Stomatal control during desiccation in the resurrection plant *Xerophyta humilis*

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Submitted in partial fulfillment of the degree of Bsc (Hons) in Botany

October 2007.

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Abstract

Stomatal apertures on leaves of the resurrection plant *Xerophyta humilis* were monitored microscopically in order to characterize stomatal regulation during a dehydration time course. In addition, the effect of exogenous application of the stress hormone ABA on stomatal regulation was followed. *X. humilis* stomatal regulation appears to be initially similar to that typical of desiccation sensitive plants, but differed in that stomata did not all close at once but at a slower rate to control the drying rate of the plant, this gave time for protection mechanisms to be laid down. The signal hormone ABA was found to have strong stomatal control on the adaxial surfaces of leaves but weak control on the abaxial leaf surfaces, thus it is difficult to say that ABA regulates the process until RWC of below 50%, where stomatal apertures open as a result of shrinkage of guard cells due to loss of water.

Introduction

One of the major environmental stresses that limit the productivity of plants is water availability. Since plants are sessile organisms, they have evolved a number of different strategies to help them cope with the challenges of water deficit stress (Bartels and Salamini, 2001). One of these strategies is desiccation tolerance, which is a common phenomenon in seeds but rare in vegetative tissues of angiosperms (Berjak et al., 2007). Desiccation tolerance has been defined as the ability of a plant or plant part to tolerate drying until its internal water potential comes into equilibrium with the atmospheric relative humidity i.e. the plants loses more than 95% of cellular water (reviewed by Farrant, 2007). Most angiosperms are desiccation sensitive and are not able to with stand extreme water loss. Such plants avoid excess water loss by using mechanisms that maintain water potentials higher than that of the surrounding environment in an attempt to continue functioning under water stressed conditions (Mundree et al., 2002). The few

desiccation tolerant plants that do occur are known as resurrection plants (Gaff, 1971) and use a different strategy: these plants lose all their free water under water stress conditions, but avoid experiencing the stress associated with this loss. Upon rehydration, they resume full metabolic activity (Mundree et al., 2002; Farrant, 2007).

For a plant to be desiccation tolerant it has to be able to limit the damage incurred due to the loss of water, repair any damage that has occurred and also be able to maintain its physiological integrity when in the dry state (Oliver et al., 1998). There are two main categories of plants that are able to tolerate desiccation; the lower order plants (bryophytes and lichens) that can withstand the total loss of free water at any rate (usually these plants lose water rapidly) and the angiosperms that will only survive such severe water loss if drying rates are slow (Oliver et al., 1998). The lower order plants tend to accrue damage on drying but use repair mechanisms as a strategy to survive desiccation, while the slow drying of resurrection plants allows time for the laying down of protective mechanisms, although ultimately some repair is inevitable (reviewed in Vicre et al., 2004; Le and McQueen-Mason, 2006; Farrant, 2007).

Understanding the mechanisms that resurrection plants use to survive desiccation is important for many reasons. However a compelling applied reason is the observation that if we are able to understand the mechanisms of protection used by such plants to survive water deficit stress, these can be bioengineered to produce drought tolerant crops that would sustain our food source even in water limited environments (Bartels and Salamini, 2001).

The plant hormone ABA (Abscisic acid) has been shown to play a part in the initiation of many of the mechanisms of desiccation tolerance; such as alterations of relative growth rates of various plant parts (Taiz and Zeiger, 2002), improving water transport (Ramanjulu and Bartels, 2002), reducing transpiration rates by control of stomatal apertures (Schroeder et al., 2002), induction of the synthesis of protective proteins (Bray, 1993; Campalans et al., 1999) and upregulating antioxidative responses against oxidative damage (Mugo et al., 1999; Jiang and Zhang, 2001). ABA is also required under 'normal'

non-stress conditions for optimal plant functioning and development (Himmelbach et al., 2003). One of the ways that desiccation sensitive plants try to stop water loss in the face of environmental water stress is to close their stomata, a response driven by ABA (Gaff and Loveys, 1992). Under conditions of drought stress ABA is synthesized by the roots (since this is where soil water status is sensed), and exported to the shoots, where it initiates a series of biochemical reactions that facilitate stomatal closure (Finkelstein and Rock, 2002). Essentially, ABA stimulates an increase in cytosolic Ca^{2+} which activates anion channels that mediate K^+ and other anions' release from the guard cells (Schroeder et al., 2002). The net result of the loss of solutes from the guard cells is the reduction of turgor and volume of the guard cells resulting in guard cell shrinkage and stomatal closure.

While desiccation sensitive plants attempt to prevent water loss, in part by stomatal regulation, resurrection plants, since they are able to tolerate desiccation, do not appear to regulate water status. It has been noted that during initial soil drying, leaf water content of many resurrection plants species is maintained at near full turgor (Sherwin and Farrant, 1996; Farrant et al., 1999; Vander Willigen et al., 2001). However once soil water has been depleted (and presumably protection mechanisms have been laid down in the plant tissues), they rapidly lose water from the leaves through the opening of stomata in what has been postulated to be an active loss of water from these plant tissues (reviewed by Farrant, 2007). However, the degree of stomatal regulation and what regulates the process before the opening of stomata to allow for complete loss of any free water from the plant tissues is not yet known.

The aim of this project is to characterize the pattern of stomatal opening and closing during drying of the resurrection plant, *Xerophyta humilis*, and to establish whether ABA plays a part in stomatal regulation. This monocot, poikilochlorophyllous (i.e. it loses its chlorophyll, and dismantles photosynthetic apparatus in the dry state) plant, is a relatively well studied species, in which physiological, biochemical and molecular studies have been conducted to date (e.g. Dace et al., 1998; Farrant, 2000; Cooper and Farrant, 2002; Collett et al., 2004; Illing et al., 2005).

Materials and methods

Plant material

Whole plants of *Xerophyta humilis* that were collected in the Pilansberg Nature Reserve, Northwest Province of South Africa were grown in a mixture of river sand, potting soil and peat in a greenhouse with no supplementary lighting and under 30% shadecloth as previously described (Dace et al., 1998). For experimental purposes fully hydrated plants were transferred to a constant environment room in which conditions were maintained at 50% relative humidity with a 14h photoperiod and 16°C (dark): 25°C (light) temperature cycle. The light intensity was 350 μmol m^{-2} s^{-1}.

Dehydration

Slow drying of whole plants was achieved by withholding water from the soil and allowing the plants to dry naturally. In order to facilitate microscopical examination of stomatal status, it was important to examine on a given day, plants that were at a range of different water contents. To obtain such a range of plant water contents (from full turgor to an air-dry state), the timing of withholding of water was staggered among the plants.
In order to test the effects of ABA on stomatal regulation two trays of plants were watered with a 500 ml solution containing 100 μM of ABA, while two other trays that were watered with 500 ml of water were used as controls. Plants were then left to dry naturally.

Water content determination

The water content (WC) was determined gravimetrically by oven drying at 70°C for 48 h and is expressed as gH_2O/g dry $mass^{-1}$. Relative Water Content (RWC) was measured using the standard formula: RWC = water content / water content at full turgor, expressed as a percentage.

Stomatal assays

The plants were subjected to several time courses of drying during which stomatal apertures and numbers were monitored at several different stages (RWC) along the

drying curve. At each RWC sampled, a leaf was removed from the plant, half of it was used to determine WC and RWC, and the other half was freeze dried using liquid nitrogen and used to monitor stomatal apertures and numbers per given leaf area on the adaxial and abaxial leaf surfaces by processing by Scanning electron microscopy (SEM). A fully analytical Leica / Leo Stereoscan 440 digital scanning electron microscope (England) fitted with a Fisons LT7400 Cyro Transfer System (Germany) was used. To standardize the stomatal counts the width and the length of each electron micrograph image were standardized and using the magnification at which each micrograph was taken, the area (mm^2) of each image was worked out.

For the ABA tests, adaxial and abaxial leaf surfaces were painted with clear nail varnish to obtain stomatal imprints. Once dry, nail varnish peels were removed from leaves and placed on slides, these were used to monitor stomatal apertures and numbers, using an Olympus CX 21 compound microscope at 400× magnification. These did not photograph well and thus no images are shown.

Statistical analysis

Where appropriate, statistical analyses were conducted using the Statistica 7 analytics software package.

Results

Figure 1 shows the time course of dehydration of *Xerophyta humilis*. Although standard deviations were large, the trend in RWC was that it declined at a gradual rate for the first 3 d of drying, from 100% RWC to approximately 65% RWC. After this, water was lost rapidly between days 3 and 4. This rapid decline was a regular feature of the dehydration time-course (see also Fig. 5). During this period of rapid water loss 35% of the total amount of leaf tissue water was lost from the plant. After the period of rapid water loss, water was lost at a steady but slightly faster rate of about 10% a day compared to the first 3 days until day 7 when the plants reached an air-dry state.

In the hydrated plants, stomata were aggregated within and along the edges of the furrows on the abaxial leaf surfaces (Fig. 2A), but were randomly distributed on the adaxial leaf surfaces (Fig. 2B). As the plants lost water, leaf shrinkage and folding caused the ridges to come closer together and the stomata to become sunken (arrowed) on the abaxial leaf surfaces (Fig. 2C), while on the adaxial leaf surfaces stomata aggregated into rows (arrowed) (Fig. 2D). On the abaxial leaf surfaces of desiccated leaves the furrows were reduced to slits due to shrinkage caused by the lack of water in the epidermal cells and no stomata were visible (Fig. 2E), while on the adaxial leaf surfaces ridges had started to form and the stomata had become sunken (Fig. 2F). These changes in anatomy made counting of stomata with open apertures difficult.

Stomatal counts per given area showed that there were generally more stomata visible on the adaxial surfaces of leaves than the abaxial surfaces (Fig. 3A, B). At near full leaf turgor on the adaxial leaf surfaces, numbers as high as 450 stomata per given area measured were visible. The visible stomatal numbers remained above 100 stomata per given leaf area until approximately 50% RWC was reached after which the number of stomata visible per given area declined to zero, when the leaf tissue was dry (Fig. 3A). On the abaxial leaf surfaces (except for a single outlier at 250 stomata per given area, the number of stomata visible were below 150 stomata per given area (Fig. 3B). When the leaves had lost 95% of their water, no stomata were visible on the abaxial leaf surfaces. The number of stomata visible decreased with decreasing RWC. This correlation was more significant on the adaxial leaf surfaces ($r = 0.7630$, $p = 0.00006$) than on the abaxial leaf surfaces ($r = 0.5644$, $p = 0.0077$). However, because stomata were difficult to see in the electron micrographs at lower RWC the data shown in Figures 3A and 3B may be erroneous.

Figures 4A and 4B show that on both the adaxial and abaxial surfaces of leaves the number of stomata visibly open per given area, decreased as the RWC of the leaves decreased. However, the adaxial leaf surfaces showed a strong linear relationship ($r^2 = 0.565$, $r = 0.7517$, $p = 0.0009$), while the relationship on the abaxial leaf surfaces was very weak ($r^2 = 0.095$, $r = 0.3082$, $p = 0.1741$). On the adaxial surfaces, at RCW close to

100%, almost all the visible stomata were open, the highest number of visible stomata at almost full turgor water content was approximately 450 stomata (Fig. 3A) and Figure 4A shows that the highest number of stomata open relative to the number of stomata visible was approximately 350 stomata per given area. For both the abaxial and adaxial leaf surfaces, very few stomata were visible when the leaf tissue was dry, which hampered the ability to assess stomatal apertures. This method is thus flawed and the alternative method of using nail varnish imprints was used for the subsequent experiments.

Figure 5 shows the time course of dehydration of *X. humilis* plants supplied with ABA and plant without the hormone. From day 0 to day 1 both the plants supplied with ABA and those without (control plants) had a similar decrease in RWC from 100% RWC to 94.1% RWC for plants supplied with ABA and 94.5% RWC for the control plants. From day 1 to day 3 the control plants had a steady decrease in their RWC to 77.1% RWC, while the ABA supplied plants maintained the high RWC as the RWC decreased by less than 3% to 91.9% RWC by day 3. Both the control and ABA supplied plants rapidly lost water between day 3 and day 4, 32.6% for the control plants and 38.3% for the plants supplied with ABA. The plants supplied with ABA continued to lose water rapidly and by day 6 the leaves of these plants had reached an air-dry state. The control plants on the other hand lost water at a slower rate than the ABA supplied plants from day 4 onwards and had not reached an air-dry state by day 6 because of this. The difference between the two drying curves was however not significant ($p = 0.891304$).

The use of nail varnish peels in the ABA tests allowed more accurate measurement of stomatal numbers and aperture characteristics. However, while this technique enabled counting, the peels did not photograph effectively and thus only the numerical data are given (Fig. 6). On the adaxial leaf surfaces, the control plants showed that when the plants were hydrated almost all the stomata were open (94% of the stomata in the field of view of the microscope at 400× magnification, at 100% RWC and 86% at 93.94% RWC). Below 90% RWC the percentage of stomata open decreased markedly to 36% at 81.16% RWC and 24% at 40.96% RWC, after which the percentage of stomata open increased again as the plants became desiccated (Fig. 6A). The plants supplied with ABA showed

that, the stomata had a distinct response to the ABA, closure of stomata at high RWC. Only 14% of the stomata were open at 100% RWC and the percentage of stomata open decreased to 8% at 90.62% RWC. As the RWC continued to decrease, the percentage of open stomata increased again after RWC had declined below 60% RWC and 68% of the stomata were open when the plants had reached an air-dry state. On the abaxial leaf surfaces stomata did not show a distinct response to ABA (Fig. 6B). The percentage of open stomata in the control plants decreased from 25% at 100% RWC to 0% at 88.31% RWC, this was followed by an increase in the percentage of open stomata once RWC dropped below 50%, with the highest percentage of open stomata of 60% at 41.25% RWC. On the abaxial leaf surfaces, the plants supplied with ABA showed similar stomatal movements as the control plants. At 100% RWC only 9% of stomata were open and this value decreased to 0% by 91.52% RWC. As the RWC decreased below 50% the percentage of stomata open increased to a high of 51% at 30.38 RWC (Fig. 6B). Both the leaf surfaces in both the control and ABA supplied plants showed a trend of stomatal closure between 100% and approximately 50% RWC and then stomatal opening at RWC less than 50%.

Discussion

Since both series of drying curves (Fig. 1 and Fig. 5) showed initial slow drying (days 1-3), followed by a rapid decline between days 3 and 4, then an air-dry state is reached, suggests that this is a real trend in this species. This is what has been reported previously for this species (Farrant, 2000). Initially water is lost at a slower rate as plants adjust to the lack of soil water availability and a RWC at near full turgor is maintained (Fig. 1). *X. humilis* being an angiosperm requires drying to take place at a slow rate to allow time for the laying down of protective mechanisms (Oliver et al., 1998; reviewed in Vicre et al., 2004a; Farrant, 2007). After day 3, water is lost at a faster rate, 35% of the total amount of free leaf tissue water in the space of one day, presumably because protection mechanisms have been laid down in the plant tissues. Previous research has found that in angiosperms such protective mechanisms play an important role in the survival of

resurrection plants as it limits the damage incurred during drying (Sherwin and Farrant, 1998; Farrant, 2000; Vander Willigen, 2001).

In most angiosperm species most of the stomata are found on the abaxial surfaces of leaves as a strategy to reduce the amount of water lost during transpiration as the abaxial surface does not get direct sunlight (Taiz and Zeiger, 2002). However the opposite is the case for *X. humilis* (Fig. 3A, B). This can be explained by the fact that the leaves of *X. humilis* are flat and grass-like (Vicre et al., 2004a), and during dehydration the leaf blades fold in half along the midrib exposing only the abaxial surfaces of the leaves to sunlight. This folding of leaves with dehydration is not only an adaptation of desiccation tolerant plants to minimize damage from light (and consequently free radical stress) in dry tissues (Sherwin and Farrant, 1998; Farrant, 2000; Vicre et al., 2004a), but it has been suggested to reduce water loss by reducing the transpiring surface. Most of the stomata on the abaxial surface could however be inside the furrows that are prominent features on the abaxial surfaces of *X. humilis* (Fig. 2G, H), which gives the impression that there are fewer stomata found on the abaxial leaf surface than on the adaxial leaf surface. While this strategy makes biological sense for the plant in terms of water conservation (at least initially, while protection mechanisms are laid down), it hinders the counting of stomata and visualization of their apertures. Thus we do not attach much confidence in the data shown in Figures 3 and 4.

Previous work on resurrection plants (other than *X. humilis*) has shown that stomatal pores are open in both hydrated and desiccated plants (Schwab et al., 1989; Vicre et al., 2004a; Moore et al., 2007b). In the hydrated state, the stomata are open to allow for CO_2 uptake, however at the same time, water is lost from the plant, but since water is available from the soil, the plants stay hydrated (Taiz and Zeiger, 2002). As the plant reaches a desiccated state the loss of water results in considerable shrinkage of the stomatal guard cells, which leaves the stomatal pores open resulting in what is postulated to be an active loss of water from the plant tissue (Vicre et al., 2004a; Moore et al., 2007b; reviewed by Farrant, 2007). The results shown in Figure 4A and B show that stomatal pores are open when *X. humilis* is in the hydrated state, but in the desiccated state the stomata are shown

to be closed. This may be due to the fact that as the plant dries down monitoring of stomatal apertures becomes difficult using SEM. The sunken stomata on the adaxial leaf surfaces and those in the furrows on the abaxial leaf surfaces could actually be open, but could not be seen in the electron micrographs used in this study. Thus, the use of SEM for the purpose of stomatal counts and monitoring of stomatal apertures may be flawed.

The second set of experiments in which nail varnish was used to gain stomatal impressions allowed more accurate counting and characterization of stomatal apertures. In these experiments the presence of high percentages of open stomata in both the hydrated and desiccated plants of the control plants supports the observations of previous work that showed that in the desiccated state stomata were opened passively (Schwab et al., 1989; Vicre et al., 2004a; Moore et al., 2007b). *X. humilis* seems to initially behave like desiccation sensitive plants, where in the hydrated state stomata are open, but once the plants roots sense a decrease in availability of soil water, stomata close, even though the RWC is still quite high (Fig. 6A, B). The closure of stomata allows for *X. humilis* to slow down the drying rate, and maintain the RWC at a high level (Fig. 5), as the plant lays down protective mechanisms. Stomata in the control plants continue to close up to approximately 50% RWC, below which ABA control on stomata is lost and stomata become open again (Fig. 6A, B). This opening of stomata at what is still relatively high RWC (50%) is totally opposite to what happens in most desiccation sensitive plants. The opening of stomata at these water contents may be because of physical changes to the leaf anatomy brought about by drying. Severe water loss from the plant causes the epidermal layers to fold and shrink, the stomatal guard cells also shrink and this results in open stomatal pores (Moore et al., 2007b). This trend is probably indistinguishable in the SEM data due to inability to see open stomata, however just looking at the data by eye, a similar trend could possibly be predicted (dotted line), (Fig. 7A, B). The percentage of open stomata on the abaxial leaf surfaces for both the ABA supplied and control plants, when the plants were hydrated was very low compared to the adaxial leaf surfaces. This was presumably because most of the stomata that would be open would be in the furrows where the moisture environment can be better controlled to reduce the amount of water lost during CO_2 take up (Fig. 6B). The plants supplied with ABA initially had their

stomata closed (Fig. 6A and B), which shows a clear response of stomata to ABA in the hydrated state, however below 60% RWC ABA no longer has an effect on the stomata as the stomatal pores open up due to the mechanical stress caused by severe water loss. This ABA-induced stomatal control is more evident on the adaxial leaf surfaces than on the abaxial leaf surfaces. This is probably because in *X. humilis* most of the stomata are on the adaxial leaf surfaces. Thus, there was no significant difference between the drying curves of the ABA supplied plants and the control plants (Fig. 5), because the RWC was taken from whole leaves, which does not distinguish adaxial and abaxial stomatal control. Therefore it is difficult to say whether ABA controls the process of stomatal regulation before stomata open at RWC below 50%.

Conclusions

This study showed that the degree of stomata regulation before the opening of stomata to allow for the complete loss of any free water from the plant tissues in *X. humilis* is initially similar to that in desiccation sensitive (DS) plants. However, unlike DS plants which shut all stomata at once, *X. humilis* increases the number of closed stomata at a slower rate in order to allow the plant to dry down slowly (Fig. 1, Fig. 5 and Fig. 6A, B). It could not be shown whether ABA actually controlled the process of stomatal regulation before the stomata open at RWC below 50% due insignificant differences between drying down curves of the ABA supplied plants and control plants. These results are however based only on those stomata that were visible with testing methods used. The use of methods that would allow all stomata to be clearly visible even when the plant has reached an air-dry state would provide a clearer picture on the degree of stomata regulation before the opening of stomata brought on by guard cell shrinkage due to severe water loss.

Acknowledgements

I would like to thank Jill M. Farrant and Keren Cooper for their assistance and advice on this study, as well as the 2007 Botany Honours class for their support and encouragement.

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Figure legends

Fig. 1.

Decrease in relative water content with time of *X. humilis* plants subjected to several time courses of drying. The relative water content (RWC) is represented as a percentage of the full turgor water content. The plotted data points are means of 3 leaves taken from 3 separate plants and the standard deviations of these means are represented by error bars.

Fig. 2.

Scanning electron micrographs, showing the change in leaf surfaces of *X. humilis* during dehydration. Hydrated abaxial (A) and adaxial (B) surfaces, abaxial (C) and adaxial (D) surface at 50% RWC and Desiccated abaxial (E) and adaxial (F) surfaces. Stomata along the edges of furrows (G) and within furrows. Key: F, furrows; R, ridges; st, stomata. Scale bars: a-h, 120μm.

Fig. 3.

Scanning electron micrographs were used to count the number of stomata relative to the RWC on the adaxial (A) and abaxial (B) leaf surfaces, during dehydration of *X. humilis*. The plotted points are the number of stomata per given area from micrographs of single leaves taken from plants at different points along the drying curve of *X. humilis*. (# = number of).

Fig. 4.

Scanning electron micrographs were used to count the number of stomata that were open relative to the RWC on the adaxial (A) and abaxial (B) leaf surfaces of during dehydration. The plotted points are the number of stomata open per given area from micrographs of single leaves taken from plants at different points along the drying curve of *X. humilis*. (# = number of).

Fig. 5.

The effect of ABA on stomatal regulation was tested by monitoring the decrease in relative water content with time in *X. humilis* plants supplied with a 500ml solution containing 100μM of ABA (♦). As controls (■), *X. humilis* plants supplied with 500ml of water were used. The relative water content (RWC) is represented as a percentage of the full turgor water content. The plotted points are means of 3 leaves taken from 3 separate plants and the standard deviations of these means are represented by error bars.

Fig. 6.

Nail varnish peels of stomatal imprints were used to monitor stomatal apertures and numbers on the adaxial and abaxial leaf surfaces of X. humilis plants supplied with a 500ml solution containing 100μM of ABA (♦) and control (■), *X. humilis* plants supplied with 500ml water. The plotted points represent the percent of stomata open relative to the number of stomata in the field of view at 400× magnification.

Fig. 7.

Scanning electron micrographs were used to count the number of open stomata relative to the RWC on the adaxial (A) and abaxial (B) leaf surfaces of *X. humilis* during dehydration. The plotted points are numbers of open stomata per given area from micrographs of single leaves taken from plants at different points along the drying curve of *X. humilis*. The dotted lines show trends predicted by eye, (# = number of).

Fig. 1.

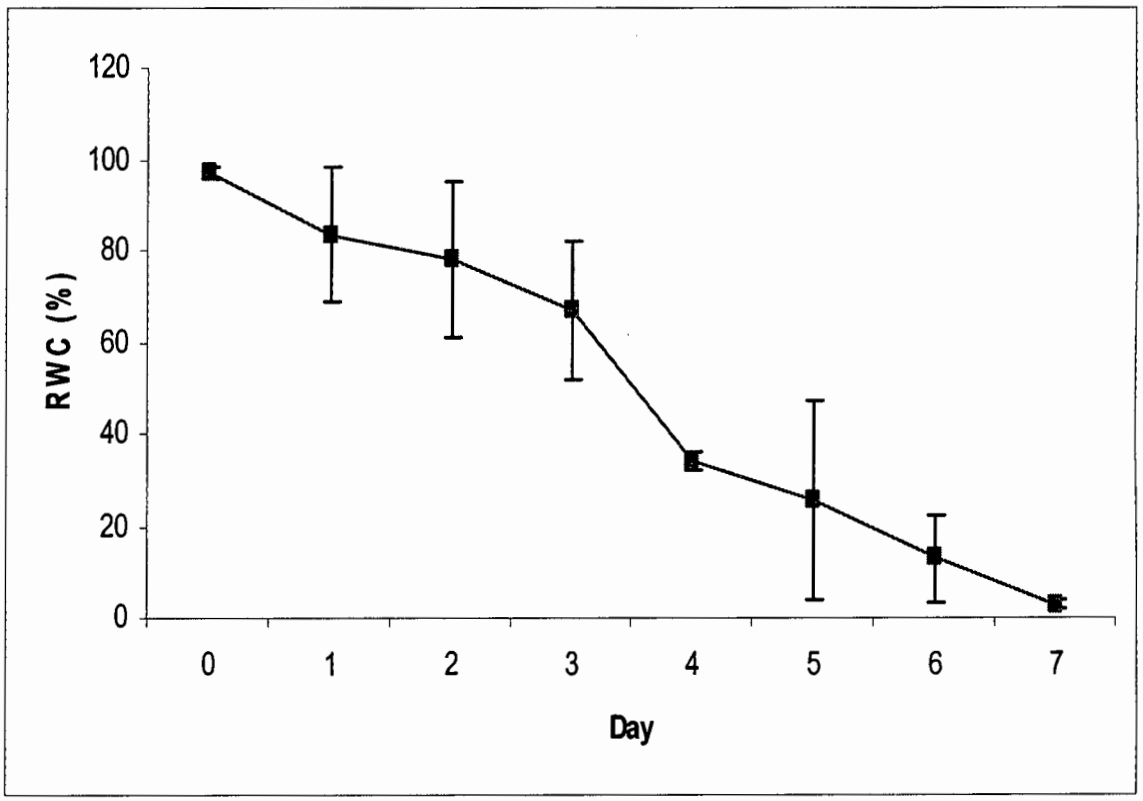
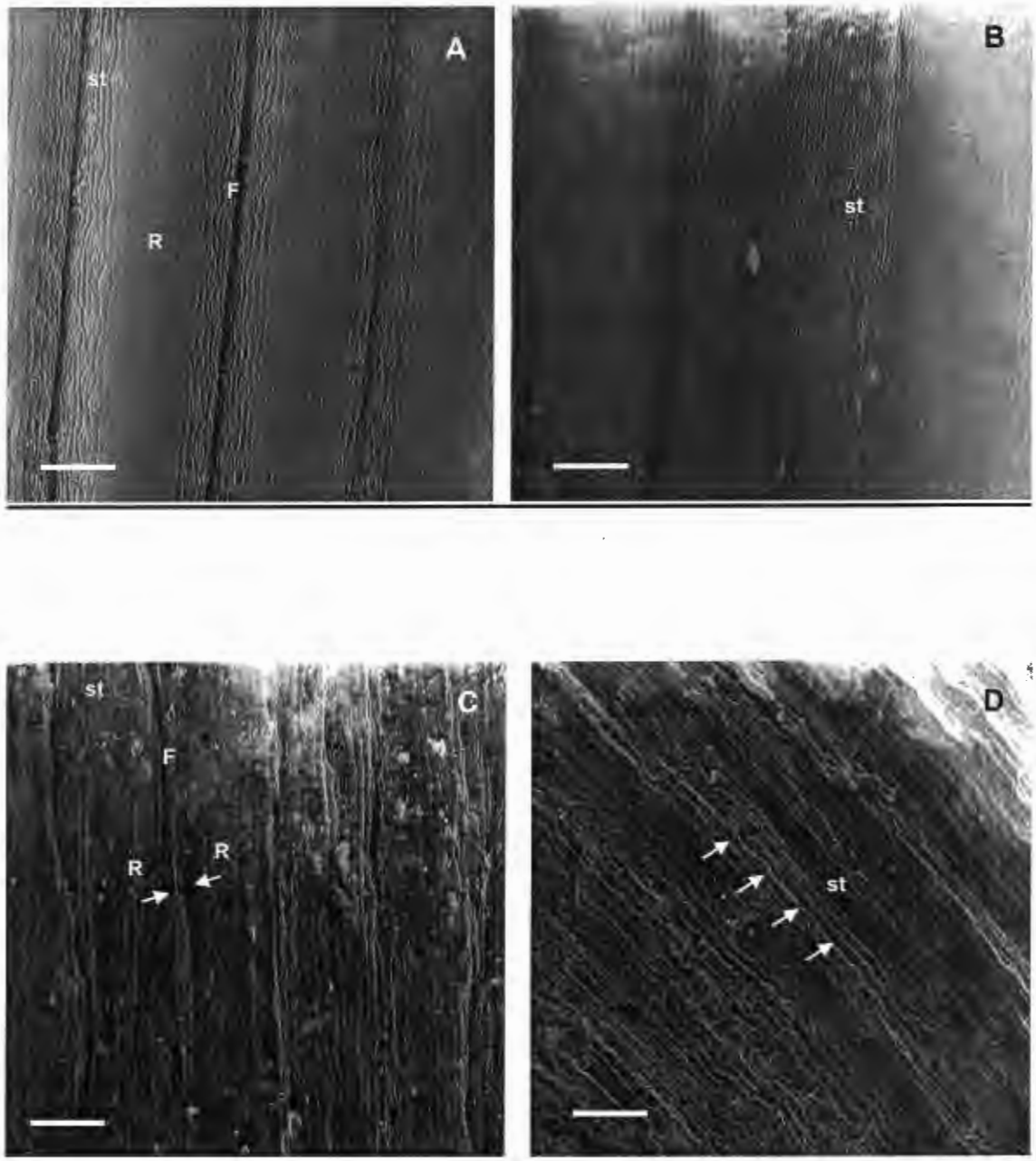
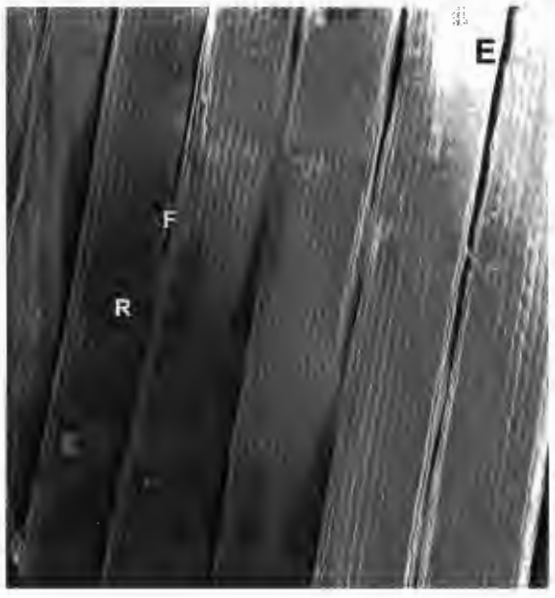
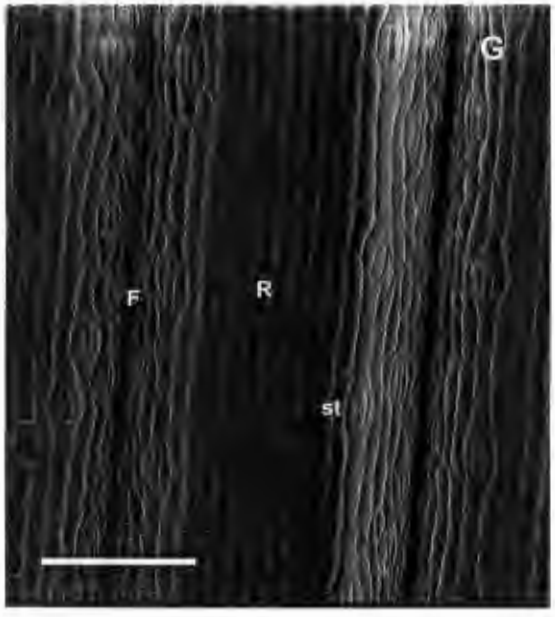


Fig. 2.




E
F
R


F


G
F
R
st

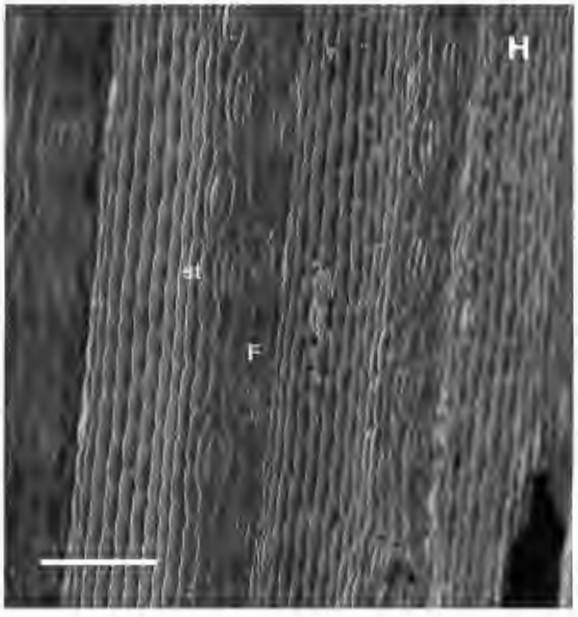
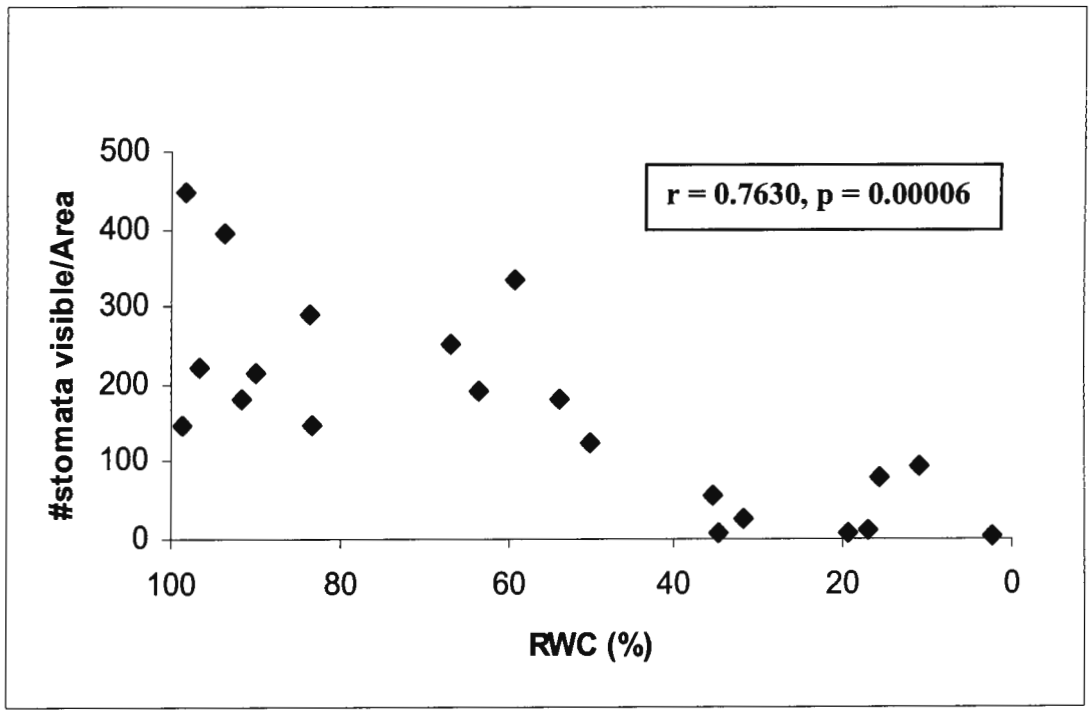

H
st
F

Fig. 3.

A


#stomata visible/Area
500
400
300
200
100
0
100
80
60
40
20
0
RWC (%)
r = 0.7630, p = 0.00006

B

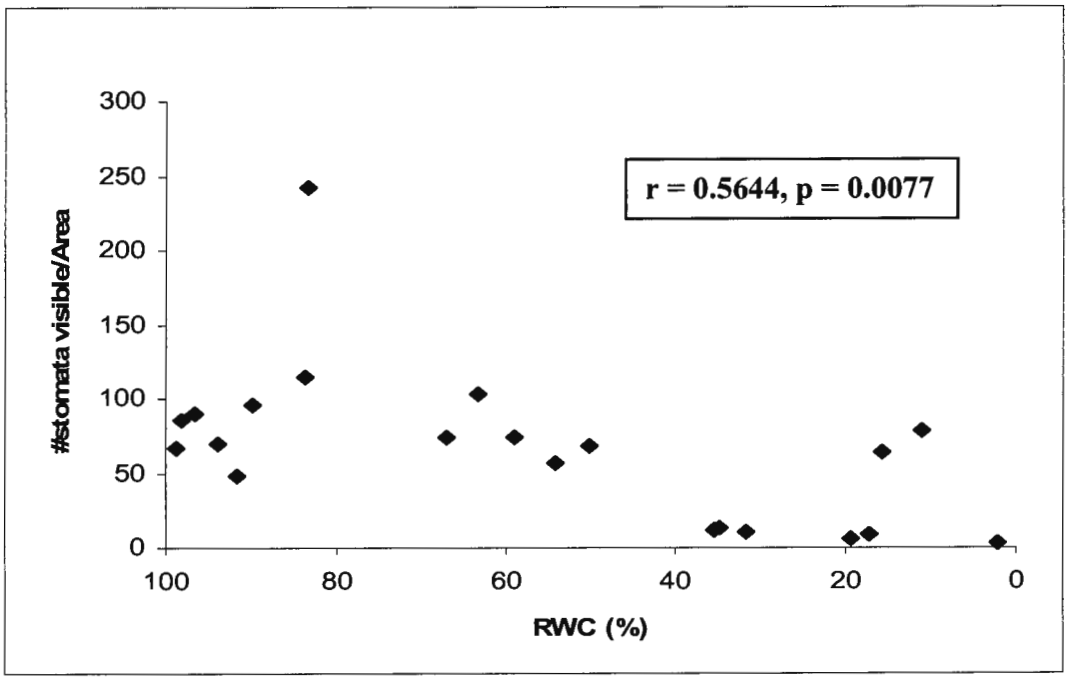
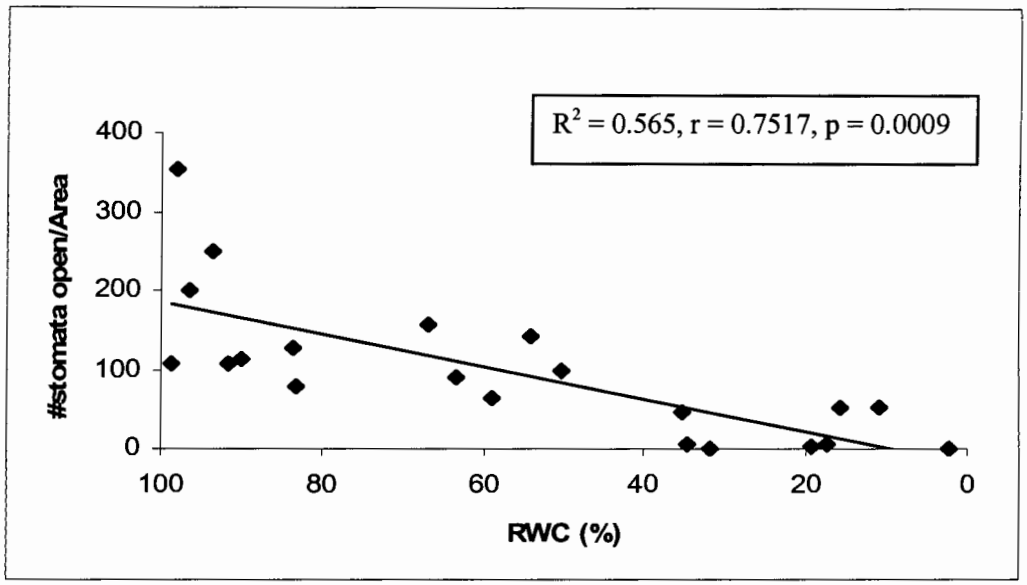

#stomata visible/Area
300
250
200
150
100
50
0
100
80
60
40
20
0
RWC (%)
r = 0.5644, p = 0.0077

Fig. 4.

A


R² = 0.565, r = 0.7517, p = 0.0009
#stomata open/Area
400
300
200
100
0
100
80
60
40
20
0
RWC (%)

B

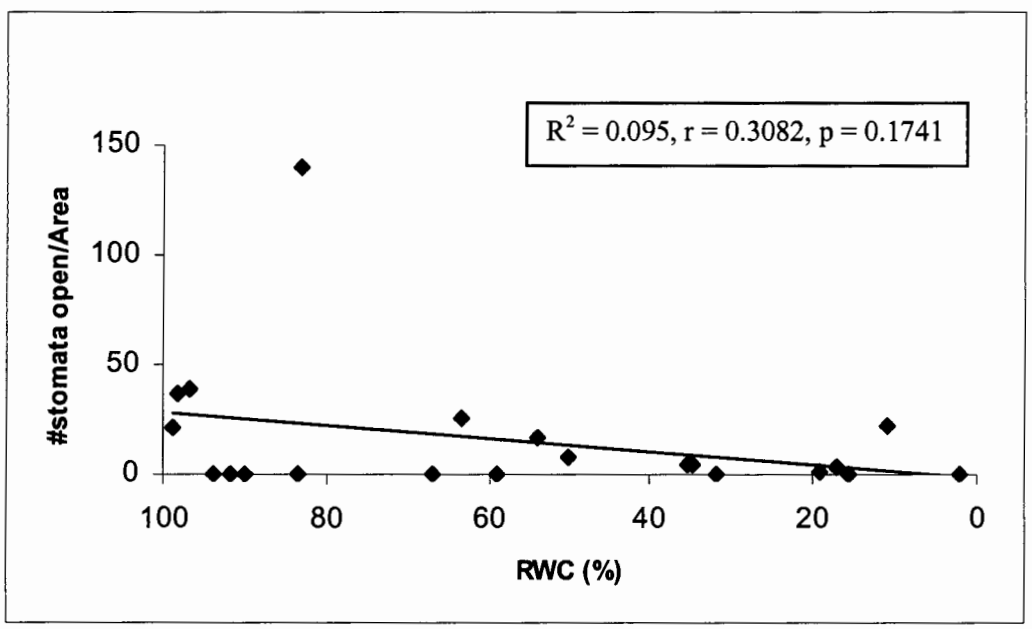

R² = 0.095, r = 0.3082, p = 0.1741
#stomata open/Area
150
100
50
0
100
80
60
40
20
0
RWC (%)

Fig. 5.

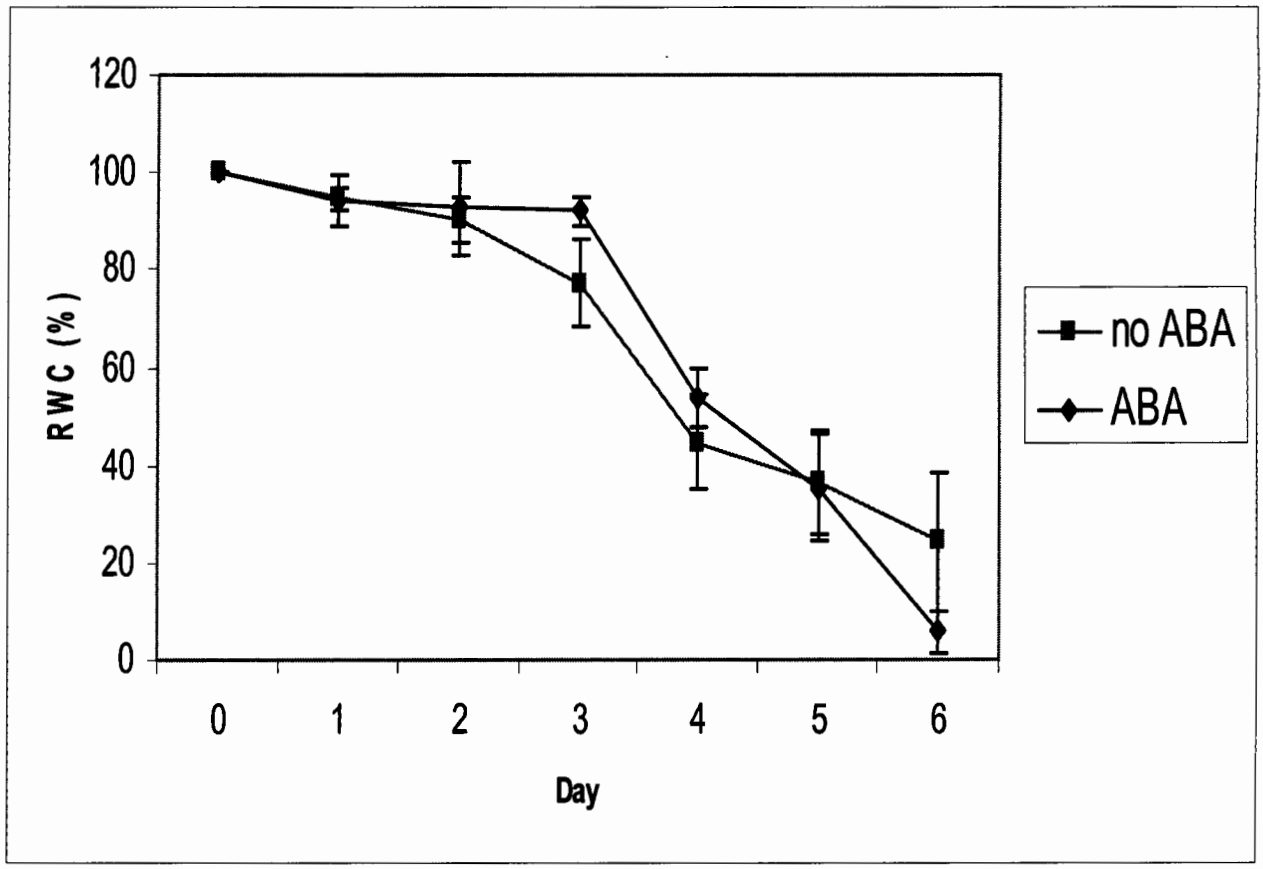
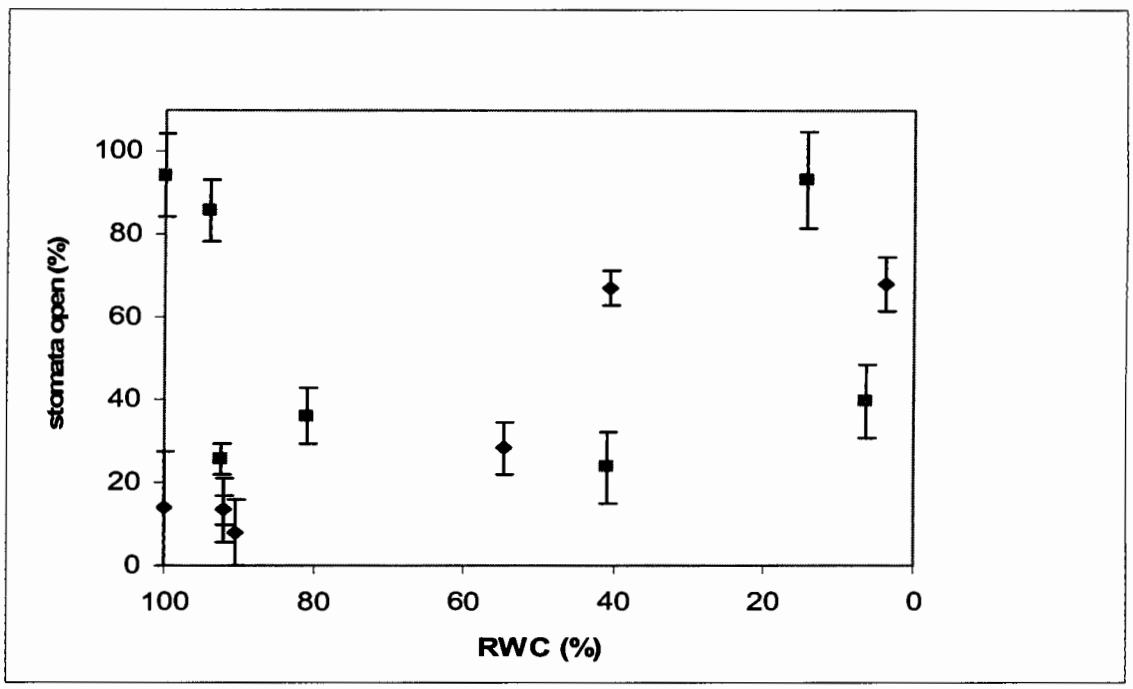


Fig. 6.

A


stomata open (%)
100
80
60
40
20
0
100
80
60
40
20
0
RWC (%)

B

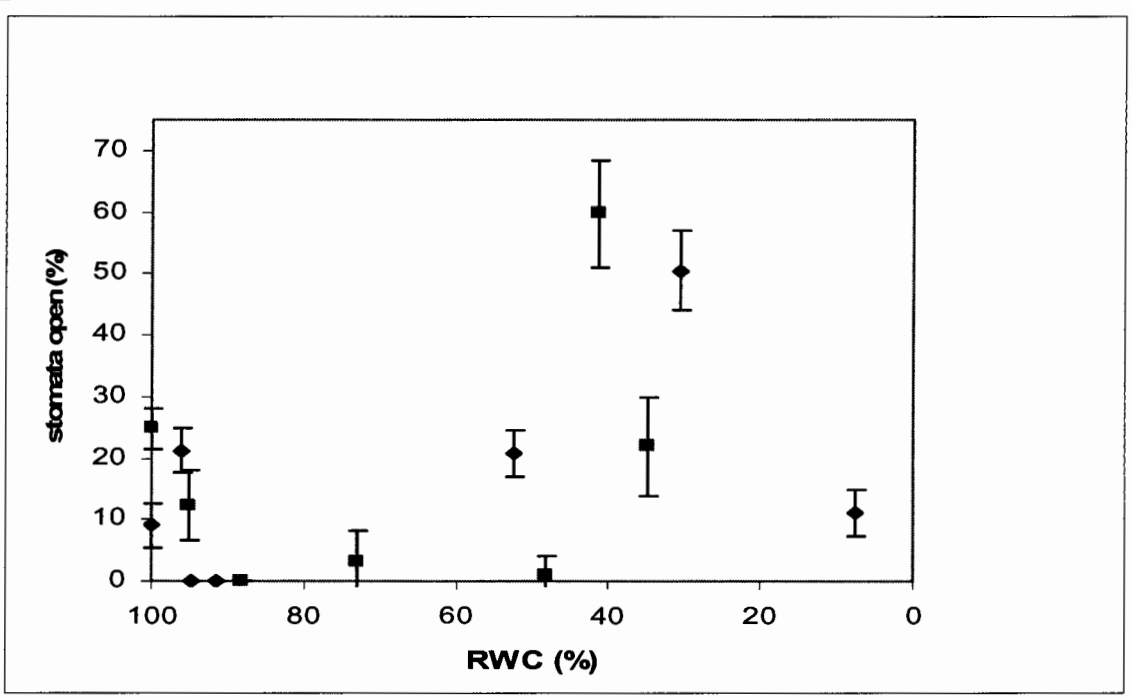
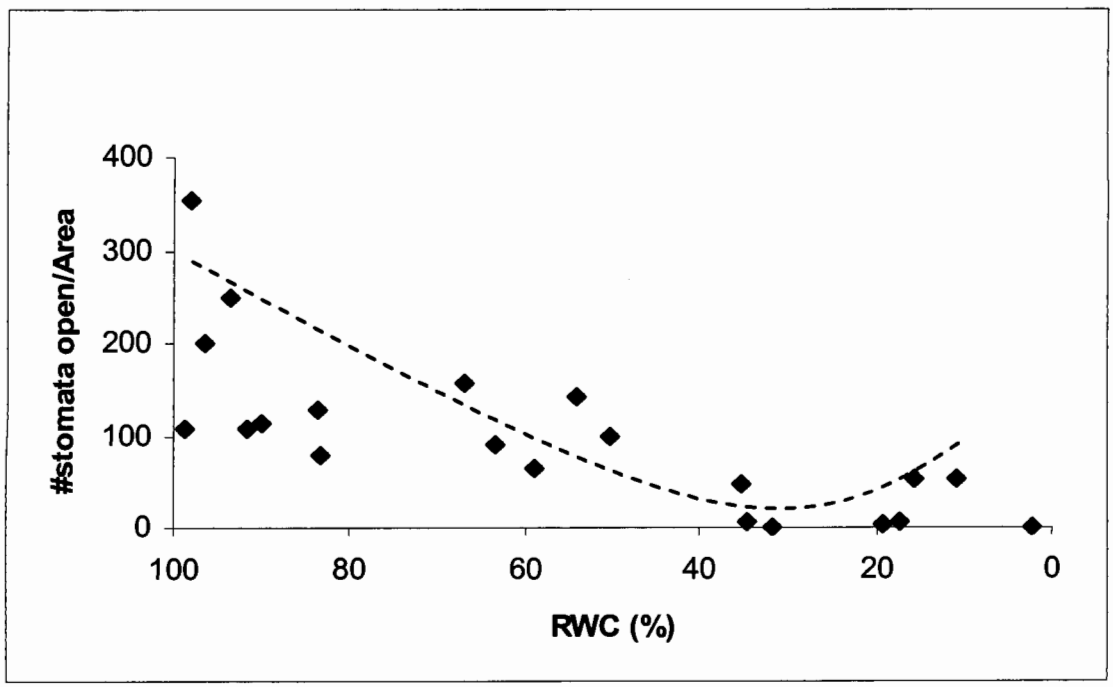
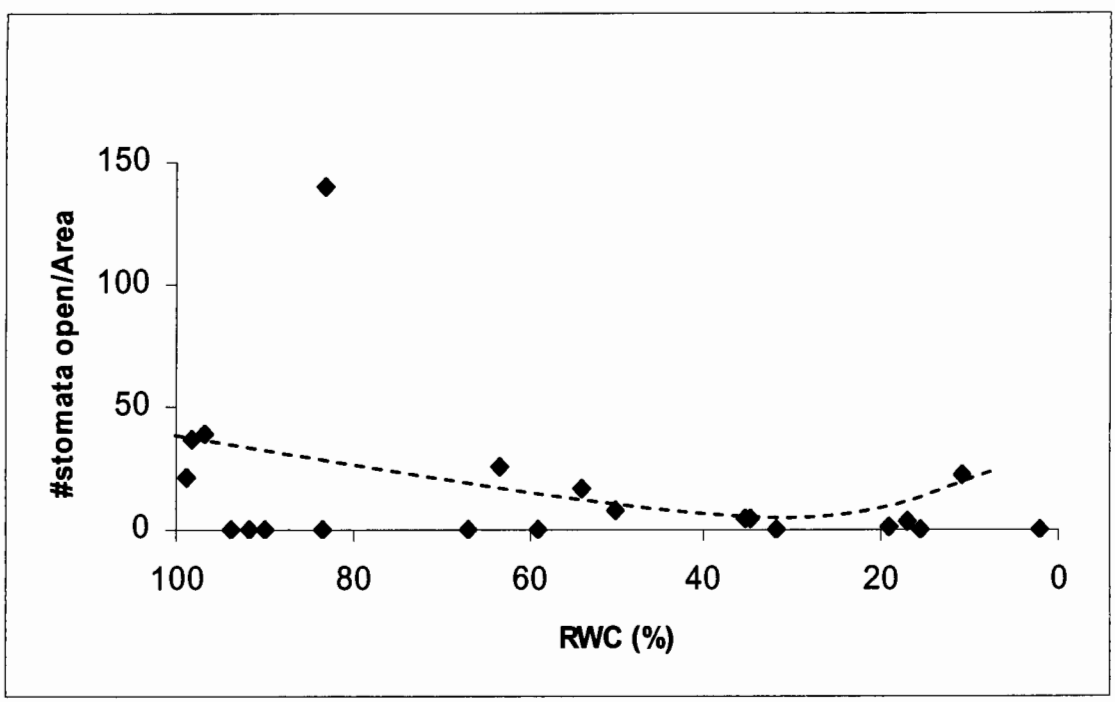

stomata open (%)
70
60
50
40
30
20
10
0
100
80
60
40
20
0
RWC (%)

Fig. 7.

A


400
300
200
100
0
#stomata open/Area
100
80
60
40
20
0
RWC (%)

B


150
100
50
0
#stomata open/Area
100
80
60
40
20
0
RWC (%)