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FROST TOLERANCE AND HARDENING OF DIFFERENT VARIETIES OF THE LEAF SUCCULENT SPECIES COTYLEDON ORBICULATA IN RELATION TO ITS REGIONAL AND GEOGRAPHICAL DISTRIBUTION IN SOUTHERN AFRICA.

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ABSTRACT:

Abstract. A preliminary study of the ability of a leaf succulent, Cotyledon orbiculata, to tolerate subzero freezing temperatures was undertaken. All five of the different varieties, var. orbiculata, var. oblonga, var. dactylopsis, var. spuria and var. flanaganii, were tested. These different varieties represent a full range of low temperature environments, coming from frost free areas as well as areas that receive regular and severe frost. Conductivity of electrolyte leakage from punched discs (6 mm diam.) was used as an immediate measure of low temperature damage resulting from cooling leaves in the laboratory.

During cooling (-3° C/h) leaves were removed at successive temperatures (10° C, 4° C, -2° C, -8° C, -11° C and -11° C (2hr later)), after which they were thawed (6 °C/h), and measured for the amount of electrolytes leaked out of punched discs into distilled water for a period of one minute. To determine the critical temperature at which an absolute concentration of electrolytes leaked correlated to killing of a leaf, an additional method of measurement of leaf weight loss was carried out. At 30% weight loss, 50% browning of the leaf occurred, this 30% correlated to approximately 20 μS/g/ml leaked out of a punched disc. Critical killing temperature was thus determined at that point, during electrolyte leakage increase, where 20 μS/g/ml was leaked. Measurement of electrolytes leaked on the basis of mass proved to be an adequate measure of damage, since there was a strong correlation in concentration of electrolyte leakage between measurement on the basis of mass and measurement on the basis of exposed surface area of the disc.

Before undergoing any cold hardening, some of the varieties from the colder regions of the country showed a surprisingly high tolerance to subzero temperatures (e.g. -9.8° C in the base of the leaf of var. orbiculata from Witwatersrand (1770m)), while other varieties from warmer regions tended to be sensitive to these temperatures. There was thus the possibility of frost being an important factor limiting the distribution of the different varieties of this species as well as being a factor promoting speciation of C. orbiculata. However after undergoing a cold hardening treatment (10° C for four days, 3° C for three days) the degree of scatter was reduced thus throwing doubt into this hypothesis. Populations from warmer areas (e.g. var. orbiculata from Hout Bay, elev. 60m) showed tremendous increase in their cold hardening ability. For a decrease in growth temperature of 10° C frost hardening was somewhat less

than the 1.7 °C found in the cacti Coryphantha vivipara of North America (Nobel 1981) with an increase in resistance of 0.8 °C, 1.4 °C, and 1.2 °C for the base, middle, and tip of the leaf respectively.

The fact that young leaves and the tips of mature leaves were found to be sensitive to freezing temperatures even after cold hardening, led to the formulation of the hypothesis that succulent plants are confined to protected microhabitats (e.g. rocky outcrops) because of the fact that these sensitive parts result in frost drought symptoms similar to those caused by water stress. Thus this plant needs to avoid frost in order to maintain high enough water economy to survive long drought periods. Observations from the field (van Jaarsveld 1989 pers com) confirm this characteristic pattern of avoidance of frost. This has bearing on all succulent flora of Southern Africa.

INTRODUCTION

Despite the fact that succulent plants in Southern Africa are known to occur at high altitudes which undergo winter freezing temperatures, they are generally held to be highly susceptible to frost (Cowling 1985). Levyns (1962) associates the high incidence of succulent plants on the coastal fringes of the Karoo-Namib Region with the low likelihood of severe frost during winter. In the south eastern Cape, the distribution of succulent Karoo vegetation was observed to be strongly associated with relatively frost free regions (Cowling and Hoffman, pers obs, in Cowling 1985). In the same way Werger (1978) suggests that the lack of succulents on the high peaks of the western Karoo could be due to the increased incidence of frost at such altitudes.

Similarly studies on desert succulents of the Sonoran Desert, North America, reveal that low wintertime temperatures can be the most important factor influencing their distribution (Shreve 1914). Of the 65 species of arborecent ceroid cacti in the Sonoran Desert, only three species (Stenocereus thurbei, Carnegia gigantea and Lophocereus skhottii) occur further north than the frost line, and frost damage is common for all three at the northern most part of their ranges in Arizona (Turnage and Hinkley, 1938; Nobel 1985). It is suggested that Carnegia gigantea is able to extend the furthest north due to the heavy spinescence on the apex of the stem, cutting down long wave radiation into the clear open sky at night (Nobel 1980). Results from biochemical studies involving the staining of the chlorenchyma cells of the cacti, confirmed the correlation found between frost tolerance and the northern most limit of their geographical distribution or the altitude at which they occur (Nobel 1982).

So why does one find such a general absence of succulent flora in colder regions? Nobel (1982) suggests two possibilities for cacti which might be applicable to the succulent flora of Southern Africa. Firstly succulents have a high cellular water content, resulting in fairly high osmotic potentials which could limit their protection from freezing. Another possibility might be the inability to undergo frost hardening as the plant is exposed to decreasing environmental temperatures during winter.

Southern Africa offers a unique opportunity for studying frost tolerance of plant species in relation to their geographical distribution around the country. Figure 1a shows the average annual number of frosty nights * (i.e. nights during which the minimum temperature fell below 0 °C). Notice that the coastal belt is practically free of frosty nights and that the greatest frequency of this condition is found on the edge of the great escarpment. This map must not be confused with the average length of the frost period, since it merely gives the number of frosty nights irrespective of the time of year; the average length of the period during which frost occurs is much longer (Figure 1b). Nevertheless the lines are similar to the former map and shows that frost persists longest on the edge of the great escarpment. It should also be noted that the absolute minimum winter temperatures also follow these contours. It should be mentioned though, that these maps do not adequately represent the distribution of frost, as there are so many factors (e.g. topography) affecting the occurrence of frost so that one finds the regional distribution of frost becomes very patchy. Numerous instances can be cited where two sites within short distances of less than a kilometre differ as to the incidence of frost on account of the situation in a valley and against a slope. For example, the north facing Magaliesberg ridges hardly ever experience frost (north slopes receive more solar radiation

* **In Southern Africa it very rarely happens that the diurnal minimum temperature occurs during the daytime.**

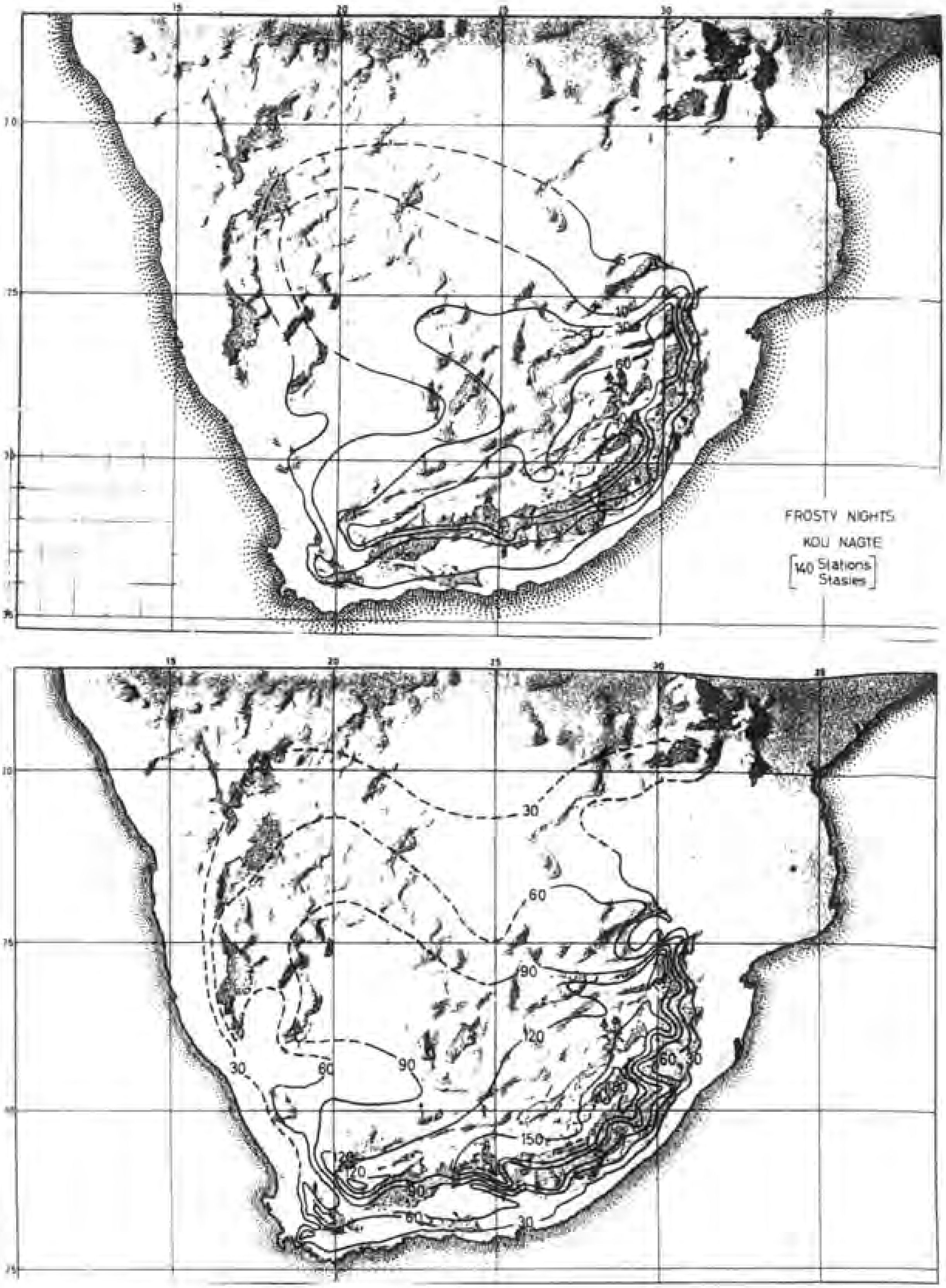


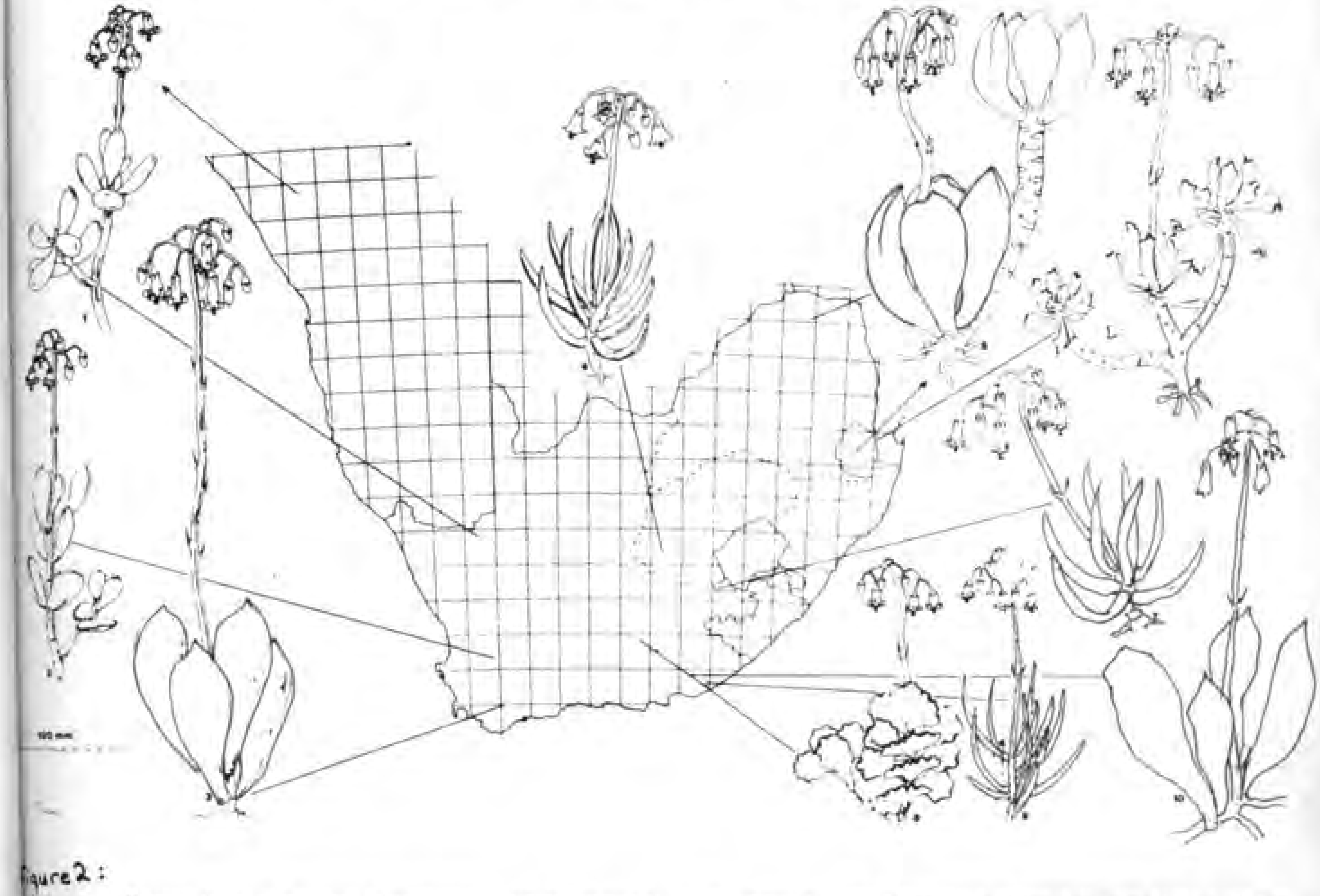
Figure 1.

Fig. 1 (a) (above) Average annual frequency of days with minimum temperature below 0˚C.

(b) (below) Duration of frost period in days (in Weather Bureau report 1965(8)).

during the day than south facing slopes, and are thus less prone to frost), whilst in the valleys it is almost a nightly occurrence during winter (Weather Bureau 8, 1965). Despite this high occurrence of frost, very little is known about tolerance of representatives of the Southern African flora to these conditions, especially with regards to the succulent flora.

This study is therefore an attempt to investigate the cold tolerance levels in the widely occurring leaf succulent species, Cotyledon orbiculata (Crassulaceae). This species is especially interesting as it is an extremely variable plant occurring as five different varieties (revised by Tölken 1979), each variety varying in its form and distribution throughout Southern Africa (Figure 2). The different varieties range in their geographical



igure 2 :

. — Extreme forms in the range of variation of **Cotyledon orbiculata:** — var. **orbiculata:** 1 *(Schlieben* 21); 2 *(Stirton & Boucher* 6136); 8 *(Tölken* —**var. spuria:** 3 *Tölken* 5501). — var. **dactylopsis:** 4 *(C.A. Smith* 4612). — var. **oblonga:** 5 *(Galpin* 10309); 6 *Tölken* 5576); 7 *(Gerstner* in PRE 32695); *Tölken* 5521). — var. **flanaganii:** 9 *(Flanagan* 1317). (after Tölken 1977) distribution, from the frost free areas of the coastline and tropical areas, to the more severe frost zones of the upper escarpment. It is strongly held that frost resistance is a genetically determined ecophysiological trait which is expressed under environmental constraints (Sakai and Larcher 1987). The gene pool of every population contains a range of variation enabling it to survive a degree of change in the environment. The degree of scatter in resistance among the progenies is a characteristic for a species and provides a measure of its scope for adaptation and selection (Eiga and Sakai 1987). The fact that some of the varieties of this species occur in severe frost areas while others do not, might suggest that extreme low temperatures are an important factor promoting the speciation of this species into colder regions. Alternatively the fact that these plants are able to exist in frost zones might not necessarily be related to cold resistance, but rather to their existence in more favourable microhabitats in frost zones, such as rocky outcrops.

Also vitally important with respect to understanding frost resistance of plants, is the ability of the plant to be able to frost harden itself as the plant is subjected to successively lower environmental temperatures with the approach of winter. This might be an even more important indication of the degree of scatter between populations of the different varieties. It also gives a good indication of the plasticity of a population to be able to adapt to frost conditions.

This study is thus concerned with answering the following questions:

1. Do the different varieties of the leaf succulent species <u>Cotyledon</u> <u>orbiculata</u> have some degree of tolerance, before cold hardening, to subzero temperatures?

2. If so, what is the degree of scatter between the populations of the different varieties in their frost resistance? And more importantly, is there any correlation between the frost resistance of each population and their geographical distribution with regards to frost and non frost regions?

3. Do these leaf succulents exhibit any degree of frost hardening, how do these compare with the hardening ability of the stem succulent cacti of North America?

4. What is the degree of scatter between populations in their resistance to subzero temperatures, after undergoing hardening? And again, how well does this frost resistance (after hardening) of each population correlate to their natural geographical distribution with regards to frost and non-frost zones?

5. Are there any differences in the frost resistance within the individual plant itself, e.g. are young leaves or buds more sensitive to the cold?

METHODS AND APPARATUS

All five varieties of Cotyledon orbiculata were obtained from their respective parts of the country (Table 1). In addition to this, seven different populations of the variety Cotyledon orbiculata var. orbiculata were obtained from their respective geographical regions of the country (Table 1). This sample of plant populations adequately represents a full range of low temperature environments, originating from areas that are frost free to areas that receive frequent and severe frost (see Table 1). The different varieties were replanted in Kirstenbosch Botanical Gardens (34° 0'S, 18° 30'E) and left to acclimatise for at least 2 months (from June to August) before the start of the experiment. So at the start of the experiment all the different varieties were coming from equal environmental conditions. This however is open to error due to the plants being exposed to the natural environmental conditions, ideally they should have been kept in a controlled environment. Nevertheless, these conditions were not severe enough to have any major altering effect on the plants. Before the start of the experiment, stems of each variety bearing fresh mature leaves were cut and kept in a controlled temperature of 20 °C for a week.

The cooling apparatus for the experiment involved the use of an alcohol bath where the alcohol was pumped through a cooling unit (model DLK 300). The rate of cooling was controlled by a valve which altered the flow rate of alcohol through the cooling unit. Temperature of the alcohol was constantly monitored using a copper constant thermocouple connected to a digital thermometer (model Fluke 2175A).

The cooling rate during the experiment was chosen to represent the natural situation as closely as possible. It was decided that a cooling rate of 3 °C/h, starting at 10 °C and dropping to -11 °C (with a 2h freeze time at -11 °C) was an accurate simulation of

VARIETY	SITE (latitude,longitude,elevation)		# OF FROSTY NIGHTS	*ABSOLUTE MINIMUM WINTER TEMPERATURE (°C)
orbiculata	Roodepoort Transvaal	(26°50'S,27°50'E,1770m)	30-60	-8
orbiculata	Greytown Natal	(28°50'S,30°45'E,1100m)	30-60	-8
orbiculata	Quolora Mouth Transkei	(32°30'S,28°15'E,20m)	5	0
orbiculata	Hout Bay S.W. Cape	(34°0'S,18°10' 60m)	5	0
orbiculata	Little Karoo	(33°50'S,20°20'E,900m)	10-30	-7
orbiculata	Namaqualand	(29°30'S,16°50'E,100m)	5	0
orbiculata	Mlaula Swaziland	(26°30'S,32°10'E,350m)	5	0
oblonga	Bethelsdorp S.E. Cape	(33°30S,25°30'E, 50m)	5	0
spuria	Little Karoo	(33°50'S,20°20E,900m)	10-30	-7
dactylopsis	Bloemfontein O.F.S.	(29°0'S,26°0'E,1500m)	30-60	-8
flanaganii	Kei Valley E. Cape	(32°30'S,28°20'E,100m)	5	0

*** Estimated from weather data of the closest possible weather station.**

Table 1 The different varieties of Cotyledon orbiculata that were obtained from their respective geographical distributors throughout the country. Relevant to this study is the number of frosty nights and absolute minimum winter temperatures (from Weather Bureau 8, 1965) occurring in these different geographical regions. Note altitude is directly related to frost severity.

the cooling rate experienced in a frost zone, close to the ground. This is a fairly slow cooling rate and thus only extracellular freezing will occur. Intracellular ice formation occurs when the cooling rate is too fast or when temperatures drop below -10 °C (Steponkus 1981). The rate of thawing is also vitally important as different thawing rates result in different types of damage to the cell. If thawing is rapid, as often happens when the sun shines in a clear sky following a frosty night, the ice melts quickly, so that the membrane is unable to reabsorb the melted water quickly enough, before it drains from the cells and even might escape from the plant through cracks in the epidermis (Bleasdale 1973). If thawing is slow, then the water from the melting ice is taken back through the membrane (providing it is not damaged) and the cytoplasm returns to its original state. Thus a relatively slow thawing rate of 6 °C/h was chosen so that cells were able to recover adequately. Thawing rate was controlled by either warming the alcohol with a heating element, or if the leaves were removed before reaching -11 °C (see later analysis of method Pg 13) then they were taken to a 0 °C room and allowed to thaw there for the appropriate time.

The leaves were sealed individually in thin plastic bags to prevent any contact with the alcohol. All air was sucked out of the bag so that there was no air layer between the leaves and the plastic and the alcohol.

In order to test the level of tolerance in these leaves, a method of electroconductivity was used. Based on leakage, Dexter et al. (1932) developed an electrical conductivity method for evaluating the cold hardiness of plants, due to the fact that an enhanced ion efflux is usually accompanied by freezing injury (Levitt, 1980). In this experiment discs (6mm diam) were punched out of the leaf, washed briefly with distilled water, dried again, and then dropped into 10 ml of distilled water. After one minute the conductivity was measured using a conductivity meter (platinum electrodes 1 cm apart. ~~make~~ Jenway model 4070). However, this technique is open to error as the use of distilled water causes a natural osmotic gradient, so that any electrolytes leaked are not necessarily due to damage of the cell membrane by freezing. It has been shown that ion leakage, after injury, also occurs from alive intact cells due to the natural osmotic gradient between the distilled water and the cell (Palta et al 1977a and 1977b). Furthermore the ion leakage has also been found to be a function of the duration of shaking the samples in distilled water (Palta 1977a). This is why I standardized the reading to one minute after the disc is dropped into the water, so that there was insufficient time for natural diffusion to have taken place, and thus only electrolytes from damaged cells resulted in any substantial conductivity reading. Ideally it would have been best to have used a buffer solution, so that no osmotic gradient between the cell and the surrounding solution existed. However this was not possible due to the time factor as it would have involved working out a buffer solution for each different variety. The advantage of using this conductivity method is that it obtains an immediate measure of damage of a large number of samples relatively quickly.

The problem, however, is that it is difficult to know what amount of electrolytes leaked coincides with lethal temperatures. In previous studies the lethal temperature was determined by taking the point at which 50% of the total ions are leaked. This however seems to be based on the assumption that 50% ion leakage corresponded to 50% of the dead cells in injured tissue (Zhu and Liu 1987). Palta et al (1977a) has found that this is an invalid assumption. It was therefore necessary to carry out an additional study that correlated the amount of damage suffered by the leaf to the amount of electrolytes leaked. Note that I used an absolute amount and not a relative percentage of electrolytes leaked, which is thus open to some error as I am making the assumption that all the different varieties have very similar concentrations of electrolytes in their cells at the start of the experiment.

ANALYSIS OF METHOD:

By extracellular freezing (as occurs in this experiment) the cooling rate is slow enough to allow water to diffuse out of the cells into the intercellular spaces. This is caused by the different water potential between supercooled water inside the cell and ice outside, which in effect creates a kind of dehydration similar to the mechanism by which plant cells react to water stress (Ashina 1978, in Skre 1988). I have therefore chosen to look at percentage water loss from the leaf as a means for determining the critical killing temperature at which a certain amount of electrolytes are leaked.

To start with, I took pairs of leaves that were opposite to each other on the stem: one leaf for measuring the percentage weight lost (i.e. dehydration), and the other for measuring the amount of electrolytes leaked. This was repeated for each of the 11 plants (5 varieties, including 7 different populations of var. orbiculata) in replicates of three. At successive temperatures during the cooling process, these being 10 °C, 4 °C, -2 °C, -8 °C,-11 °C and -11 °C (2 hour duration), the three pairs from each of the 11 plants were removed. Unfortunately not all of the 11 plants could be repeated for all the different temperatures, so that in some cases the results remain somewhat incomplete. One leaf of each pair was placed in a room (20 °C, 50% RH, 10h day/14h night) for the measurement of dehydration, while the other leaf, after a recovery period of 24 hours, was used to measure the amount of electrolytes leaked. Any leaves that had the misfortune of coming into contact with the alcohol were damaged, and thus discarded. Measurement of electrolytes was carried out as described above, while measurement of percentage weight loss was carried out by measuring the initial mass and the mass after 1 week. This percentage weight loss was correlated to the percentage browning of the leaf. Where more than half of the leaf went brown, it was decided that the leaf had been killed. From observations it was decided that at 30% weight loss the majority of leaves had more than 50% browning. Thus 30% weight loss was regarded as the cut off point.

Weight loss was then correlated against the concentration of electrolytes leaked (µS/g/ml) for the different plants. It was found that at 30% weight loss, the amount of electrolytes leaked ranged from 16 µS/g/ml to 23 µS/g/ml) for the different varieties, with a mean of 19 µS/g/ml (S.E= 2.35). 20 uS/g/ml was thus used as the cut off point for the killing temperature, Figure 3 indicates that this was a reasonable choice. There may however be some degree of error in this choice due to the fact that the leaves of the different varieties were of different sizes and shapes and thus might have resulted in different rates of dehydration during the one week recovery period.

So far measurement of the amount of electrolyte leakage has been made only on the basis of mass. There is however the possibility that electrolytes leaked per gram is not necessarily an accurate way of expressing the amount of electrolyte leakage. Most of the electrolytes measured probably came from the exposed cells and the cells just beneath these, and not from the cells in the middle of the disc which are incorporated in the measurement of mass of the disc. Therefore measurement on the basis of exposed surface area of each disc was thought possibly to be a more accurate way of measuring damage done to each cell. It was tested to see whether there was any difference between these two different ways of expressing electrolytes leaked. There was however no difference found between them, in fact there was a very strong correlation between these two measures (see Figure 4, $p < 0.001$). Therefore measurement of electrolytes leaked on the basis of mass was regarded as an accurate measure of damage incurred by the cells.

Measure of frost tolerance before and after hardening:

Having determined at what level of electrolyte leakage, killing of the tissue occurs (20 µS/g/ml) it was possible then to measure the critical temperature at which each plant is resistant to damage.

For measurement of tolerance before hardening the same results that were obtained for the method analysis were used to determine critical temperature. To try and get some idea of the variation within one leaf, electrolytes leaked from a disc punched out from the base, middle and tip of the leaf were measured separately. With the decrease in temperature, a linear regression line was worked out from the point of electrolyte leakage increase to the point where the increase levelled off again. The temperature corresponding to 20 µS/g/ml was regarded as the critical temperature of resistance (Figure 5). This is somewhat different from other studies where either 50% leakage of electrolytes or the point of inflection (where a small change in temperature brings about the largest change in relative electroconductivity) is taken as the point corresponding to the critical temperature (Su et al 1987).

To determine the ability of these plants to undergo frost hardening, the plants were subjected to a period of cold hardening treatment. Plants were kept in a dark 10 °C room for 4 days, and then in a dark 3 °C room for 3 days (Nobel 1982 found that the halftime for cold hardening in response to a sudden

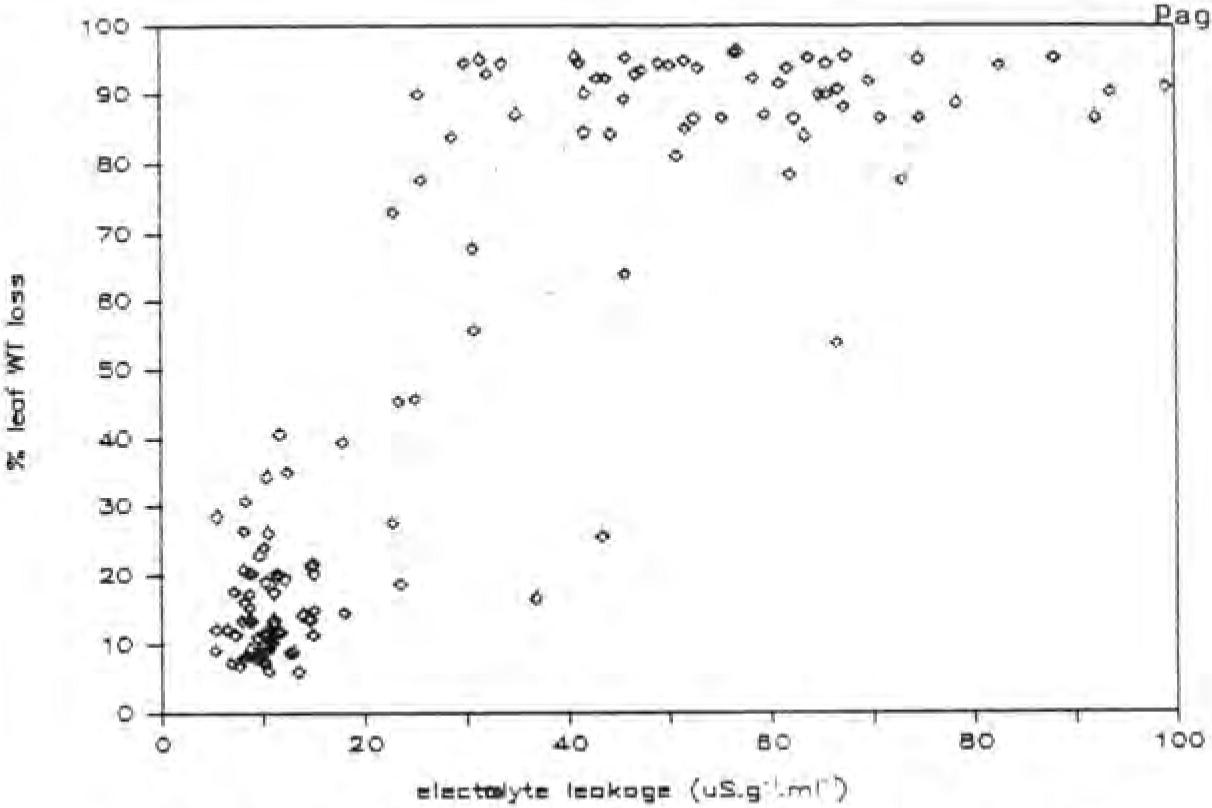


Figure 3. Predicted electrolyte leakage at 30% leaf weight loss (regarded as the lethal level). Range between 16 and 23 uS/g/ml for the different varieties, with a mean of 19 uS g ml (S.E.=2.3). Therefore 20 µS/g/ml regarded as the critical amount.

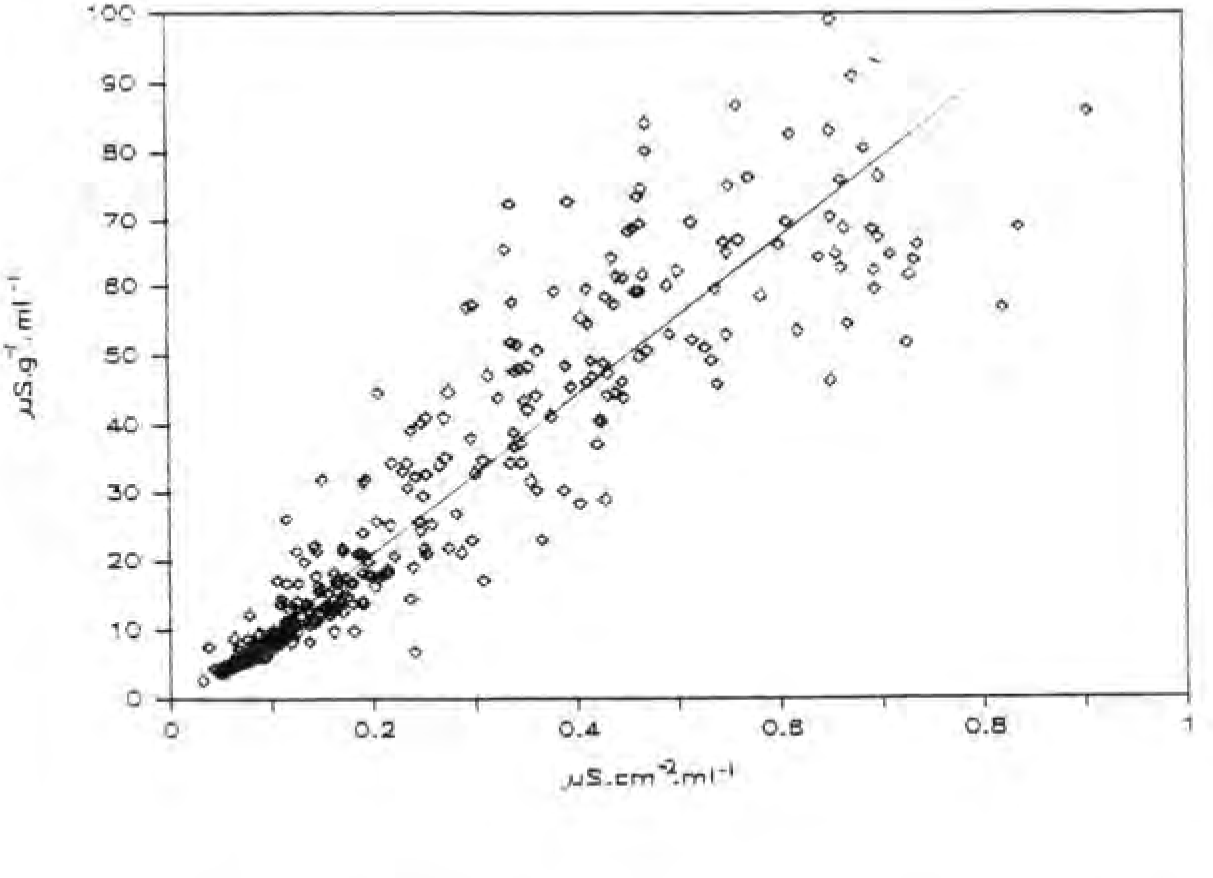


Figure 4. Electrolyte leakage measured on basis of mass vs. electrolyte leakage measured on basis of exposed surface area of punched disc (r2 = 0.89, n = 334).

decrease in growth temperatures is 3-4 days for various agaves and cacti). Once the leaves had undergone this treatment, the same procedure as for the non-hardened plants was repeated. The same amount of 20 µS/g/ml was regarded as the critical temperature for both hardened and non-hardened experiments. Only mature leaves were used for experimentation.

To determine the variation within a plant, young and middle aged leaves from the var. orbiculata of the Little Karoo and the Transkei coast were tested for resistance. This was only tested after the hardening treatment.

It is important to mention that the comparisons made between different varieties, and between leaves of the same plant were done merely on the basis of observation, no statistical tests were performed.

RESULTS

Frost Tolerance before hardening:

Figure 5 shows an example of how the critical temperature was determined. Note the difference between the base, middle and tip of the leaf, where the tip proves to be the most sensitive. The results for the rest of the plants are summarised in Table 2. The r^2 values for the regressions lines ranged mostly between 0.7 and 0.95. The slope of the regression line (i.e. the rate of electrolytes leaked) did not seem to be connected to the level of resistance of the plant in any way, and so was not considered.

The level of resistance ranges from a considerable -9.8°C for the base of the leaf of var. orbiculata from the high elevations of Roodepoort, to the tip of the leaf of var. spuria from the Little Karoo with a resistance of only -1.9°C.

The results also reveal that before any increase in electrolyte leakage that the different varieties have very similar concentrations of electrolytes. This clarifies the assumption made of all varieties having similar concentrations of electrolytes at the start of the experiment, and thus comparisons made between the different varieties are valid.

Frost tolerance after hardening:

Table 3 summarises the critical temperatures of the 11 different plants studied, after a treatment of frost hardening. There is an increase in almost every plant in their resistance, with the exception of var. orbiculata from Greytown and Roodepoort (but only at the base of the leaf) and a few others that stayed more or less the same in their resistance. There was an average increase in resistance (including all the different varieties) of -0.8 °C, -1.4 °C, and -1.2 °C for the base, middle, and the tip of the leaf respectively, as the growth temperature is reduced by 10 °C.

Variation of tolerance within the plant:

From Tables 2 and 3, it is evident that there is variation even within the leaf. The tip seems to be the most sensitive, even after hardening, followed by the middle and then the base of the leaf. Results of comparisons of resistance of younger and older leaves, reveal that in both varieties of var. orbiculata from the Little Karoo and the Transkei, the younger leaves are the most sensitive (Figure 6). The very young bud leaves of var. orbiculata from the Transkei had a very low resistance of -2.4 °C even after hardening.

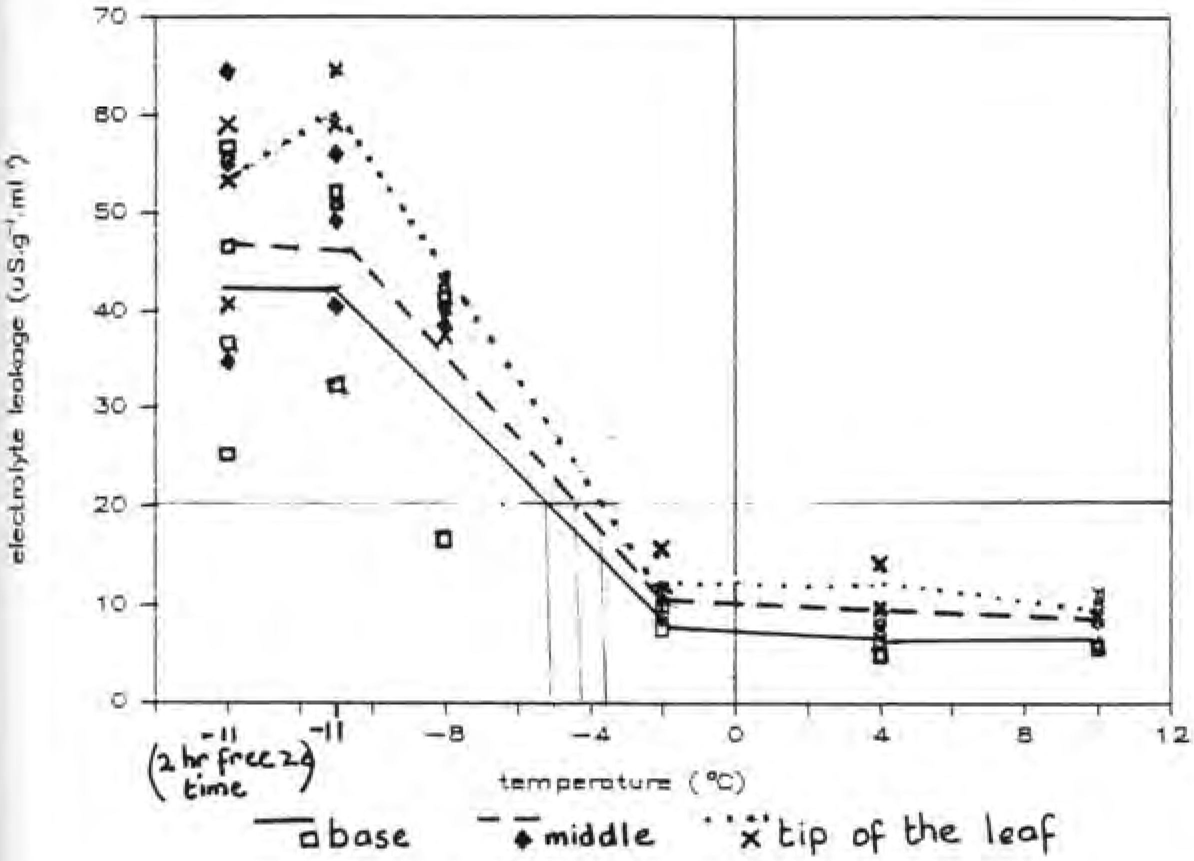


Figure 5. Critical low temperature tolerance of var. oblonga of the Eastern Cape (elev.50m) before undergoing cold hardening. Critical temperature is determined for the base (r^2 = 0.73, slope -3.6), middle (r^2 = 0.94, slope -4.3) and the tip (r^2 = 0.95, slope -5) of the leaf at that point where 20 µS/g/ml of electrolytes are leaked out of the punched discs into distilled water for a period of one minute. Slope was taken from the point of increase in leakage to where there is a levelling off again.

		CRITICAL KILLING TEMPERATURE (°C)		
VARIETY	**SITE (ELEVATION)**	**BASE OF LEAF**	**MIDDLE OF LEAF**	**TIP OF LEAF**
orbiculata	Roodepoort (1770m)	-9.8	-5.6	-6.2
orbiculata	Greytown (1100m)	-8.3	-3.8	-3.3
orbiculata	Transkei Coast (20m)	-5.0	-3.4	-4.2
orbiculata	Hout Bay (60m)	-4.3	-3.1	-3.0
orbiculata	Little Karoo (900m)	-3.3	-2.7	-2.4
orbiculata	Namaqualand (100m)	-3.3	-3.3	-3.6
orbiculata	Swaziland (350m)	-4.5	-4.4	-3.9
oblonga	S.E. Cape (50m)	-5.1	-4.1	-3.6
spuria	Little Karoo (900m)	-3.5	-2.3	-1.9
dactylopsis	Bloemfontein (1500m)	-5.2	-4.5	-3.4
flanaganii	Kei Valley (100m)	-2.6	-2.7	-2.3

Table 2 These are results of frost tolerance in mature leaves of the different varieties of <u>Cotyledon</u> <u>orbiculata</u> before undergoing any hardening treatment (room temperature 20°C for 1 week, 10h day/14h night). The critical killing temperature was determined for the 3 separate parts of the leaf at that temperature where 20 µS/g/ml of electrolytes were leaked, out of the punched disc, into distilled water for 1 minute (see method).

		CRITICAL KILLING TEMPERATURE (°C)		
VARIETY	**SITE (ELEVATION)**	**BASE OF LEAF**	**MIDDLE OF LEAF**	**TIP OF LEAF**
orbiculata	Roodepoort (1770m)	-9.2	-8.8	-7.9
orbiculata	Greytown (1100m)	-5.9	-5.7	-4.7
orbiculata	Transkei Coast (20m)	-6.1	-5.2	-4.1
orbiculata	Hout Bay (60m)	-7.1	-6.6	-5.6
orbiculata	Little Karoo (900m)	-5.0	-4.8	-4.9
orbiculata	Namaqualand (100m)	-3.3	-4.3	-4.5
orbiculata	Swaziland (350m)	-5.3	-5.6	-4.6
oblonga	S.E. Cape (50m)	-6.5	-6.5	-5.2
spuria	Little Karoo (900m)	-7.2	-6.6	-5.9
dactylopsis	Bloemfontein (1500m)	-8.4	-7.4	-6.5
flanaganii	Kei Valley (100m)	-6.5	-6.3	-5.8

Table 3 These are results of frost tolerance in mature leaves of the different varieties of Cotyledon orbiculata after a process of hardening (4 days in 10 C room, 3 days in 3°C room). The critical killing temperature was determined for the 3 separate parts of the leaf, at that temperature where 20 µS/g/ml of electrolytes were leaked, out of the punched disc, into distilled water for a period of one minute (see method).

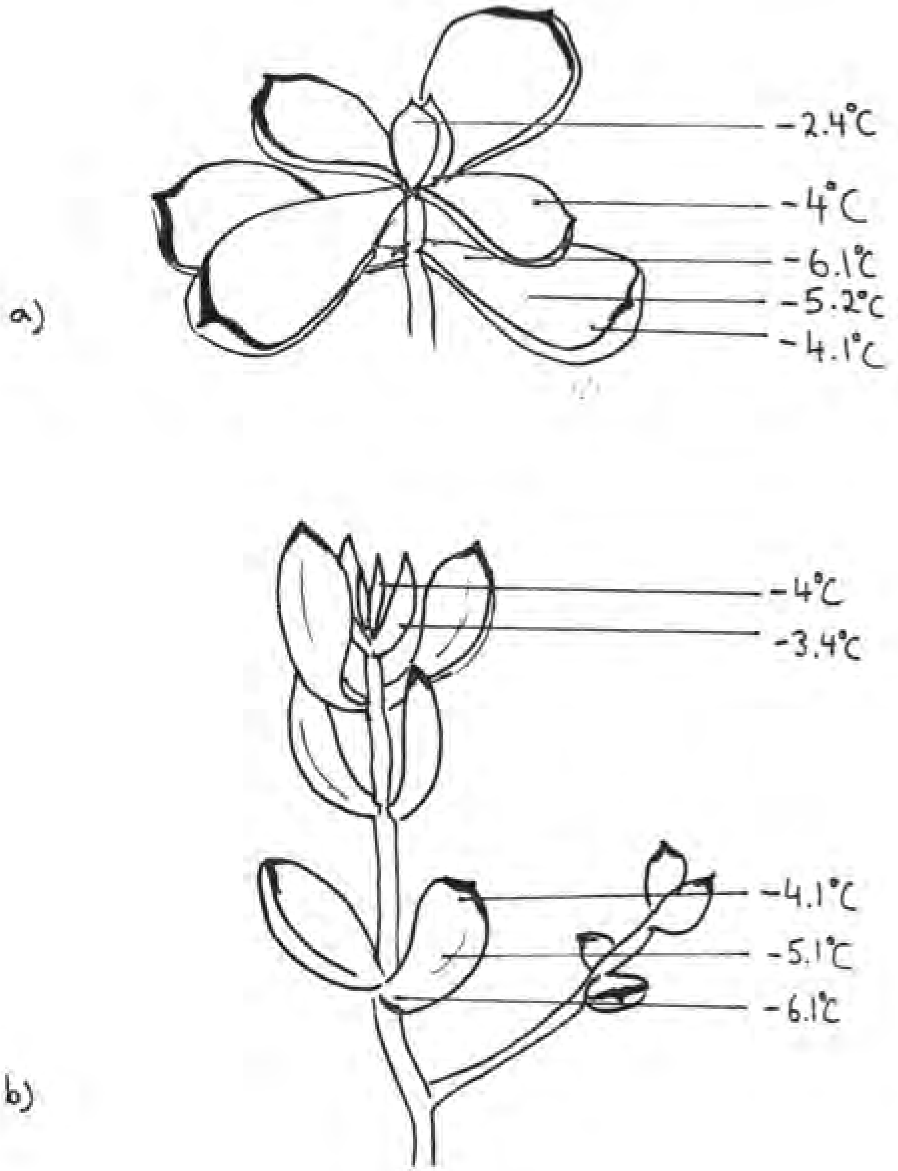


Figure 6. Variability in tolerance between different aged leaves and different parts of the leaf after undergoing hardening, in *C. orbiculata* var. *orbiculata* from the

(a) Transkei (elev. 20m) and

(b) Little Karoo (elev. 900m)

DISCUSSION:

Despite the current dogma that succulent plants are very sensitive to freezing temperatures, it was evident from the results that this particular succulent species does show some degree of resistance to these temperatures. However, there was a tremendous degree of variability in the resistance of these leaves, with some varieties being quite sensitive while others show substantial resistance. This variability was especially evident in plants that had not undergone any hardening treatment (see Table 2). It is important to note that when making comparisons between the different varieties, that there is in fact a great degree of variability in resistance within one particular leaf itself, with the base of the leaf inevitably being more resistant than the middle and tip of the leaf. Thus when making a comparison between varieties, it should be between similar parts of the leaf.

Frost tolerance before hardening:

It is evident from the unhardened plants (Table 2), that var. orbiculata from Roodepoort (elev. 1770m) had the greatest degree of resistance in the base, middle and tip of the leaf (-9.8 °C, -5.6 °C and -6.2 °C respectively). The only other plants to have a resistance greater than -5 °C (i.e. more negative) were var. orbiculata from Greytown (elev. 1100m), var. dactylopsis from Bloemfontein (elev. 1500m) and var. oblonga (elev. 50m), but this was only in the base of their leaves and not at the middle and tip. The resistances for the remaining plants all fall between 0 °C and -5 °C and therefore can be regarded as being fairly sensitive to freezing temperatures. I would predict that in a reasonably severe frost these plants would suffer injury or even death. These results of freezing resistance in unhardened plants would thus seem to indicate that there is some correlation between the natural geographical distribution of the plant and its ability to resist subzero temperatures. The three plants that occur in the most severe frost areas, var. orbiculata (from Roodepoort), var. orbiculata (from Greytown), and var. dactylopsis appear to be the three most resistant plants, while the plants from the lower elevations seemed to be more sensitive (with the exception of var. oblonga). Can one thus deduce that the minimum winter temperature is limiting the distribution of these different population, and possibly be an important factor promoting the speciation of this plant? I would be very cautious in making such a deduction for two reasons. Firstly the two varieties var. orbiculata and var. spuria from the Little Karoo (elev. 900m), which one would expect to be reasonably frost hardy since they come from relatively frosted areas, proved to be some of the most sensitive plants. This was especially so for var. spuria, which was resistant only up to -1.9°C at the tip of its leaf. The other reason is that it is possible that var. orbiculata (from Roodepoort) and var. dactylopsis had a slight advantage over the other varieties, since they were obtained most recently out of all the plants and so might have not had enough time to acclimatise to the more moderate environment of Kirstenbosch Botanical Gardens (Also note that they were obtained at the start of winter). It is quite possible that these two plants needed longer than two months to undergo a sufficient dehardening process.

Nevertheless the fact that some of these plants are very sensitive before hardening does have some major ecological implications. During the early phases of winter, before the plants have undergone any substantial hardening, an occurrence of a sudden severe frost might have devastating effects. This might have some bearing on the microhabitats where these plants are generally found. Van Jaarsveld (1989 pers com) has observed in collecting this plant species that they are generally found on rocky outcrops. Rocky habitats favour survival in the winter months as they provide protection against frost (Steenberg and Lowe 1969) as well as predation (Osmond et al 1987). In addition to this C. orbiculata seems to dominate more on the northern slopes (Van Jaarsveld pers com 1989) which receive more solar radiation (in the southern hemisphere) during the day, and thus have fewer frost occurrences. For a more thorough understanding of frost tolerance in these plants, we need to turn to their ability to undergo frost hardening. This is often a more accurate way of distinguishing between different varieties.

Frost tolerance after hardening:

The results in Table 3 reveal that this plant is able to undergo some degree of hardening. After the frost hardening treatment (a drop of 17 °C growth temperature) the response ranged from no increase in resistance in some (e.g. the base of the leaves of var. orbiculata of Roodepoort and Greytown), to an increase of -4.3 °C in var. spuria (at the middle of the leaf). Nobel (1981) in his studies on cold hardening of cacti in North America found that Coryphatha vivipara, which occurs from Mexico to Canada, showed an increase of -1.7 °C in its resistance as the growth temperature was reduced by 10 °C. The average increase in resistance for Cotyledon orbiculata (including all the different varieties) was somewhat less for a reduced growth temperature of 10 °C (-0.8 °C, -1.4 °C and -1.2 °C for the base, middle and tip of the leaf respectively). It is however difficult to make such a comparison as there was variability between the different varieties in their frost hardening ability.

Looking at the frost hardening ability of the different varieties individually, reveals some interesting results. There appears to be no definite correlation between the frost hardening ability of the plants and their geographical distribution. In fact some of the most marked increases in hardening ability occur in varieties from frost free regions; e.g. var. flanaganii (elev. 100m), var. orbiculata (from Hout Bay, elev. 60m). Both of these varieties show substantial resistance to freezing temperatures after undergoing hardening, which in fact compare quite closely to some varieties from much higher elevations; e.g. var. dactylopsis (elev. 1500m). Nevertheless the two most resistant varieties in all three parts of the leaf after hardening, remain as before, these being var. orbiculata (Roodepoort, elev. 1770m) and var. dactylopsis (elev. 1500m). However, var. orbiculata (from Greytown, elev. 1100m), which one would expect to also show a high resistance after hardening, actually showed a decrease in its resistance at the base of the leaf, and a slight increase at the middle and tip of the leaf, and thus remains fairly sensitive to freezing temperatures (I make this observation hesitantly, as experimental error could have caused this result). Another variety that one would expect to increase in resistance substantially after hardening, but did not, was var. orbiculata (from the Little Karoo, elev. 900m), which still remained fairly sensitive to freezing temperature (-5 °C, -4.9 °C, -4.9 °C for the base, middle and tip). As predicted results that did seem to show some correlation between ability to frost harden and geographical distribution, were var. spuria (elev. 900m) which showed a marked increase in its resistance. Var. orbiculata (from Namaqualand, elev. 100m) and var. orbiculata (from Swaziland, elev. 350m) showed an expected small increase in resistance, and thus remained fairly sensitive to freezing temperatures.

The one feature that becomes very evident from these frost hardening results, is that there is less variability between the different varieties in their tolerance to freezing temperatures after hardening than before hardening. This is emphasised by some varieties from lower frost free elevations (e.g. var. flanaganii and var. orbiculata from Hout Bay) that have shown a great affinity for frost hardening so that their tolerance to freezing temperatures is similar, if not higher, to that of varieties from higher elevations. Thus the possibility of frost being an important factor limiting the geographical distribution of the different varieties becomes less convincing. One must therefore ask, why do these plants have this intrinsic ability for adaptation to freezing temperatures irrespective of their geographical distribution? It has been suggested (van Jaarsveld pers com) that possibly the degree of tolerance seen in this succulent species irrespective of its geographical distribution, is evidence of adaptation to cold conditions during the last glacial period. This is widely open to debate.

Resistance variability within the plant itself:

I have already mentioned the apparent difference in resistance between the base, middle and tip of the leaf, with the base in almost all cases being the most resistant, while the tip tends to be the most sensitive. This is apparent both before and after hardening. The fact that the tip of the leaf is sensitive to freezing temperatures even after undergoing hardening, for almost all of the varieties, is of consequence due to the fact that the tip is the most exposed part of the leaf and is thus bound to suffer damage during a severe frost.

Looking at the different aged leaves within a plant (Figure 6a and b) the results reveal that even after hardening, the younger leaves in both the varieties tested, are more sensitive to freezing than the mature leaves. This is especially true for the young bud leaves of var. orbiculata (from Transkei) which are very sensitive with a critical temperature of -2.8 °C. The plant therefore needs to protect its bud leaves in some way. One possibility might be that it only produces new young leaves at the beginning of the warm season. It is also possible that C.

orbiculata is able to show some degree of avoidance of frost. Beck et al (1987) found in giant rosette plants, occurring in alpine regions of East Africa, that upon cooling, the single adult leaves nyctinastically bend inwards to form what they term a night bud. This acts as an insulation mechanism for the more sensitive bud leaves in the middle. This nyctinastic movement of the rosette leaves is supposedly brought upon from a loss of cell turgor of the cells from central region of the lower side of the leaf (since they receive more cooling stress), upon loss of turgor the cells cannot collapse and hence presumably force the leaf towards the central axis. It is possible that this might be occurring in C. orbiculata to some extent as I have observed that when these plants were in the 3°C room during hardening that the mature leaves of some of the plants had bent inwards towards the stem and thus covered the young bud leaves. In the natural environment this inward bending of the leaves would also mean that these plants are cutting down their exposed surface for the emittance of long wave radiation into the clear open night sky. This mechanism for avoidance of freezing temperature would need to be tested in the field.

The sensitivity in these young leaves might be similar to the sensitivity experienced by young seedlings. Unfortunately I was unable to test any seedlings for their tolerance, but if they are as sensitive as these young bud leaves, which I predict they are, then it would mean they require some sort of protection from these low temperatures during germination, especially because they will be closer to the ground and thus experience far more severe freezing temperatures than an adult plant. Nobel (1984, 1988) found for cacti and agave seedlings nurse plants or a sheltered microhabitat is of vital importance for the survival of these seedlings (I would suggest that nurse plants and sheltered microhabitat sites play an important role for the survival of seedlings of C. orbiculata). The tolerance of seedlings still needs to be tested.

An unanswered question:

The question that still remains unanswered though is why, if this leaf succulent species is able to show resistance to relatively cold freezing temperatures after undergoing hardening, does one find that it is generally restricted to rocky outcrops and often in greater abundance on the north facing slopes, which are characteristic of frost protected areas? A possible reason for this might be that even though the plant as a whole is resistant to frost conditions, the young leaves and tips of mature leaves (also most probably seedlings) remain sensitive to freezing temperatures, even after undergoing hardening. I suggest this is of major consequence to the rest of the plant, as it results in a frost-drought condition within the plant, similar to that caused by water stress (Tranquillini 1981). To a succulent plant this will have devastating long-term effects as it is essential that they have a high water content so as to survive long drought periods. This disturbance to the water economy of the plant due to frost, might therefore be a reason for <u>Cotyledon</u> <u>orbiculata</u> being confined to the more favourable microhabitats of rocky outcrops. This has bearing on other succulent plants of Southern Africa.

Further recommendations:

The topic of frost tolerance of succulent plants in Southern Africa requires many more studies before we can gain a true understanding of role of frost. Basically any study on this topic is new information as it is such a neglected topic. Firstly, studies should be orientated to the tolerance of succulent seedlings to frost conditions. Seedling establishment is one of the most crucial phases in the life cycle of a plant, and yet there have been no ecophysiological studies carried out on these seedlings. Related to this is the importance of shade plants and microhabitats for the survival of seedlings. Work is presently being carried out in this field of study (R. Bueckman, M.Sc student).

Also we need a greater understanding of the biochemical and physiological processes at the cell level to fully understand how succulents are responding to cold (i.e. What are the implications of having such a high cell water content?).

Very pertinent to this study when making comparisons between varieties and trying to relate their frost tolerance ability to their geographical distribution is possibly to look at the polyploidy of the different varieties. It has been suggested that a greater size in polyploidy might be directly related to the frost tolerance of a plant (Sakai and Larcher 1987). This might also help answer some questions on the climatic history of these plants.

Lastly of vital importance, is to combine physiological studies with what is found in the field. Possibly even to carry out some physiological experiments in the natural environment, e.g. test the effect frost has on the water potential of these succulent plants along an altitudinal gradient.

This subject of frost tolerance of succulents in Southern Africa still remains unexplored, and so I encourage any further studies that might help broaden the understanding of these unique and fascinating plants.

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