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DROUGHT-INDUCED CHANGES IN CHLOROPHYLL FLUORESCENCE IN FLORISTS' CINERARIA AND A XEROPHYTIC PROGENITOR

by

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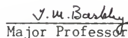

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Plants inhabiting xeric areas have evolved a remarkable variety of morphological and physiological defenses against the effects of water deprivation. Many attempts have been made to identify these traits and the factors that control their expression. However, water stress has many effects on plant growth and metabolism (18) and has lead to a myriad of adaptive strategies at many levels. Since drought resistance is a complex of many morphological and/or physiological characteristics, the heritability of adaptive traits is likely the result of multiple gene traits, which code for a complex of physiological parameters. Gauhl (7) has suggested that a plant's physiological attributes are under the control of selective pressures that reflect the plant's evolutionary history within a given habitat. Thus, physiological divergence in response to environmental changes may be intimately associated with species differentiation and the success of a species within a given habitat. This line of reasoning has given rise to the use of exotic germplasm as a source of stress-tolerant traits (17, 18, 24).

Sullivan and Ross (27) state that a major problem in selecting for drought tolerance has been identifying desirable factors for selection. Kramer (15) describes drought tolerance as a plant's ability to withstand appreciable water loss and still maintain growth and photosynthesis at low water potentials. Furthermore, Heisey and Milner (13) suggest that "of all the physiological processes that can be measured quantitatively in a plant, its photosynthetic rate is probably the most crucial." Therefore, it is likely that a plant's photosynthetic response to water deprivation may serve as a useful criterion in selection for drought tolerance. If so, the heritability

of drought tolerance may also be followed at the photosynthetic level.

SELECTION OF AN APPROPRIATE PLANT GROUP

Finding a suitable plant group to test the idea of physiological divergence as an adaptive response to environmental changes, as well as addressing the inheritance of physiologically adaptive traits, is a difficult task unless (i) the ancestors of the group are known and extant and (ii) pertinent ecological information is available. For this reason, we chose to address these ideas in the cultivated complex, Florists' Cineraria (*Senecio cruentus*).

The genus *Senecio* (Asteraceae) is a large cosmopolitan group containing about 2300 species, mostly perennial herbs or sub-shrubs. The section *Pericallis* consists of ten species endemic to the Canary Islands, plus a complex of cultivars termed the Florists' Cineraria (hereafter called cineraria). Cineraria is believed to have developed into a distinct complex within about 50 years after their initial introduction into cultivation at Kew (1). A taxonomic study by Midcap (19) aimed at identifying the progenitors of cineraria suggests that cineraria is a multi-species complex derived from *S. cruentus*, *S. heritieri*, *S. tussilaginus* and perhaps others. Midcap's conclusions were based on a chemosystematic study where flavanoid biochemistry was used to identify species boundaries and species relatedness. The progenitor species, in addition to being biochemically unique, also exhibit ecological differences.

The Canary Islands are geologically recent. Their volcanic origin has given rise to topographic variation that ranges from sea level up to 3700 meters, creating a diversity of ecological niches. The climate

ranges from warm-subtropical to cool-montane, with the temperature extremes buffered by the surrounding ocean. Interestingly the progenitors exhibit habitat variation, which correlates with water availability. *Senecio cruentus* and *S. tussilaginus* are both mesophytes, occurring in open areas and woodland fringes. In contrast, *S. heritieri* occupies lower, open, xeric slopes. Thus, we expected that *S. heritieri* may possess drought tolerant attributes based on the distributional patterns exhibited by this species. In addition, the expression of these traits may be manifested in the cultivated complex. Therefore, this investigation is aimed at mapping changes in the photosynthetic physiology of *S. heritieri* and cineraria when water stress is slowly applied through soil drought. Since cineraria is a fully developed group of cultivars that are derived from a restricted gene pool and are morphologically and physiologically distinctive from the putative ancestors, the complex provides a convenient model to address evolutionary events at the physiological level.

STRESS-INDUCED CHANGES IN THE PHOTOSYNTHETIC PROCESSES

Photosynthesis can be viewed as an integrated set of reactions consisting of (i) the primary photochemical events of light capture, (ii) oxidation/reduction and phosphorylation reactions and (iii) the carbon reduction reactions. Each set of reactions represents many sites potentially inhibited by water stress. The water stress induced decline in net photosynthesis is a consequence of both stomatal closure causing increased constraint on CO_2 diffusion and a decreased chloroplast activity (non-stomatal inhibition) (4). Ogren and Oquist (21) have shown that in the early stages of water deprivation, the

decline in CO_2 uptake may be attributable to both stomatal and non-stomatal factors. However, the further decline in CO_2 uptake observed as water stress continues is the result of non-stomatal inhibition. Furthermore, the set of reactions targeted by water stress may be species-specific. Characterizing water stress effects *in vivo* at the sub-cellular level is difficult. However, chlorophyll fluorescence provides a non-invasive assay that can monitor the detrimental influence of environmental factors.

Smillie (25) has shown that injury resulting from heat stress causes damage to photosystem II (PS II), as inferred by the change in variable fluorescence. Furthermore, these results suggest that measurement of the variable component of fluorescence *in vivo* provides a method to monitor the onset of cellular heat injury, which allows for a ranking of plants in order of heat resistance. Govindjee (9) and Havaux et al. (11) found that electron transport through PS II was hindered by water stress in *Nerium oleander* and *Nicotiana tabacum* based on similar interpretations of stress-induced changes in fluorescence. This suggests that PS II is a vulnerable site to heat and drought induced injury. However, Genty et al. (8) and Conroy et al. (5) found that PS II was not targeted by water stress in *Gossypium hirsutum* and *Pinus radiata* since variable fluorescence was not altered. Schreiber (23) obtained similar results in *Arbutus unedo* and suggested that drought stress causes a failure of the Calvin cycle to act as an electron acceptor. This interpretation agrees with the findings of Mayoral et al. (18) and Becker and Fock (2), who showed that several Calvin cycle enzymes are inhibited at low water potentials in wheat and maize. Perhaps one reason for the lack of consensus concerning the

primary target of water stress on the photosynthetic apparatus is due inpart to the different plant species used in each of these studies. Thus, the genetic and physiological potential of these plants may be inherently different.

Chlorophyll fluorescence is a relatively new tool to plant breeding and has been applied sparingly in this context. A major objective of this study is to evaluate the application of this tool in systematic studies, where ranking plants according to drought tolerance is a goal. Our results will speak more to the physiology of certain Senecios rather than to the systematics of the genus. In brief, we found that fluorescence induction may provide a rapid method to monitor the onset of drought-induced injury. However, a ranking of relative drought tolerance was not possible from induction kinetics alone.

MATERIALS AND METHODS

Plant Materials. Seeds of Seneceio heritieri (from Jardin De Aclimatacion, Puerto De La Cruz, Tenerife, Espana) and Florists' Cineraria varieties; Cindy Mix (cv one), Tourette Mix (cv four), Improved Festival (cv five), and Ball Florist Selected Mix (cv seven) (Ball Seed Co., Chicago, Ill.) were germinated in a greenhouse before being transplanted to pots and transferred to a growth chamber. Pots were constructed of PVC pipe 10.2 cm inside diameter and 10 cm deep. Plants were grown on a mixture of sand, perlite, and peat. Roots and soil were constrained in pots with Nitex nylon cloth (H.R. Williams Mill Supply Co., Kansas City, Mo.) placed between the pot and a PVC sleeve. A 16 hr photoperiod, beginning at 6 am, was used in the growth chamber. Light intensity was 150 umol m^{-2} s^{-1}. Day temperature was 16C and night temperature 9C. Plants were watered to field capacity every 2-3 days with distilled water and every third watering with Hoagland's nutrient solution. Prior to drying cycles, plants were watered with nutrient solution (Figs 1-3) or placed on foam blocks (26) (Oasis brand) for 6 days (Figs 4-8). The foam blocks were continually saturated with nutrient solution to maintain the soil at near field capacity. The drying cycles were initiated by suspending watering or removing plants from water columns. Drying cycles were ended by rehydrating the soil to field capacity with distilled water. The number of plants sampled in each experiment is given in the figure legends.

Leaf Water Potential. Leaf water potential was monitored by the dew point method with Wescor thermocouple psychrometers (Wescor Inc.,

Logan, UT). Leaf discs 7mm diameter were taken from leaves adjacent to the leaf being used for fluorescence measurements. Water potential was measured immediately after fluorescence assays.

Net Photosynthesis. _In vivo_ rates of photosynthesis were estimated by measuring CO_2 exchange rates (ADC infrared CO_2 analyzer) from intact leaves. A chamber (Parkinson leaf chamber) was placed over a known area of leaf tissue. CO_2 exchange rates were taken in the growth chamber prior to the dark adaption period.

Fluorescence Measurements. The kinetics of chlorophyll fluorescence induction were measured using a home-built fluorimeter (10), which was modified for intact leaf tissue with fiber optics. An excitation beam from a 300 W projector lamp was passed through a 450 nm broad band interference filter (Detric Optics) and focused onto the leaf using fiber optics (Dolan Jenner Industries). Fluorescence was collected by a second fiber optics bundle and monitored by a photomultiplier tube protected with a 680 nm narrow band interference filter. Plants were dark adapted for 30 minutes prior to fluorescence measurements. A single upper canopy leaf was monitored through the duration of the drying cycle. Light intensity at the leaf surface was 14nE cm^{-2} s^{-1}.

RESULTS

Water stress was induced using a drying cycle regime in which soil was pulse-watered daily to field capacity. Then, at a specified time, the daily watering was suspended. Net photosynthesis was monitored each day during the water deprivation period. Fig. 1 shows the response of net CO_2 gas exchange from cineraria and *S. heritieri*. Upon visible wilting, net gas exchange was similarly halted in both groups.

In addition to net CO_2 exchange, we monitored the induction kinetics of chlorophyll fluorescence. In short, plants were adapted to darkness by a 30 min dark incubation. Dark adaption provides for the dissipation of electrons allowing electron carriers to exist in the oxidized state, thereby relaxing the photosynthetic apparatus. When a leaf was illuminated following dark-adaption, the fluorescence emission displayed characteristic changes known as the Kautsky effect (Fig. 2 [inset]). The Kautsky effect is seen as a set of reproducible transients in fluorescence intensity that occur when dark incubated, photosynthetic tissue is illuminated with continuous light. The changes in fluorescence intensity reflect the start-up of the photosynthetic process and should be regarded as primarily reflecting excitation and energy conversion at PS II. However, due to the functional connection of PS II to the other components of the photosynthetic apparatus, fluorescence yield is an indirect indicator of the whole photosynthetic process (23). Papageorgiou's (22) designation of fluorescence transients (O-I-D-P-S-M-T) are used in this study to describe the characteristic induction phases and to convey the drought-induced changes in fluorescence. Interpretation is complex and

will be addressed in more detail below. Suffice it to say at this point, fluorescence emission between the onset of illumination and the peak P (fast changes) are due to photochemistry, while the subsequent transients (slow changes) are due to enzymology, perhaps owing to the light-dependent activation of Calvin cycle enzymes.

Fig. 2 shows the fluorescence induction kinetics from well-watered and stressed plants. The fluorescence kinetics of S. heritieri were similar to those of cineraria before and subsequent to water deprivation. All of the kinetic parameters of chlorophyll fluorescence induction were quantified and plotted as a function of the duration of the drying cycle. These included O, P, the P:O ratio (variable fluorescence), and the maximum slope of decline from P (RQ). Only one kinetic parameter, RQ, was observed to correlate with the onset of wilting and the loss of net photosynthesis. RQ refers to the maximal rate of fluorescence quenching from the major peak P (5). Fig. 3 shows the gradual decline in RQ that occurred during the water deprivation period. These results suggest that RQ could reflect the water status of the plant, thereby aiding in a study aimed at mapping the onset and recovery of stress-induced changes in photosynthetic tissue.

Gardner and Nieman (6) showed that leaves undergo a daily rhythm of water status. As stomata open, transpiration occurs and a water deficit is generated in the leaves. In well-watered systems, water absorption by roots resupplies the leaves such that the water deficit is moderate. In drying experiments such as ours (exemplified in Figs. 1 and 3), water resupply is increasingly slow as soil moisture progessively declines throughout the duration of the drying cycle. Therefore, if RQ were a monitor of leaf water status, we might expect

daily oscillations in RQ that decrease in magnitude as drought stress continues and becomes more severe reflecting the findings of Gardner and Nieman (6).

Fig. 4 shows the daily oscillations of RQ in plants maintained in soil that was continuously watered through a water column system (days 1 & 2). RQ was monitored at morning (8am), midday (1pm) and evening (8pm) intervals. RQ reached a minimum at midday in all plants, followed by a recovery the next morning. The drying cycle was initiated on day 3 following the morning measurements. Between days 4 and 5, there was no morning recovery. In addition, RQ continued to decline as predicted until water was added. Upon water addition, RQ began to recover by midday and leaves that were wilted before watering had regained turgidity by that evening. However, RQ did not recover to pre-stress levels during that period. Therefore, the photosynthetic apparatus may have incurred irreversible damage resulting in the depressed RQ observed in turgid leaves. Furthermore, variability in RQ can be attributed to the timing of readings within each daily cycle. This implies that major changes in RQ in water-sufficient plants represents reversible changes.

In subsequent experiments, we addressed this variability in RQ by measuring RQ at only one time during the day (morning). Fig. 5 shows the results of such an experiment, in which fluorescence parameters and leaf water potentials were monitored during a drying cycle initiated by removing plants from water columns. At the initiation of the drying cycle, the water status of each plant depended on several variables including total transpiration, root growth and the area of soil surface uncovered. Therefore, the initial leaf water potential of each plant

was not uniform at the start of the drying cycle. These factors also influenced the rate of water removal from the soil and thereby the time required for each plant to complete the cycle. We have addressed these problems by plotting the change in water potential as a function of the time to complete the drying cycle. The difference in drying rate between individuals within a group contributed to the standard deviation observed at any one time point. However, it is clear that the decline in RQ correlates with the change in leaf water potential.

The data from Fig. 5 are replotted in Fig. 6 to better assess the relationship between the change in leaf water potential and the decline in RQ. A regression coefficient for the regression line is given for each plant group to denote the degree of confidence expressed by the relationship between RQ and the change in leaf water potential. Interestingly, a 40 percent reduction of RQ corresponded to a change in leaf water potential of -5 to -9 bars in cineraria while the same reduction in RQ resulted in a -14 bar change in *S. heritieri*. Thus, a greater change in leaf water potential was required to produce a similar decline of RQ in the progenitor relative to cineraria. This suggested that the photosynthetic physiology of these two groups responded differently to drought-induced alterations.

DISCUSSION

In this report, we have used chlorophyll-fluorescence induction to follow drought-induced changes at the photosynthetic level. Three major points are noteworthy. First, water deprivation did not alter the variable portion of fluorescence. Rather, the rate of quenching from P (RQ) declined as a result of soil drought. As will be discussed later, this suggests that PS II is not targeted by water stress. Second, diurnal oscillations of RQ were observed in well-watered plants. Furthermore, water stress resulted in the cessation of the daily cycling. Following the addition of water, RQ did not return to prestress levels. The implication of these observations are discussed within the context of reversible and irreversible damage to the photosynthetic apparatus. Third, and perhaps most interesting from a taxonomic point of view, the stress-induced decline in RQ correlated with a decrease in leaf water potential. Thus, the use of fluorescence in breeding programs may warrant consideration.

Several studies have used chlorophyll fluorescence to monitor the effects of drought on photosynthetic processes (5, 8, 9, 11, 21). The assumption is that stress-induced changed in the photosynthetic apparatus will be reflected in corresponding changes in the fluorescence induction kinetics. Analysis of these changes should then yield information on the site and extent of the stress-induced damage. However, at this time, an unambiguous interpretation of these transients is not possible. Nonetheless, basic interpretations are afforded as a result of increasing investigations into the nature of fluorescence induction. A brief explanation of the designated

transients and their relevance to photosynthetic processes follows. A more complete description is available elsewhere (16).

After dark incubation, the electron transport acceptors of PS II should be in the oxidized state. Upon illumination, there is an initial rise in fluorescence to a level designated O. This rise occurs within msec and is thought to represent fluorescence emission by excited chlorophyll molecules occurring before the absorbed light energy has migrated to the reaction centers. The subsequent rise of fluorescence from O is seen as a set of reproducible transients, which comprise the variable component of fluorescence. Variable fluorescence can be represented as the ratio of P:O. The rise from O to a minor peak I is thought to represent the migration of electrons to the primary electron acceptor of PS II. When the acceptor is reduced, fluorescence emission is increased as a result of the transient block in PS II photochemistry. A further rise to a major peak P is correlated to a decline in O_2 evolution and is caused by a transitory block of electron transport through PS I. Such a block would pile up electrons in the electron transport chain leading to an increased reduction of PS II acceptors. Since fluorescence arises from PS II, this transient block is seen as an increase in fluorescence. Furthermore, the block in PS I activity that causes the rise to P is probably due to a lack of $NADP^+$ acting as electron acceptors. Thus, the decline from P, (RQ), is mediated by the reoxidation of NADPH in the Calvin cycle with CO_2 acting as the final substrate.

STRESS INDUCED CHANGES IN FLUORESCENCE

Havaux et al. (11) and Govindjee (9) have reported that water

stress leads to a decrease in variable fluorescence owing to a decrease in the level P. These investigators suggest that the decrease in the P:O ratio is due to damage caused on the water oxidation side of PS II. This effect is overcome when leaf discs are pre-incubated with electron donors to PS II (11). Bradbury and Baker (3) have shown that damage on the reducing of PS II resulting from photoinhibition is also seen as a decline in the P:O ratio. In contrast, Fig. 2 shows that variable fluorescence was unaffected by water stress in Senecio heritieri and cineraria suggesting that PS II is not targeted by water stress. The reduction of net photosynthesis (Fig. 1) did, however, correlate with a decline in RQ (Fig.2 & 3).

Conroy et al. (5) and Hetherington and Smillie (12) have observed a gradual decline in RQ during dehydration suggesting a preferential slowing of electron flow subsequent to PS II supporting the view that photophosphorylation is the primary target of water stress. From our data, it can not be determined whether the chloroplast coupling factor or the Calvin cycle is preferentially targeted since a decline in either could produce the decline in RQ.

In addition, it is unlikely that the decline in RQ is attributable to a stress-induced decline of internal CO_2 owing to stomatal closure. The dark adaption period prior to the fluorescence assay should cause stomatal closure in both water-stressed and well-watered plants. Thus, internal CO_2 concentrations would be similar at the end of the dark incubation period regardless of stress treatment. Wong et al. (28) have shown that water stress reduced the rate of CO_2 assimilation and leaf conductance similarly such that intercellular partial pressures of CO_2 remained constant. Furthermore, Nakamoto et al. (20) monitored

chlorophyll fluorescence induction in isolated spinach chloroplasts that were exposed to varying concentrations of CO_2. There . showed that varied CO_2 caused changes in the induction transients subsequent to RQ. Therefore, we argue that the changes in RQ observed prior to water deprivation and the continual decline in RQ induced by water deprivation is the result of non-stomatal factors.

DIURNAL OSCILLATIONS OF RQ

Fig. 4 shows that RQ undergoes daily oscillations in water-sufficient plants. The oscillations of RQ may reflect daily oscillations in leaf water potential observed by Gardner and Nieman (6). However, Kalt-Torres et al. (14) showed that there are also daily rhythms in assimilate export rate and enzyme activities that relate to diurnal changes in photosynthesis. It is likely that a combination of both is responsible for the reversible midday decline of RQ since changes in water potential may be closely linked to assimilate export rates and enzyme activity. The cessation of these rhythms and the subsequent decline in RQ observed during increasing drought may reflect irreversible alterations. This suggestion is supported by the fact that RQ did not return to pre-stress levels after water addition, even though leaves were no longer visibly wilted. Thus, measurement of the daily cycles of RQ may aid in monitoring the reversible and irreversible changes in photosynthesis observed in natural conditions.

FLUORESCENCE AND WATER POTENTIAL

Although the underlying factors controlling fluorescence induction are not well understood, we have shown that soil drought causes a

decline of RQ during water deprivation (Fig. 2). In cineraria and *S. heritieri*, RQ was the only transient altered. This may reflect similar sites of stress-induced inhibition. However, this is not necessarily the case since similar changes in RQ are equally attributable to changes in photophosphorylation or carbon reduction. Nonetheless, analysis of induction kinetics alone does not reveal a marked difference in the response of these two groups at the photosynthetic level. When leaf water potentials are taken in conjunction with fluorescence measurements (Fig. 5), it is clear that the the decline in RQ is closely tied to the decrease in plant water status. Further analysis of these data (Fig 6) reveals an interesting relationship that can be seen by comparing the change in water potential for each group when RQ is reduced by 40 percent (arbitrary). The xerophyte, *S. heritieri*, requires a greater change in leaf water potential (-14 bars) than cineraria (-5 to -9 bars) to cause a similar decrease in RQ. This suggests that if alterations in the photosynthetic physiology reflect the drought tolerance of a species, cineraria may be perceived as the more drought-sensitive group.

In this context, chlorophyll fluorescence may aid in identifying the underlying genetic factors controlling the photosynthetic response of water-stressed plants. From this study, the relative contribution of physiological traits from *S. heritieri* can not be determined without similar information from the other possible progenitors of cineraria. Nonetheless, we have shown that there is a differential response to water deprivation in these two groups. Furthermore, we have demonstrated that chlorophyll fluorescence warrants consideration as a useful tool to assess relative drought tolerance. However, the use of

fluorescence in this manner may be restricted to controlled environments where other factors are not allowed to contribute to the stress-induced changes. Furthermore, measurement of other related physiological parameters may also be necessary.

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Figure Legends

Fig. 1. Net photosynthesis of Senecio heritieri, cv ONE and cv FOUR monitored during a period of water deprivation (cultivar names are as describes in materials and methods). Measurements from wilted leaves are indicated by the arrows. Each point represents a single determination taken from the same leaf during the drying period.

Fig. 2. Room temperature chlorophyll fluorescence transients from leaves irradiated with 450 nm light subsequent to a 30 min dark adaption period. Only the variable portion of fluorescence is shown. The same leaves used for net photosynthesis determinations are represented. The inset denotes a fluorescence transient with characteristic fluorescence levels (O-I-D-P) observed in Florist's Cineraria and S. heritieri. RQ is the maximal rate of fluorescence quenching (maximum slope) from the point P.

Fig. 3. Percent of the initial rate of quenching (RQ) during a drying cycle experiment as described in material and methods. Trial 1 represents the %RQ monitored on a daily basis from those plants represented in Figs. 1 and 2. Trial 2 represents a different set of individuals. Closed symbols represent measurements taken from wilted leaves. Each point is an average of two readings taken from the same leaf. ($P \leq .05$)

Fig. 4. The rate of quenching (RQ) at 8am, 1pm and 8pm. Plants received water during days 1 and 2. At time "A", water was withheld. At time "B", water was added to end the drying cycle. Each point is an average of two

readings taken from the same leaf. ($P \leq .05$)

Fig. 5. The change in leaf water potential (Ψw) and percent of initial RQ monitored during a drying cycle. Water deprivation began on day 0 and was ended after all plants within a group had wilted. Each point represents an average of three different plants. Standard deviation is indicated by the vertical bars.

Fig. 6. A replot of the data obtained from the experiment described in Fig. 5. The regression coefficient (r) of the linear-regression line is shown at the top of each graph. Arrows represent the change in leaf water potential corresponding to a 40% decrease from the initial RQ.

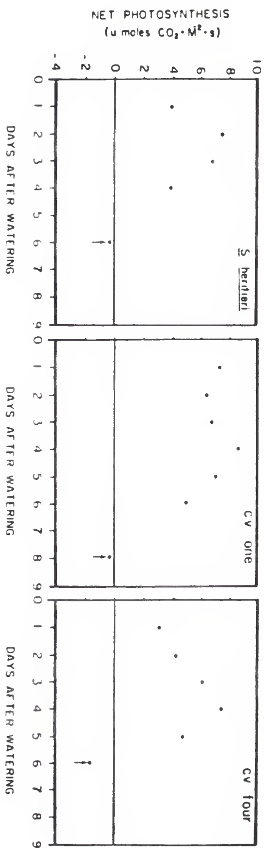
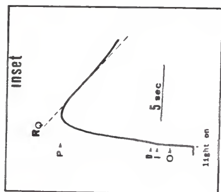


Fig 1



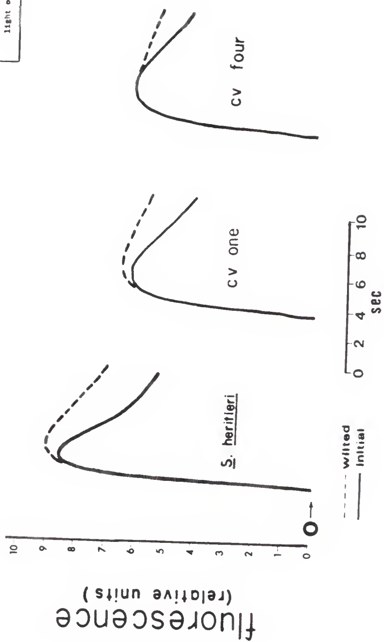


Fig 2

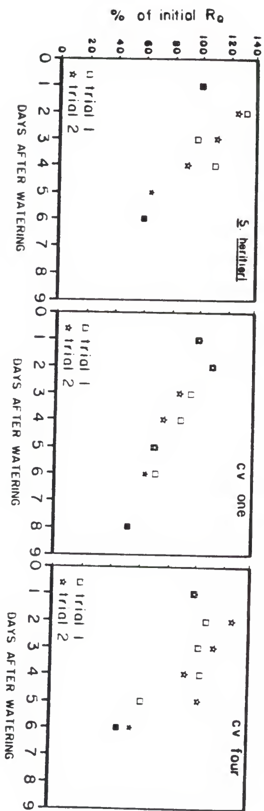


Fig 3

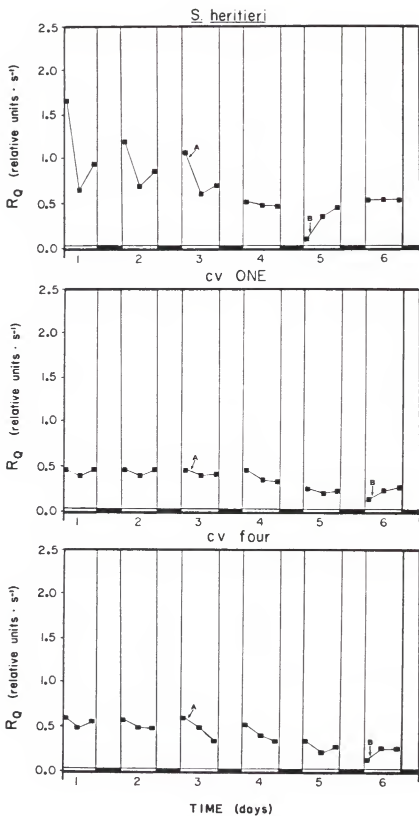


Fig 4

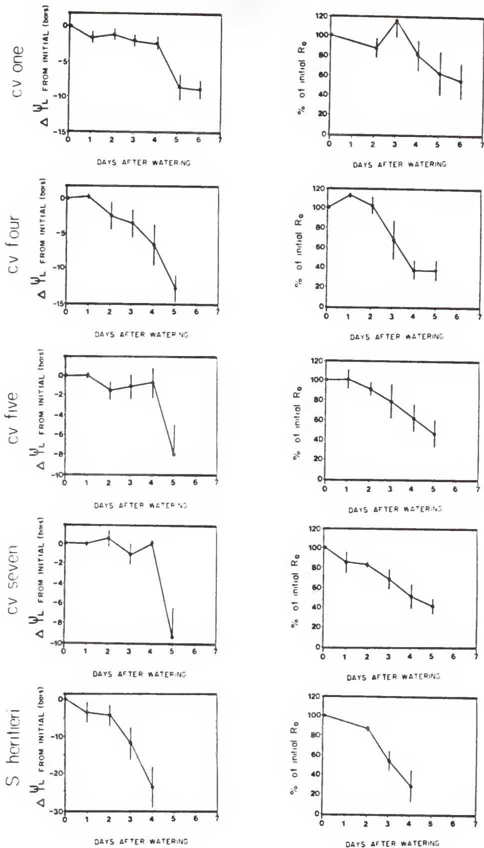
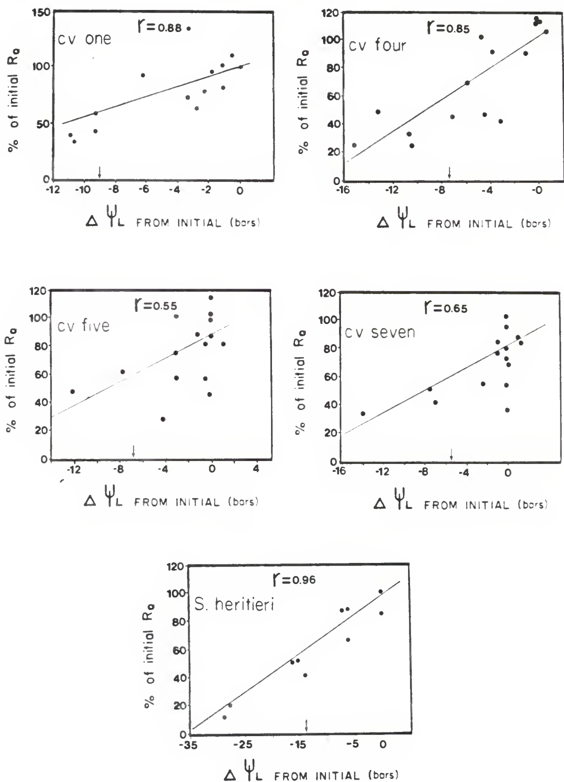


Fig 5

Fig 6



DROUGHT-INDUCED CHANGES IN CHLOROPHYLL FLUORESCENCE IN FLORISTS' CINERARIA AND A XEROPHYTIC PROGENITOR

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ABSTRACT

Water stress leads to a decline in net photosynthesis and altered chloroplast activity. Furthermore, the set of chloroplast reactions targeted by water stress may be species specific. We have characterized water-stress induced changes in the cultivated complex Florists' Cineraria (*Senecio cruentus*) and a progenitor species (*Senecio heritieri*). When stress was slowly applied by soil drought, net photosynthesis and chlorophyll fluorescence transients were altered similarly at wilting. Variable fluorescence was unchanged while the rate of fluorescence quenching declined. This suggests that water stress does not target photosystem II electron transport. Interestingly, the xerophytic progenitor required a greater change in leaf water potential to produce a similar change in the rate of fluorescence quenching relative to Florists' Cineraria. If alterations in the photosynthetic physiology reflect the relative drought tolerance of a species, the cultivated complex may be perceived as more drought sensitive.

In addition, we observed a diurnal cycling in those fluorescence transients influenced by water deprivation. This correlates with the diurnal changes in plant water status, suggesting that fluorescence induction may provide an indirect assay of plant water status.