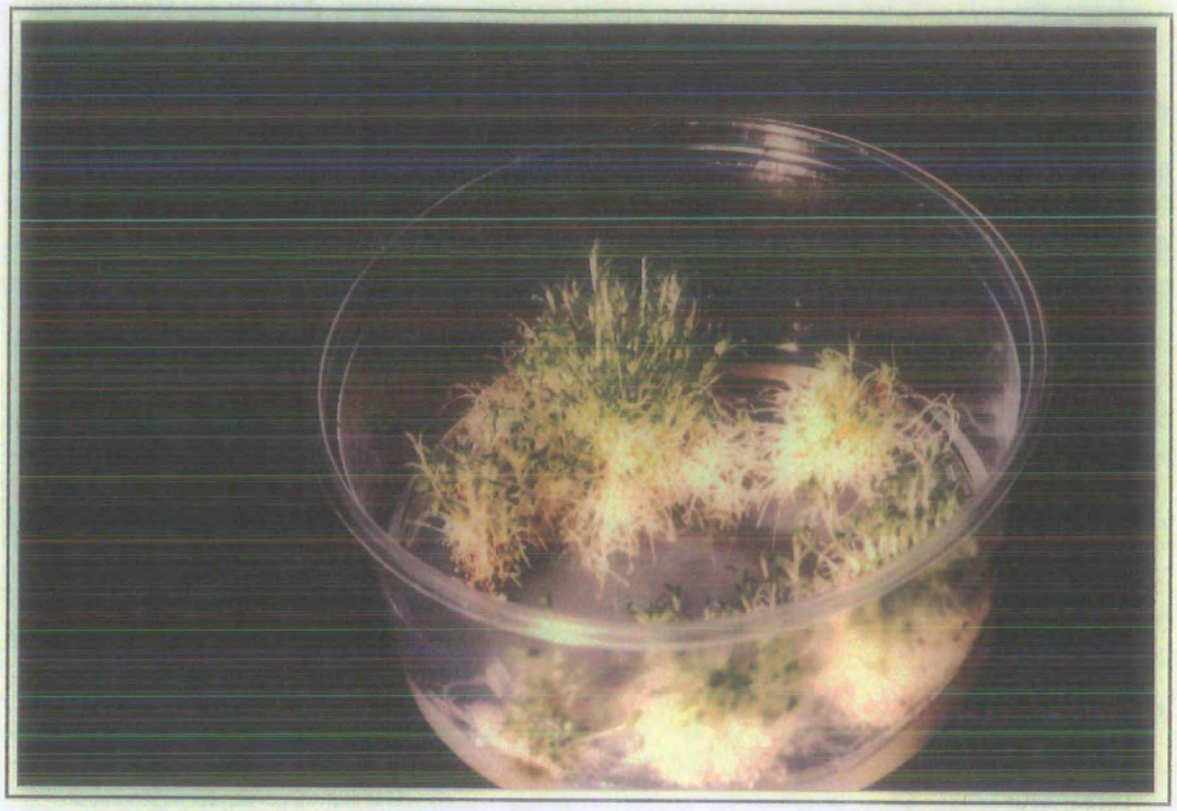


Plate 4 Culture of *Dionysia lamingtonii*

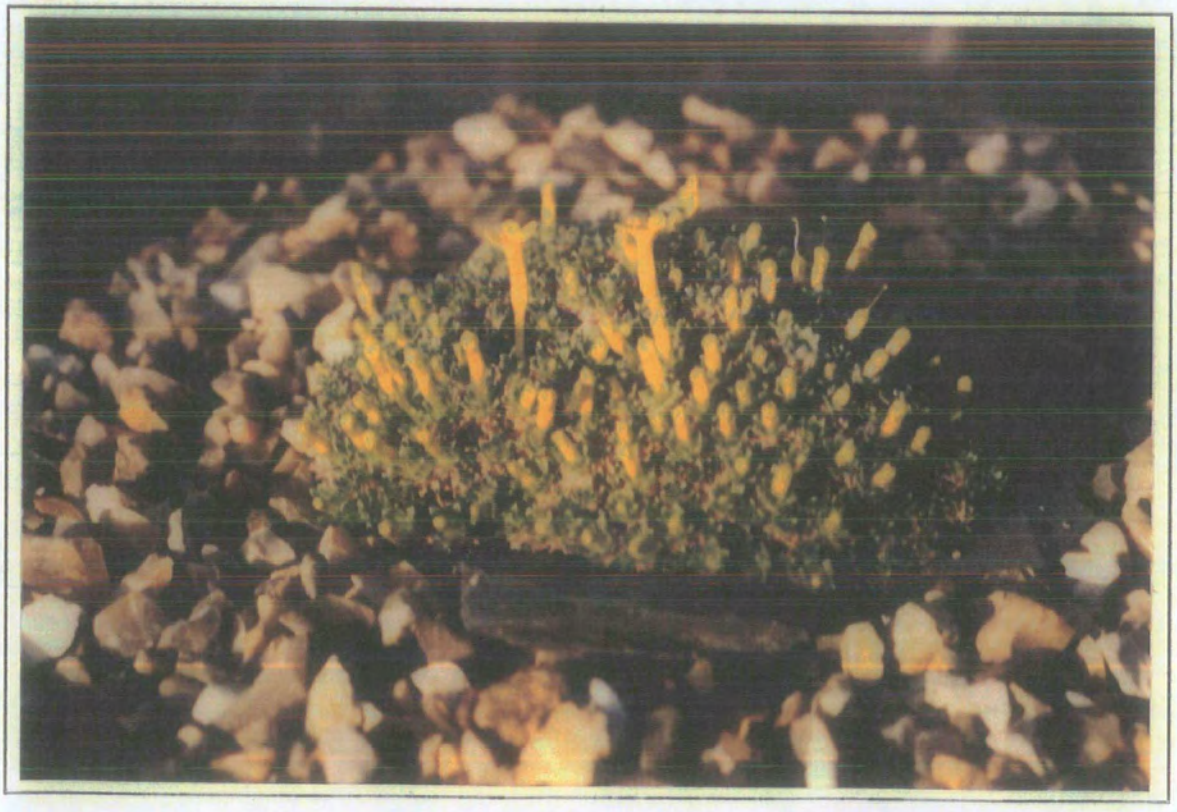


Plate 5 *Dionysia lamingtonii* Established from Culture

Dionysias were cultured in 8oz plastic food containers (Bella Containers [W. K. Thomas Ltd., Chessington, Surrey]) , which permitted the exchange of gases, including water vapour, between the culture vessel and the growing environment. Cultures were stressed by allowing the loss of water vapour from the containers. Stressing occurred gradually as the volume of agar shrank and water uptake by the plant material became more difficult. Physiological changes were found to result from water stress.

Table 2 Formulations of Tissue Culture Media (mg/2l)

Chemical	MS1	MS2	MK
NH_4NO_3	1650	1650	1598.8
KNO_3	1900	1900	
$NH_4H_2PO_3$			123.7
$CaCl_2$	332.2	332.2	332.2
$MgSO_4$	180.7	180.7	180.7
KH_2PO_4	170	170	
$FeSO_4.7H_2O$	27.8	27.8	27.8
Fe Na EDTA			36.7
Na_2EDTA	37.26	37.26	
$MnSO_4.H_2O$	16.9	16.9	16.9
$ZnSO_4.7H_2O$	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2
KI	0.83	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	
$CoCl_2.6H_2O$	0.025	0.025	0.025
myo-inositol	100	100	100
nicotinic acid	0.5	0.5	0.5
pyridoxine.HCl	0.5	0.5	0.5
thiamine.HCl	0.1	0.1	0.1
glycine	2.0	2.0	2.0
sucrose	30000	30000	30000
IBA		0.1	
BAP		2.0	
agar	7000	7000	7000

MS1 =Murashige and Skoog Basal Medium.
MS2 =Murashige and Skoog Basal Medium with hormones.
MK =Minimal Potassium Medium.

2.3 The Weaning of Explants

Material from unstressed cultures were taken and treated as sources of cuttings for conventional propagation. Lower leaves were removed during this process and these were

retained to determine the stomatal densities for individual propagules as previously described.

Thirty cuttings were inserted into 1-5 ml Eppendorf pipette tips half filled with a substrate composed of:

25%	perlite
25%	chick grit
25%	sharp sand
12.5%	John Innes No. 2 compost
12.5%	Loamless compost

The compost was soaked by immersing the tubes in water, these were then pressed into a sand bench in an alpine house so that the cuttings were 1cm below the surface; this provided both shade and a stable temperature. The tops of the tubes were covered with horticultural grade fleece.

The tubes were examined periodically for the appearance of roots or death of the propagule. Death was judged to be the point were the propagule had degraded to a point were rooting was no longer possible.

2.4.1 The Determination of Stomatal Densities

Leaves of approximately the nominated size (to ensure a standard stage of development) were removed and placed

adaxial side down on an adhesive slide, created using Sellotape. The leaves were then flattened out using a seeker. One drop of acetone was applied to the leaf surface to dissolve the farina and to soften the acetate sheet which was then applied. The acetate was worked into the surface using the tip of a finger before applying an even pressure via a microscope slide for 30 seconds. The specimen was than allowed to dry. The slide was removed and the acetate sheet peeled away under running water. The acetate sheet was then dried and trimmed to leave the area of the leaf impression.

The leaf imprints were fastened to a slide using Sellotape. The imprints were then projected through a photographic enlarger and compared to the nominated standard size leaf. Selected specimens were viewed under the microscope. Imprints which were unclear, or had large areas omitted, were rejected.

A Panasonic M5 video camera was focused down the eye-piece of an Olympus BH-2 microscope and the image displayed on a 21" television screen. A micrograticule slide was viewed and the zoom lens of the camera adjusted until 0.2mm equalled 135mm under the x10 lens. Four grids 135m by 135mm were drawn on the acetate sheet and fixed to the screen. Specimens were viewed under the microscope. Stomata were counted in each 0.2 mm by 0.2mm square ($0.04mm^2$) and recorded. Incomplete areas and squares containing vascular tissue were noted. The midrib was discounted.

Maps for the densities of the specimens were prepared using Table Editor and Pagemaker4. The imprints were projected onto the maps and the limits of the leaves defined. Three dimensional maps were prepared using Landscape Explorer on a Paragon 486 DX33 computer with 4Mb of memory. Stomatal densities were then analysed using Minitab.

2.4.2 Tests for the randomness of stomata

To test for the possible clustering of stomata, maps of the relative positions of stomata and glands were prepared. The resulting maps were analysed using the point-centred quarter method; this is an ecological method used to determine the density of species in an area and is applicable only to random distributions. Nearest-neighbour frequencies for stomata and glands were also calculated from the data. The numerical relationship of farinal glands to stomata was also investigated.

The video camera, television and microscope where set-up as previously described. Under the x10 objective the camera zoom lens was adjusted so that 143.5mm equalled 0.2mm. An acetate sheet was attached to the screen and details of stomates and farinal glands in a 200x210mm area recorded. This represented 0.08mm^2 of leaf surface. Four leaves were selected from each treatment and replicated four times. The acetate maps were transferred to paper using a photocopier.

The position of the glands were used as the centre for the point-centred quarter method. Vertical and horizontal lines were drawn through the central points and the distance of the nearest stoma in each of the blocks was recorded. The average distance was then taken. The maximum number of points were taken from each map avoiding the multiple recording of any stoma. Distances were plotted against gland and stomatal densities. Actual and calculated stomatal densities were compared using a chi-square statistic.

Nearest-neighbour frequencies were calculated for both stomata and glands. A stoma was selected on the map and the nearest stoma to this found, the nearest stoma to this point was then located. The process was continued avoiding multiple measurements of any pore. The stomatal density was calculated and results pooled according to density; means and standard deviations were then calculated. The same process was used to calculate the nearest-neighbour measurements for glands. Graphs were then plotted using Cricket Graph.

Nearest-neighbour measurements were also used to evaluate stomatal ordering was calculated according to Clark and Evans (1954, in Korn (1993)).

2.4.3 The Determination of Pore Length for Different Stomatal Densities

The video camera, television and microscope where set-up as previously described. Under the x40 objective the camera zoom lens was adjusted so that 135mm equalled 0.2mm. A suitable area free of flaws was chosen and the stomatal density recorded.

The 0.04mm^2 area was viewed under the high power (x40)lens and six clear stomata selected, four from the corners and two from the centre of the field, and their pore lengths recorded. Six areas of differing density were examined from each leaf, with five replicates per treatment. A scale was established using a micrograticule slide. The x40 lens was found to give a magnification of x7000.

The stomatal density was regressed against pore length for each treatment and the fits and residuals stored. Stomatal density was plotted against the residuals but a curved relationship was not apparent. A regression line was then fitted to the data and graphs plotted.

2.4.4. The Effect of ABA and Wilting on Potassium Accumulationin Guard Cells of Unstressed Material.

Epidermal strips may be stained with sodium cobaltinitrite to show the presence of potassium in epidermal cells. A yellow precipitate of potassium cobaltinitrite is formed in the presence of potassium and may be treated with ammonium sulphide to give a black precipitate of cobalt sulphide; this precipitate is easier to assess under the light microscope. Results may be expressed in terms of a 5-step scale based on percentage area stained (Raschke and Fellows, 1971), or illustrated using photomicrographs (Mansfield and Jones, 1971).

Leaves from unstressed plant material were removed and floated on ABA solutions of 1mg/l, 10mg/l, and 40mg/l ABA for two hours under Philips TLD 30W/29 tubes (7420 lux) at 25 °C. Others were allowed to become flaccid on the bench for forty minutes. All leaves were then incubated on distilled water for two hours under the same conditions in order to stimulate stomatal opening, through an accumulation of potassium. The slow rate of CO_2 diffusion in water results in conditions favourable for stomatal opening (Mansfied and Jones, 1971). Epidermal strips were then removed for the determination of the potassium content as follows:

A solution of sodium cobaltinitrite was prepared by diluting 2ml of glacial acetic acid with 13ml of distilled water, into

which was dissolved 5.5g of sodium cobaltinitrite. Epidermal strips were removed and dipped in acetone to remove farina from the surface. The strips were then dipped in distilled water (DW) to remove the acetone, before being placed in phials of DW in order to remove potassium present on the epidermal surface. Epidermal strips were washed twice, washings being removed with a Pasteur pipette. The water was replaced with ice-cold sodium cobaltinitrite. This was left for thirty minutes before washing three times with ice-cold DW. The water was removed and replaced with 1% ammonium polysulphide solution for two minutes. The strips were washed, and mounted in DW and examined under the microscope for evidence of cobalt sulphide precipitate.

Further experiments were carried out on leaves from pot grown material, pot grown material stressed for forty minutes on the bench and leaf material grown on media supplemented with additional calcium (Table 3).

2.4.5 The Effect of ABA on Stomatal Aperture

The removal of epidermal strips may cause changes to the stomatal aperture due to the change of pressure when removed from the underlying and connecting mesophyll tissue. Further changes occur with the unavoidable rupturing of epidermal cells, the turgor of which exerted a counter pressure on the guard cells, Weyers and Meidner (1990). These effects,

however, should be constant for all the material sampled allowing a comparison between treatments to be made.

Epidermal strips require to be fixed prior to examination to prevent changes occur after the conclusion of the experiment. However immediate and subsequent changes in aperture have been found when strips were plunged into absolute alcohol (Heath, 1950; Meidner, 1981; Hsiao and Fisher, 1975 [in Weyers and Meidner, 1990]); with epidermal strips being apparently influenced by light and the duration of exposure to the fixative.

Leaves of *in vitro* raised *Dionysia aretiodes 'Phyllis Carter'* were removed and floated abaxial surface down in distilled water, for four hours, under illumination (7420 lux) at 25 °C. Leaves were then transferred to solutions of 1, 10 and 40 mg/l ABA for two hours under the same conditions.

After two hours the leaves were removed and placed in ice-cold FAA in the dark until examination. The experiment was staggered so that all samples spent approximately one hour in the fixative prior to examination. FAA was found to remove the farina from the leaf surface. Leaves were washed in distilled water. Epidermal strips were removed, mounted in water, and examined under the light microscope at x40 magnification.

The image was displayed on a television screen as previously described, giving a magnification of x8000. Pore length and pore width were measured using graph paper.

Pore aperture was calculated as for an ellipse:

$$pi\ (ab)$$

where a and b are the radii of the long and short axes.

2.5 Analysis of Farina from Dionysia Species

Leaves were removed from the plant material, *pot grown (de novo)* and *in vitro,* and dipped in acetone to remove the farina. Leaves were then sandwiched between an acetate sheet and Sellotape. Leaf areas were ascertained by printing an enlargement of the leaf material using a photocopier, and weighing the resultant duplicates against a standard. The acetone was allowed to evaporate from the farina sample which was then redissolved in 0.2ml acetone.

Sample thin layer chromatography (TLC) plates (Whatman 250um silica gel on aluminium, 20x20cm plates) were run using farina from *Dionysia bornmuelleri* and *Dionysia mira* in 10% acetic acid in chloroform, to determine loading in relation

to leaf area. Loading was set at a sample of farina from 5.56cm^2 of leaf material.

Samples of:

Dionysia archibaldii
Dionysia aretiodes 'Phyllis Carter' -pot grown
Dionysia aretiodes 'Phyllis Carter' -unstressed
Dionysia aretiodes 'Phyllis Carter' -stressed
Dionysia aretiodes 'BBF'
Dionysia aretiodes 'Gravetye'
Dionysia aretiodes 'Paul Furse'
Dionysia bornmuelleri
Dionysia denticulata -in vitro culture
Dionysia denticulata -pot grown
Dionysia hissarica
Dionysia mira
Dionysia tapetoides
Dionysia tapetoides H1146
Dionysia teucriodes

were prepared as described.

TLC plates were allowed to run until the solvent had moved 160mm up the plate, or until the solvent front started to break up, which ever occurred first.

Plates were allowed to dry and were then viewed and photographed under UV light at 366 nm.

Samples of farina in acetone were also examined using a Cecil CE 6602 scanning spectrophotometer

2.6.1 Determination of Cations in Leaf Material

Leaf material from unstressed, stressed, minimal potassium media and pot grown specimens, was removed and dried in an oven at 60°C overnight and stored in a desiccator until required.

0.01g replicates of material were taken and ashed at 500 °C for six hours in crucibles washed in 4% nitric acid. 0.3ml of 6MHCl was added to dissolve the cold ash, and this was pipetted into 1.5 ml Eppendorf tubes. The solutions were evaporated to dryness in a vacuum oven (80°C and 400mm Hg) for one and a half hours. 0.1ml of 6MHCl was added and the solutions boiled in a water bath for twenty minutes, before being cooled in ice-cold water. 0.9ml of distilled water was added before again boiling for twenty minutes. The solutions were again cooled, and then centrifuged. Sample solutions were prepared by diluting 0.4ml of the original solutions to 1ml.

Solutions for analysis were prepared as follows:

Sodium -0.1ml of the final solution was diluted to 1ml.

Potassium -0.25ml of the final solution was diluted to 1ml.

Calcium/ Magnesium -0.1ml of 10% lanthanum solution was added to 0.1ml of the final solution, which was then diluted to 1ml with distilled water.

Potassium and sodium were determined by atomic emission using an Instrument Laboratory aa/ae spectrophotmeter 357. Calcium and magnesium were determined by atomic absorption. Standards were prepared following MAFF (1986).

2.6.2 The Effect of pH and Calcium Concentration on Cation Accumulation

The experiment was repeated using additional material from *in vitro* cultures which had been grown under modified calcium and/or pH regimes (Table 3).

Table 3 Calcium and pH Modified Media

Culture Media	$CaCl_2$ mg/l	calcium level	pH
W	166.1	x1	6.8
X	830.5	x5	5.7
Y	830.5	x5	6.8
Z	1661.0	x10	5.7

3 RESULTS AND STATISTICAL ANALYSES

3.1 The Weaning of Explants

The survival ability of the thirty propagules was investigated, of which eight were found to root. The number of propagules for which stomatal densities were obtained was smaller due to difficulties obtaining leaves at the same stage of development and obtaining suitable imprints. Stomatal densities were again found to be variable (Fig 2).

Fig 2 Stomatal Densities Derived from Propagules

SPECIMEN	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
2	**212**	**18.406**	**4.351**	*)
4	13	24.231	4.585	(-*-)
5	64	33.750	7.892	(*
6	**160**	**15.694**	**4.256**	*)
7	36	50.639	6.193	(*)
9	32	23.750	9.105	(*)
10	10	38.000	7.732	(-*-)
12	75	19.467	4.455	(*)
14	32	34.000	4.392	(*)
19	15	26.400	6.220	(-*)
21	65	23.985	13.206	(*)
22	35	37.543	8.621	(*)
a*	***187***	***5.064***	***1.632***	*)*
c*	***281***	***3.954***	***1.435***	*(**
d*	***117***	***3.556***	***1.170***	*)*
POOLED STDEV =		5.004		15 30 45

* denotes propagules initially discounted due to size
Figures in bold type indicate propagules which rooted

Table 4 Dates of Rooting or Removal of Propagules

Propagule	Rooting	Removal
2	22/6	
6	29/6	
7		28/7
9		25/8
10		5/8
12		21/7
14		29/6
19		14/7
21		25/8
22		6/7
a	22/6	
c	22/6	
d	22/6	

Although a general trend for the early rooting and late death of propagules was observed (Table 4) some early deaths and late rooting were also found. The date of death was not found to be correlated to stomatal density.

The small number of acceptable samples for analysis, particularly for those which rooted, was found to cause statistical problems. The first regression was found to indicate that stomatal density played little role in propagule rooting and that the individual cuttings and leaf samples were more important in determining outcome (Figs 3 and 4).

Fig 4 REGRESSION OF ROOTING, EXCLUDING ADDITIONAL DATA

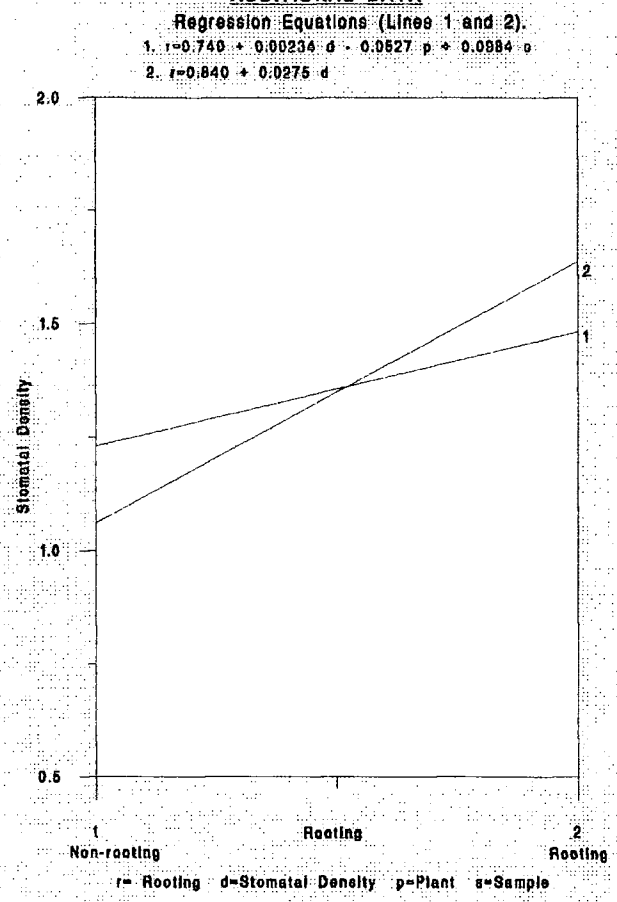


Fig 5 REGRESSION OF ROOTING, INCLUDING ADDITIONAL SAMPLES

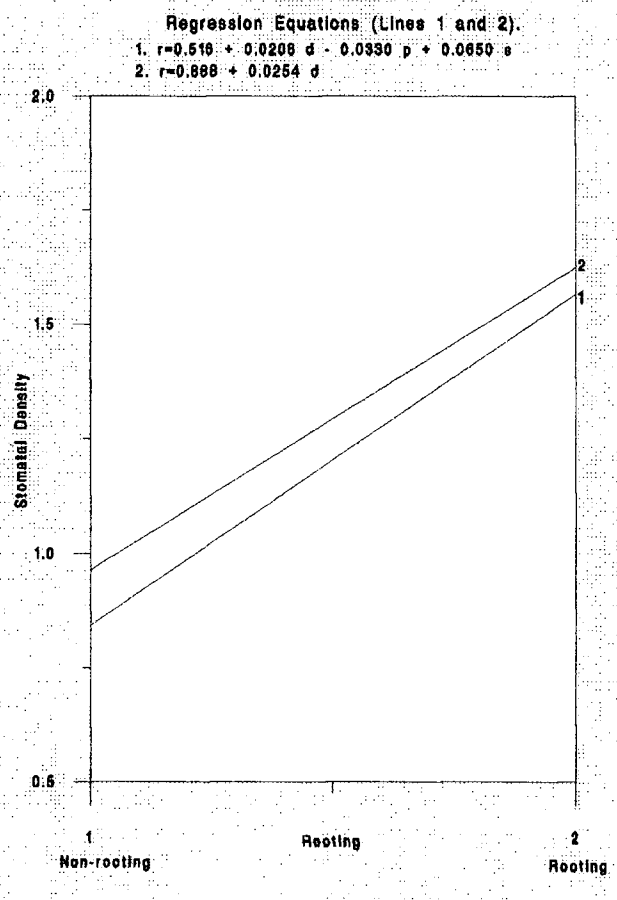


Fig 3 Best Fits for Regressions Excluding Undersized Material

Best Subsets Regression of r

Vars	R-sq	Adj. R-sq	C-p	s	d	p	s
1	86.3	86.3	285.1	0.18132			X
1	68.1	68.0	1473.8	0.27665		X	
1	20.3	20.2	4589.0	0.43699	X		
2	90.5	90.5	9.7	0.15075		X	X
2	86.8	86.7	254.5	0.17813	X		X
2	71.1	71.0	1277.9	0.26341	X	X	
3	90.6	90.6	4.0	0.14993	X	X	X

d=stomatal density, p=propagule, s=sample.

It was decided to investigate, and then include details from, undersized material. Stomatal densities are known to increases during leaf development and then drop as leaf area increases. Ticha (1982) found that peak values occurred at low levels of leaf area expansion. Stomatal densities for the undersized material were found to be low, it was deemed likely that stomatal densities would decrease rather than increase. The results were included to contrast with the previous analysis.

Fig 6 Best Fits for Regressions Including Undersized Material

Best Subsets Regression of r

Vars	R-sq	Adj. R-sq	C-p	s	d	p	s
1	38.9	38.9	1772.4	0.31397	X		
1	7.2	7.1	3312.6	0.38697		X	
1	0.1	0.0	3657.4	0.40150			X
2	64.4	64.4	535.1	0.23970		X	X
2	58.6	58.6	817.0	0.25851	X		X
2	44.7	44.6	1493.9	0.29889	X	X	
3	75.4	75.3	4.0	0.19941	X	X	X

d=stomatal density, p=propagule, s=sample.

The individual effect of density, measured by r^2, when considered alone was found to be higher than the other variables when the additional results were included. The model most closely fitting the results would still include the leaf sample and propagule, as well as density (Figs 5 and 6). However, density would appear an important factor in determining the chances of a propagule rooting, when the additional results are included. Given the low level of propagule establishment and the effect of adding additional results, it would be beneficial to repeat the experiment on a larger scale in order to precisely determine the effect of stomatal density upon rooting.

3.2 The Determination of Stomatal Densities

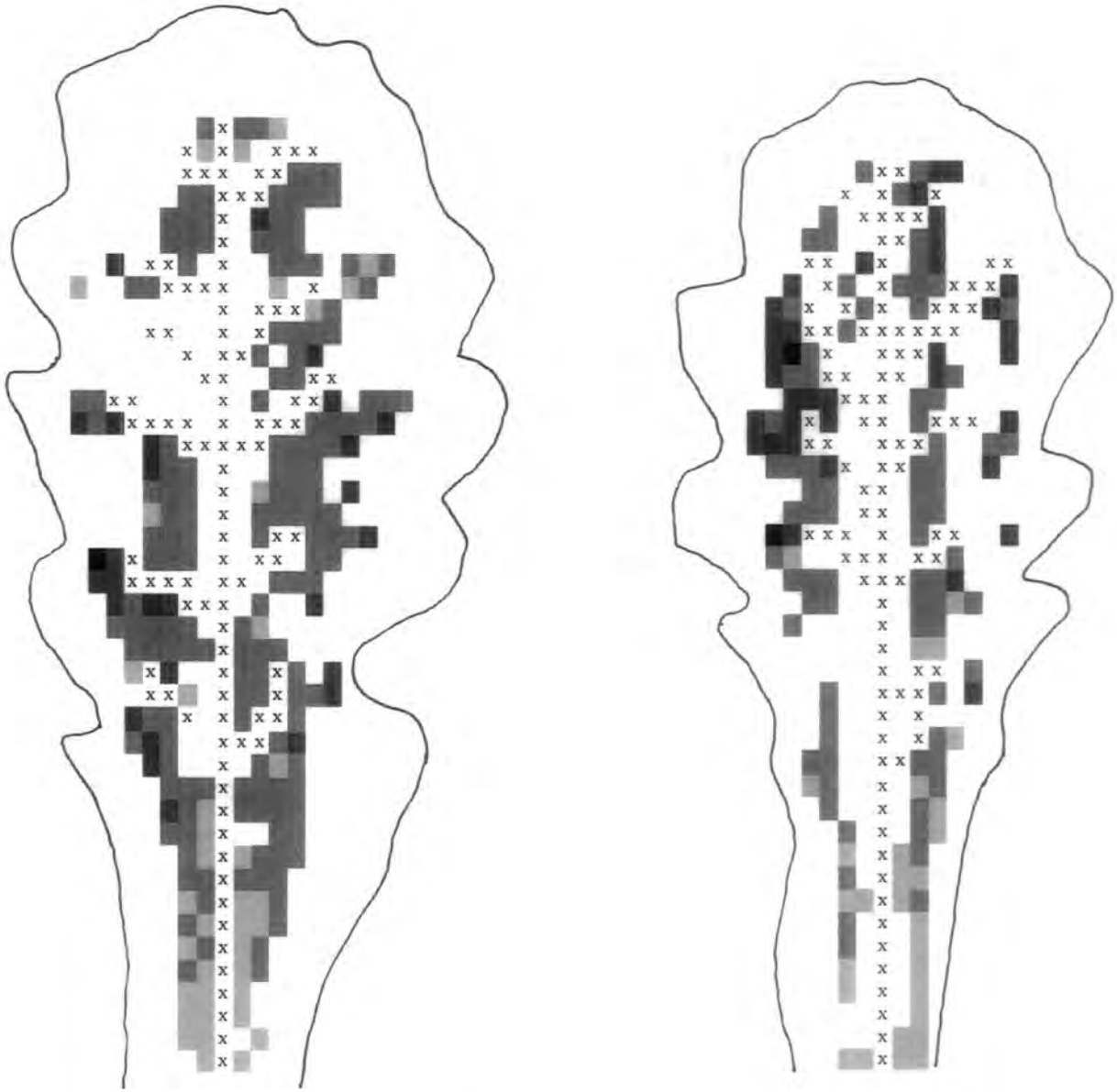
Large variations in stomatal densities were found within treatments. The variation between leaves from pot grown plants was more limited (but still significant) than the variation in stressed and unstressed *in vitro* grown material (Table 5).

Table 5 Means and Standard Deviations for Stomatal Density ($0.04mm^2$ squares) from Whole Leaf Counts

	Pot	Stressed	Unstressed
	7.92 (±2.71)	12.50 (±4.51)	14.03 (±7.07)
	7.22 (+2.61)	9.50 (±3.95)	15.44 (±5.33)
	7.79 (±2.26)	11.10 (±4.08)	8.30 (±2.88)
	8.39 (±3.47)	8.56 (±4.08)	6.64 (±2.31)
	9.47 (±3.56)	5.64 (±1.88)	9.51 (±3.31)
	9.26 (±3.97)	12.26 (±2.78)	14.32 (±5.16)
	8.45 (±3.39)	11.38 (±3.42)	11.68 (±4.57)
	10.67 (±2.96)	7.03 (±2.07)	
		10.38 (±2.73)	
Pooled	8.42 (±3.20)	10.11 (±4.69)	10.97 (±5.43)

Control of stomatal density appears to be lost during *in vitro* culture, with greater variation in stomatal densities compared to results from whole plants. The inability to control stomatal densities during micropropagation has been reported for a number of species (Pearson, personal communication) and has been linked with excessive water loss from micropropagules during weaning.

Cultures of *Dionysia aretiodes 'Phyllis Carter'* were found to produce very variable stomatal densities on the surface of leaves, with higher and lower stomatal densities recorded compared to pot grown material (Figs 7-21). Stressed cultures of *Dionysia aretiodes 'Phyllis Carter'* produced density patterns which were highly variable but with a slight trend to lower densities compared to unstressed material (Appendix 1). It may be postulated that the wide range of stomatal densities found in plants grown *in vitro* may result



	No data.
	0-5 stomata per 0.04mm square.
	6-10 stomata per 0.04mm square.
	11-15 stomata per 0.04mm square.
	16-20 stomata per 0.04mm square.
	21-25 stomata per 0.04mm square.
X	Possition of vascular tissues.

Fig 7 Maps of Stomatal Distribution -Pot Grown Material

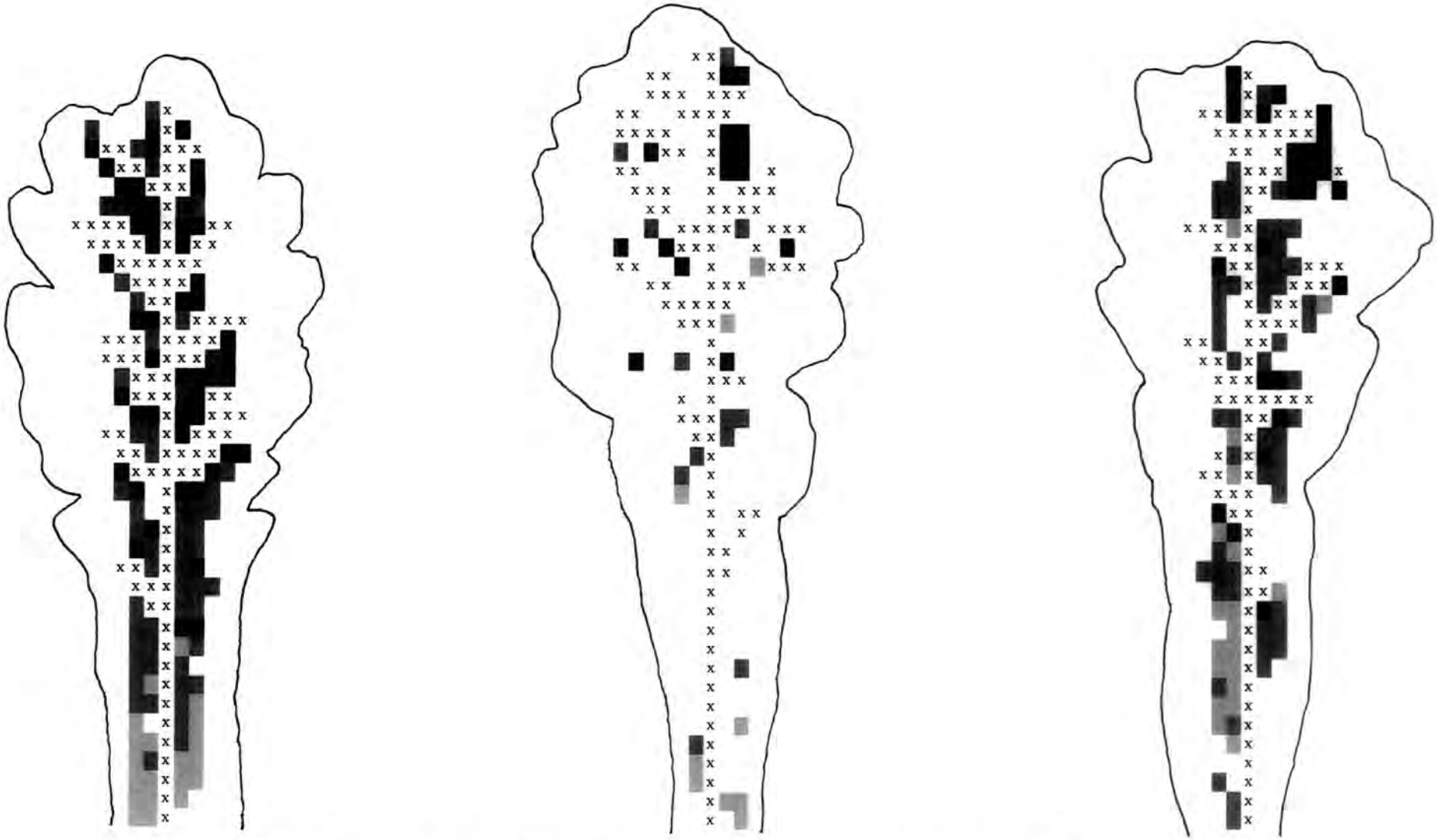


Fig 8 Maps of Stomatal Distribution -Pot Grown Material

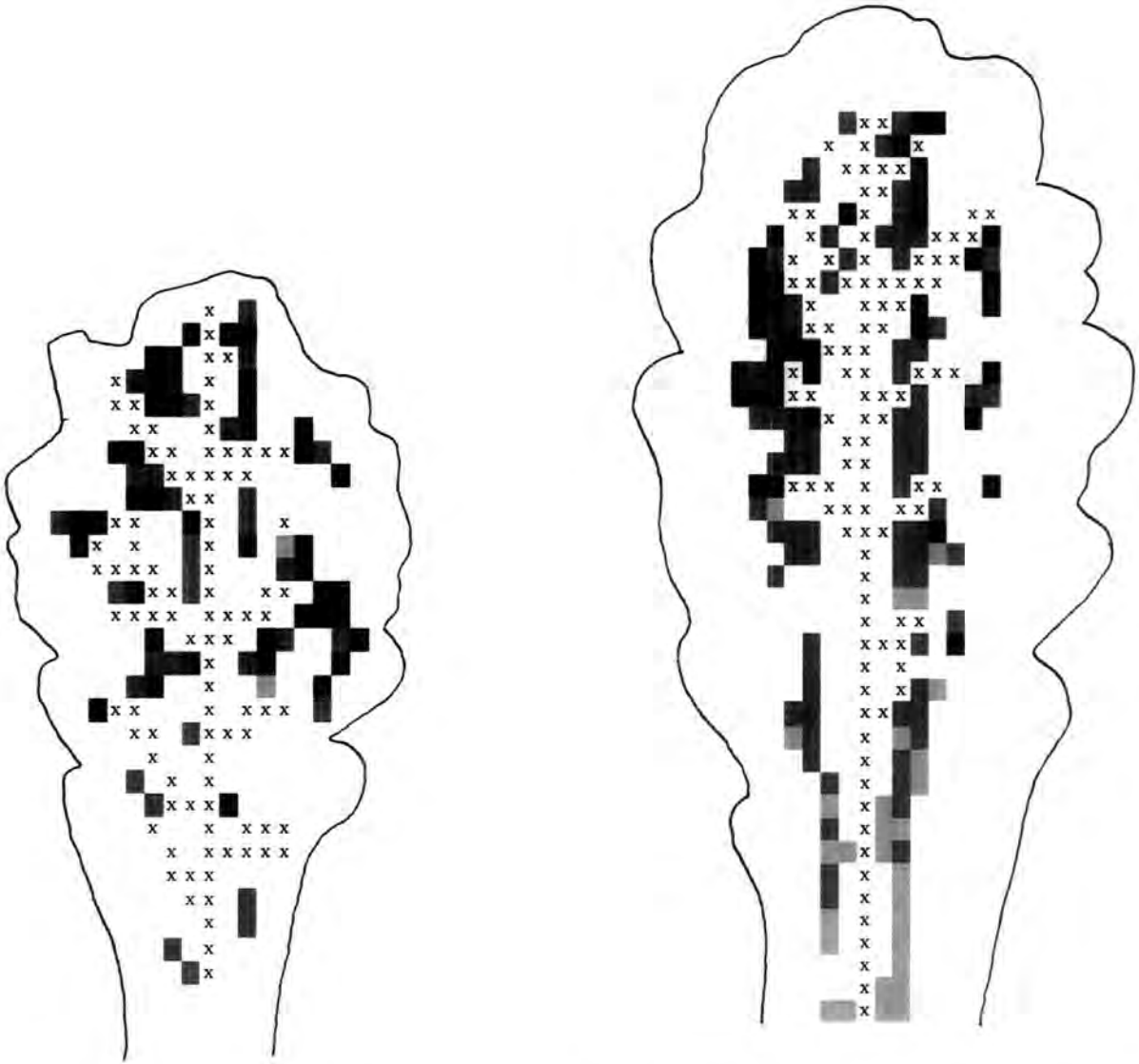
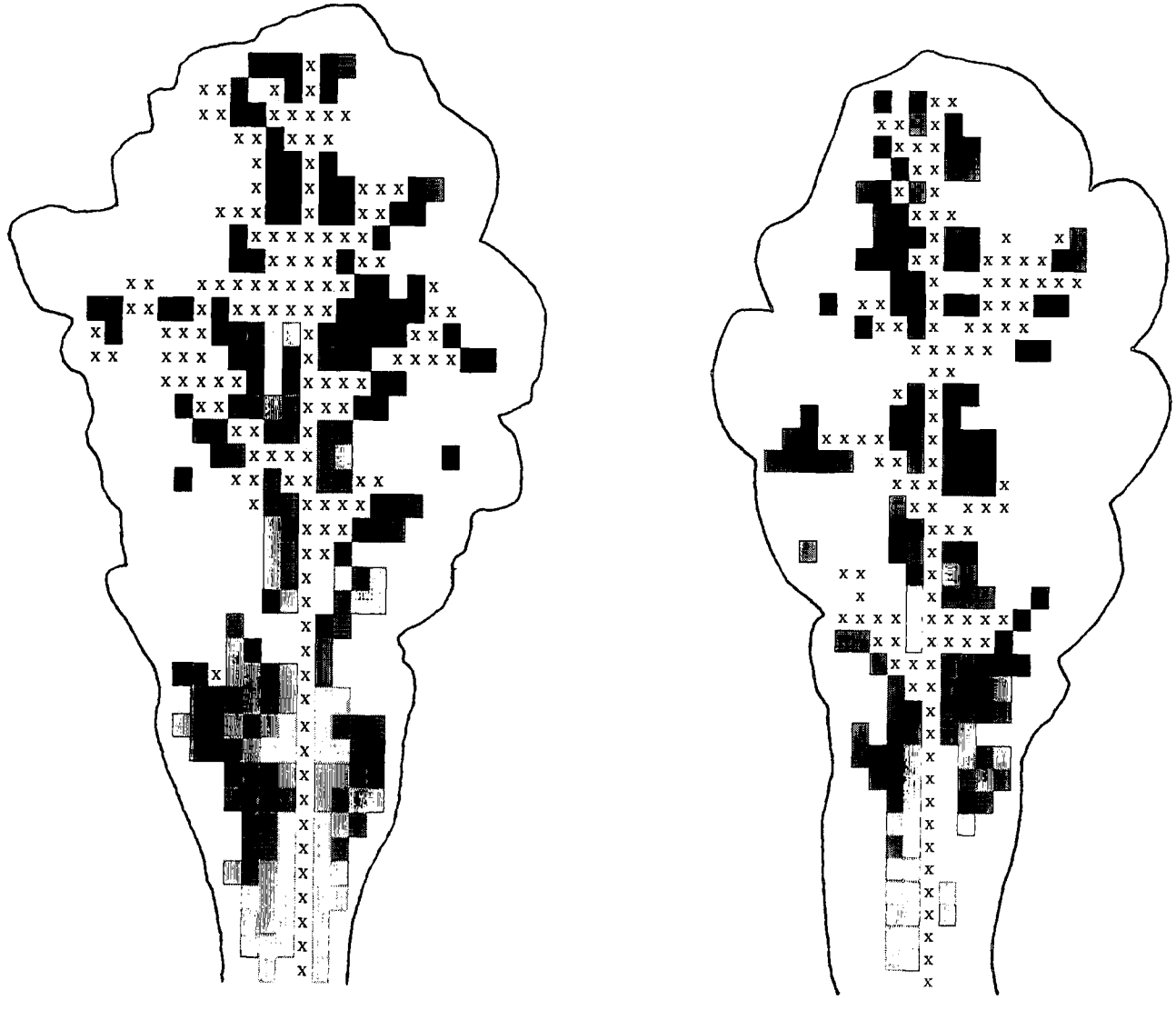


Fig 9 Maps of Stomatal Distribution -Pot Grown Material



	No data.
	0-5 stomata per 0.04mm square.
	6-10 stomata per 0.04mm square.
	11-15 stomata per 0.04mm square.
	16-20 stomata per 0.04mm square.
	21-25 stomata per 0.04mm square.
X	Possition of vascular tissues.

Fig 10 Maps of Stomatal Distribution -Unstressed Cultures

Fig 11 Maps of Stomatal Distribution -Unstressed Cultures

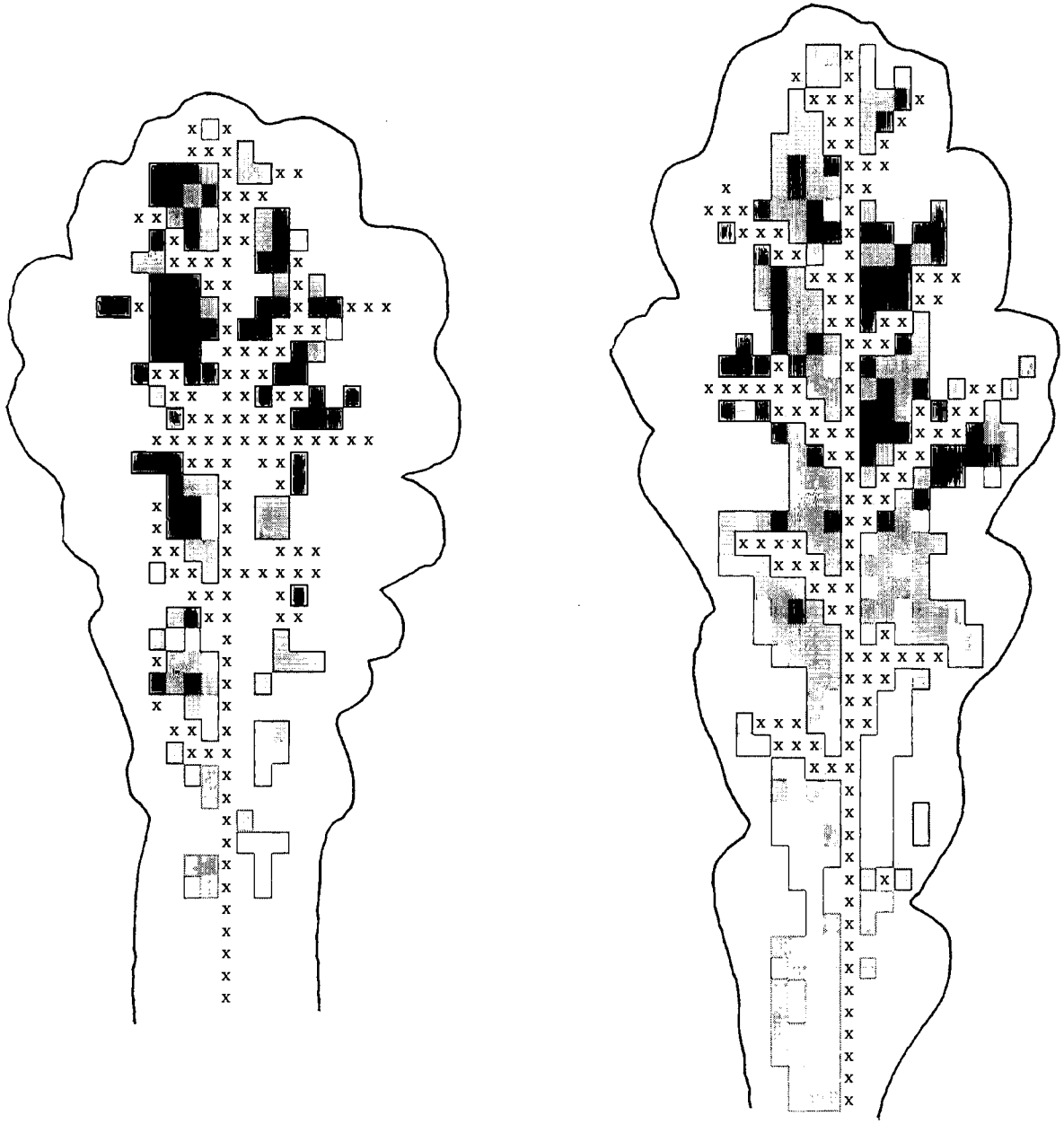
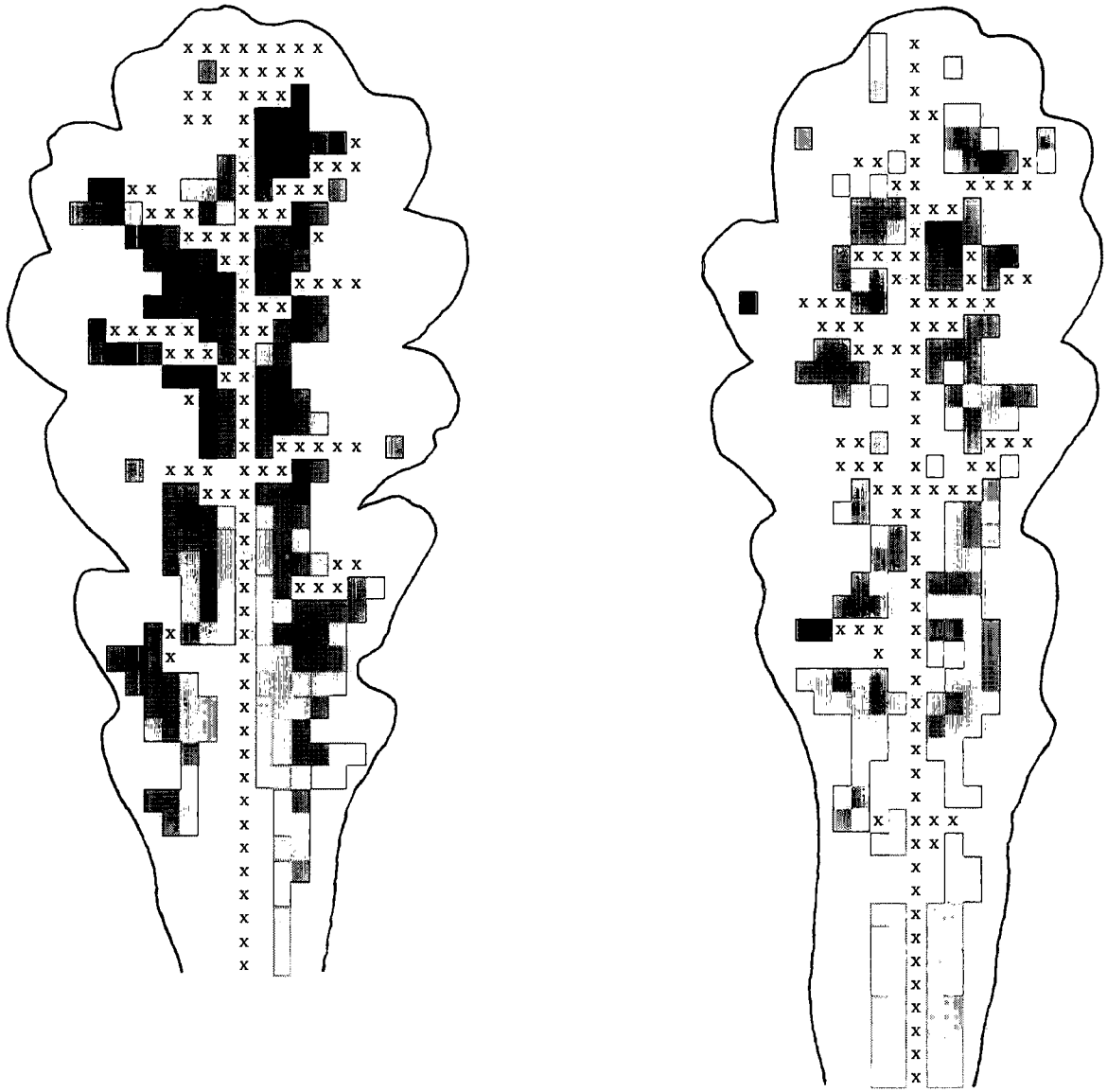


Fig 12 Maps of Stomatal Distribution -Unstressed Cultures



	No data.
	0-5 stomata per 0.04mm square.
	6-10 stomata per 0.04mm square.
	11-15 stomata per 0.04mm square.
	16-20 stomata per 0.04mm square.
	21-25 stomata per 0.04mm square.
X	Possition of vascular tissues.

Fig 13 Maps of Stomatal Distribution -Stressed Cultures

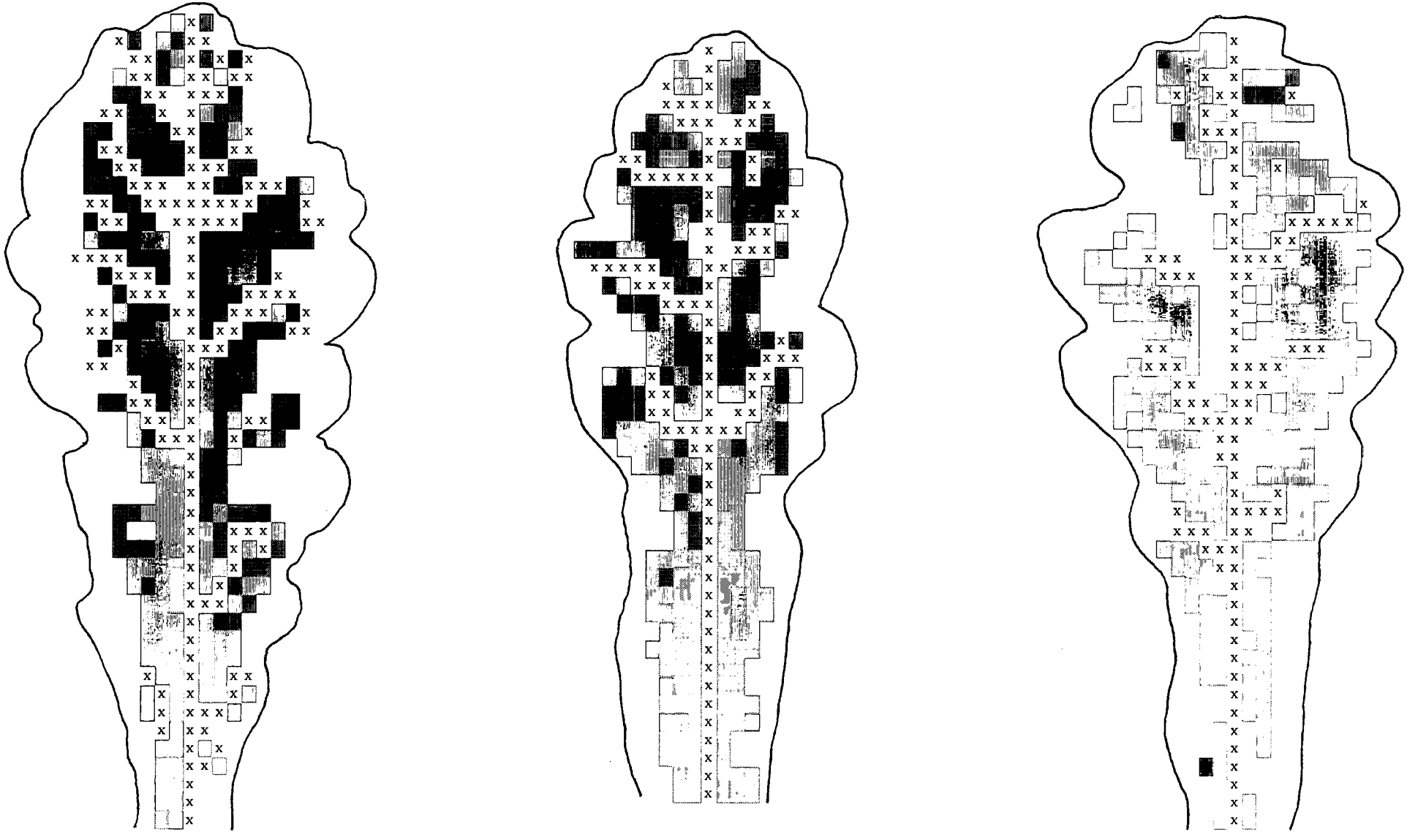


Fig 14 Maps of Stomatal Distribution -Stressed Cultures

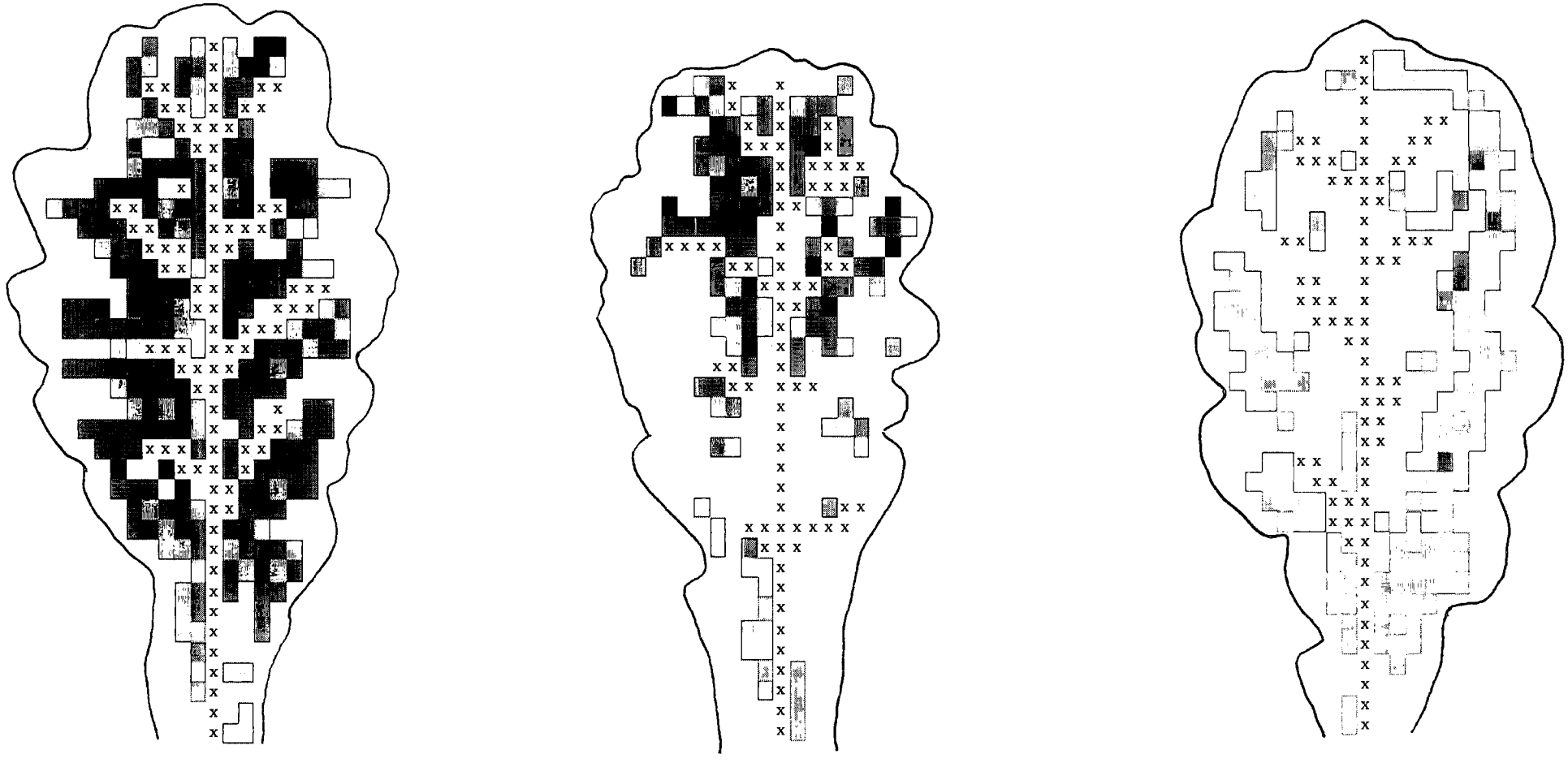


Fig 15 Maps of Stomatal Distribution -Stressed Cultures

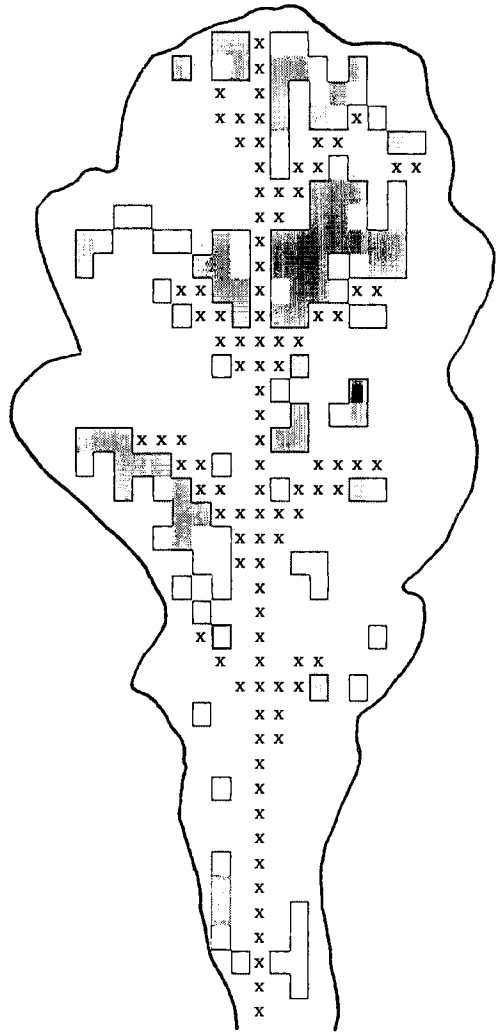


Fig 16 Map of Stomatal Distribution -Stressed Culture

Key to 3-D Stomatal Density Maps

P = PETIOLE

16- Stomata /0.04mm²

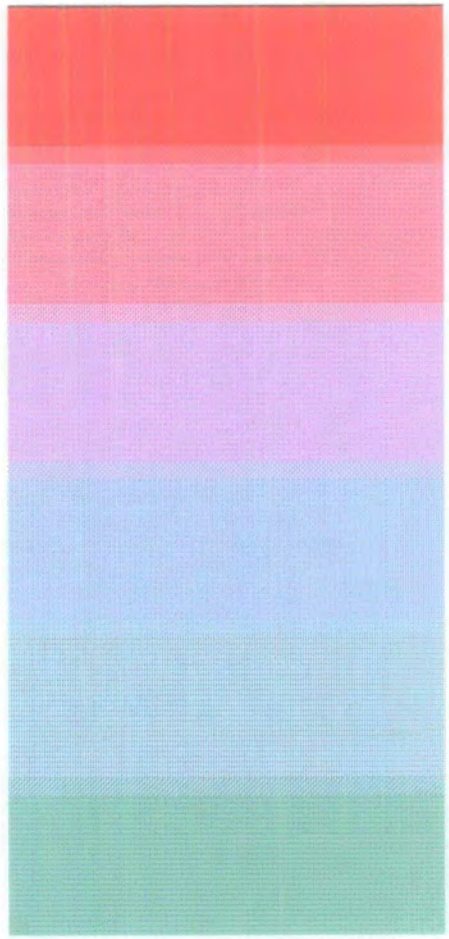
11-15 Stomata /0.04mm²

6-10 Stomata /0.04mm²

0-5 Stomata /0.04mm²

No Data

Back Ground



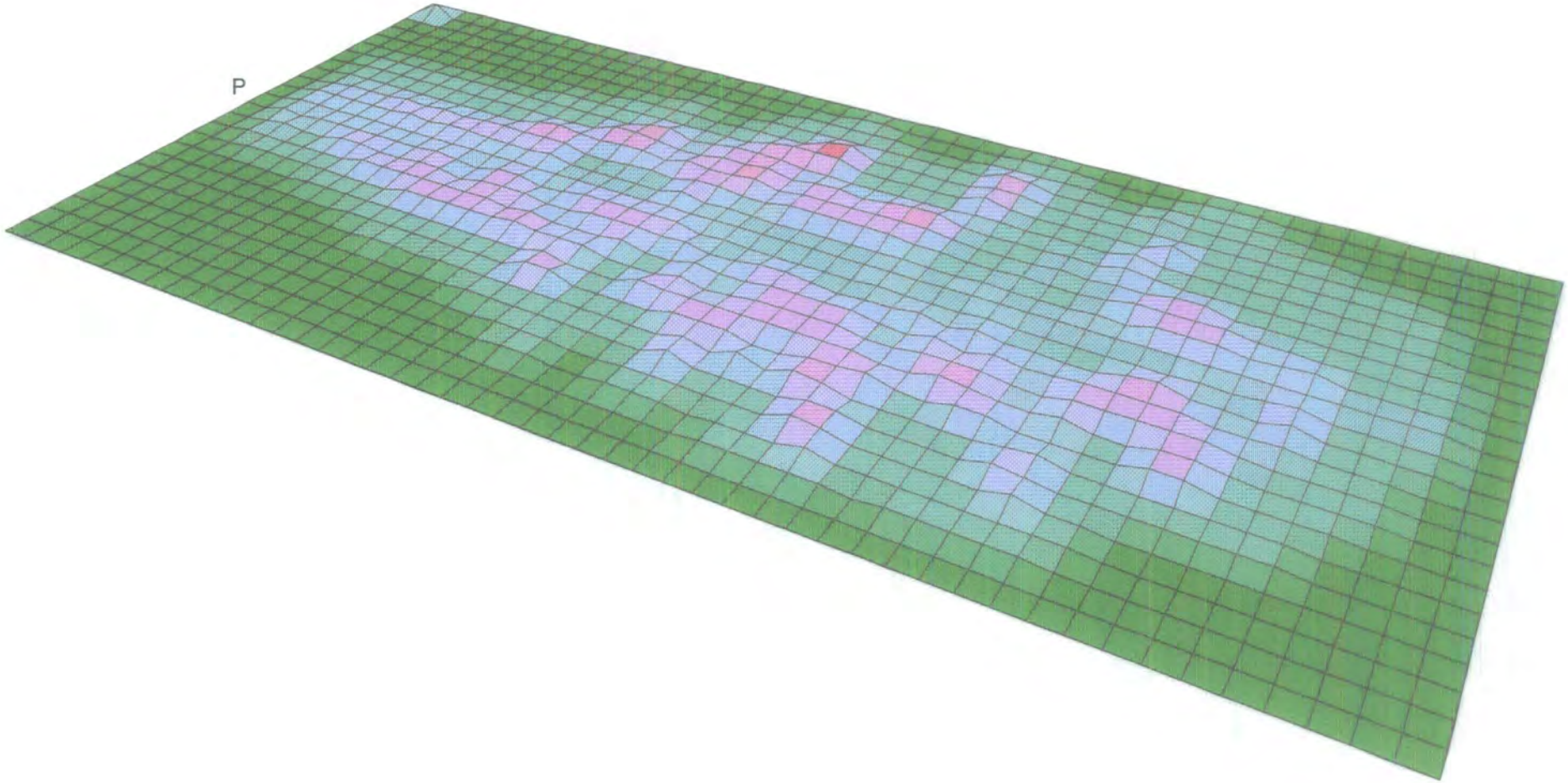


Fig 17 Map of Stomatal Frequency -Pot Grown Material

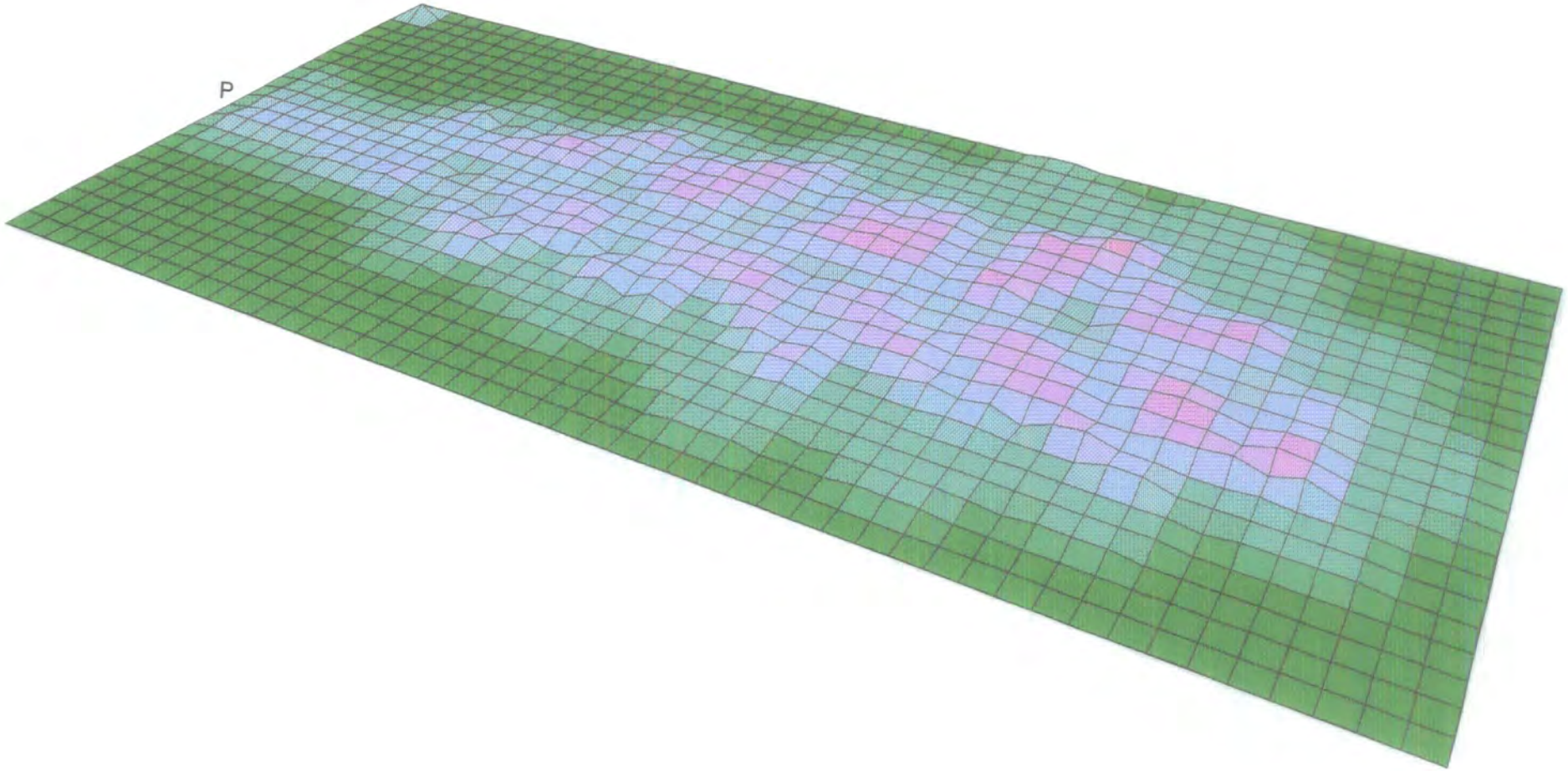


Fig 18 Map of Stomatal Frequency -Unstressed Material of Low Density

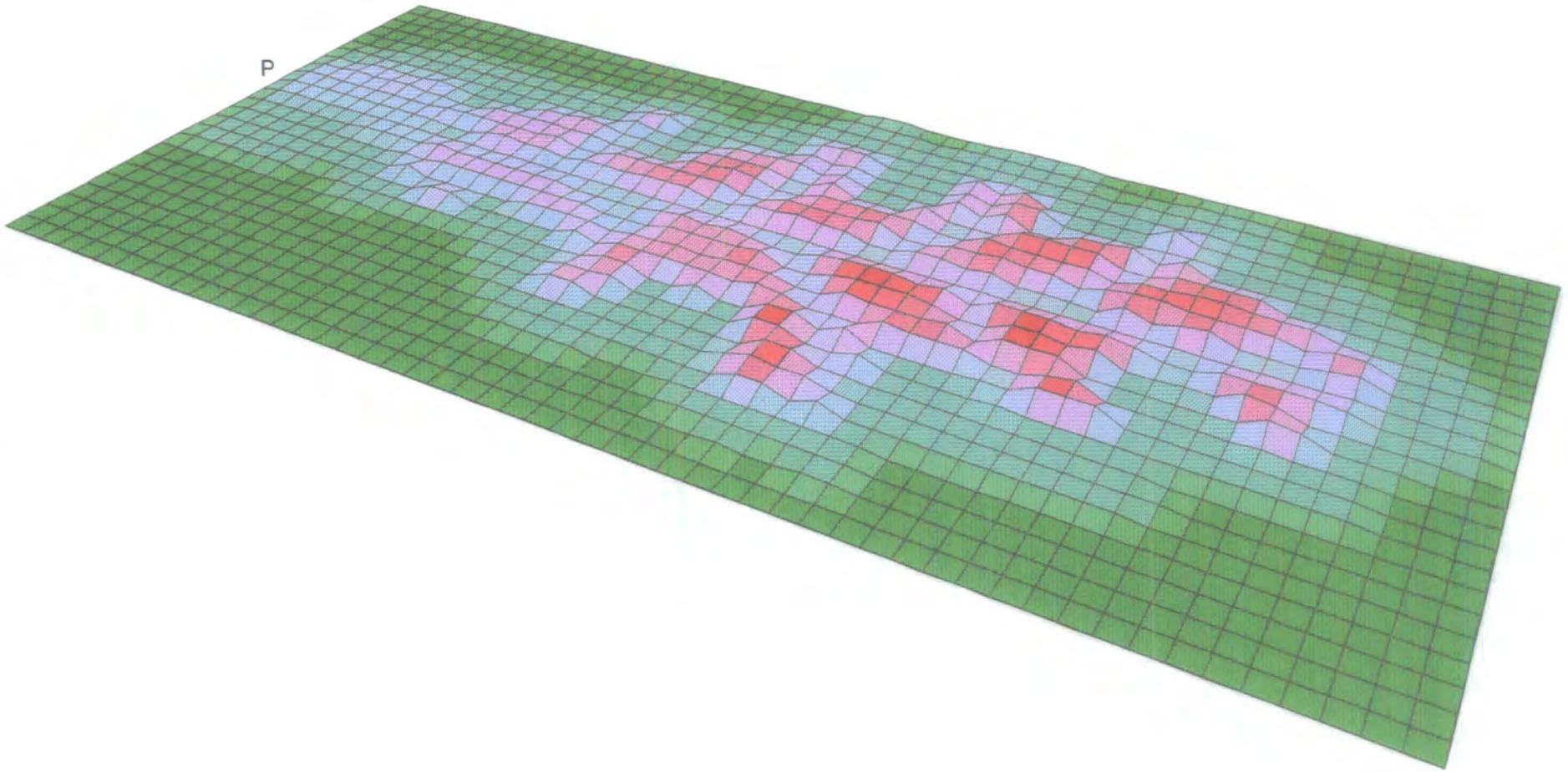


Fig 19 Map of Stomatal Frequency -Unstressed Material of High Density

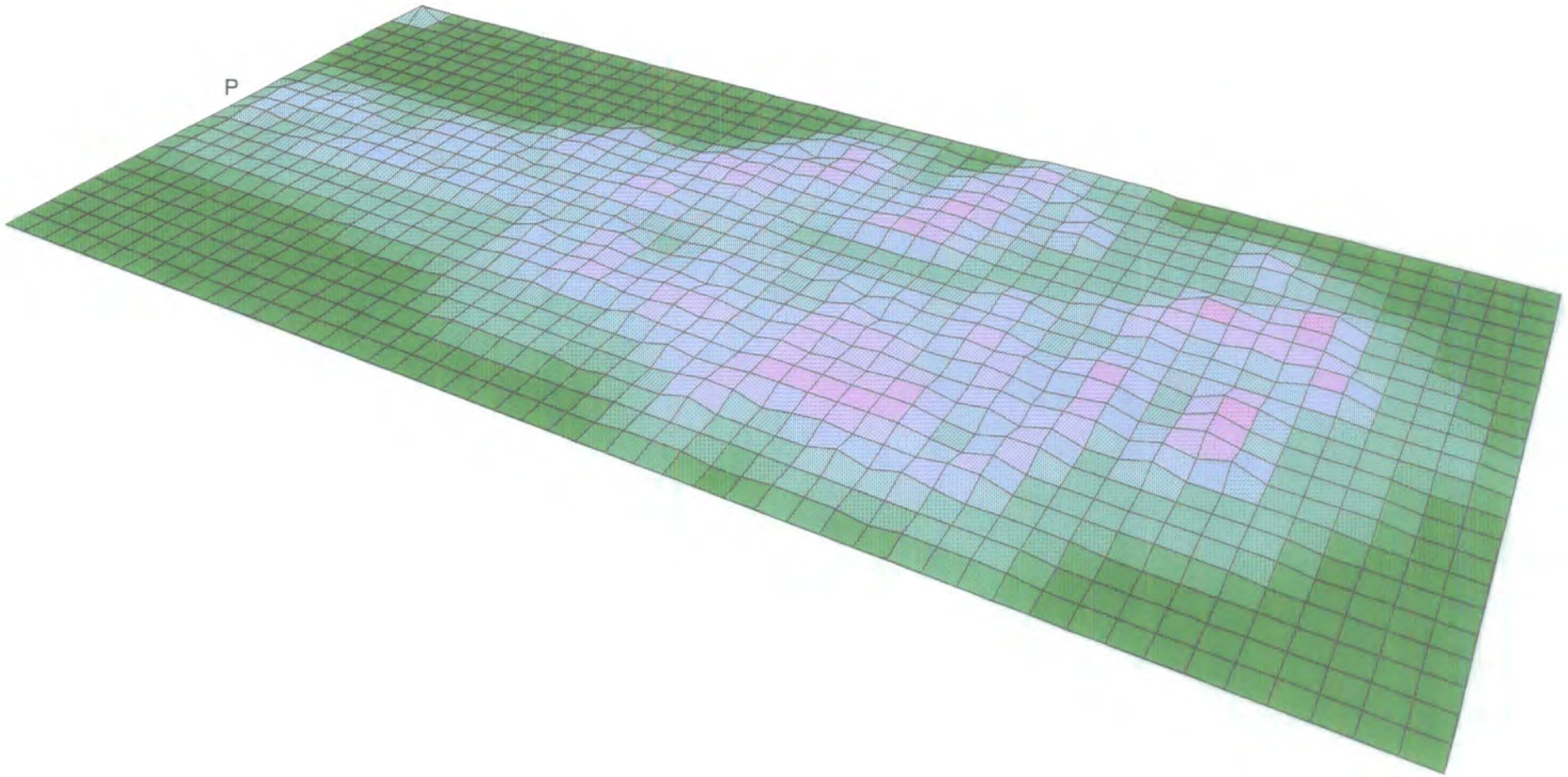


Fig 20 Map of Stomatal Frequency -Stressed Material of Low Density

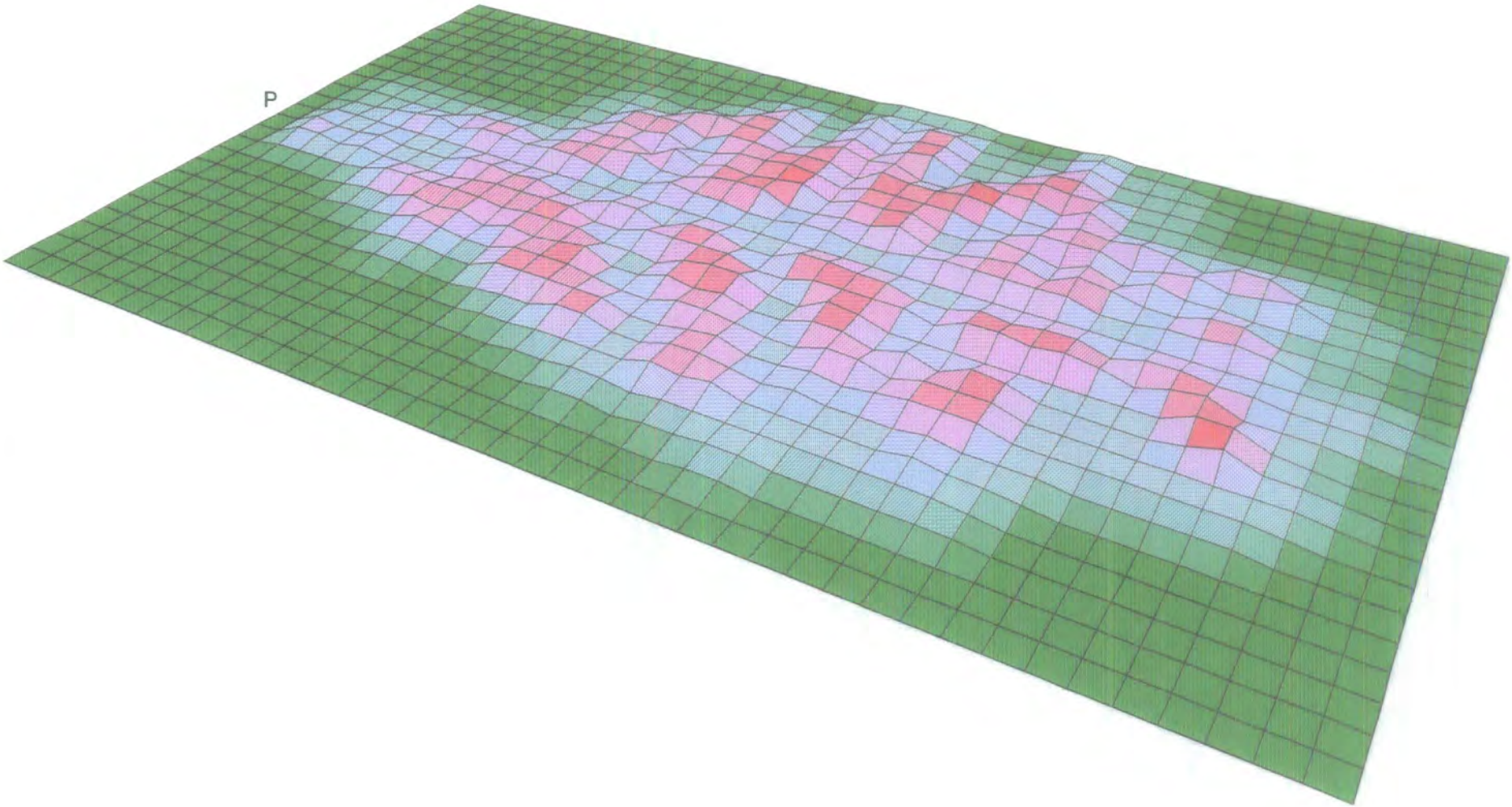


Fig 21 Map of Stomatal Frequency -Stressed Material of High Density

in significant differences in water loss, which might have an affect upon weaning of micropropagules.

3.3 Tests for Randomness of Stomata

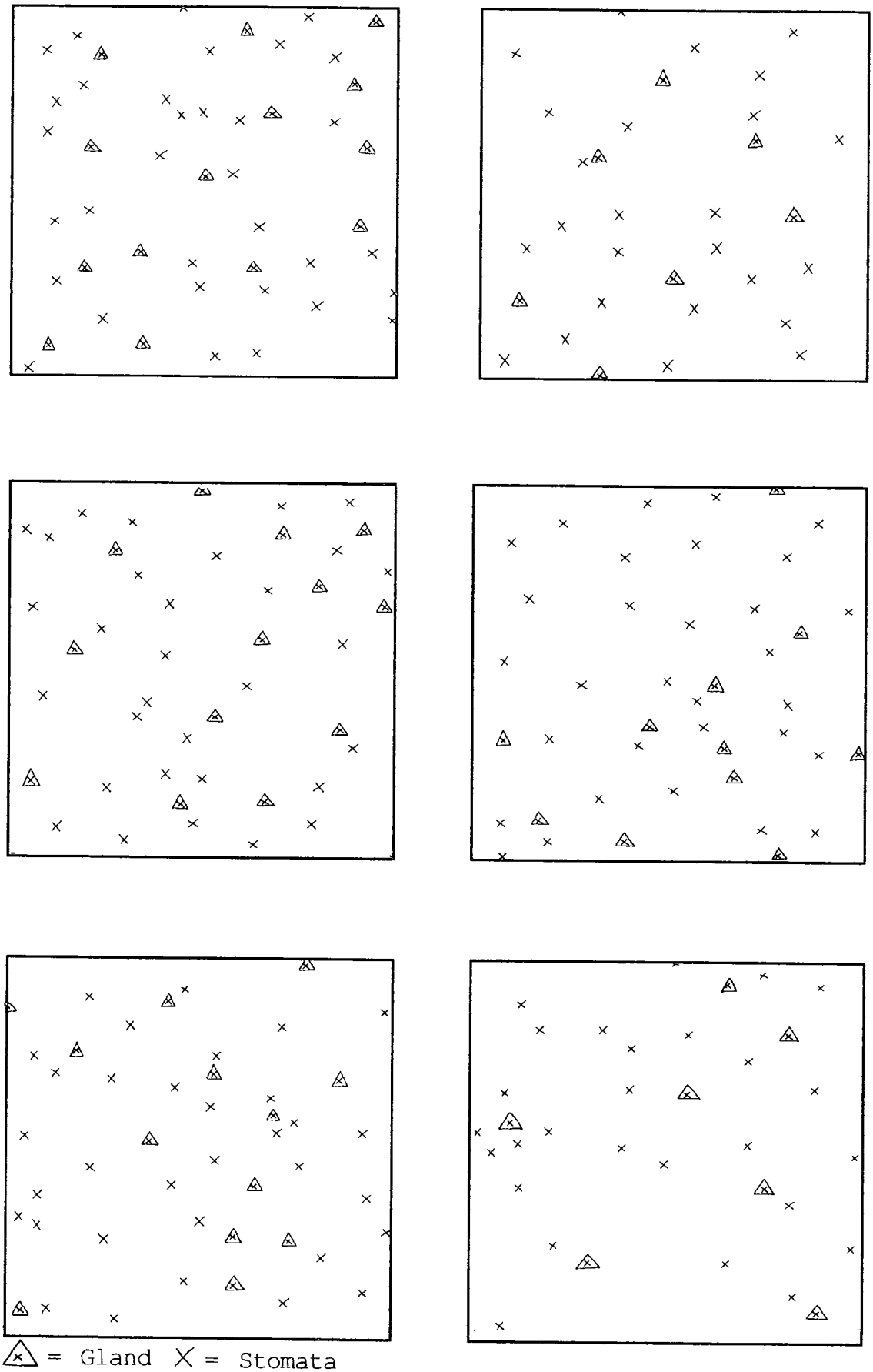
Maps of stomatal and gland distribution (Figs 22-24) were analysed using a range of methods.

Table 6 Analysis of Variants for Actual Stomatal Densities Compared to those Calculated from Point-Centred Quarters.

Treatment	Chi-square	Significance
Pot	11.52	NS
Stressed	19.64	NS
Unstressed	97.06	***

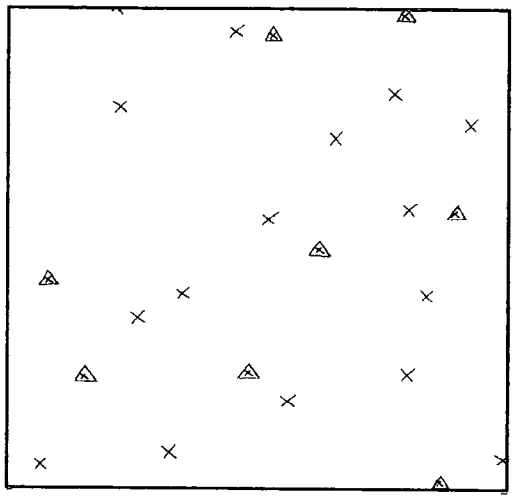
*** significant at the 0.1% level

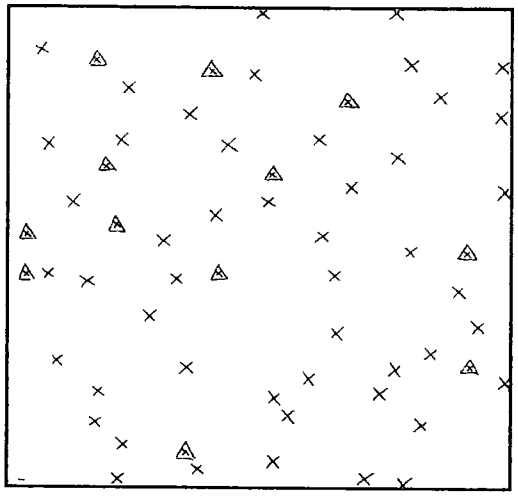
Fig 22 Examples of Distribution Maps for Glands and Stomata -Pot Grown Material

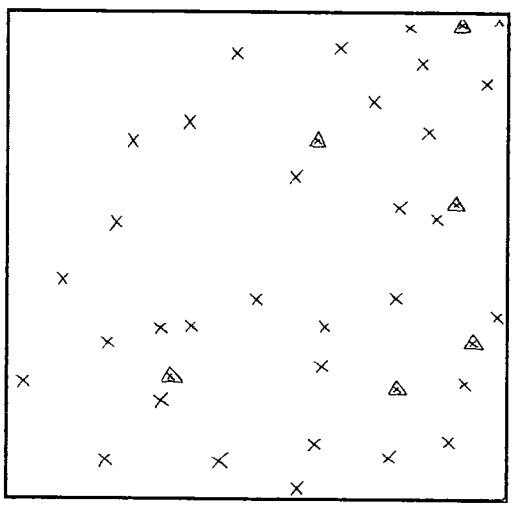


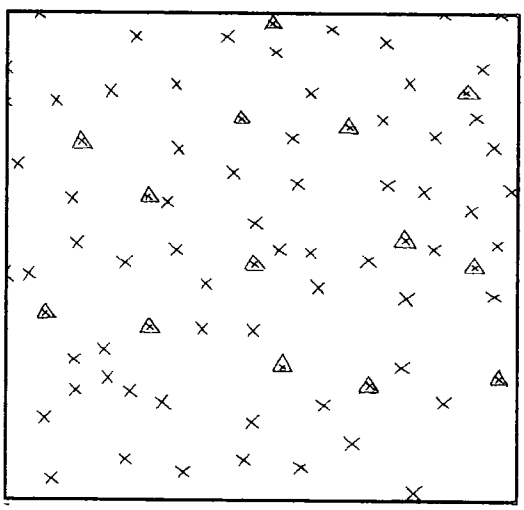
= Gland × = Stomata

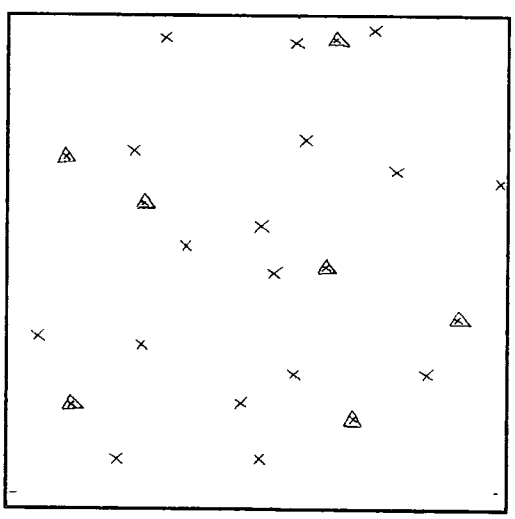
Fig 23 Examples of Distribution Maps for Glands and Stomata -Unstressed Cultures











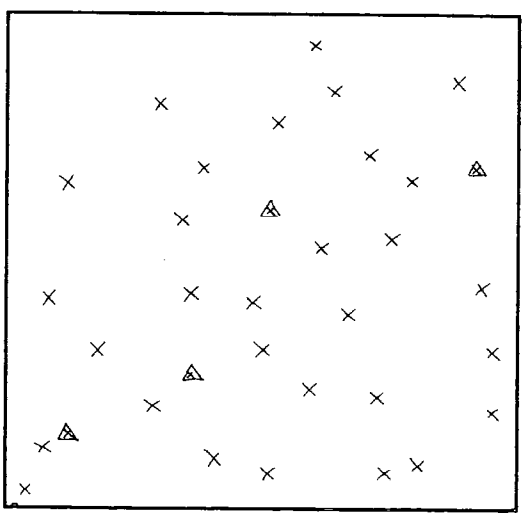


Fig 24 Examples of Distribution Maps for Glands and Stomata -Stressed Cultures

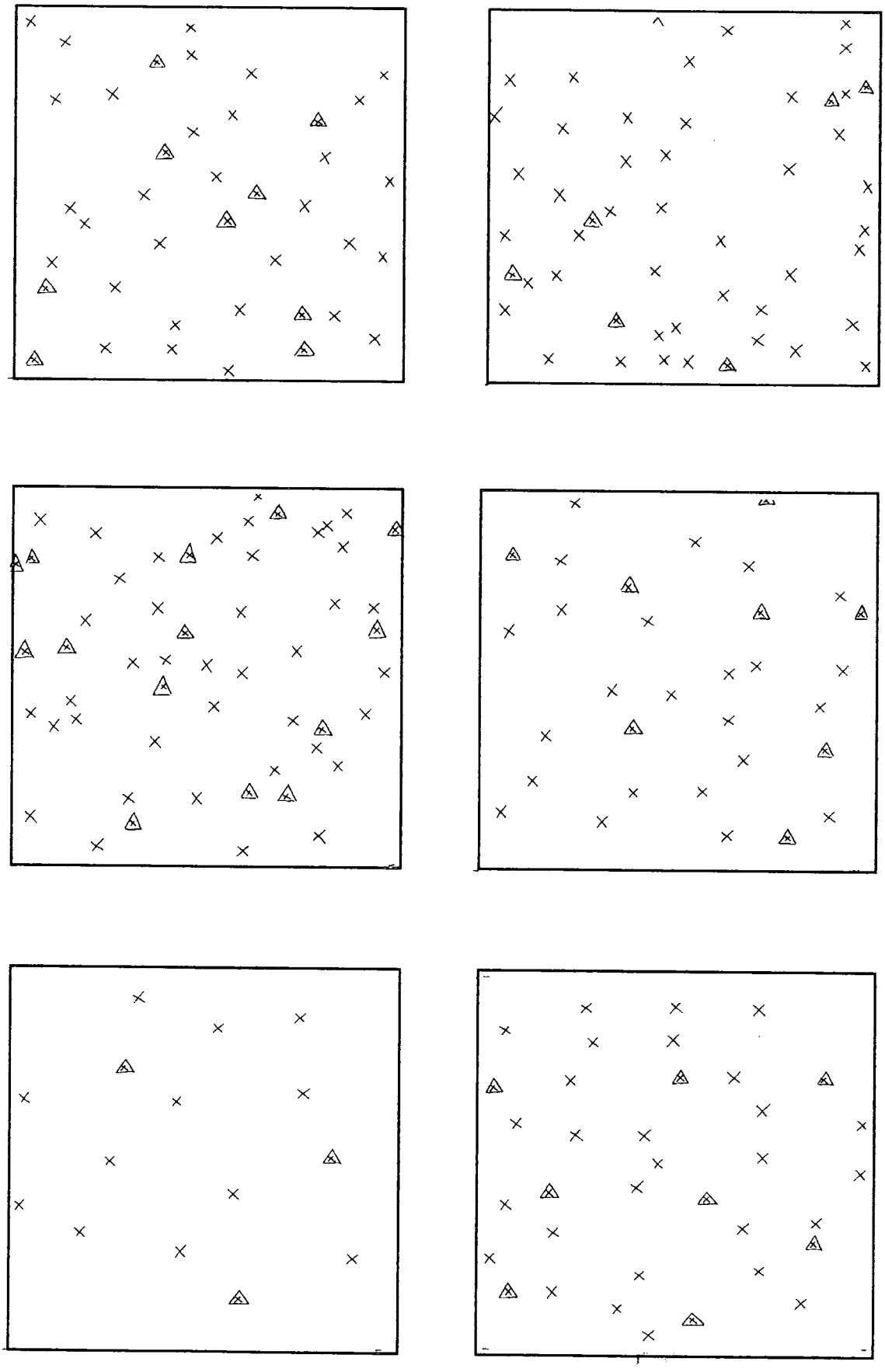


Table 7 R Values for Stomatal Ordering.

	Stomata	Glands
Unstressed	0.79	0.81
Stressed	0.81	0.81
Pot	0.78	0.83

R= 2.19 -perfect ordering
R= 1.00 -random array
R= 0.00 -clustering of loci.

R=1(square root d)
where:
R= non-scalar unit.
1= average nearest-neighbour distance
d= density $= \frac{\text{stomata in field}}{\text{area of field}}$

The results from the point-centred quarter method show that there is no significant difference between the calculated and actual stomatal numbers for the pot grown and stressed treatments (Table 6). This indicates that there is very good evidence to suggest that stomata are randomly distributed and not clustered. The high level of significance for the unstressed plant material would indicate that the stomata are not randomly distributed in this treatment and therefore show clustering.

These results were not supported by further analysis from nearest-neighbour measurements. Nearest-neighbour measurements plotted against stomatal density supported a straight line for pot grown material, and therefore no

clustering was apparent. The greatest curve, showing clustering, was from the stressed treatment; a curved line relationship was also found for unstressed cultures (Figs 25, 27 and 29).

Analysis showed good agreement for the position of glands between stressed and pot grown treatments. Good fits were found for exponential lines within gland plots but with a steeper curve for the unstressed material compared to other treatments. Clustering of glands is therefore apparent in each treatment (Figs 26, 28 and 30).

The point-centred quarter method is open to criticism as a method to determine randomness as the glands were used as the central point for the analysis, random points should have been selected. This might explain the differences found between methods. The results illustrate the interaction of both gland and stomatal structures. In the pot grown and stressed material only one plot supports a line with a high r^2 value. The point-centred quarter measurements for the pot grown material show a relationship with stomatal densities, as the distance between a gland and the surrounding stomata increase, stomatal density decreases (Figs 35 and 36). In the stressed material (Figs 33 and 34) , however, the measurement between glands and surrounding stomata show a correlation with gland density. As a distance between a gland and its surrounding stomata increases then gland density decreases. Unstressed cultures (Figs 31 and 32) show a

Fig 25 STOMATAL DENSITY AND NEAREST-NEIGHBOUR MEASUREMENTS FROM UNSTRESSED MATERIAL

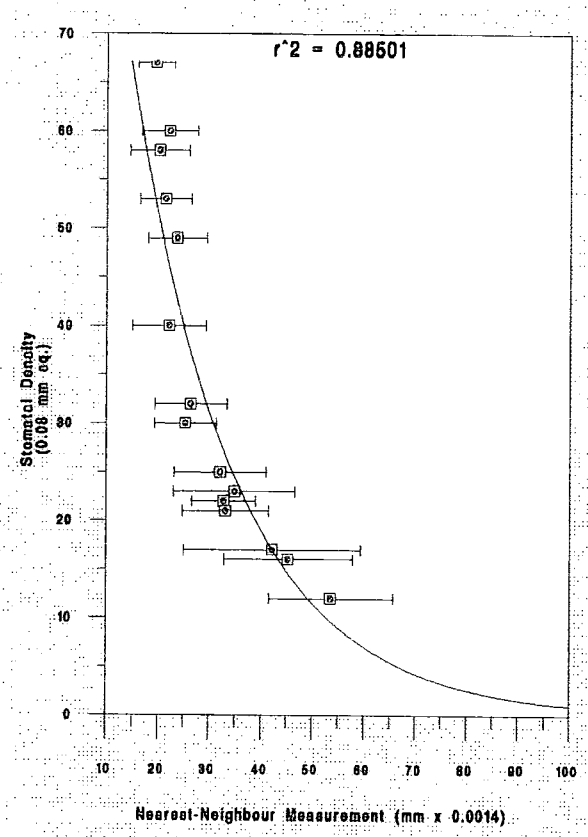


Fig 26 GLAND DENSITY AND NEAREST-NEIGHBOUR MEASUREMENTS FROM UNSTRESSED MATERIAL

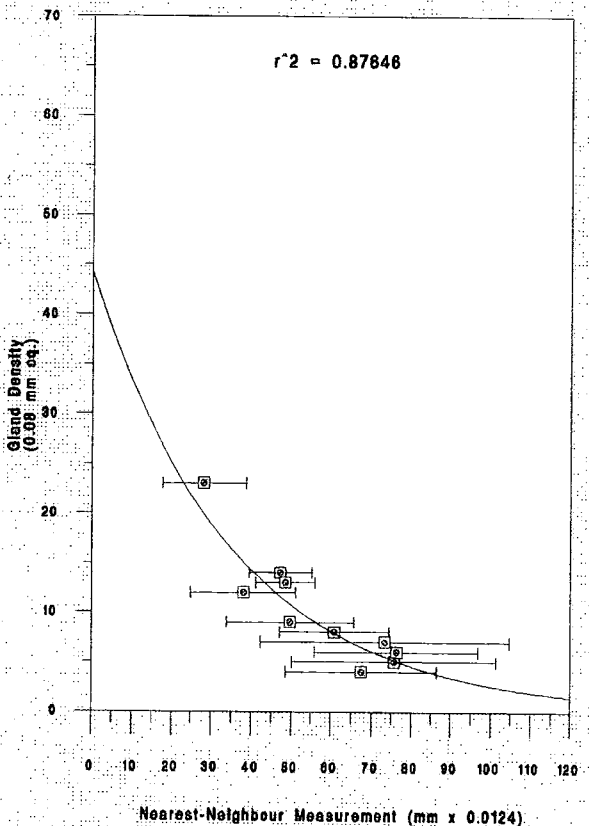


Fig 27 STOMATAL DENSITY AND NEAREST-NEIGHBOUR MEASUREMENTS FROM STRESSED MATERIAL

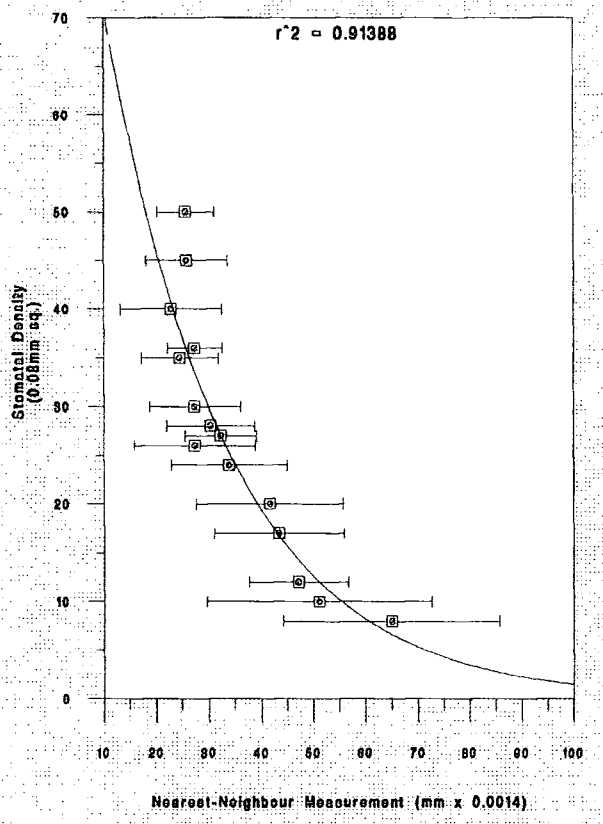


Fig 28 GLAND DENSITY AND NEAREST-NEIGHBOUR MEASUREMENTS FROM STRESSED MATERIAL

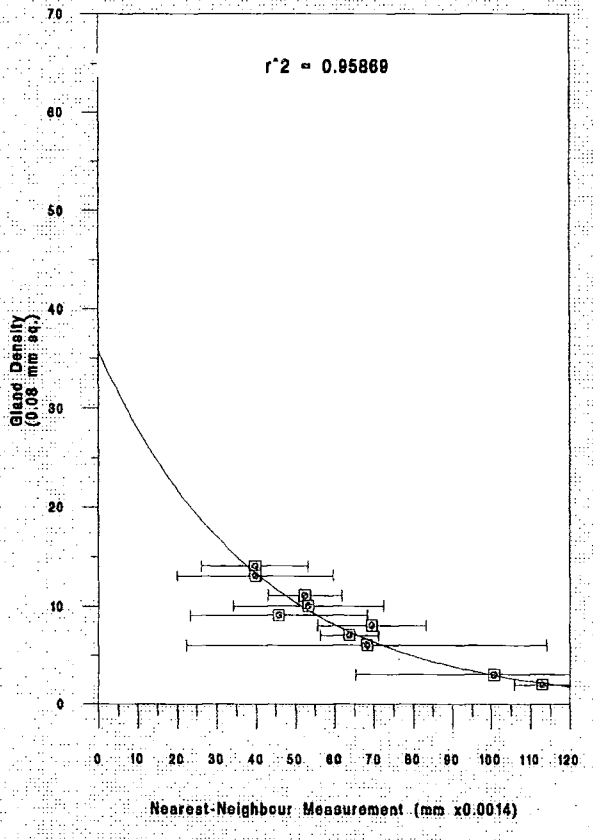


Fig 29 STOMATAL DENSITY AND NEAREST-NEIGHBOUR MEASUREMENTS FROM POT GROWN MATERIAL

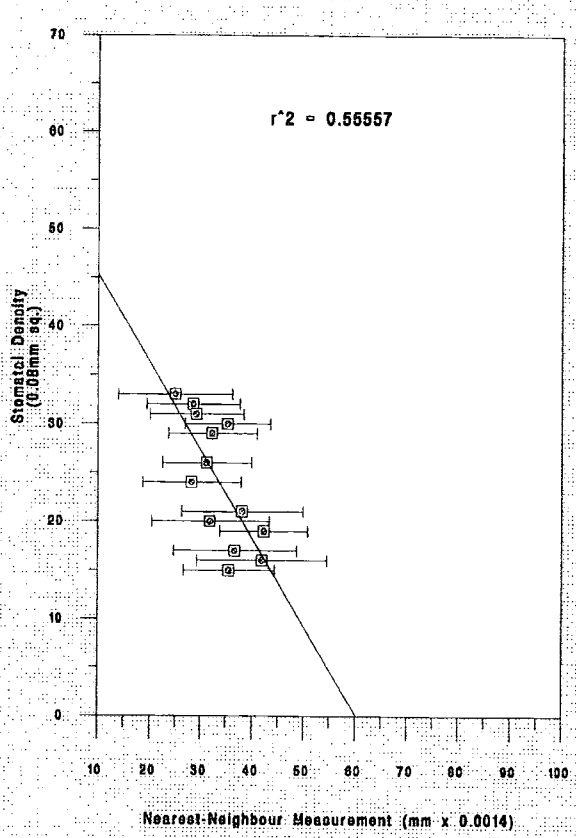


Fig 30 GLAND DENSITY AND NEAREST-NEIGHBOUR MEASUREMENTS FROM POT GROWN MATERIAL

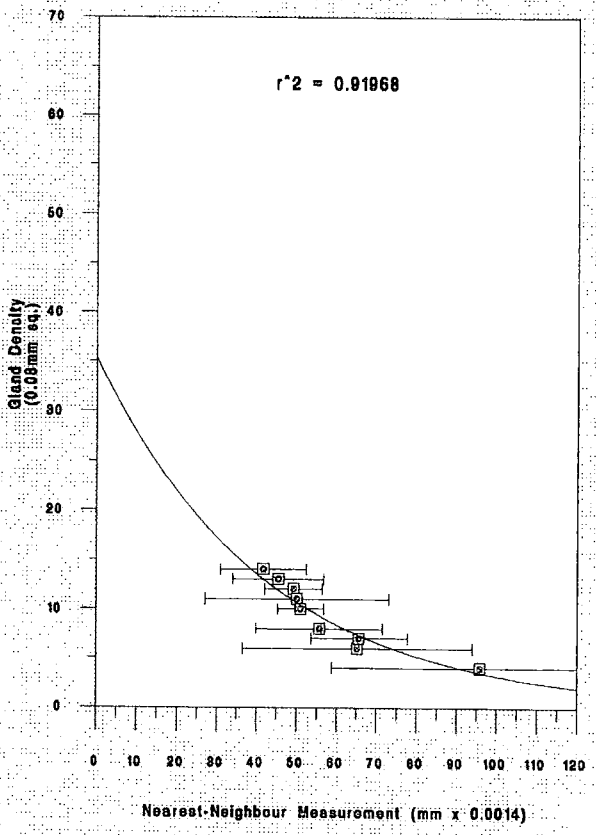


Fig 31 STOMATAL DENSITY AND POINT-CENTRED QUARTER MEASUREMENTS, UNSTRESSED MATERIAL

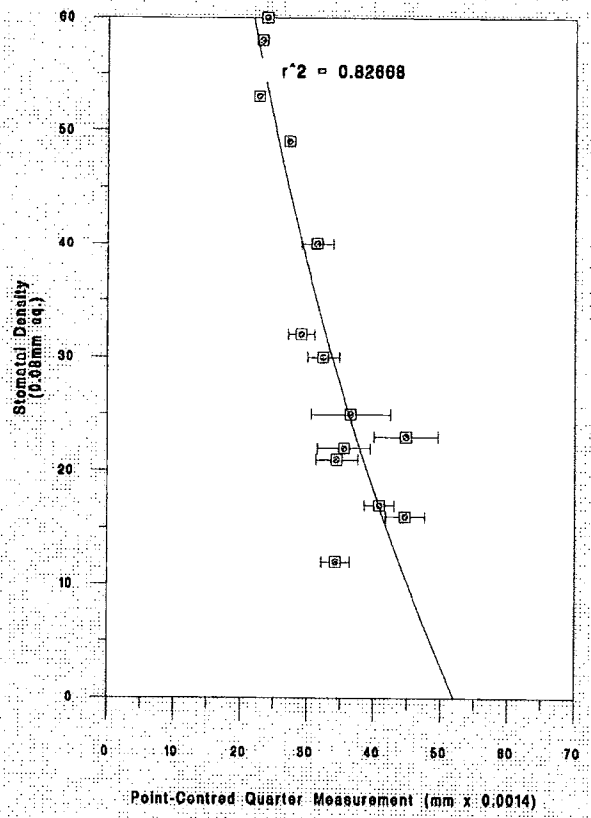


Fig 32 GLAND DENSITY AND POINT-CENTRED QUARTER MEASUREMENTS, UNSTRESSED MATERIAL

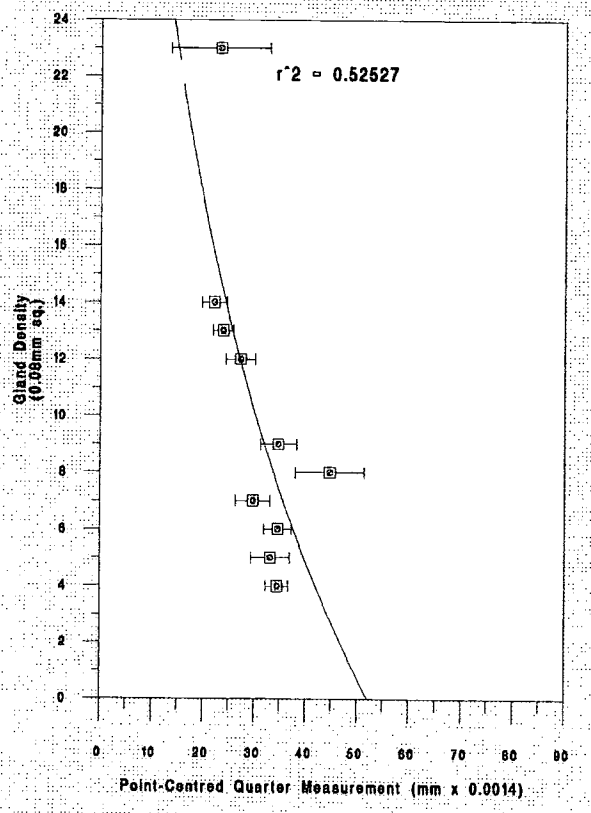


Fig 33 STOMATAL DENSITY AND POINT-CENTRED QUARTER MEASUREMENTS, STRESSED MATERIAL

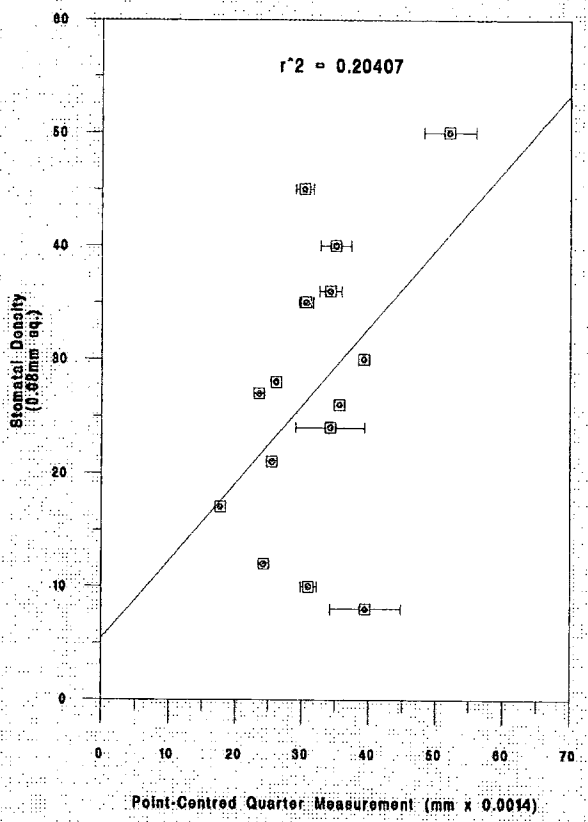


Fig 34 GLAND DENSITY AND POINT-CENTRED QUARTER MEASUREMENTS, STRESSED MATERIAL

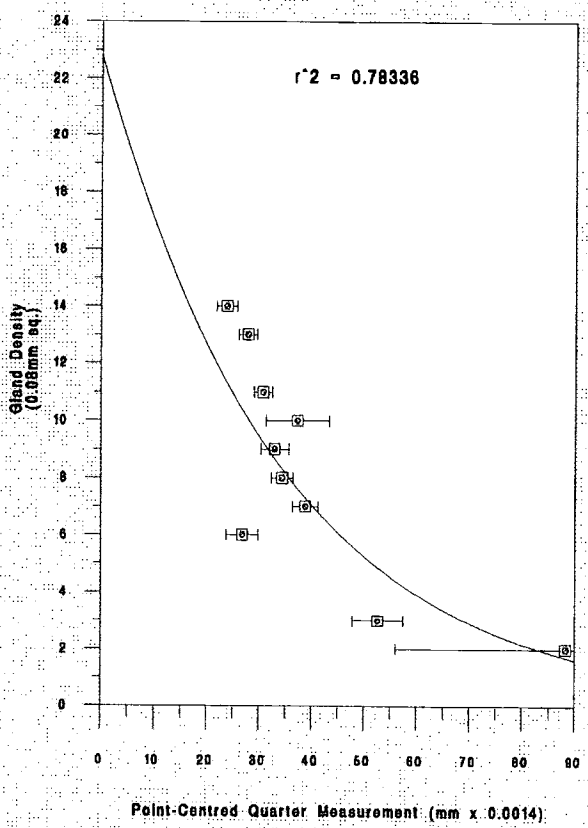


Fig 35 STOMATAL DENSITY AND POINT-CENTRED QUARTER MEASUREMENTS, POT GROWN MATERIAL

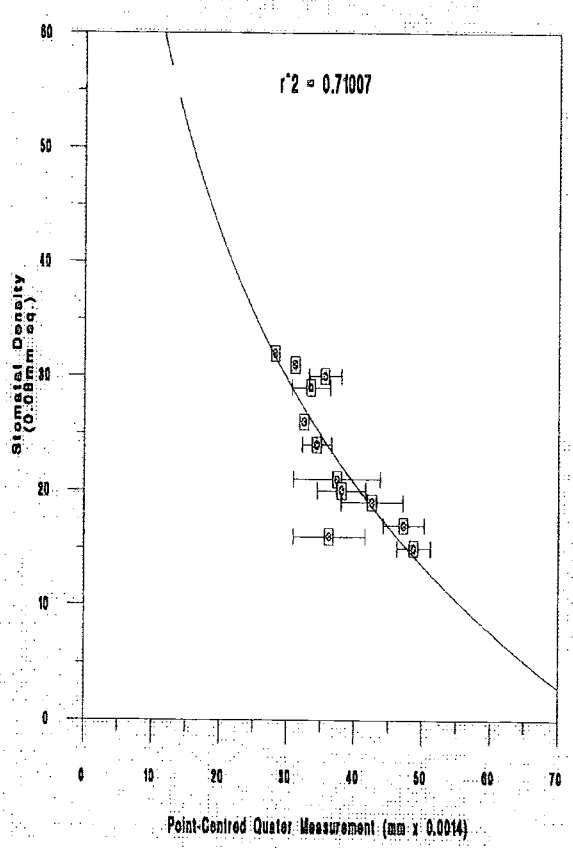
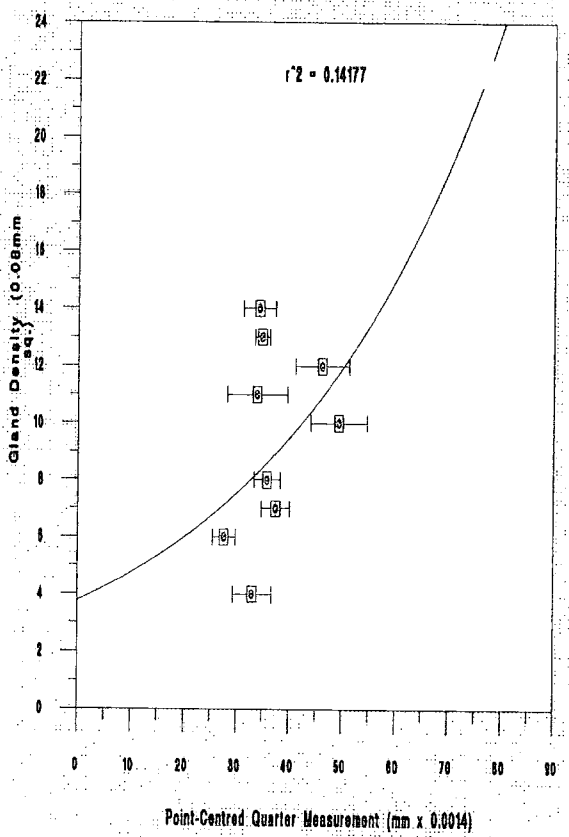


Fig 36 GLAND DENSITY AND POINT-CENTRED QUARTER MEASUREMENTS, POT GROWN MATERIAL



relationship with both gland and stomatal densities, as the distance between the gland and the surrounding stomata rise, both gland and stomatal densities drop slightly.

No significant differences were found using the method of Clark and Evans (op cit.), which suggested that some clustering occurred in all treatments and for both leaf structures (Table 7). Korn (1993), however found that R values were not very useful in distinguishing between models of stomatal patterning.

Analysis of Stomatal and Gland Densities.

The densities of stomata and glands in given areas were recorded and regressed using Minitab (Table 8).

Table 8 Best Subsets Regression of Glands

Vars	R-sq	Adj. R-sq	C-p	s	density	t'ment
1	41.3	40.1	1.6	3.1296	X	
1	0.1	0.0	33.6	4.0828		X
2	42.1	39.5	3.0	3.1443	X	X

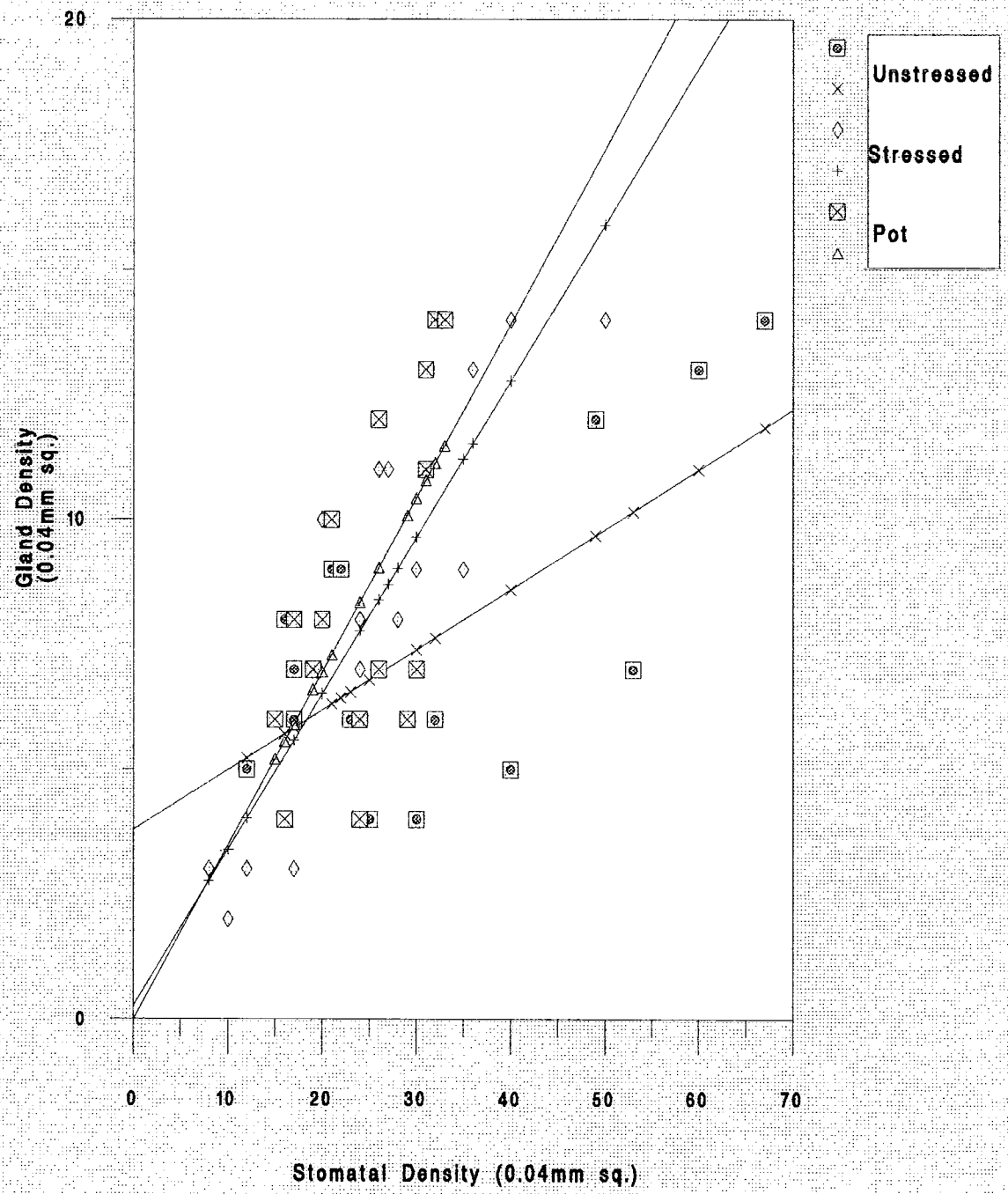
The regression equation which most closely fits the data is that of the number of glands against the actual stomatal density (Table 8). This gave the lowest C-p value. Treatments

had no significant effect (Appendix 2). High r^2 values were obtained for the regression lines particularly for the stressed cultures. The data, with regression lines, were plotted.

Figure 37 shows that farinal glands increase in number with increasing stomatal densities. Similarities were noted between the regression line for the stressed and pot grown material, with a less acute line for unstressed cultures.

It would appear that fewer farinal glands are produced in culture under conditions of high humidity, but when subject to water stress new leaves were found to produce a greater number of farinal glands. It may be postulated that the more numerous farinal glands increase the boundary layer of the leaf material and therefore reduce water loss. Woolly farina production may also limit the loss of water from the leaf. Although thin layer chromatograms of farina, standardised to leaf area, showed few significant differences between treatments. Quantitative and qualitative differences were found between pot grown and *in vitro* cultures of *D. denticulata* (Plate 21).

Fig 37 STOMATAL AND GLAND DENSITIES



3.4 The Determination of Pore Length for Different Stomatal Densities

There would appear to be a trend between stomatal frequency and pore length which has been noted in other species, Pallardy and Kozlowsky (1979). Liang et al (1975).

Pore length decreases as stomatal density increases with similar regression lines for pot grown and unstressed cultures (Figs 38 and 39). Stressed material appear to show a smaller pore length for a given density with a shallower regression line (Fig 40). However the r^2 values are relatively low and do not support further analysis of the data.

Fig 38 PORE LENGTH AGAINST STOMATAL DENSITY. POT GROWN.

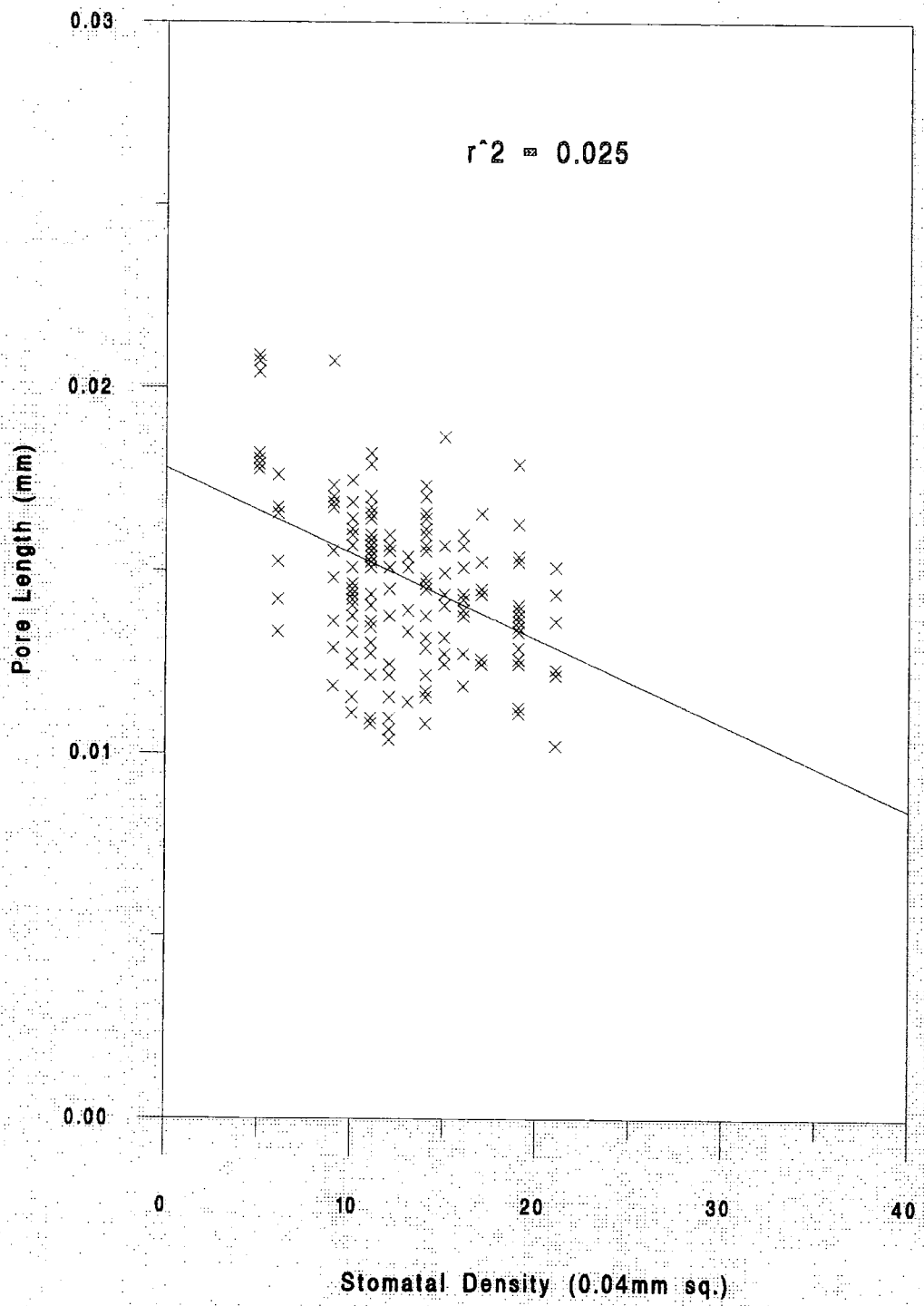


Fig 39 PORE LENGTH AGAINST STOMATAL DENSITY. UNSTRESSED

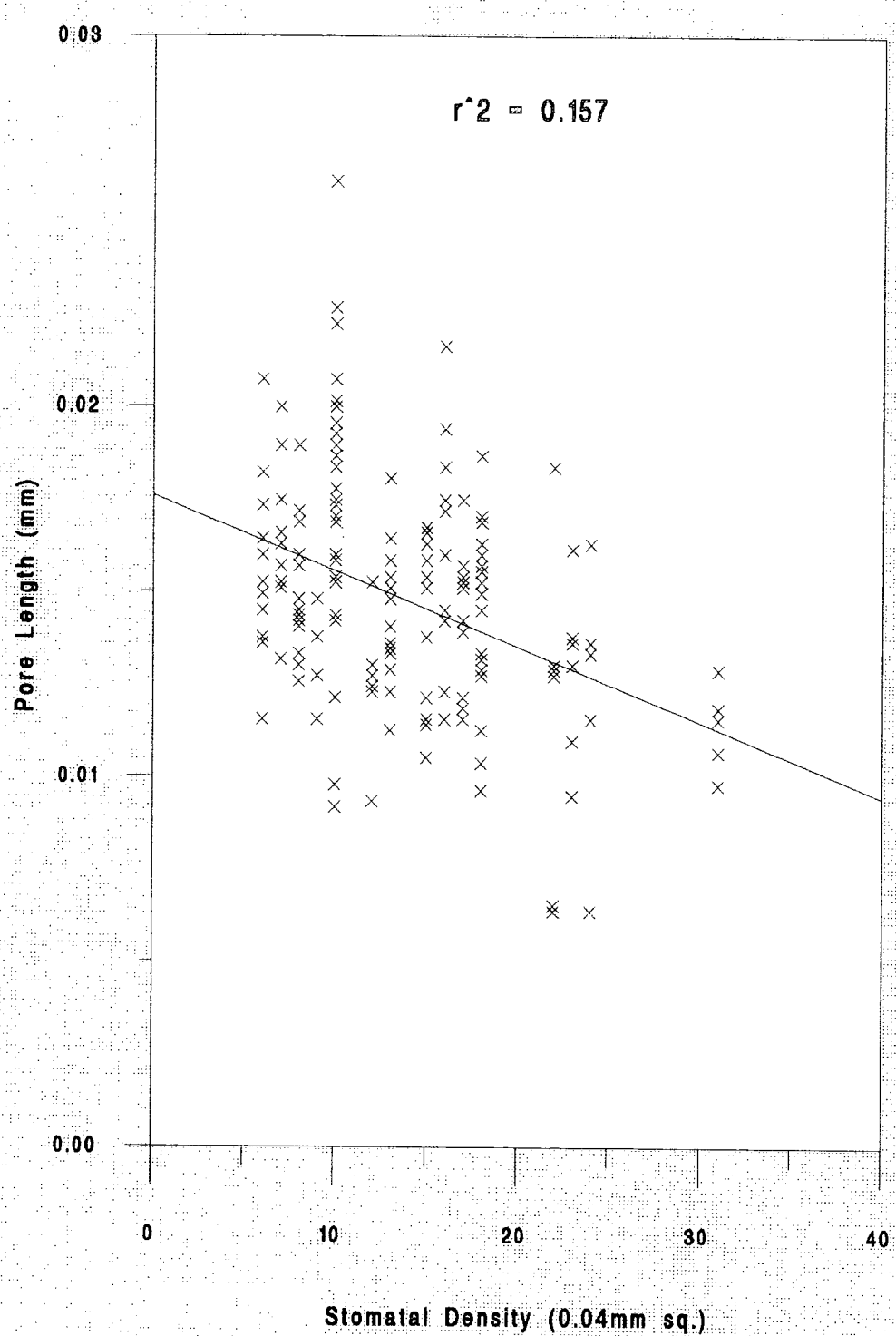
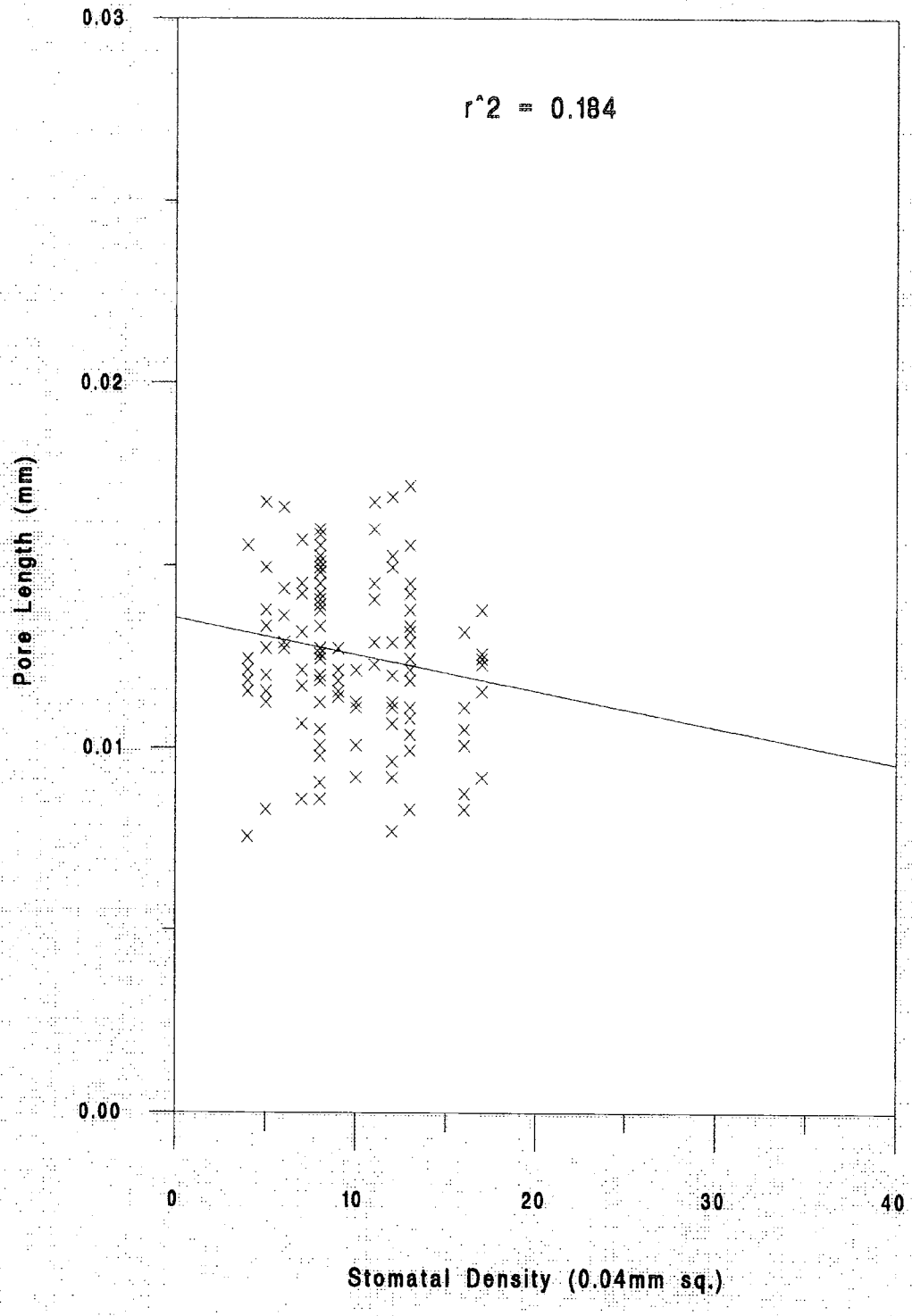


Fig 40 PORE LENGTH AGAINST STOMATAL DENSITY. STRESSED CULTURE.



3.5 The Effect of ABA and Wilting on Potassium Accumulation in Guard Cells of Unstressed Material

Potassium accumulation may be scored according to the percentage area stained black with cobalt sulphide within the guard cells (Raschke and Fellows, 1971). On examination the epidermal strips of *Dionysia aretiodes* proved highly variable, differing significantly from the results anticipated from the literature. Potassium accumulation was not showed by a uniform black precipitate of cobalt sulphide as anticipated, but appeared as bands of grey colouration. This may be due in part to sunken stomata with overlapping epidermal cells (Plates 6 and 7). It was therefore not possible to grade the results against percentage area stained as there were both quantitative and qualitative differences.

The results were recorded on video tape and later analyses using Microscale TC/ITM version 2.0. An image from the video tape was fixed and analysed. Individual stomates were taken and areas high-lighted, details of the area and position on the grey scale recorded. Two or three sets of data were recorded for each stoma giving an indication of the relative degree of staining in different areas of the guard cell.

The results were analysed by calculating the inverse of the grey 1-250 scale from the computer as a 1-25 scale. This

Plate 6

Stoma of *Dionysia aretiodes* with Covering Epidermal Cells

Note shift of focus. Bar =1um.

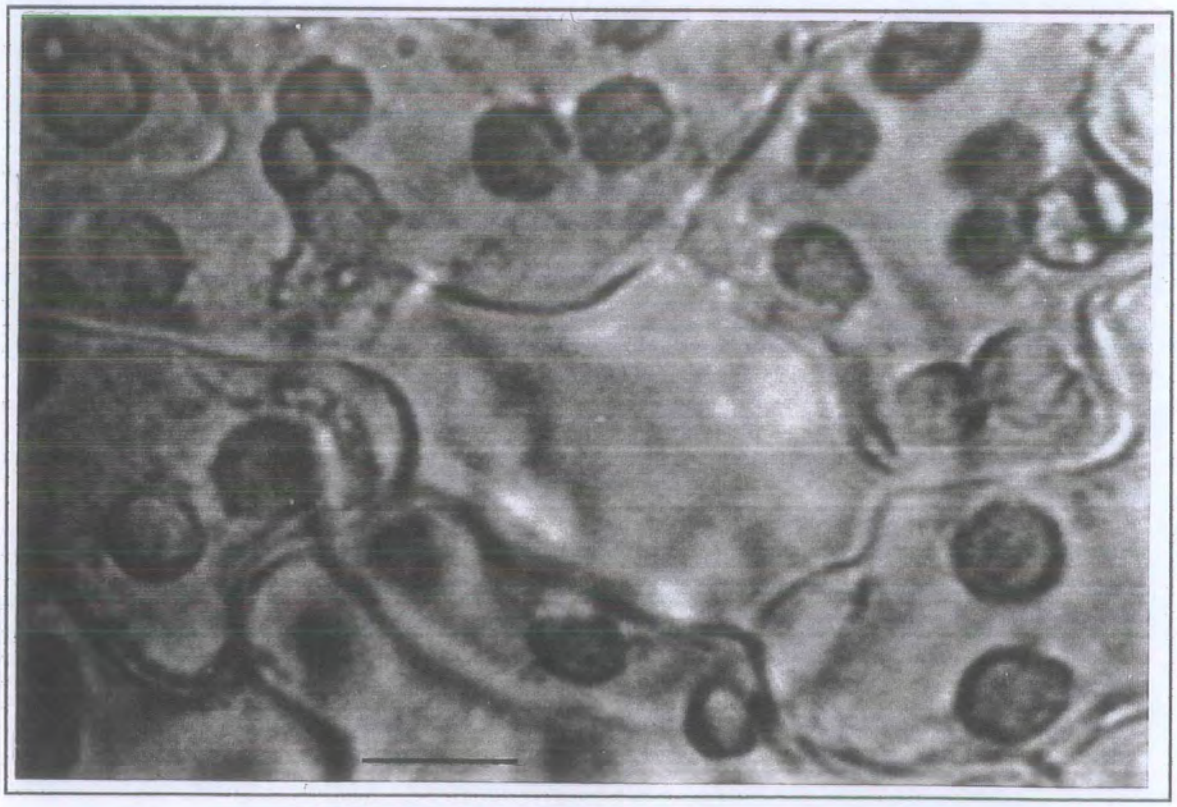


Plate 7

was multiplied by the area covered and the result given as a percentage of the guard cell area (Table 9).

Table 9 Potassium Concentration of Guard Cells Expressed as a Percentage*

	Pot	Flaccid	ABA 40mg/l
	22.9	44.4	15.9
	34.4	50.7	18.1
	23.6	53.1	21.5
	16.3	30.7	
	22.5	56.6	
	26.9	49.5	
	24.1	42.8	
	22.7	32.6	
	27.2	32.0	
	24.6	53.9	
	31.3	43.6	
	35.8	36.0	
	45.6	38.3	
		22.0	
		27.1	
		35.3	
Mean	27.5	40.5	18.5
Stdev	7.6	10.4	2.8

* where complete staining with black precipitate =100%

It was noted by observation and from the calculations that substantial accumulations of potassium occurred in the guard cells of leaves allowed to become flaccid on the bench (Plate 12), and that some accumulation had occurred in the pot grown control material (Plate 15). However little staining occurred in samples exposed to ABA, even at the highest concentration of 40mg/l (Plates 8-11). The analysis of the results from the ABA treatments proved less reliable. The examination of the epidermal strips through the microscope resulted in light diffraction causing the stomatal throat and perimeter of the guard cells to be delimited by a dark area.

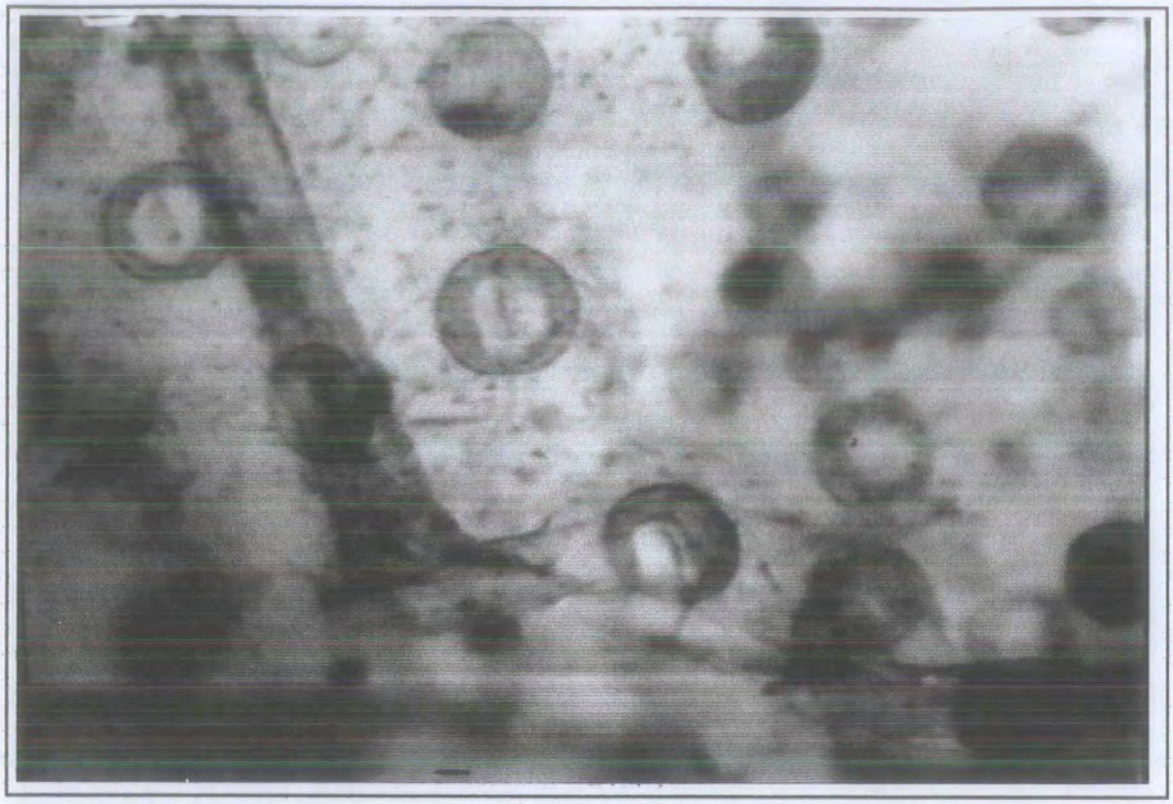


Plate 8 Potassium Staining in Unstressed Material -Control

Note the low level of potassium accumulation. Bar =1um.

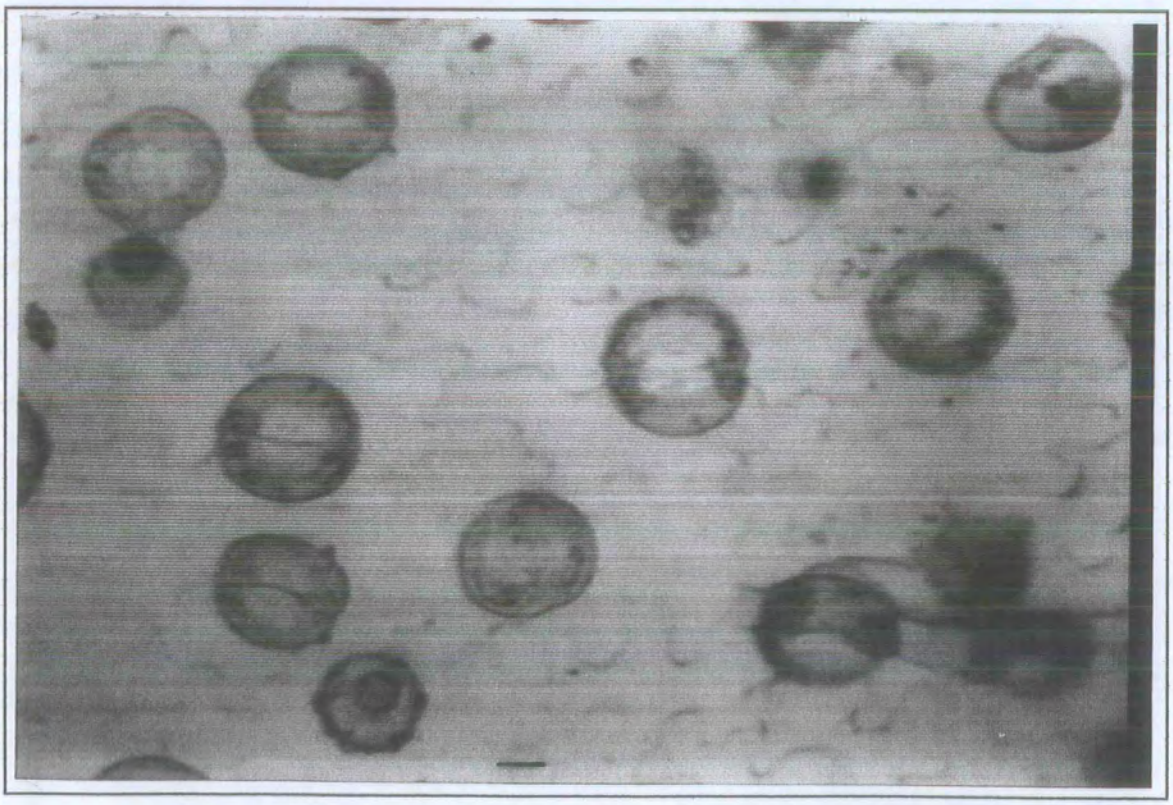


Plate 9 Potassium in Unstressed Material -1mg\l ABA

Note the minimal accumulation of potassium. Bar =1um.

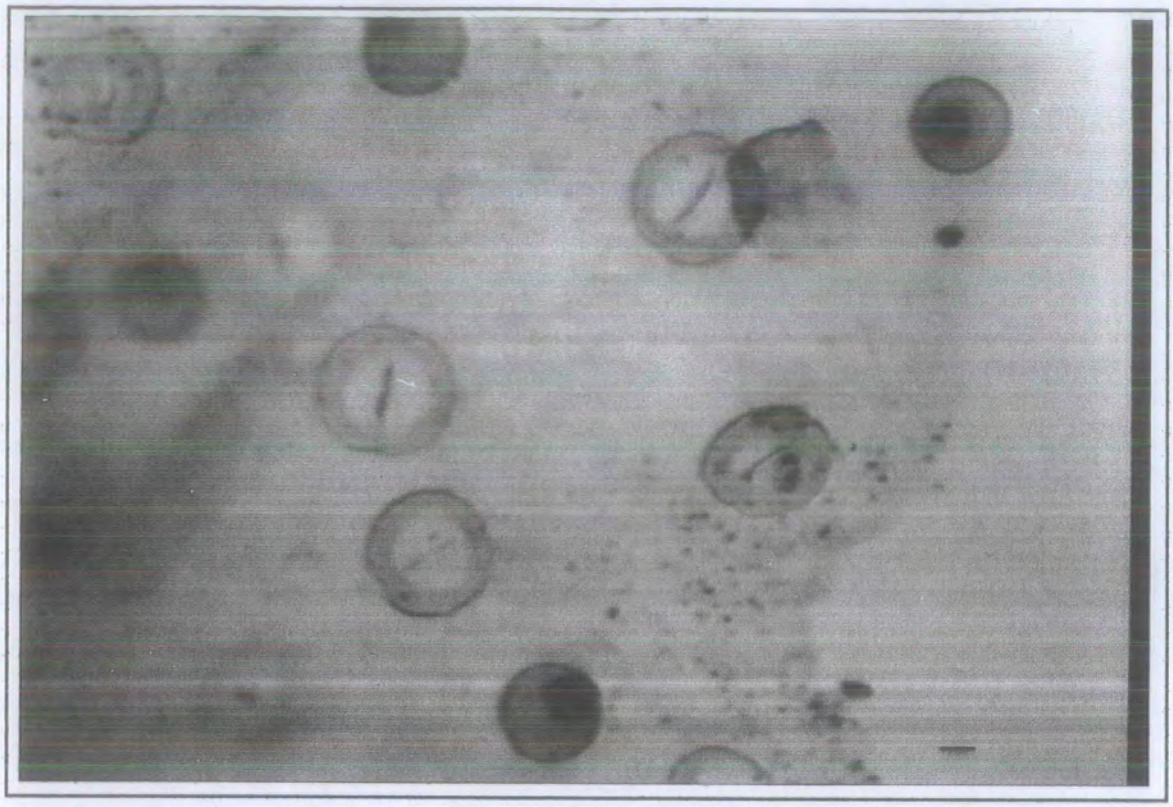


Plate 10 Potassium Staining in Unstressed Material -10mg/ABA
Note low levels of potassium accumulation. Bar =1um.

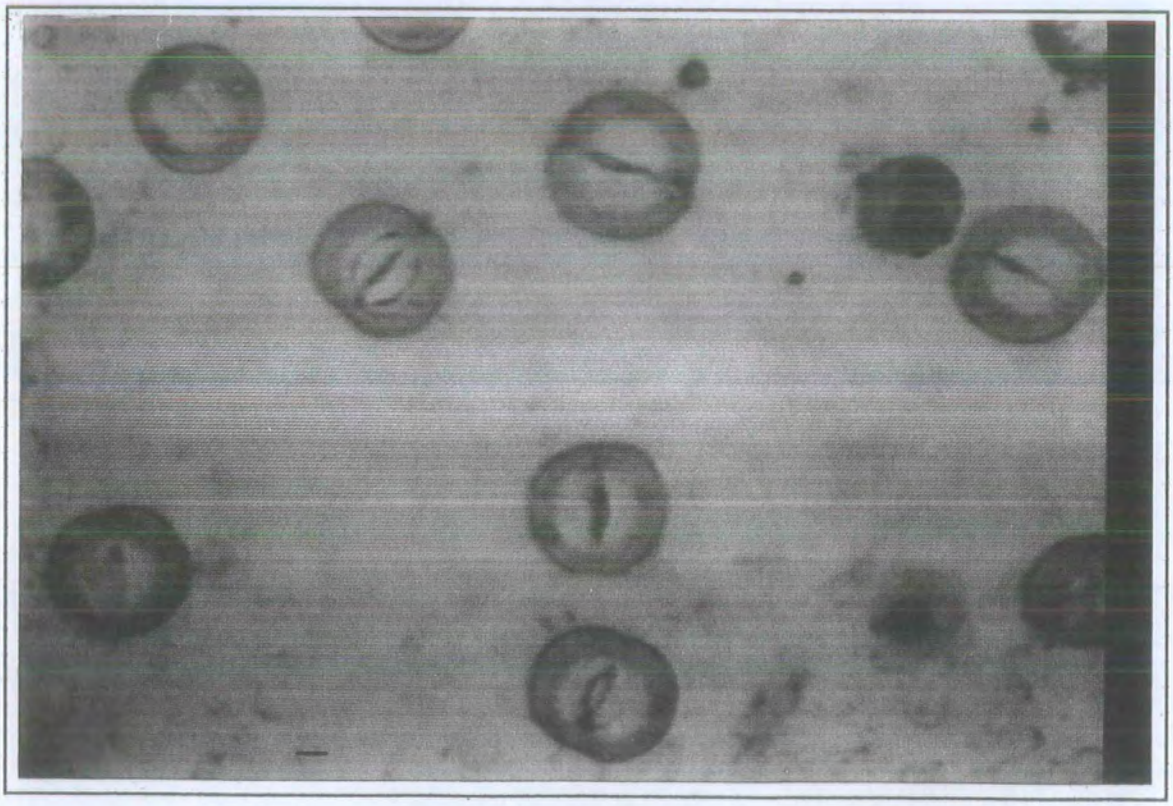


Plate 11 Potassium Staining in Unstressed Material -40mg\l ABA
Note low levels of potassium accumulation. Bar= 1um.

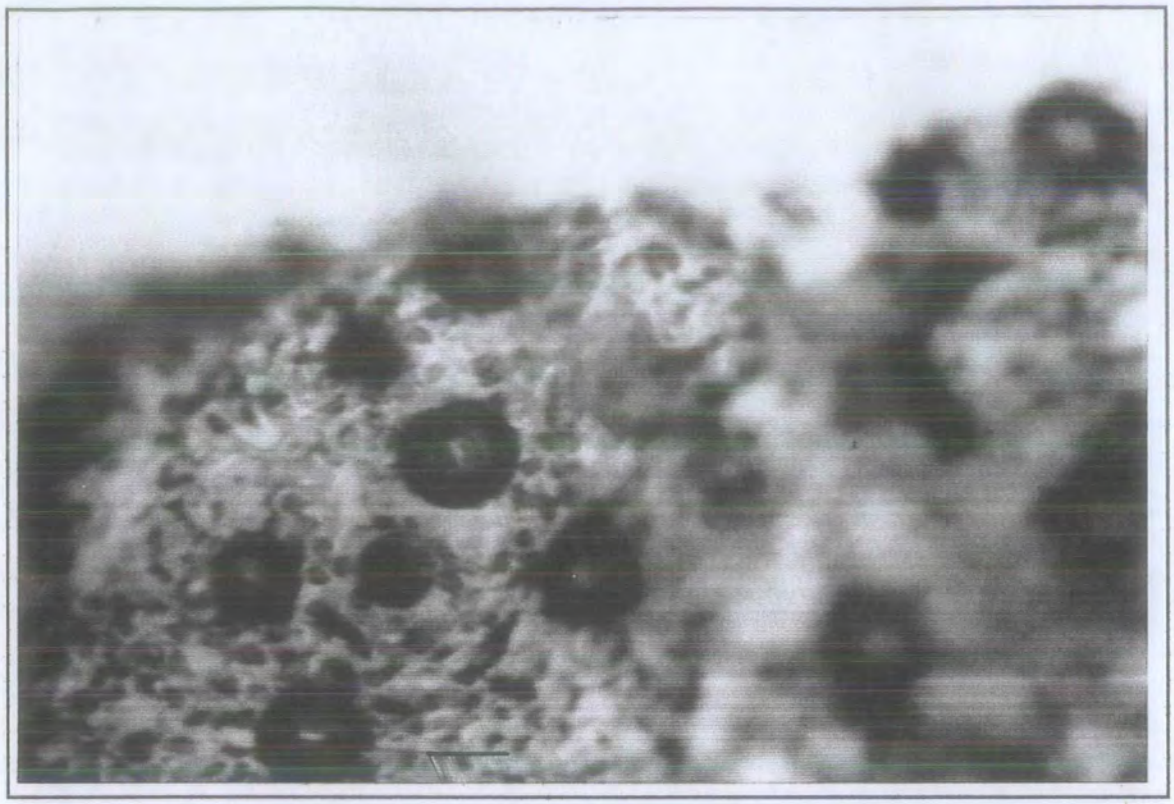


Plate 12 Potassium Staining in Unstressed Material -Flaccid

Note high potassium accumulations in guard cells and chloroplasts. Bar =4um.

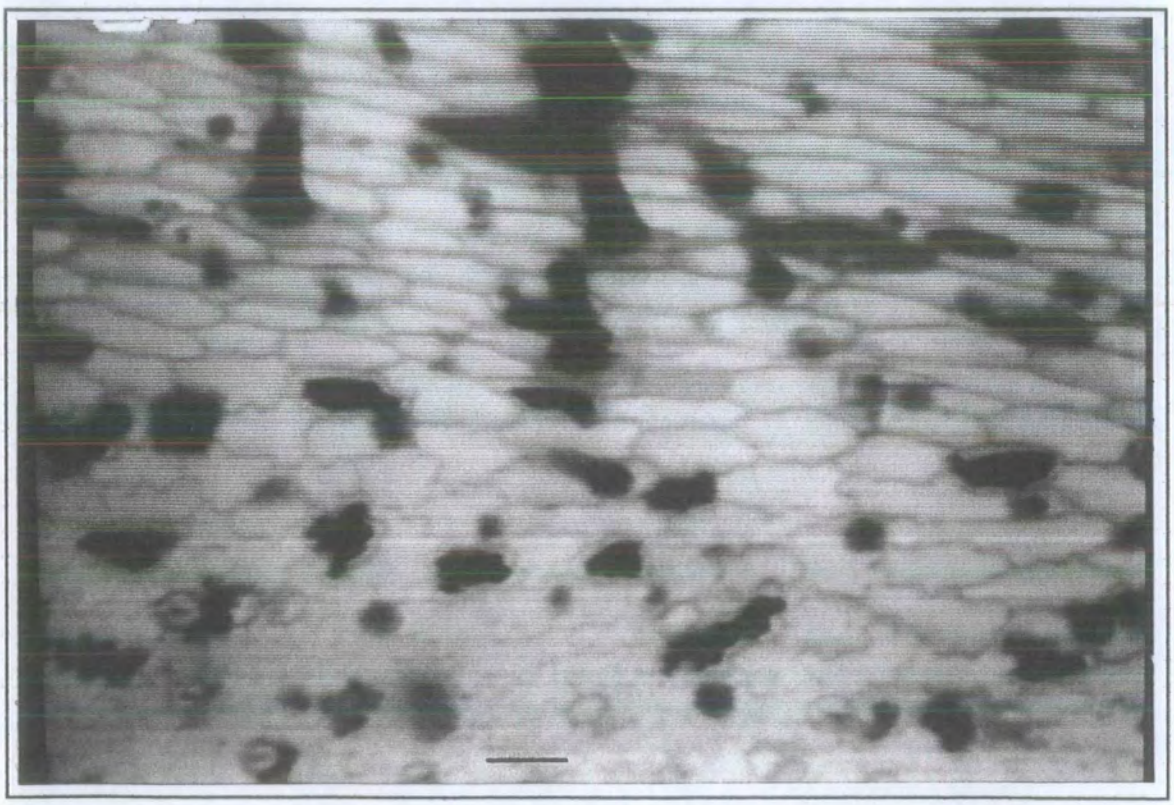


Plate 13 Potassium Staining in Pot Grown Material

Note potassium staining in epidermal cells and trichomes. Bar =10um.

Plate 14 Potassium Staining in Epidermal Hairs of *Dionysia aretiodes* *'Phyllis Carter'* -Pot Grown

Bar =3um.

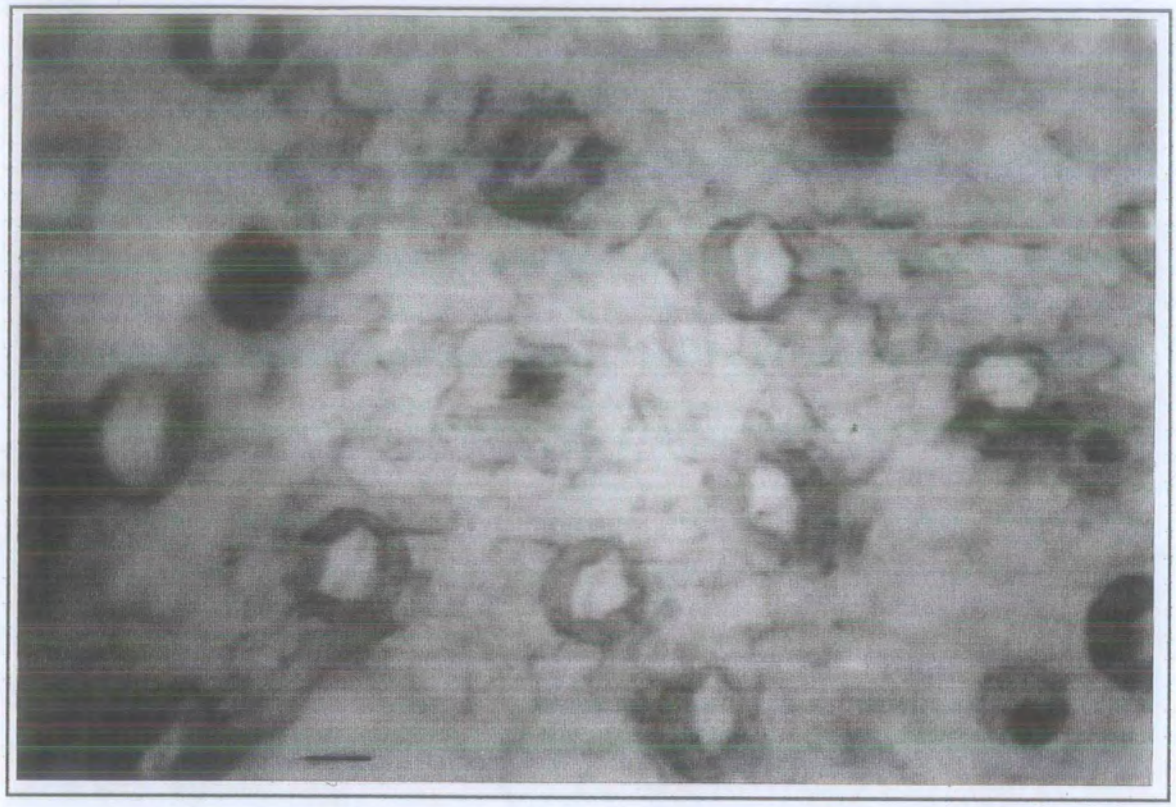


Plate 15 Potassium Staining in Pot Grown Material

Note low accumulations of potassium. Bar =1um.

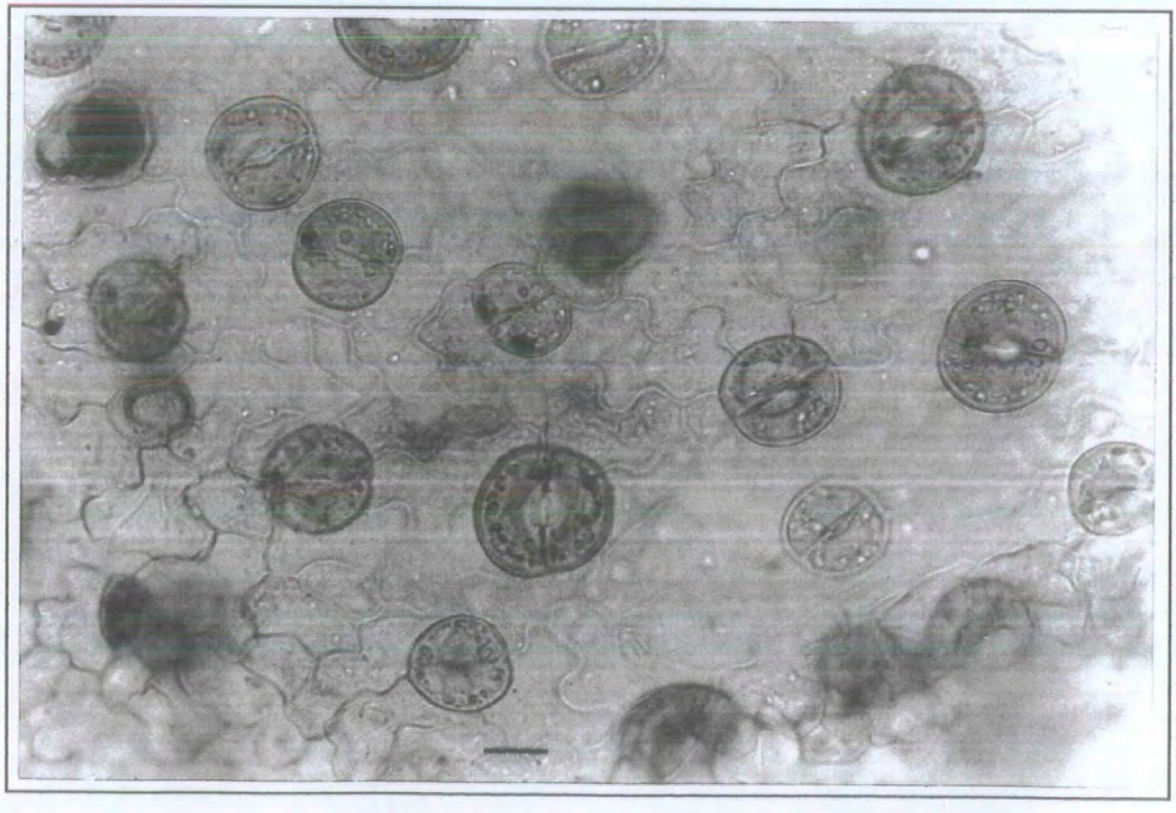


Plate 16 Potassium Staining in Two Day Old Propagules

Note low accumulations of potassium. Bar =1um.

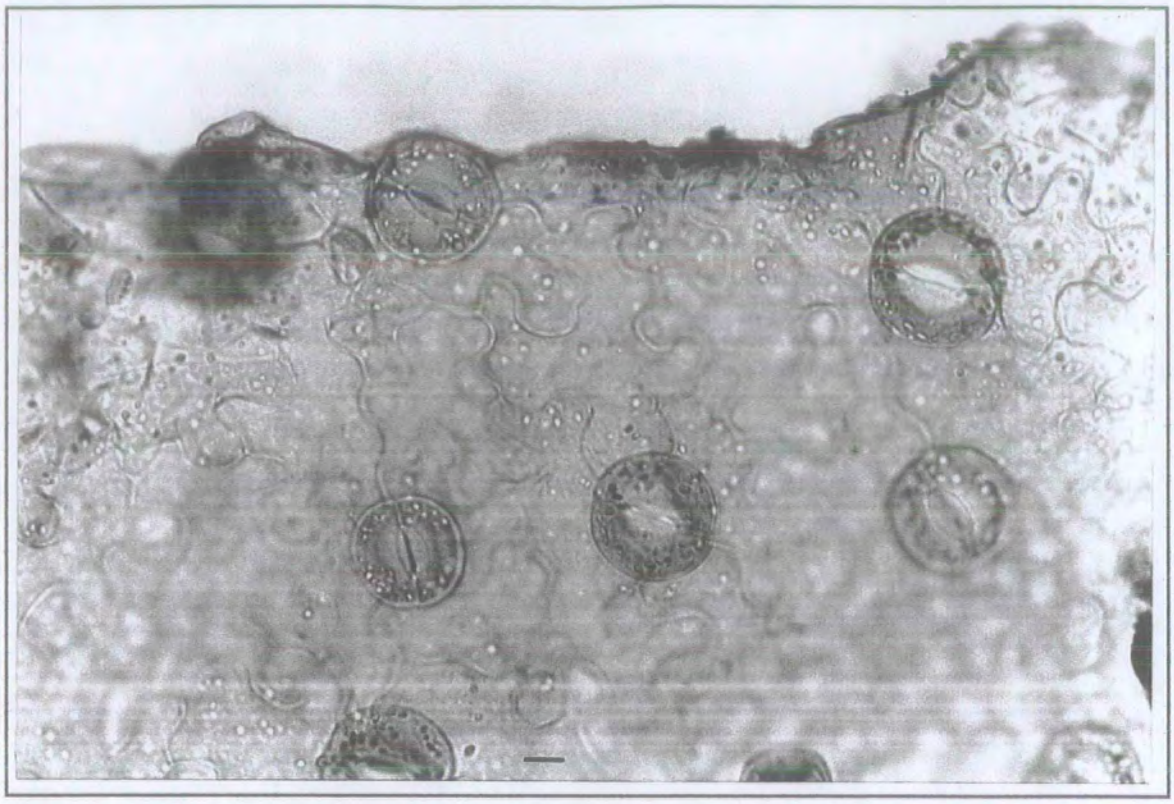


Plate 17 Potassium Staining in Unstressed Cultures -Media X

Note minimal accumulations of potassium. Bar =1um.

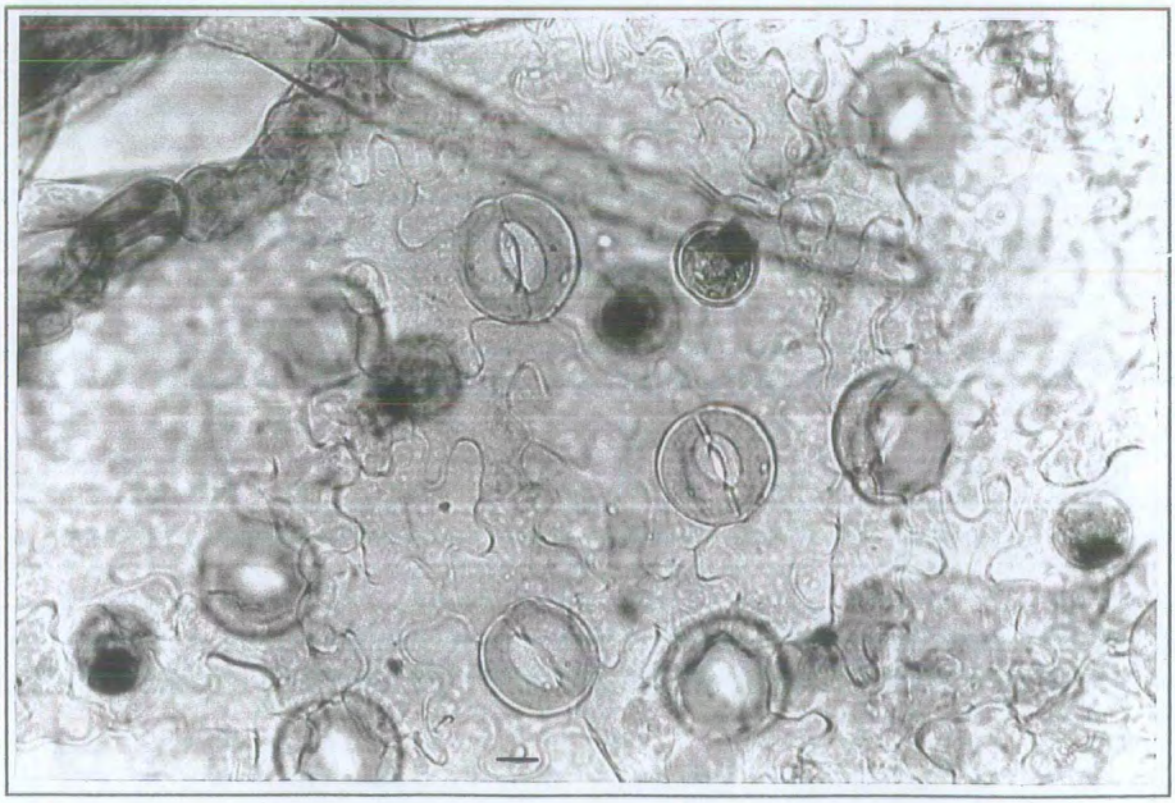


Plate 18 Potassium Staining in Unstressed Cultures -Media Z

Note minimal accumulations of potassium. Bar =1um.

Due to the very light staining occurring in the ABA treatments these ares were picked-up preferentially by the computer programme giving an over-estimation of the amount of potassium present.

No additional accumulation of potassium was evident from cultures supplemented with five or ten times the initial calcium content (Plates 17 and 18). The results are presented as photographs taken from the video, or in the case of later studies, as photomicrographs.

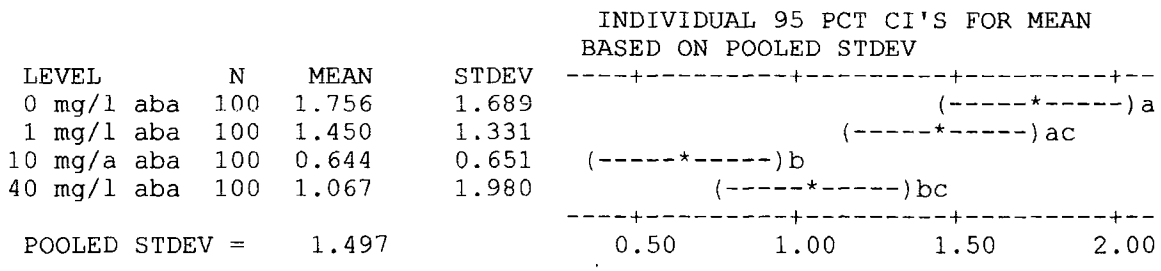
3.6 The Effect of ABA on Stomatal Aperture

Table 10 The Effect of ABA on Stomata

Conc.ABA mg/l	Pore Length	Pore diameter	Pore aperture
0	**5.025** (1.342)	**0.465** (0.408)	**1.756** (1.689)
1	**3.498** (1.074)	**0.531** (0.419)	**1.450** (1.331)
10	**5.235** (1.500)	**0.171** (0.183)	**0.644** (0.651)
40	**5.131** (1.238)	**0.266** (0.430)	**1.067** (1.980)

Figures in bold type =means.
Figures in brackets =standard deviations.

Fig 41 Analysis of Variance for the Effect of ABA on Stomatal Closure



Different lettering shows at least a **level of significance between treatments.

Table 11 Effect of Treatments on Stomata

Treatment	Pore Length	Pore diameter	Pore aperture
In vitro	**5.025** (1.342)	**0.465** (0.408)	**1.756** (1.689)
Pot Grown	**7.621** (1.602)	**0.344** (0.305)	**2.056** (1.892)
Propagation material	**8.5275** (2.920)	**0.505** (0.416)	**3.174** (2.874)
Wilted (*in vitro*)	**11.695** (2.104)	**0.745** (0.434)	**6.811** (4.008)
Wilted (pot grown)	**10.648** (1.983)	**0.536** (0.341)	**4.446** (3.449)

Figures in bold type =means.
Figures in brackets =standard deviations.

Fig 42 Analysis of Variance for the Effect of Treatments on Stomatal Closure

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
in vitro	100	1.756	1.689	(--*--)a
pot grown	100	2.056	1.892	(--*--)a
propagule	100	3.173	2.874	(--*--)b
wilted 1	100	6.811	4.008	(--*--)c
wilted 2	100	4.445	3.449	(--*--)d

POOLED STDEV = 2.921

2.0 4.0 6.0 8.0

Different lettering shows at least a **level of significance between treatments.

wilted 1 =*in vitro* raised leaf wilted on the bench.
wilted 2 =pot grown leaf wilted on the bench.

The results show that stomatal length is very variable, and that ABA and other treatments have relatively little effect on pore width, and consequently pore aperture. ABA was found to cause a decrease in stomatal aperture with concentrations up to 10mg/l, but an application of 40mg/l of ABA caused the pore aperture to increase; possible through damage to the stomata. Material which had been treated as propagation material for two days showed a greater stomatal aperture than *in vitro* material. *In vitro* leaves, allowed to wilt on the bench, showed the widest mean aperture.

3.7 Analysis of Farina from Dionysia Species

It was found that there were few differences between TLC plates, or survey scans, for the material from *Dionysia aretiodes 'Phyllis Carter'* (Plate 21, Figs 43-46). One

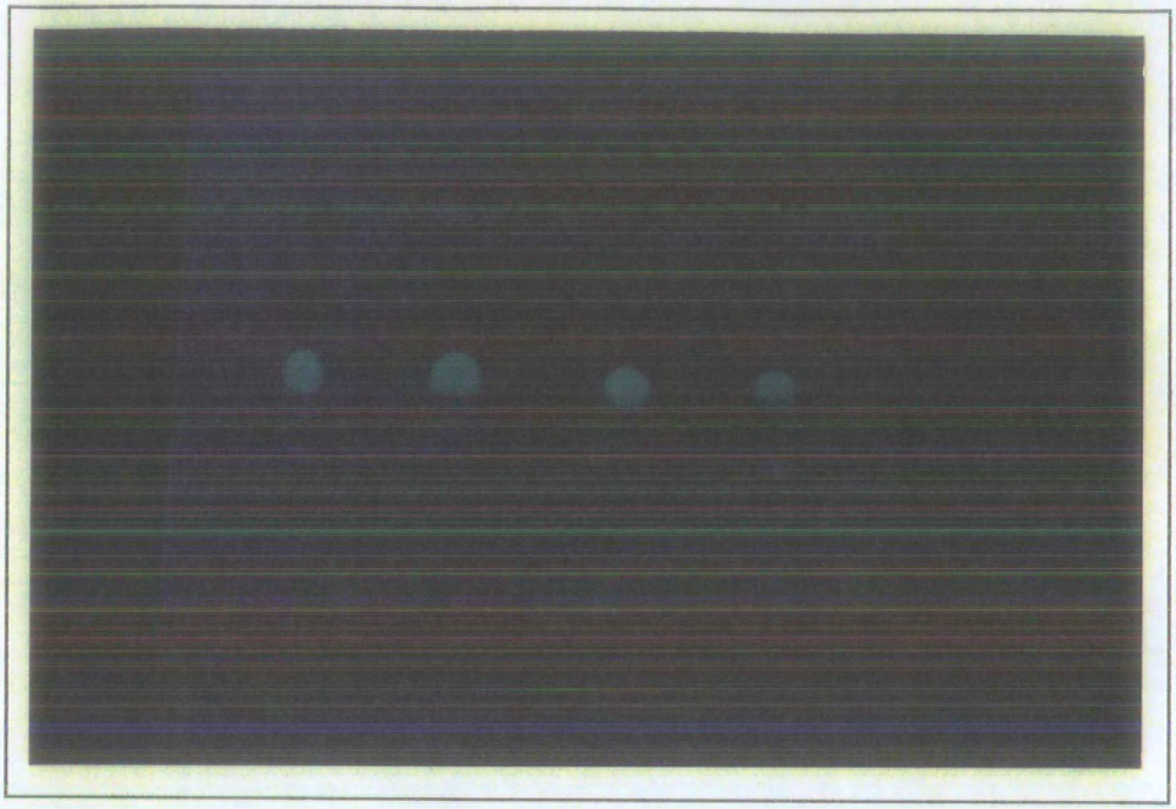
compound reflecting UV light at an rf value of 0.52 was present in the pot grown material (and other cultivars of *D. aretiodes* [Plate 20]) that was not present in the *in vitro* raised material.

The only other comparison between whole plants and *in vitro* material was made with D. *denticulata*; here the loading rates differed because of a lack of plant material. The samples from pot raised material represent only 7.6% of the required loading. There appears to be several differences between the two samples; a reflective compound (Rf 0.85) increased in the unstressed material whilst an extra absorbing fraction was present at Rf 0.81 in the pot grown material. The bright reflective band near the solvent front was absent from the pot-grown material but this may have been due to the lower loading of this farina fraction (Fig 21).

It appears that the farina produced by *Dionysia* species is, in many cases, more complex than that found in the genus *Primula*. Large variations were found to occur between closely related species.

It would appear that substantial differences occur between *D. denticulata* material, and some differences within *D. aretiodes' Phyllis Carter'*. Further studies of other species would be useful, as it seems there is some evidence for biochemical changes when material is cultured *in vitro*.

Plate 19 Farina Samples from TLC Plate 1



1 2 3 4

1 *Primula florindae*

2 *Dionysia mira*

3 *Dionysia bornmuelleri*

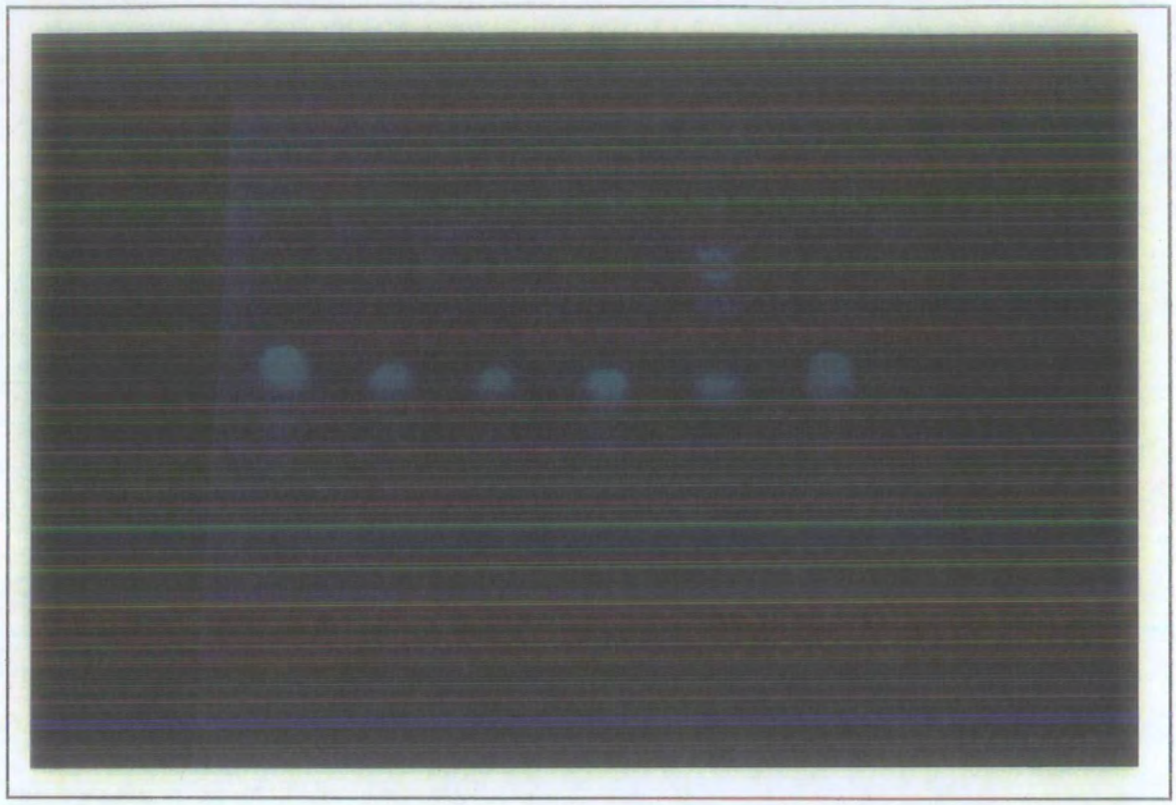
4 *Dionysia teucriodes*

Plate 20 Farina Samples from TLC Plate 2

1 2 3 4 5

1 *Dionysia bornmuelleri*
2 *Dionysia aretiodes 'Phyllis Carter'* -Pot Grown
3 *Dionysia aretiodes 'BBF'*
4 *Dionydia aretiodes 'Paul Furse'*
5 *Dionysia aretiodes 'Gravetye'*

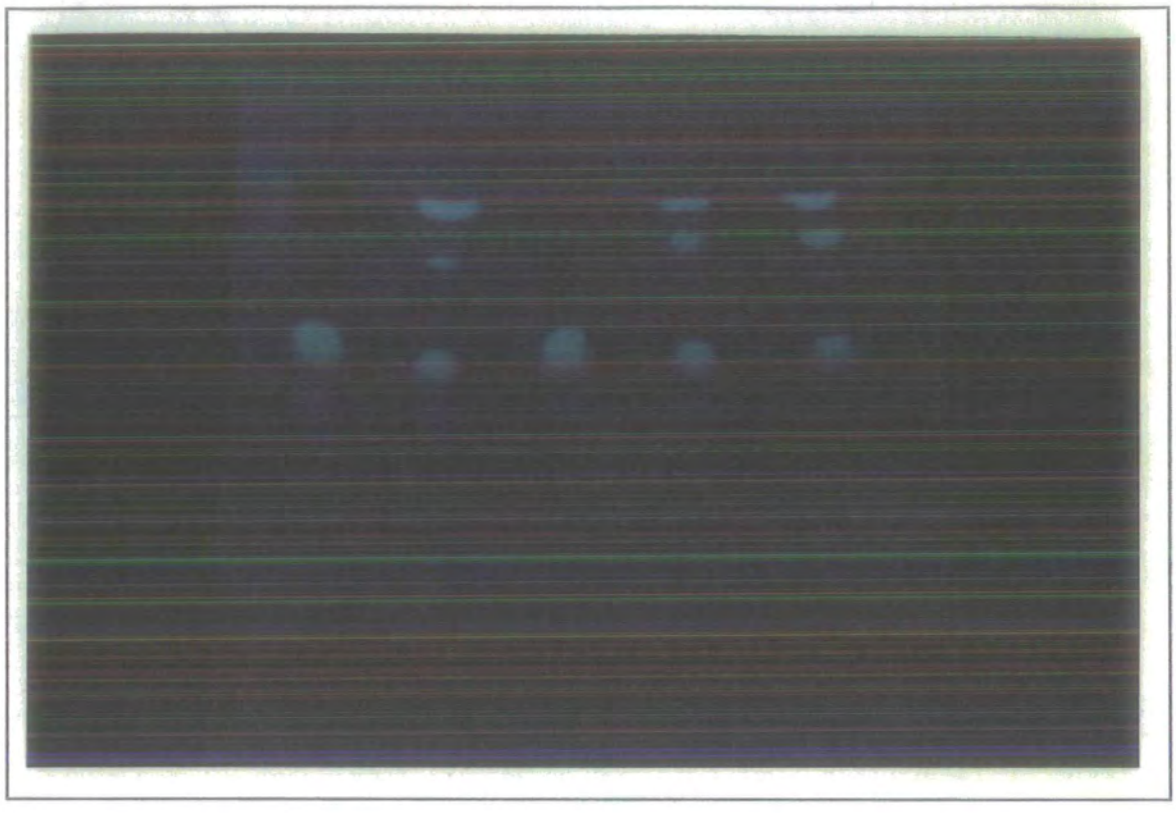
Plate 21 Farina Samples from TLC Plate 3



1 2 3 4 5 6

1 *Dionysia bornmuelleri*
2 *Dionysia aretiodes 'Phyllis Carter'* -Pot Grown
3 *Dionysia aretiodes 'Phyllis Carter* -Stressed'
4 *Dionydia aretiodes 'Phyllis Carter'* -Unstressed
5 *Dionysia denticulata* -Unstressed
6 *Dionysia denticulata* -Pot Grown

Plate 22 Farina Samples from TLC Plate 4



1 2 3 4 5

1 *Dionysia bornmuelleri*

2 *Dionysia hissarica*

3 *Dionysia archibaldii*

4 *Dionysia tapetoides*

5 *Dionysia tapetoides H1146*

Plate 23 *Dionysia mira* Showing Farina

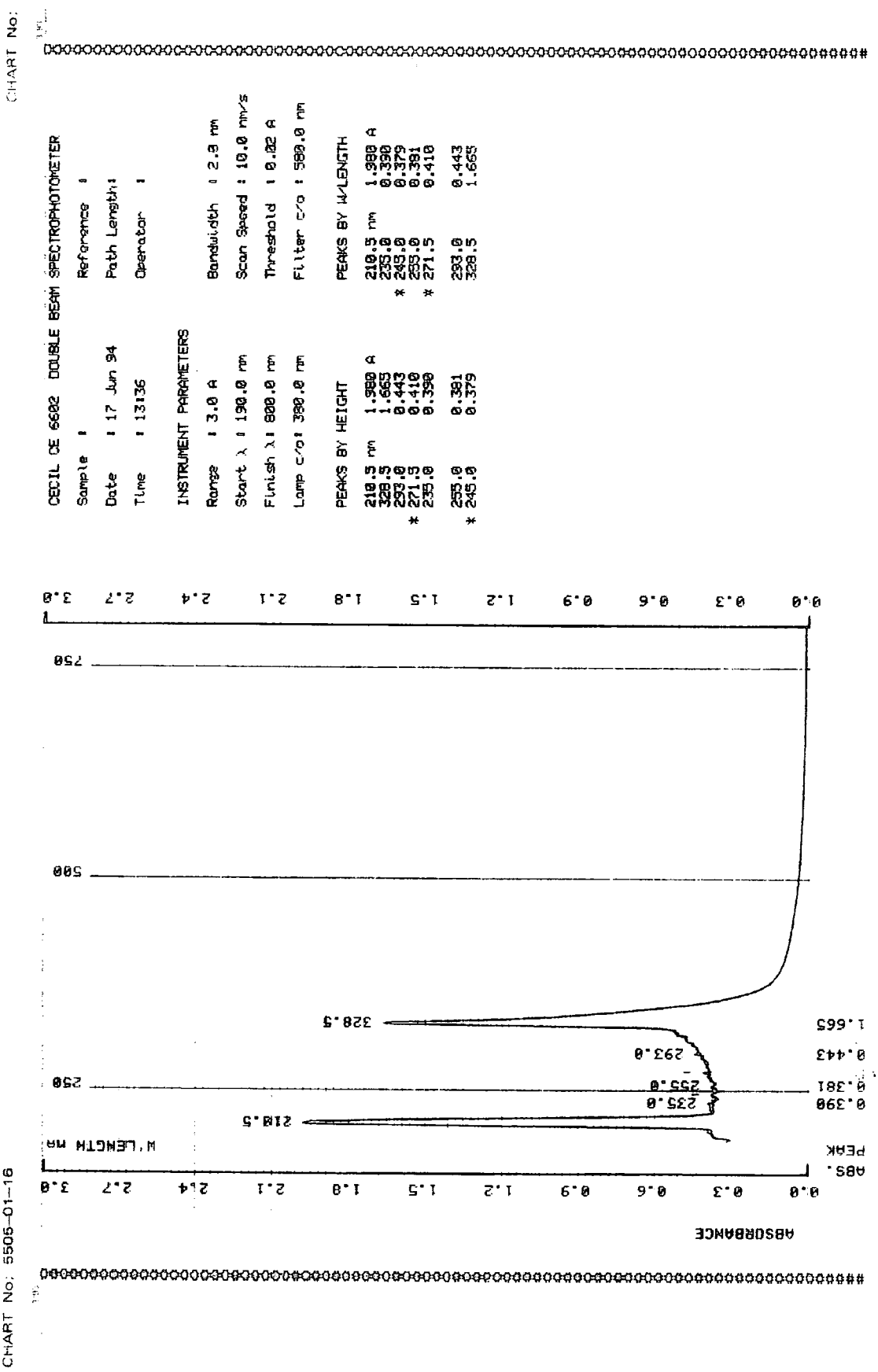


Fig 43 Spectrum of Farina from *Dionysia aretiodes* -Pot Grown

Fig 44 Spectrum of Farina from *Dionysia aretiodes* -Pot Grown

Fig 45 Spectrum of Farina from *Dionysia aretiodes* -Stressed

Fig 46 Spectrum of Farina from *Dionysia aretiodes* -Unstressed

Fig 47 Spectrum of Farina from *Dionysia bornmulleri*

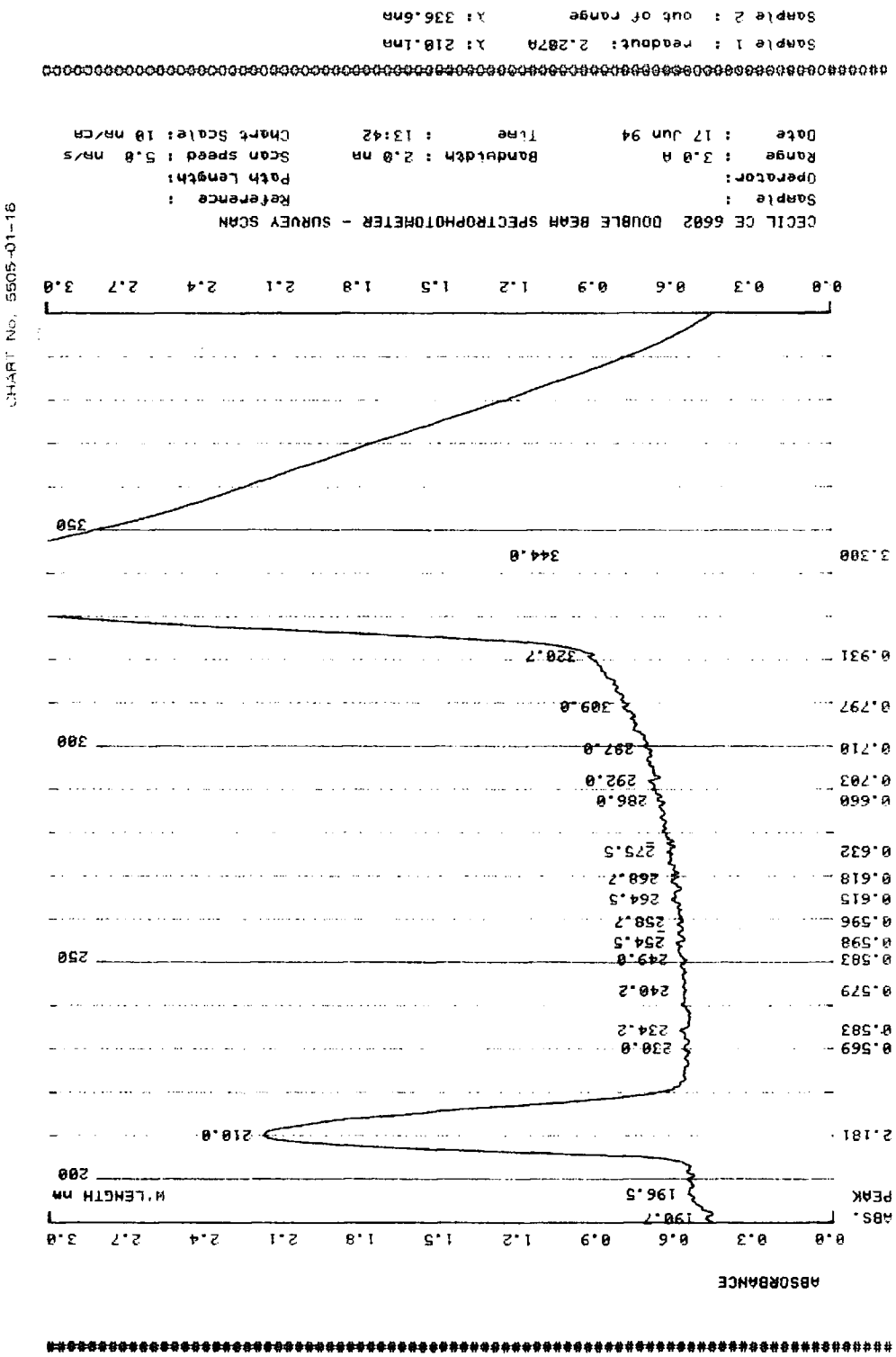


Fig 48 Spectrum of Farina from *Dionysia hissarica*

Fig 49 Spectrum of Farina from *Dionysia mira*

3.8.1 Determination of Cations in Leaf Material

Table 12 Potassium and Sodium Concentrations in Leaf Material

	Potassium			Sodium		
	pot	stressed	unstressed	pot	stressed	unstressed
	14.35	20.76	13.87	1.2150	5.3625	6.630
	14.81	24.93	17.71	1.6775	8.9500	5.570
	15.14	26.47	16.18	2.5375	8.7400	7.755
	12.08	19.63	22.55	1.5500	8.8150	7.760
	*	9.55	20.14	*	3.6275	8.035
Mean	14.10	20.27	18.10	1.74	7.10	7.15
Stdev	1.38	6.63	3.38	0.56	2.46	1.04

Table 13 Calcium and Magnesium Concentrations in Leaf Material

	Calcium			Magnesium		
	pot	stressed	unstressed	pot	stressed	unstressed
	6.98	4.1950	3.3550	3.45	1.25	1.00
	6.74	3.8850	3.7100	2.75	1.22	1.18
	8.80	4.1975	4.9750	3.35	1.32	1.30
	7.08	4.5625	4.5775	2.78	1.12	1.38
	*	3.1350	3.9925	*	0.62	1.02
Mean	7.40	4.00	4.12	3.08	1.11	1.18
Stdev	0.95	0.54	0.65	0.37	0.28	0.16

Table 14 Cation Concentrations from Plants on the Minimal Potassium Media

	Minimal Potassium Media			
	Potassium	Sodium	Calcium	Magnesium
	1.64	5.32	2.22	0.68
	0.35	2.12	2.43	0.32
	1.64	5.54	2.66	0.72
Mean	1.21	4.33	2.44	0.57
Stdev	0.74	1.92	0.22	0.22

It was found that stressed and unstressed cultures behaved very similarly in their cation accumulation, so that stressing cultures *in vitro* caused no appreciable change. Samples from pot grown material were significantly different from those raised *in vitro* (Tables 12 and 13, Figs 50-54).

Potassium levels were found to be similar across the treatments and were not statistically different (Appendix 4). Stressed cultures accumulated 20.3mg, unstressed cultures 18.1mg with the pot grown plants showing the lowest accumulation at 14.1mg/g (Fig 50).

Sodium levels increased fourfold in cultured material, and showed approximately one half the content of calcium and one third the content of magnesium compared to whole plants. Pot grown material was found to be statistically different from the stressed and unstressed cultures, which were not statistically different (Appendix 4, Figs 51-54)).

When potassium was minimised in the media the accumulation of all cations dropped to approximately half that of the other cultured material, while potassium content was negligible at 1.2mg/g. The minimal potassium culture declined from initiation, appearing to recycle nutrients from the old to the new leaves. Death followed a small number of subcultures (Table 14, Fig 54).

3.8.2 The Effect of pH and Calcium Concentrations on Cation Accumulation

Table 15 Calcium Concentrations in Leaf Material from Modified Media

	Calcium				
Media	U	W	X	Y	Z
	3.1400	2.5225	5.0925	8.5225	12.7025
	3.6200	3.2825	8.0075	6.1925	5.6600
	5.1975	2.1075	8.2050	10.3000	16.0450
	3.3125	2.4825	10.1300	10.8350	18.8350
	3.7175	2.4825	11.6925	7.6100	13.6900
					18.7900
Mean	3.80	2.58	8.63	8.69	14.29
Stdev	0.82	0.43	2.49	1.91	4.93

Table 16 Sodium Concentrations in Leaf Material from Modified Media

Sodium					
Media	U	W	X	Y	Z
	4.9675	1.7225	0.9750	2.2675	1.1000
	5.1500	1.5500	2.3725	2.0075	0.2325
	5.6500	1.3450	2.0775	2.0375	1.5775
	8.5450	1.5325	1.4225	2.3825	1.0775
	6.3250	1.3325	1.0925	1.8300	1.7075
					2.1375
Mean	6.13	1.50	1.59	2.10	1.30
Stdev	1.45	0.16	0.61	0.22	0.66

Table 17 Magnesium Concentrations in Leaf Material from Modified Media

Magnesium					
Media	U	W	X	Y	Z
	1.3100	0.7550	0.7275	0.9625	0.9975
	1.6700	0.9425	0.8225	0.8350	0.4600
	1.2925	0.7175	0.9075	1.1725	0.9650
	1.4525	0.8175	1.1400	0.9125	0.9475
	1.1550	0.8500	1.1750	0.9375	0.9350
					1.1350
Mean	1.38	0.82	0.95	0.96	0.91
Stdev	0.20	0.09	0.20	0.13	0.23

Table 18 Potassium Concentrations in Leaf Material from Modified Media

	Potassium				
Media	U	W	X	Y	Z
	12.82	16.54	10.67	21.84	14.35
	15.24	17.75	10.38	7.71	6.33
	22.69	12.76	13.94	19.16	14.49
	19.72	16.24	18.27	16.67	15.30
	19.89	15.08	10.91	15.56	15.53
					16.03
Mean	18.07	15.67	12.83	16.19	13.67
Stdev	3.97	1.89	3.36	5.32	3.65

Increased calcium levels, or adjustments to pH, in the media did not result in a statistically significant alteration in potassium levels (Appendix 5 [Table 18, Fig 55]).

Magnesium and sodium levels appeared to drop by approximately one third in all treatments, with no significant difference between calcium enriched, or pH modified, media (Appendix 5 [Tables 16 and 17, Figs 56 and 58]).

Calcium levels appeared unaltered by pH, but more than doubled with increased calcium levels in the media. No statistical difference was found between five or tenfold enrichment, though this may be due to the small number of samples (Appendix 5 [Table 15, Fig 57]).

Fig 50 POTASSIUM ACCUMULATION IN LEAF MATERIAL

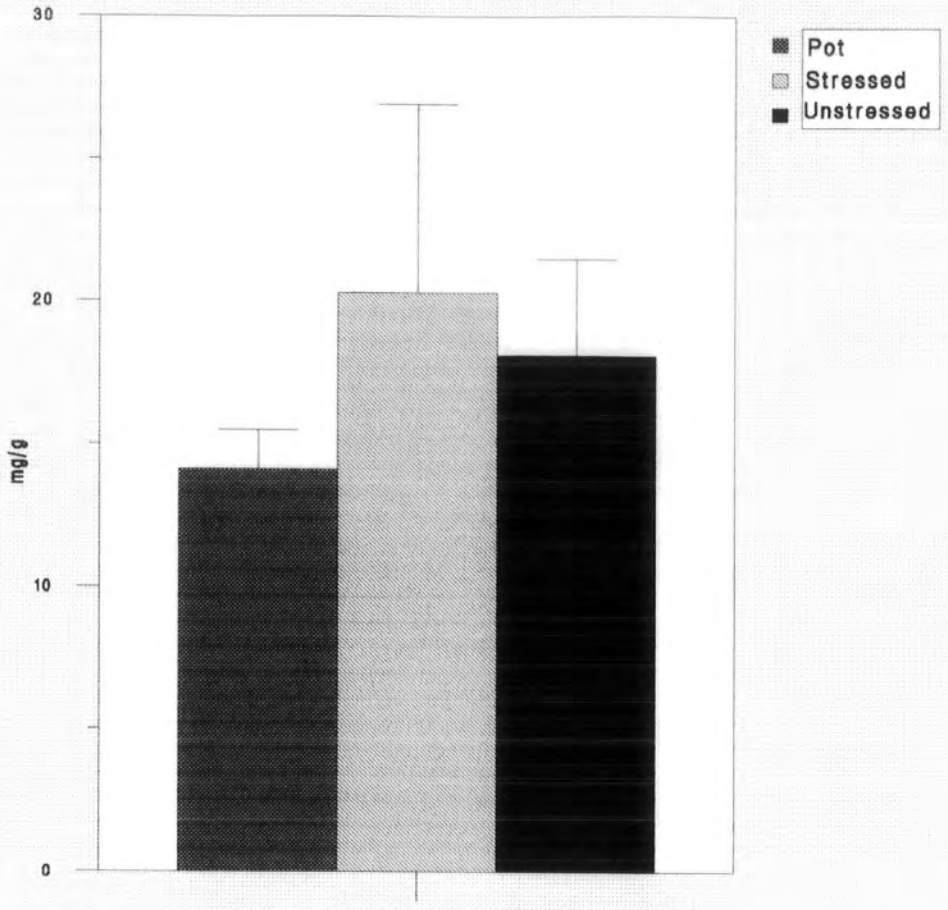


Fig 51 SODIUM ACCUMULATION IN LEAF MATERIAL

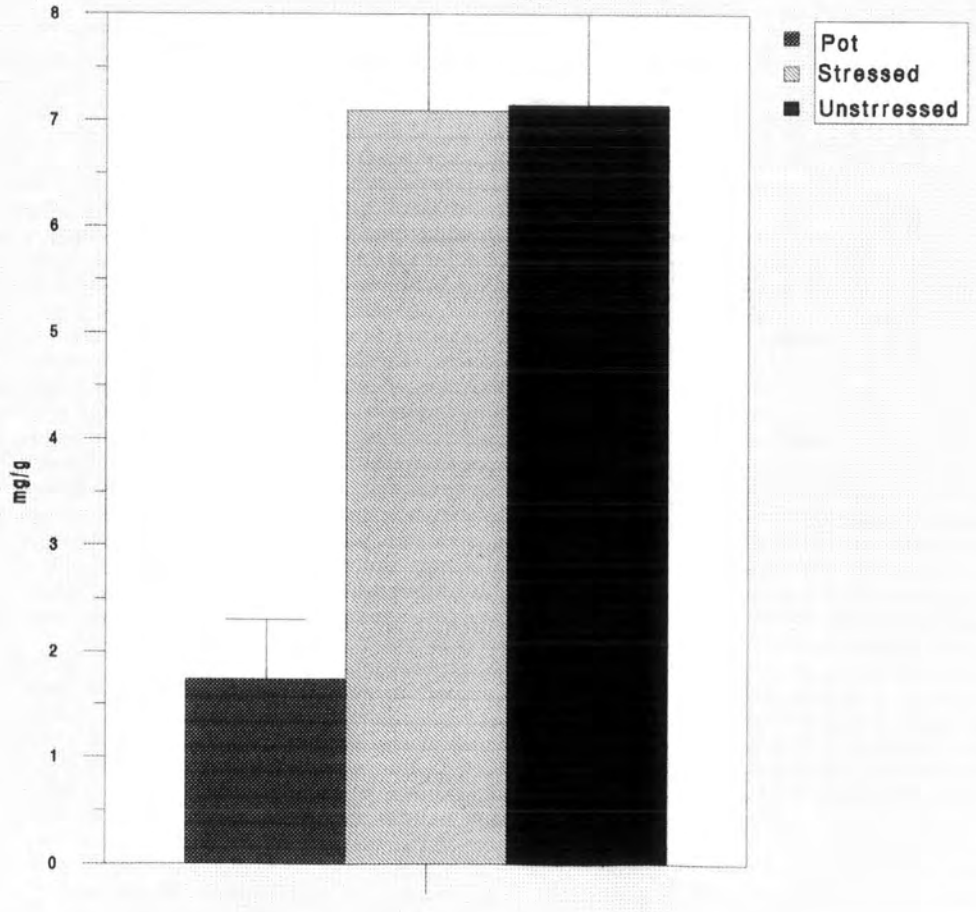


Fig 52 CALCIUM ACCUMULATION IN LEAF MATERIAL

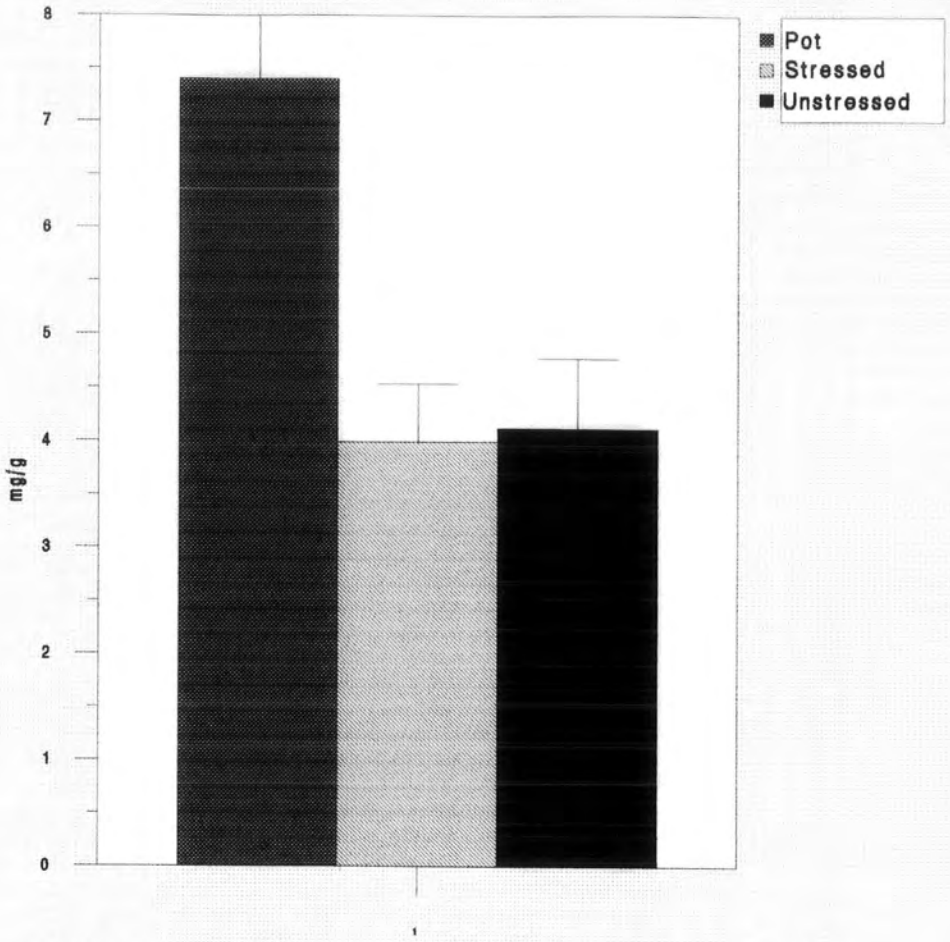


Fig 53 MAGNESIUM ACCUMULATION IN LEAF MATERIAL

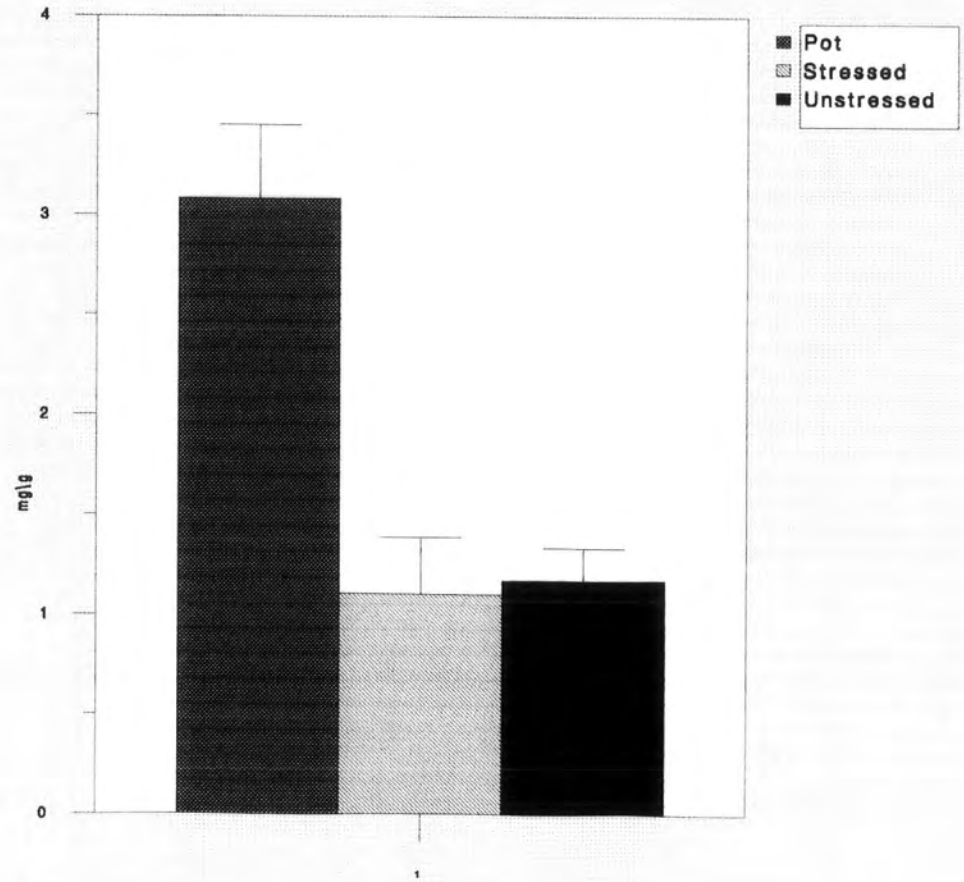


Fig 54 CATION ACCUMULATION IN MINIMAL POTASSIUM MEDIA

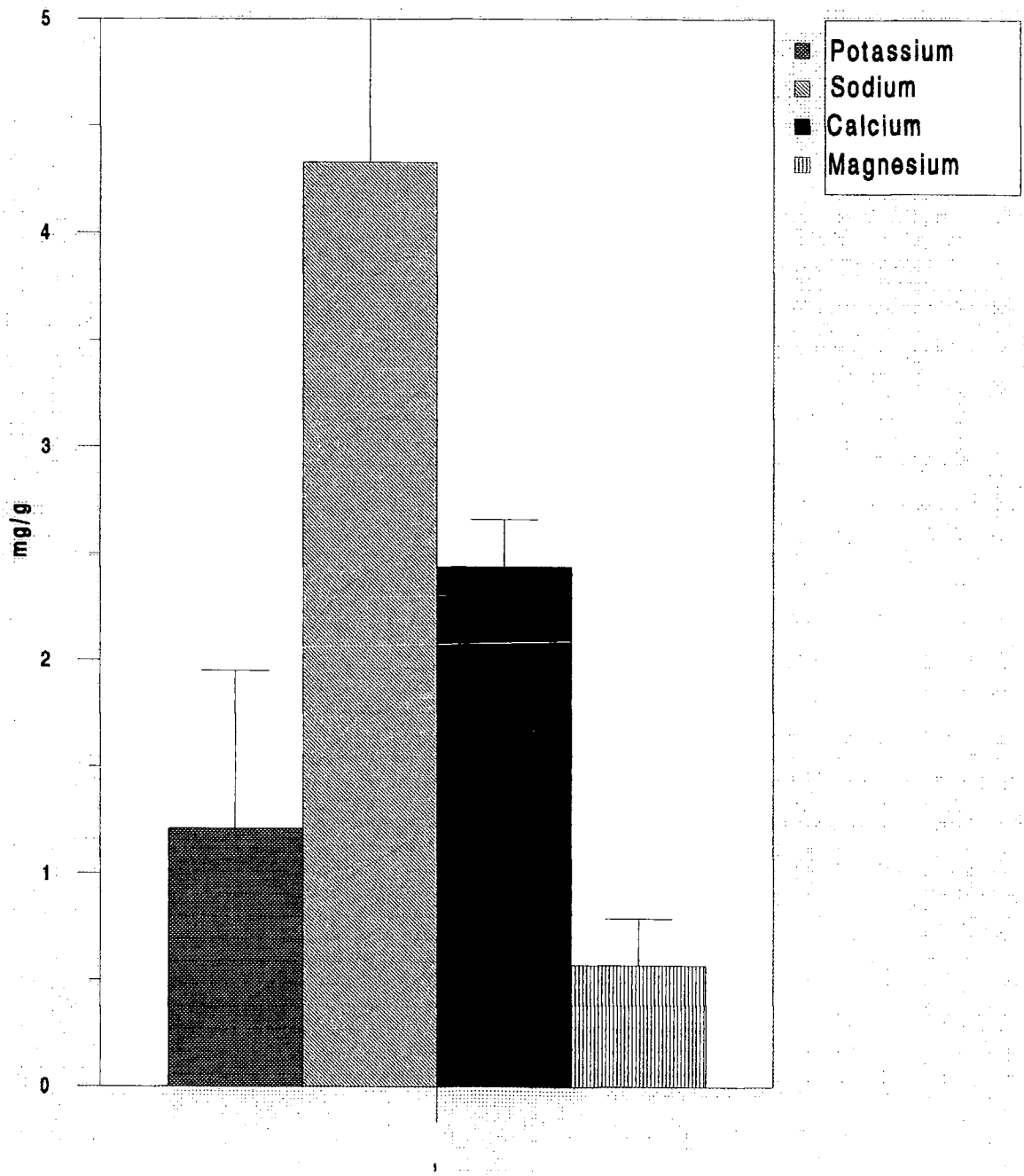


Fig 55 POTASSIUM ACCUMULATION IN LEAF MATERIAL FROM MODIFIED MEDIA

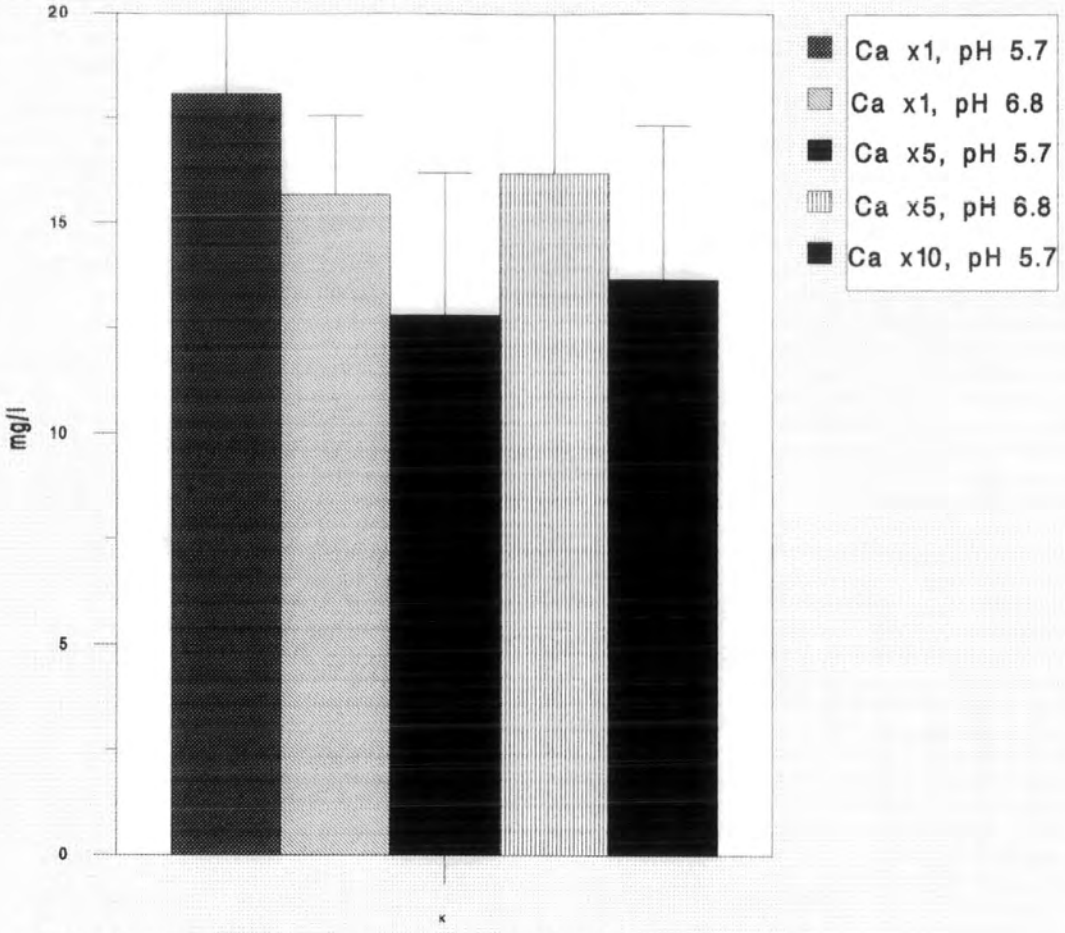


Fig 56 SODIUM ACCUMULATION IN LEAF MATERIAL FROM MODIFIED MEDIA

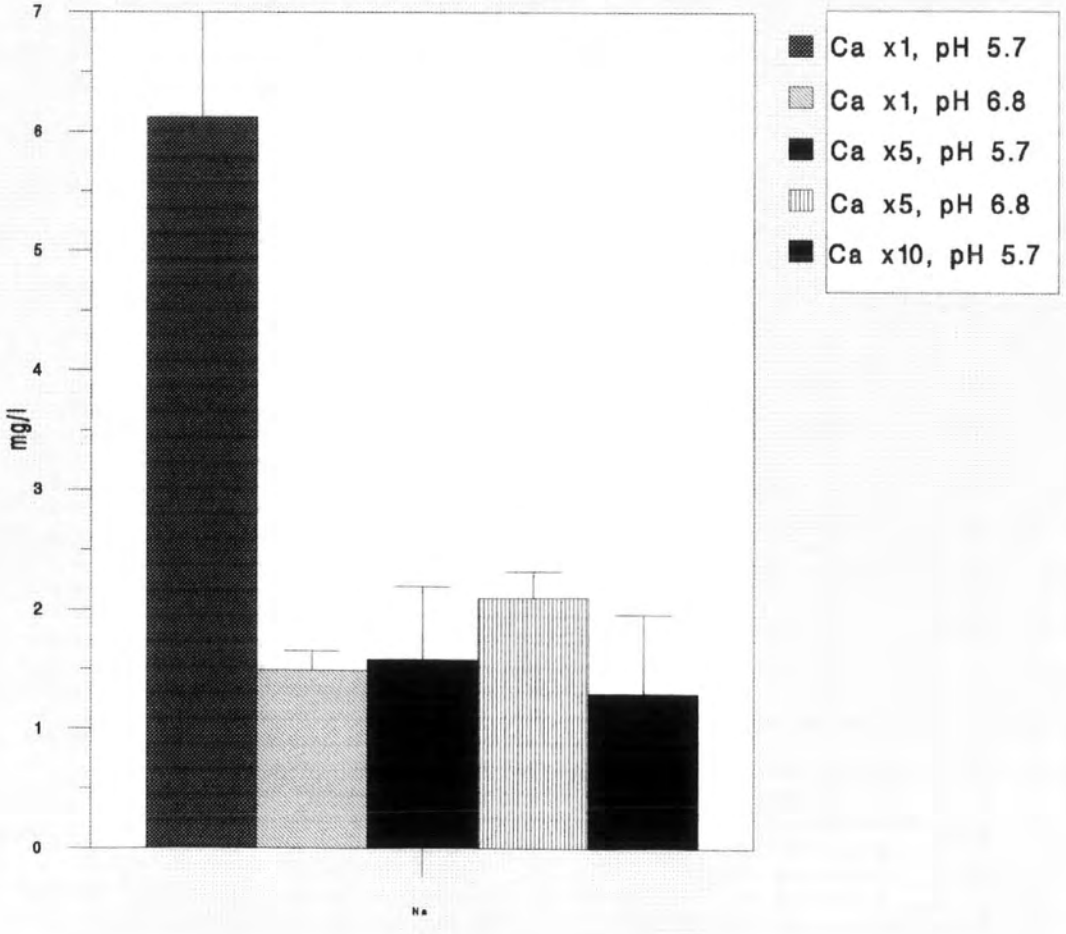


Fig 57 CALCIUM ACCUMULATION IN LEAF MATERIAL FROM MODIFIED MEDIA

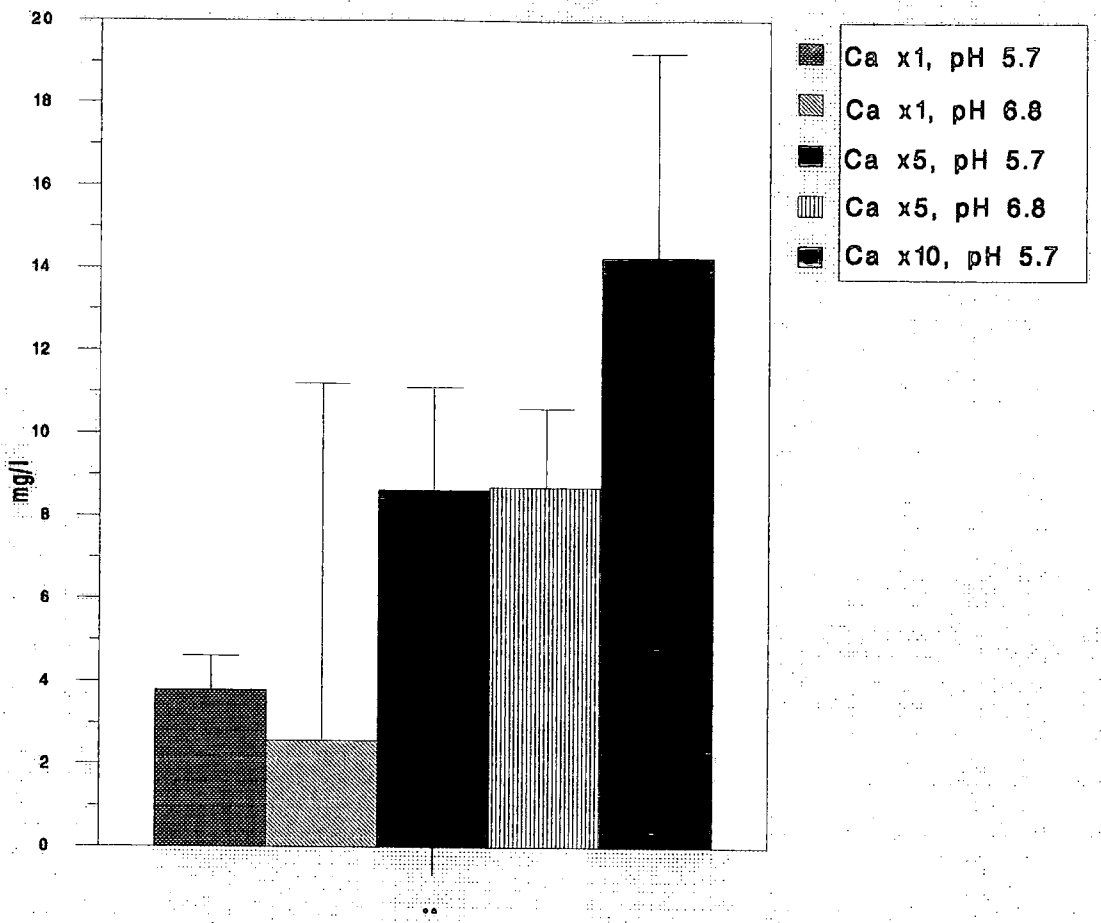
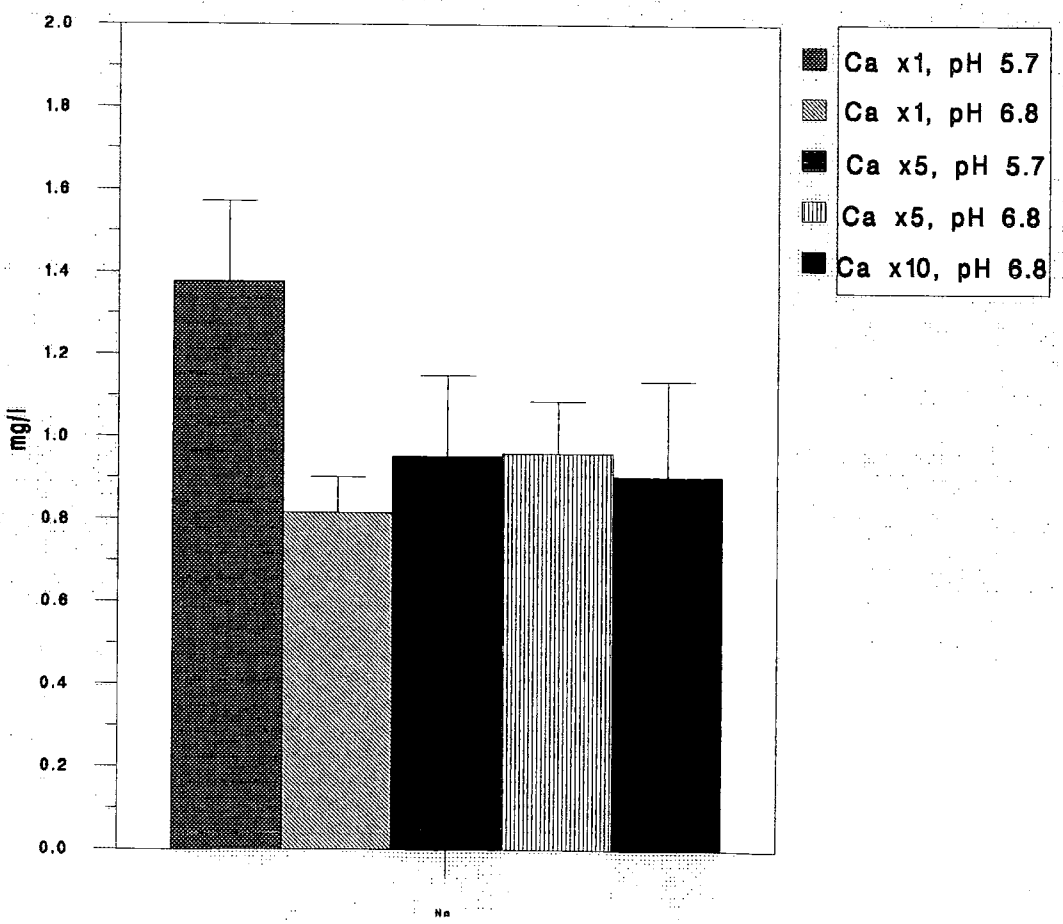


Fig 58 MAGNESIUM ACCUMULATION IN LEAF MATERIAL FROM MODIFIED MEDIA



4 DISCUSSION

4.1 Stomatal Density Studies

The control of stomatal density has been shown to be lost during *in vitro* culture with leaves of very high and low stomatal densities being produced. Stomatal densities may play a key role in regulating the amount of water loss from the plants leaf surfaces. This is particularly importance to the weaning of micropropagules, which have only limited ability to replenish water lost from evapo-transpiration.

Weaning experiments (Section 3.1) have indicated that stomatal density plays some role in the survival, or death, of propagules. Propagules with low densities appeared to favour establishment, possibly by reducing water loss from the leaf surface. Propagules showing stomatal densities comparable to, or greater than, pot grown plants material failed to root. Other differences in the epidermal surface affecting water loss, ie clustering and the number of glands, and the clustering of stomata, may help explain why only propagules of abnormally low stomatal densities survived to root.

4.2 Studies into the Interaction of Stomata and Farinal Glands

The results of the point-centred quarters experiment appears to show a changing influence of stomata and farinal glands on the nature of the development of the epidermis. It appears that the glands show a differing affect on development according to treatment. Sachs and Novoplansky (1993) discovered that for leaves of *Peperomia obtusifolia* glands were formed prior to stomatal development but could later change to form stomata. A negative correlation between glands and the frequency of additional glands was also found.

The nearest-neighbour measurements show that the glands in all treatments show clustering; no apparent inhibition of one gland by another takes place. The number of glands would appear to have no effect on the closeness of neighbouring stomata in pot grown material. In stressed cultures, however, the distance between glands and stomata were closely correlated to gland density, with no apparent relationship to stomatal density. Unstressed cultures showed an intermediate response with both gland and stomatal densities affecting the proximity of stomata to farinal glands. Some inhibition of stomatal development by farinal glands would appear to take place.

Glands appear to show a strong inhibitory effect on stomatal development in stressed cultures, no effect in pot grown

material and some limited inhibition in unstressed cultures. Clustering of both glands and stomata are apparent from the nearest-neighbour work, with the exception of the stomatal distribution for pot grown material, where a straight line relationship prevails; presumably because no stomatal inhibition by glands occurs.

An inhibitory mechanism to account for these findings is still lacking.

It is interesting to note that the chi-squared experiment high-lighted the unstressed culture as differing between the known and calculated stomatal densities; other treatments were not significant. The number of glands produced in relation to stomatal densities in the unstressed cultures was also lower than that of the other two treatments; stressing increased the number of glands which developed. Lower gland densities might be thought to show an inhibitory effect on additional gland formation, however greater clustering is also found for this treatment with the nearest-neighbour measurements. Water stress *in vitro* resulted in the inhibition of growth and root development for two or three subcultures following transfer to fresh media; therefore this is not a suitable hardening technique prior to weaning.

The proximity of farinal glands and stomata are likely to have an effect upon water loss. It may be postulated that where stomatal development is inhibited by the development of

glands, water loss might increase due to a reduction in the boundary layer adjacent to the stomata. This would appear to conflict with the results as this pattern developed under conditions of water stress. The farina of Dionysias is woolly, however, and small distances between glands may still be covered by this material.

Farina itself is a complex mixture of substances, whose composition appears to undergo some changes during *in vitro* culture. The effect of these changes on water loss is unknown.

The stomatal pore constricts the vapour flux pathway according to pore width, with water shells forming outside the stomata. Interference with water loss may occur if these shells overlap, this is likely to be negligible unless the stomata are less that three times stomatal pore width apart (Parlange and Waggner, 1970). The pore length of *Dionysia aretiodes* compared to stomatal density has been shown to be variable, with regression lines of low r^2 values. Even with the higher pore length values for a given stomatal density, in pot grown material where stomatal distribution appears not to be clustered, interference between pores is unlikely to occur. In cultured material stomata were found not to have a random distribution. It is therefore possible that clustering may reduce the distances between stomates to a point where interference may occur between water shells of adjacent stomata, particularly as control of stomatal density has been

shown to be lost during culture, resulting in some explants with abnormally high stomatal densities. This might result in a net reduction in water loss. It would seem possible that an increase in gland inhibition of stomata occurs during *in vitro* culture and may enhance clustering of stomata, leading to reduced water loss, through a potential water flux interference between pores.

4.3 Farina

Dionysia farina remains a puzzling, enigmatic substance, whose function remains uncertain. It seems likely that farina has an ecological or physiological role (Wollenweber, 1977), rather than being a waste substance as proposed by Blasdale (1947). The farina of *Dionysia aretiodes* and *D. tapetiodes* may have insecticidal properties as bees were observed to die after contact with these plants. This might be a defense mechanism against predation, or insect death could result in improved soil nitrogen status around the plants; however aphid damage does occur on Dionysias.

It seems probable that the woolly farina produced on *Dionysias*, differing from the powdery farina on *Primulas*, may increase the boundary layer, limit water loss by occluding the recessed stomata, or protect the guard cells and epidermis from UV light. The role of farina remains to be finally established.

Certain similarities between farina of different species may be observed from the TLC plates. It would appear that tentative groupings might be constructed thus:

(Primula florindae)
Dionysia teucriodes

D. bornmuelleri
D. mira
D. aretiodes
D. archibaldii

D. hissarica
D. tapetiodes
D. denticulata

A taxonomic role for farina was proposed by Harborne (1968), however such a taxonomy would appear at odds with the accepted classification of the genus (Table 1). Certainly the outward appearance of the plants does not correlate with apparent similarities of farinal components. The farina may have an ecological, or physiological function and may relate to habitat, or geographic distribution, rather than indicate a close genetic link between species. Further work would be required to produce a chemotaxic classification of the genus; including the recovery of separated components and their identification using spectrophotometric means.

4.4 ABA and Stomatal Function Studies

As has been stated, the response of leaf tissue to ABA is highly dependent upon the method of feeding (Trejo et al, 1993). *Dionysia* leaves were floated on ABA solutions for two hours; the major route of ABA uptake would be through the petiole section of the leaf. Direct absorption through the leaf itself would be less likely to occur given the strong water repellant nature of the farina on the leaf surface. The rate of ABA absorption may thus have been limited by the lack of evapotranspiration from the leaf. Differences in response due to feeding regimes and plant material make comparisons between research results difficult. Results suggest that sufficient ABA was absorbed through the xylem, notwithstanding metabolism in the mesophyll tissue, to cause a change in stomatal aperture. ABA causes a net efflux of potassium ions and thus causes stomatal closing. A limited reduction in stomatal aperture was found with 1mg/l ABA, with maximum closure at 10mg/l ABA. Further opening occurred at 40mg/l ABA, although this may be due to damage caused to the guard cells at this concentration. The swift decline of plants *de novo*, after root disturbance through potting, might be an acute ABA mediated reaction. Tests with sodium cobaltinitrite have shown that treatment with ABA did not increase the concentration of potassium in the guard cells, when subsequently placed in an environment to stimulate opening. It is possible that potassium was lost from the guard cells during treatment with ABA and that a similar

level restored afterwards either by active transport, or the leaking in of potassium ions from adjoining cells. In either event very little potassium appeared to present in any epidermal strips from leaves grown *in vitro*. Epidermal strips taken from pot grown material showed a slight (but not significant) increase in potassium accumulation in guard cells, although more potassium was visible in the epidermis, with some epidermal cells and bases of leaf hairs showing large accumulations. It is possible as the leaf surface proved unwettable with water, that air might have been trapped around the stomata causing a delay in the depletion of CO_2 and thus opening. Limited opening of stomata was found for both stressed and unstressed pot grown material. Large accumulations of potassium were however found for leaves from *in vitro* culture stressed on the bench and illuminated under the same conditions. Removal of epidermal strips is known to cause disruption, the experiment could usefully be repeated by incubating isolated epidermal strips, although this would remove the affect of the mesophyll.

Potassium accumulation for all material was considerably different from that expected from the literature. It is conceivable that stomata may function in this species with lower influxes and effluxes of potassium and thus limited stomatal movement under normal conditions. It may be postulated that the presence of epidermal hairs, farinal glands and sunken stomata may markedly reduce the water loss from the leaf surface preventing rapid changes in water

status. Leaves of *Dionysia mira* (Plate 23) are found to curl upward during the summer, light is reflected back from the farina and the angle of the leaf ensures that less light is absorbed. Water loss for the purpose of temperature regulation may thus be reduced. *D. aretiodes* shows leaf rolling which may give rise to similar effects (Plate 2). A lack of stomatal function has also been shown for *Gunnera tinctoria* (Osborne et al 1993) as an adaption to a wetland habitat.

When stressed on the bench *in vitro* raised material was found to produce the largest accumulation of potassium observed in the treatments. Propagules exposed to the propagation environment for two days failed to accumulate any more potassium than the *in vitro* material and may therefore show a similar state of stomatal operation.

The results of potassium accumulation in the epidermis and the measurements of stomatal aperture, show few differences to suggest a change of stomatal functionality between treatments. The wider stomatal aperture resulting from stressing *in vitro* material on the bench compared to that from stressed pot grown material suggests that any lack of change is not due to the rigidity of the guard cell wall.

4.5 Determination of Cations in Leaf Material

An analysis of cations has shown a decrease in the K:Na ratio during *in vitro* culture. Stressed and unstressed cultures have both shown an increase in sodium accumulation, and a corresponding drop in calcium and magnesium levels in leaf material.

Brainerd and Fuchigami,(1982) suggested that the change in the K:Na ratio might explain the lack of stomatal functionality of *in vitro* cultures. The change in the K:Na ratio in Dionysias occurred through the added accumulation of sodium, rather than any drop in potassium uptake. The addition of NaCl to the culture media has been shown to increase sodium levels and decrease calcium uptake *in vitro* (Wardle et al ,1981). The reduction in K/Na selectivity as a result of salt stress *de novo* may be ameliorated by the addition of calcium (Martinez and Lauchii, 1993), which may also enhance potassium concentrations (Hawkins and Lewis, 1993). The increase in calcium in the media failed to stimulate additional potassium uptake in Dionysias, but the K:Na ratio was ameliorated by a large drop in sodium uptake as a result of either the presence of additional calcium, or the increase of media pH from pH 5.7 to pH 6.8, or both. Magnesium levels dropped under all treatments, but calcium accumulation increased with increasing calcium concentrations in the media, irrespective of pH.

The results from *in vitro* studies appear to confirm results gained *de novo;* calcium competes with sodium for absorption and increases K/Na selectivity. It was found that K:Na ratio could be ameliorated, or improved above that shown by pot grown material, by increasing the calcium and/or the pH level of the media, Ca:Na ratios could be restored, or improved, by the addition of calcium to the media irrespective of pH.

The results for potassium accumulation in guard cells and stomatal apertures suggests that stomata of *Dionysia aretiodes 'Phyllis Carter'* show low levels of functionality irrespective of the growing environment *in vitro* or *de novo.* Adjustments in calcium, or pH levels, in the medium do not appear to cause any significant difference to stomatal function in Dionysias. It was not possible to test the hypothesis, in this species, that the lack of stomatal function *in vitro* is due to a decrease in the K:Na ratio.

The results show that cation levels in *Dionysia* leaves are easily manipulated by increasing the calcium concentration, and/or pH levels, of the media. If a similar response where shown by other species, media adjustment might prove a novel and useful way of hardening *in vitro* material prior to weaning. It would be desirable to test this hypothesis on a plant species whose stomatal operation is known to be more responsive than Dionysias.

The results presented here show the accumulation of cations in whole leaves, but in connection with stomatal function the epidermis is of importance and may show different accumulations as compared to the leaf as a whole. A further investigation into cation levels in the individual cells using X-ray microanalysis (Pihakaski-Maunsbach and Harvey ,1992) would therefore be useful.

CONCLUSION

The research has shown that *Dionysia aretiodes 'Phyllis Carter'* displays a lack of stomatal functionality under the range of culture conditions studied. It is postulated that the morphological adaptions to a dry environment exhibited by *D. aretiodes* have eliminated the need for normal patterns of stomatal operation.

ABA, or calcium and pH modifications to the culture media, had little affect on potassium accumulation in guard cells, or on stomatal aperture; however changes in pH or calcium concentration were shown to ameliorate K:Na ratios. Alterations of media pH and/or calcium concentration might provide a useful method of hardening *in vitro* cultures prior to weaning for species showing standard stomatal functions.

Differences in the pattern of epidermal development were observed between treatments; control of stomatal density was lost *in vitro*. The juxtaposition of glands and stomata, and stomatal density, would appear important factors in determining the survival ability of propagules from culture.

Whilst no immediate methods to improve the weaning of *Dionysia aretiodes 'Phyllis Carter'* from culture were found, further investigation of the water status of Dionysias appear warranted. Attempts to regulate the control of stomatal densities during culture, or the rooting of propagules into

compost in vitro prior to weaning, would seem profitable areas of further research. Water loss as a function of stomatal density appears to be a key factor in the weaning of propagules.

Appendix 1 Analysis of Stomatal Densities

ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	23	27235.0	1184.1	83.72	0.000
ERROR	4139	58540.6	14.1		
TOTAL	4162	85775.5			

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
pa	221	7.792	2.707	(-*-)
pb	107	7.224	2.615	(-*-)
pc	110	7.791	2.263	(-*-)
pd	137	8.394	3.473	(-*-)
pe	125	9.472	3.557	(--*-)
pf	38	9.263	3.971	(---*---)
pg	105	8.457	3.388	(-*--)
ph	78	10.667	2.962	(--*-)
sa	203	12.488	4.514	(-*)
sb	181	9.459	3.952	(-*)
sc	260	11.100	4.080	(-*-)
sd	272	8.566	4.080	(-*)
se	260	5.638	1.877	(-*)
sf	243	12.259	2.780	(-*)
sg	125	11.384	3.419	(-*-)
sh	173	7.029	2.067	(*-)
si	130	10.385	2.729	(--*-)
ua	199	14.030	7.072	(-*-)
ub	145	15.441	5.328	(-*--)
uc	226	8.296	2.882	(-*)
ud	235	6.643	2.306	(*-)
ue	181	9.508	3.309	(-*-)
uf	128	14.320	5.156	(-*-)
ug	281	11.680	4.573	(-*)

POOLED STDEV = 3.761

6.0 9.0 12.0 15.0

p=pot, s=stressed, u=unstressed.

Appendix 2 Analysis of Gland Densities

Analysis of variants for glands. Stressed v unstressed

ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	1	3.3	3.3	0.25	0.621
ERROR	28	372.7	13.3		
TOTAL	29	376.0			

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
stressed	15	8.333	4.065	(----------*----------)
unstressed	15	7.667	3.177	(----------*----------)

POOLED STDEV = 3.648

6.0 7.5 9.0 10.5

Analysis of variants for glands. Stressed v pot

ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	1	0.4	0.4	0.03	0.865
ERROR	29	399.3	13.8		
TOTAL	30	399.7			

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
stressed	15	8.333	4.065	(----------*----------)
pot	16	8.562	3.346	(----------*----------)

POOLED STDEV = 3.711

7.2 8.4 9.6

Analysis of variants for glands. Unstressed v pot

ANALYSIS OF VARIANCE

SOURCE	DF	SS	MS	F	p
FACTOR	1	6.2	6.2	0.58	0.451
ERROR	29	309.3	10.7		
TOTAL	30	315.5			

LEVEL	N	MEAN	STDEV
unstressed	15	7.667	3.177
pot	16	8.562	3.346

POOLED STDEV = 3.266

INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV

```
--+---------+---------+---------+-----
  (-------------*-------------)
          (-------------*-------------)
--+---------+---------+---------+-----
6.0       7.2       8.4       9.6
```

Appendix 3 Regressions of Gland and Stomatal Densities

Regression of glands against stomatal density in stressed cultures

The regression equation is
glands = 0.27 + 0.313 stomata

Predictor	Coef	Stdev	t-ratio	p
Constant	0.267	1.250	0.21	0.834
stomata	0.31263	0.04444	7.03	0.000

s = 1.924 R-sq = 79.2% R-sq(adj) = 77.6%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	183.20	183.20	49.48	0.000
Error	13	48.13	3.70		
Total	14	231.33			

Regression of glands against stomatal density in unstressed cultures

The regression equation is
glands = 3.79 + 0.120 stomata

Predictor	Coef	Stdev	t-ratio	p
Constant	3.789	1.381	2.74	0.017
stomata	0.12018	0.03794	3.17	0.007

s = 2.477 R-sq = 43.6% R-sq(adj) = 39.2%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	61.574	61.574	10.04	0.007
Error	13	79.759	6.135		
Total	14	141.333			

Regression of glands against stomatal density in pot grown plants

The regression equation is
glands = - 0.01 + 0.348 stomata

Predictor	Coef	Stdev	t-ratio	p
Constant	-0.010	2.904	-0.00	0.997
stomata	0.3481	0.1147	3.03	0.009

s = 2.690 R-sq = 39.7% R-sq(adj) = 35.4%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	66.620	66.620	9.21	0.009
Error	14	101.317	7.237		
Total	15	167.937			

Appendix 4 Analysis of Cation Concentrations

Analysis of variants for K levels

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
K pot	4	14.095	1.382	(-----*----)a
K stressed	5	20.268	6.628	(----*----)a
K unstressed	5	18.090	3.380	(----*----)a
K min K	3	1.210	0.745	(------*-----)b
POOLED STDEV =		4.190		0.0 8.0 16.0 24.0

Different lettering shows at least a **level of significance between treatments.

Analysis of variants for sodium levels

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
Na pot	4	1.745	0.563	(------*------)a
Na stressed	5	7.099	2.456	(-----*------)b
Na unstressed	5	7.150	1.035	(------*-----)b
Na min K	3	4.326	1.920	(-------*--------)ab
POOLED STDEV =		1.681		0.0 2.5 5.0 7.5

Different lettering shows at least a **level of significance between treatments.

Analysis of variants for calcium levels

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
Ca pot	4	7.3988	0.9483	(---*---)a
Ca stressed	5	3.9950	0.5373	(--*--)b
Ca unstressed	5	4.1220	0.6538	(---*--)b
Ca min K	3	2.4383	0.2190	(---*---)c

POOLED STDEV = 0.6597

2.0 4.0 6.0 8.0

Different lettering shows at least a **level of significance between treatments.

Analysis of variants for magnesium levels

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
Mg pot	4	3.0813	0.3705	(--*--)a
Mg stressed	5	1.1080	0.2847	(--*--)bc
Mg unstressed	5	1.1750	0.1649	(--*-)b
Mg min K	3	0.5733	0.2208	(---*--)c

POOLED STDEV = 0.2692

1.0 2.0 3.0

Different lettering shows at least a **level of significance between treatments.

Appendix 5 Analysis of Cation Concentrations on Modified Media

Analysis of variants for calcium levels

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
Ca -u	5	3.798	0.816	(-----*----)a
Ca -w	5	2.575	0.430	(----*----)a
Ca -x	5	8.625	2.486	(----*----)b
Ca -y	5	8.692	1.912	(----*-----)b
Ca -z	6	14.287	4.926	(----*---)b

Different lettering shows at least a **level of significance between treatments.

Analysis of variants for sodium levels

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
Na -u	5	6.1275	1.4502	(---*--)a
Na -w	5	1.4965	0.1621	(--*---)b
Na -x	5	1.5880	0.6131	(---*---)b
Na -y	5	2.1050	0.2197	(---*--)b
Na -z	6	1.3054	0.6593	(---*--)b

POOLED STDEV = 0.7680 — 2.0 — 4.0 — 6.0

Different lettering shows at least a **level of significance between treatments.

Analysis of variants for magnesium levels

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
Mg -u	5	1.3760	0.1952	(------*------)a
Mg -w	5	0.8165	0.0874	(------*-----)b
Mg -x	5	0.9545	0.1963	(-----*------)b
Mg -y	5	0.9640	0.1260	(------*-----)b
Mg -z	6	0.9067	0.2306	(-----*-----)b

POOLED STDEV = 0.1781 — 0.75 — 1.00 — 1.25 — 1.50

Different lettering shows at least a **level of significance between treatments.

Analysis of variants for potassium levels

LEVEL	N	MEAN	STDEV	INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV
K -u	5	18.072	3.967	(---------*---------)a
K -w	5	15.674	1.886	(---------*---------)a
K -x	5	12.834	3.361	(---------*---------)a
K -y	5	16.188	5.322	(---------*---------)a
K -z	6	13.672	3.652	(--------*--------)a

POOLED STDEV = 3.794 — 10.5 — 14.0 — 17.5 — 21.0

Different lettering shows at least a **level of significance between treatments.

References

Blanke, M. M. & Belcher, A., R. (1989). Stomata of apple leaves cultured *in vitro*. Plant Cell, Tissue and Organ Culture, 19, 85-89.

Blasdale, W. C. (1947). The secretion of farina by species of *Primula*. J. Roy. Hort. Soc., 72, 240-245.

Brainerd, K. E. & Fuchigami, L. H. (1982). Stomatal functioning in *in vitro* and greenhouse apple leaves in darkness, mannitol, ABA, and CO_2. Journal of Experimental Botany, 33, 388-392.

Buttery, B. R. & Tan. R. I. & Buzzell, J. D. & Gaynor, D. C. & Mac Tavish, D. C. (1993). Stomatal numbers of soybean and response to water stress. Plant and Soil, 149, 283-288.

Cosgrove, D. J. & Hedrich, R. (1991). Stretch-activates chloride, potassium, and calcium channels coexist in plasma membranes of guard cells of *Vicia faba L.* Planta, 186, 143-153.

Gowing, D. J. G. & Jones, H. G. & Davies, W. J. (1993). Xylem-transported abscisic acid: the relative importance of its mass and its concentration in the control of stomatal aperture. Plant, Cell and Environment, 16, 453-459.

Grey-Wilson, C. (1988). A survey of *Dionysia* in cultivation. The Plantsman, 10 (2), 65-84.

Grey-Wilson, C. (1989). The genus *Dionysia*. Alpine Garden Society, Woking, England.

Harborne, J. B. (1968). Comparative biochemistry of flavonoids: VII Correlations between flavonoid pigmentation and systematics in the family Primulaceae. Phytochem, 7, 1215-1230.

Hawkins, H-J. & Lewis, O. A. M. (1993). Combination effect of NaCl salinity, nitrogen form and the calcium concentration on the growth, ionic content and gaseous exchange properties of *Triticum aestivum L. CV Gamtoos*. New Phytol. (1993), 124, 161-170.

Kearns, E. V. & Assam, S. M.(1993). The guard-cell environment connection. Plant Physiol., 102, 711-715.

Korn, R. W. (1993). Evidence in dicots for stomatal patterning by inhibition. Int. J. Plant Sci.,154(3), 367-377.

Koshuchowa, S & Zoglauer, K & Goring, H (1990).Structure and function of stomata of plants cultured *in vitro*. Biochem. Physiol. Pflanzen.,289-299.

Lee, J. S & Bowling, D. J. F. (1993). The effect of a mesophyll factor on the swelling of guard cell protoplasts of *Commelina communis*. Journal of Plant Physiology, 142, 203-207.

Liang, H. G & Dayton, D. D. & Chu, C. C. & Casady, A. J. (1975). Heritability of stomatal density and distribution on leaves of grain Sorghum. Crop Science, 15 ,567-570.

MacRobbie, E. A. C. (1990). Calcium-dependent and calcium-independent events in the initiation of stomatal closure by abscisic acid. Proc. R. Soc. Lond. B, 241, 214-219.

MAFF (1986). Reference book 427. Third Edition. Her Majesty's Stationary Office, London.

Mansfield, T. A. & Jones, R. A. (1971). Effects of abscisic acid on potassium uptake and starch content of stomatal guard cells. Planta, 104, 147-158.

Martinez, V. & Lauchli A. (1993). Effects of Ca^{2+} on the salt-stress response of barley roots as observed by *in vivo* ^{31}P-nuclear magnetic resonance and *in vitro* analysis. Planta, 190, 519-524.

Mishin, K. E. & Rasmusson, D. C. & Moss, D. N. (1972). Inheritance and physiological effects of stomatal frequency in barley. Crop Science, 12, 780-783.

Munchow, R. C. & Sinclair, T. R. (1989). Epidermal conductance, stomatal density and stomatal size among genotypes of *Sorghum bicolor (L.)* Moench. Plant, Cell and Environment, 12, 425-431.

Murashige, T, and Skoog F. (1962). A revised medium for the rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant.,15, 473-497.

Osborne, B. A. & O'Connell, C. & Campbell, G. C. & Weyers, J. D. B. (1993) Stomata of *Gunnera tinctoria* do not respond to light, CO_2 or ABA. Abstracts of the Society for Experimental Botany Conference, Canterbury, 1993.

Pallardy, S. G & Kozlowski, T. T. (1979). Frequency and length of stomata of 21 *Populus* clones. Can. J. Bot., 57, 2519-2523.

Parlange, J-Y. & Waggner, P. E. (1970). Stomatal dimensions and resistance to diffusion. Plant Physiology, 46, 337-342.

Pihakaski-Maunsbach, F. & Harvey, D. M. R. (1992). X-ray microanalytical (EDX) investigation of potassium distribution in mesophyll cells of non-acclimated and cold-acclimated rye leaves. Plant, Cell and Environment, 15, 585-591.

Raschke, K. & Fellows, M. P. (1971). Stomatal movement in *Zea mays*: shuttle of potassium and chloride between guard cells and subsiduary cells. Planta, 101, 296-316.

Richards, J. (1993). *Primula*. B. T. Batsford Ltd, London, England.

Sachs, T. & Novoplansky, N. (1993). The development and patterning of stomata and glands in the epidermis of *Peperomia*. New Phytol., 123, 567-574.

Santamaria, J. M. & Davies, W. J. & Atkinson, C. J. (1993). Stomata of micropropagated *Delphinium* plants respond to ABA, CO_2, light and water potential, but fail to close fully. Journal of Experimental Botany, 44, 99-107.

Sallanon, H. & Tort, M. & Coudret, A. (1993). The ultrastructure of microproagated and greenhouse plant stomata. Plant Cell, Tissue and Organ Culture, 32, 227-233.

Schnepf, E. & Klasova, A. (1972). Zur feinstruktur von ol- und flavon-drusen. Ber Dtsch Bot Ges, 85, 249-258.

Smith, S. & Weyers, D. J. D. & Berry, W G. (1989). Variation in stomatal characteristics over the lower surface of *Commelina communis* leaves. Plant, Cell and Environment, 12, 635-659.

Ticha, I. (1982). Photosynthetic characteristics during the ontogenesis of leaves. 7 Stomata density and sizes. Photosynthetica, 16, 375-471.

Tardieu, F. & Zhang, J. & Gowing, D. J. G. (1993). Stomatal control by both [ABA] in the xylem sap and leaf water status: a test of a model for drought or ABA-fed field-grown maize. Plant, Cell and Environment, 16, 413-420.

Tort, H. S. M. & Coudret, A. (1993). The ultrastructure of micropropagated and greenhouse rose plant, stomata. Plant Cell, Tissue and Organ Culture, 32, 227-233.

Trejo, C. L. & Davies, W. J. & Del Mar P. Ruiz, L. (1993). Sensitivity of stomata to abscisic acid. An effect of the mesophyll. Plant Physiol, 102, 497-502.

Wardle, K. & Dixon, P. A. & Simpkins, I. (1981) Sodium accumulation by leaves of cauliflower plantlets and the effect of the mode of plant formation. Ann. Bot., 47, 635-659.

Weyers, J. D. B. & Meidner, H. (1990). Methods in stomatal research. Longman Scientific and Technical. Harlow.

Wollenweber, E. (1977). The systematic implications of flavonoid secretion by plants. Progress in Phytochemistry, 4, 53-67.

Wollenweber, E. & Schnepf, E. (1970). Vergleichende untersuchungen uber die flavonoiden exkrete von mehl -und ol -drusen bei primeln und die feinstruktur der drusenzellen. Z. Pflanzenphysiol, 62, 216-227.