ADAPTIVE SIGNIFICANCE OF PHOTOSYNTHETIC AND METABOLIC REGULATION IN *NICOTIANA TABACUM* L. PLANTS DURING DROUGHT STRESS

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PREFACE

This thesis is an analysis of the little we currently know of how four tobacco cultivars of different tolerance respond to drought stress, and how drought stress may theoretically affect plant processes. It is hoped that the strongly advocated views, many hypotheses or speculations will serve as an impetus to progress. Furthermore, no pretence is made here as to completeness in covering the literature or possible adaptive responses to drought stress.

It also highlights the complexity of the physiological and metabolical consequences of drought stress, from which it becomes clear that virtually all aspects of the plant's economy are altered. At a first glance, the myriad changes elicited by drought stress appear too numerous and diverse to be of use in the analysis of crop yield and water use, with the consequences that this complexity could easily lead one to despair of ever achieving an accurate knowledge of the system. Fortunately, as will be shown, complete understanding is not a prerequisite for the effective practical utilization of the basic concepts.

This optimism appears to be warranted as it is highly probable that the array of drought stress-induced responses in a plant are tightly orchestrated through metabolic controls and by the long-distance communication network of plant hormone, water, and assimilate transport. Therefore, in this context, it is not necessary to keep track of all the induced changes as a few well-chosen signposts will do.

I hope that this thesis will be of particular interest to researchers seeking a critical evaluation of some of the most prominent drought tolerance adaptive responses, but we also trust that it will be of interest to a wide range of plant scientists including molecular biologists, physioligists, agriculturalists, ecologists and plant breeders concerned with drought stress prone environments.

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ABSTRACT

ADAPTIVE SIGNIFICANCE OF PHOTOSYNTHETIC AND METABOLIC REGULATION IN *NICOTIANA TABACUM* L. PLANTS DURING DROUGHT STRESS.

The subject matter covered in this thesis has close affinities with that variously known as wholeplant physiology, environmental plant physiology and crop physiology, although each of these terms mean different things to different people. Emphasis was placed on integration and trying to assess quantitatively the importance of each physiological trait identified which may contribute to the drought tolerance of a tobacco (*Nicotiana tabacum* L.) cultivar, in the context of the whole plant-environmental system. Four tobacco cultivars, TL33, CDL28, GS46 and ELSOMA, in sequence of increasing drought tolerance, were subjected to intensifying drought stress under controlled environmental conditions by withholding watering. Both photosynthesis related and other drought stress-induced metabolic changes, were comparatively monitored with changes in the severity, duration and time course of the stress. This was done in order to gain more insight into the underlying biochemical mechanisms, which may bestow differential degrees of drought tolerance on tobacco cultivars and which could in practice lead to more clearly defined quantitative parameters being implemented in drought tolerance selection programmes.

When the stomatal and non-stomatal limitations to photosynthesis were evaluated, a drought stress-induced decrease in the carboxylation efficiency ($\partial A/\partial c_i$) which was less pronounced in the drought-tolerant cultivars, was observed. This coincided with a slower stress-induced increase in the CO_2 compensation point (Γ) and intercellular CO_2 concentration (c_i) in the drought-tolerant cultivars, which was due to the maintenance of higher net photosynthetic rates (A). All cultivars showed a decrease in water use efficiency (WUE) and hence an increase in the marginal cost in terms of water used to carbon gained ($\partial E/\partial A$). These changes occurred more slowly in the drought-tolerant cultivars, which was due to the maintenance of higher A values, as stomatal conductance (g) decreased more slowly in spite of higher transpiration rates (E). The relative degree of stomatal limitation (l) did not increase much (ca. 35%), nor differ significantly among the cultivars. The increase in c_i and Γ was interpreted as indicating that mesophyllic rather than stomatal factors were responsible for the drought stress-induced decrease in A, because of the decrease in the $\partial A/\partial c_i$ component of the mesophyllic photosynthetic capacity. From the results obtained when the partial photochemical reactions were determined polarographically with isolated thylakoids, it became clear that in all four cultivars drought stress induced a decline in the quantum efficiency (Φ), PSI-, PSII-, and PSI + PSII-activity. The nature and extent of this decline, however, differed between the respective cultivars. The Φ, PSII- and PSI + PSII- activity of the drought-tolerant cultivars were characterised by a fast initial decline, which subsequently declined at a slower continuous rate as the drought stress intensified. PSII-

activity was found to be more drought sensitive than PSI-activity, and even more so in the case of the drought-sensitive cultivars. The observation that PSII-activity was most affected by drought stress is corroborated by the fluorescence data which indicated that the I/P-ratio and ΔF-values of the drought-sensitive cultivars increased earlier and reached higher end values. This indicated that the drought-tolerant cultivars were characterised by a higher photosynthetic efficiency at low Ψ_L.

On evaluating some of the more biochemical drought stress-induced changes, a progressive highly significant ($p < 0.01$), but differential, increase in glutathione reductase activity was detected in all four cultivars as their leaf water potential (Ψ_L) decreased. On reaching Ψ_L of -2.51 MPa, the glutathione reductase activity of the drought-sensitive cultivars increased by 159% (TL33) and 187% (CDL28), as opposed to the 233% (GS46) and 250% (ELSOMA) in the drought-tolerant cultivars. In spite of an initial lag (up to a Ψ_L of ca.-1.5 MPa), on average, the superoxide dismutase (SOD) activity of the drought-tolerant cultivars increased by 244% while that of the drought-sensitive cultivars only increased by 161%. Contrary to all other enzyme activities monitored, the moderate increase (doubled at most) in catalase activity was more pronounced ($p < 0.05$) in the drought-sensitive cultivars. Increased ascorbate peroxidase activity was not only observed to be ca. 300-400% higher in the drought-tolerant cultivars under stress, but was also more pronounced than the increase in catalase activity. This seem to indicate that ascorbate peroxidase rather than catalase may be mainly responsible for scavenging drought stress-produced H_2O_2.

A statistically significant ($p < 0.01$) decline in both water soluble protein concentration and total number of -SH groups already occurred at a Ψ_L of ca.-0.77 MPa. The initial rate of decline in both the concentration of water-soluble protein and number of -SH groups, as well as the end-values reached by both these parameters, differed significantly between the drought-tolerant and drought-sensitive cultivars. In contrast with the fast initial decline observed in GS46 and ELSOMA at light stress levels, which stabilised and occurred more gradually as the drought stress intensified, the concentration of water-soluble protein and number of -SH groups declined at a slower but continuous rate in TL33 and CDL28 as Ψ_L decreased. At a Ψ_L of -2.51 MPa, the number of -SH groups present in GS46 and ELSOMA respectively, was 63.6% and 65.2%, and that of TL33 and CDL28 39.9% and 46.8% of their respective control values. Furthermore, the concentration of water-soluble protein of ELSOMA was still 75.5% and that of GS46, 65.4% of their controls, even at a Ψ_L of ca.-2.51 MPa, which contrasts with the corresponding values of 46.6% and 55.3% for TL33 and CDL28, respectively.

The tobacco plants adjusted largely to drought stress by the accumulation of solutes.

This resulted in a decrease in osmotic potential at full turgor (Ψ_{π}^{100}), which was more pronounced in the drought-tolerant cultivars. Drought stress-induced an increase in the proportion of bound water (B) and the volumetric bulk modulus of elasticity ($\in$), but did not alter the relative water content at incipient plasmolysis (RWC°). In spite of the latter response, due to the lower ε of the drought-sensitive cultivars, the Ψ_L at which incipient plasmolysis ($\Psi_L°$) occurred was consistently less negative in these cultivars. Furthermore, because of their higher ε (ca. 0.23 MPa for GS46, and 0.28 MPa for ELSOMA) the threshold Ψ_L values at which both ABA and proline accumulate rapidly occurred earlier in these cultivars. A substantial accumulation of both ABA and proline was observed in all four cultivars, the extent of which in the case of the proline, but not necessarily the ABA, correlated positively with the drought tolerance of the four cultivars. When, with the aid of antibodies produced against a bovine serum albumin -(±)- ABA conjugate, quantitative comparisons of the ABA concentrations in the sub-cellular compartments of GS46 were made prior to and when the plants were drought stressed, at Ψ_L of ca. -0.45 MPa, a quantitatively similar positive immunogold labelling pattern was observed in both chloroplasts and apoplast. However, at a Ψ_L of ca. -1.55 MPa a twofold drought stress-induced increase in the apoplastic ABA concentration was observed while the ABA concentration in the chloroplasts did not differ from that of the controls.

Results are also presented, which emphasise the adaptive significance of effective leaf movements and indicate that drought stress specifically influence the carotenoid composition. A strong quantitative correlation existed between the formation of zeaxanthin and the type of fluorescence quenching indicative of non-radioactive energy dissipation, which occurred to a lesser extent in GS46 and ELSOMA. Ultrastructural observations, indicated that the drought-tolerant cultivars mobilised a more than adequate starch reserve to a greater extent. Anatomical differences in terms of differences in resistance to water flow and the percentage intercellular spaces exist between the respective cultivars. Both these traits correlated positively with the slower decrease in WUE and faster recovery upon rehydration in the drought-tolerant cultivars of several of the physiological parameters monitored.

From the results presented and extensive literature search regarding the physiological changes monitored, which are discussed in relation to their physiological cost and potential drought tolerance adaptive advantage, it is clear that drought tolerance in tobacco, as is the case in other plants, is the complex consequence of many physiological, biochemical and morphological factors. Furthermore, as each of the traits examined forms part of an integrated plant response, it would seem as if plant breeders should be selecting the best sets of physiologically based adaptive traits for particular environments.

It is in this regard that the following general conclusions (the details of which and

potential selection value are given in each paper) drawn from this investigation, may be of value: Drought tolerance may be:

(i) resistance to (a) stress induced decreases in $\partial A/\partial c_i$, that could result in the maintenance of higher A rates, which is vital for fast recovery upon rewatering, or (b) -SH oxidation or -SH/-SS- interchange as best explained by a combination of the -SH/-SS- hypothesis and the concept of protein turnover,

(ii) the consequence of regulatory mechanisms such as, (a) PSII-regulation as reflected by fluorescence changes, or (b) an effective antioxidant system,

(iii) a capability to (a) predict (ABA accumulation), (b) postpone (by osmoregulation), or (c) to limit the severity (by proline accumulation) of the stress, and/or finally

(iv) the result of environmentally driven anatomical changes such as (a) differences in vessel length and diameter, or (b) differences in the percentage intercellular spaces.

OPSOMMING

DIE AANPASSINGSWAARDE VAN FOTOSINTETIESE- EN METABOLIESE REGULERING IN *NICOTIANA TABACUM* L. PLANTE GEDURENDE DROOGTESTREMMING.

Die onderwerp behandel in hierdie proefskrif het hou verband met dit wat algemeen bekend staan as heelplant-plantfisiologie, omgewings-plantfisiologie en gewasfisiologie, alhoewel elk van hierdie terme 'n ander betekenis vir elke persoon mag hê. Klem is gelê op integrering en daar is gepoog om die belang van elke fisiologiese eienskap, wat moontlik tot die droogteverdraagsaamheid van 'n tabak (*Nicotiana tabacum* L.) kultivar mag bydra, te identifiseer en in die konteks van die heelplant-omgewingsisteem kwantitatief te evalueer. Vier tabakkultivars, in volgorde van toenemende droogteverdraagsaamheid, naamlik: TL33, CDL28, GS46 en ELSOMA, is onder gekontroleerde omgewingstoestande aan droogtestremming met toenemende intensiteit, deur die weerhouding van water, onderwerp.

Beide fotosintese-verwante en ander droogtestremming-geïnduseerde metaboliese veranderinge is vergelykenderwys gemonitor met verandering in die intensiteit, duur en tydsverloop van die stremming. Dit is gedoen om 'n diepgaande insig te verkry in die onderliggende biochemiese meganismes wat moontlik in verskillende mate droogte-verdraagsaamheid aan tabakkultivars mag verleen en wat in die praktyk kan lei tot die implimentering van meer duidelik gedefinieerde kwantitatiewe parameters in programme vir die seleksie vir droogteverdraagsaamheid.

Tydens die evaluering van die stomatale en nie-stomatale beperking van fotosintese, is 'n droogtestremming geïnduseerde afname in die karboksileringseffektiwiteit ($\partial A/\partial c_i$), wat in die geval van die droogteverdraagsame kultivars minder prominent was, waargeneem. Dit het saamgeval met 'n stadiger stremming-geïnduseerde toename in CO_2-kompensasiepunt (Γ) en intersellulêre CO_2-konsentrasie (c_i), by die droogteverdraagsame kultivars weens die handhawing van hoër netto fotosinese tempos (A). Al die kultivars het 'n afname in waterverbruikseffektiwiteit (WUE) en dus ook 'n toename in die eenheidskoste van waterverbruik tot koolstof wins ($\partial E/\partial A$), vertoon. Weens die handhawing van hoër A-waardes tydens verminderde stomatale geleiding (a) het hierdie veranderinge, ten spyte van hoër transpirasietempo's (E), stadiger by droogteverdraagsame kultivars plaasgevind. Die relatiewe mate van stomatale beperking (l) het nie veel (ca. 35%) toegeneem of merkbaar tussen die kultivars verskil nie. Die toename in c_i en Γ is geïnterpreteer as aanduiding dat mesofilliese, eerder as stomatale faktore verantwoordelik was vir die droogtestremming-geïnduseerde afname in A, as gevolg van die afname in die $\partial E/\partial c_i$, komponent van die mesofilliese

fotosintesekapasiteit. Op grond van resultate verkry met behulp van die polarografiese bepaling van elektronvloeitempo van fotochemiese reaksies in geïsoleerde tilakoiedmembrane, het dit geblyk dat droogtestremming 'n afname in die kwantumeffektiwiteit (Φ), PSI-, PSII- en PSI + PSII- aktiwiteit geïnduseer het, waarvan die aard en omvang egter tussen die onderskeie kultivars verskil het. Die Φ, PSII- en PSI+ PSII- aktiwiteit van die droogteverdraagsame kultivars is aanvanklik gekenmerk deur 'n vinnige aanvanklike afname wat later teen 'n stadiger kontinue tempo afgeneem het, soos die droogtestremming geïntensifeer het. Daar is bevind dat PSII-aktiwiteit meer droogtesensitief as PSI- aktiwiteit is en selfs meer so in die geval van die droogtesensitiewe kultivars. Dié waarneming dat PSII- aktiwiteit in 'n groter mate deur droogtestremming beïnvloed word, word gestaaf deur die fluoressensie data. Deurdat die I/P-verhouding en ΔF-waardes van die droogtesensitiewe-kultivars vroeër gestyg het en hoër eindwaardes bereik het, wys daarop dat die droogteverdraagsame kultivars deur 'n hoër fotosintese-effektiwiteit by lae blaarwater-potensiaal (Ψ_L)-waardes getipeer word.

Gedurende die evaluering van sommige van die meer biochemiese droogtestremming-geïnduseerde veranderinge is 'n progressiewe, hoogs betekenisvolle ($p < 0.01$), maar differensiële toename in glutatioonreduktase-aktiwiteit met 'n afname in Ψ_L in al vier kultivars waargeneem. By 'n Ψ_L van ca. -2.51 MPa het die glutatioonreduktase-aktiwiteit van die droogtesensitiewe kultivars slegs met 159% (TL33) en 187% (CDL28) toegeneem, teenoor die 233% (GS46) en 250% (ELSOMA) in die droogteverdraagsame kultivars. Ten spyte van 'n aanvanklik vertraging (tot 'n Ψ_L van ca. -1.5 MPa), het die gemiddelde superoksied-dismutase (SOD)-aktiwiteit van die droogteverdraagsame kultivars met 244% toegeneem terwyl dié van die droogtesensitiewe kultivars met slegs 161% toegeneem het. In teenstelling met al die ander ensiem-aktiwiteite wat gemonitor is, was die matige (op die meeste verdubbel) toename in katalase-aktiwiteit hoër ($p < 0.05$) in die droogtesensiteiwe kultivars. Verhoogde askorbaatperoksidase-aktiwiteit was nie slegs ca. 300-400% hoër in die droogteverdraagsame kultivars gedurende vogstremming nie, maar was ook meer dramaties as die toename in katalase-aktiwiteit wat daarop mag dui dat askorbaatperoksidase eerder as katalase verantwoordelik is vir die opruiming van droogtestremming-geproduseerde H_2O_2.

'n Statisties betekenisvolle ($p < 0.01$) afname in beide die konsentrasie wateroplosbare proteïene en totale aantal sulfhidrielgroepe (-SH) het reeds by 'n Ψ_L van ca. -0.77 MPa voorgekom. Die aanvanklike afnametempo in die konsentrasie wateroplosbare proteiene en aantal -SH- groepe, sowel as die eindkonsentrasies en eindtotaal van dié parameters, het opvallend verskil tussen die droogteverdraagsame- en droogtesensitiewe kultivars. In teenstelling met die aanvanklike vinnige afname (gedurende ligte vogstremming) wat gestabiliseer het en baie meer geleidelik plaasgevind het tydens intense vogstremming in die geval van GS46 en ELSOMA, het die konsentrasie wateroplosbare proteïene en aantal -SH-groepe teen 'n

stadiger maar kontinue tempo oor die totale vogstremmingsbereik in TL33 en CDL28 afgeneem. By 'n Ψ_L van ca. -2.51 MPa was die aantal -SH-groepe teenwoordig in GS46 en ELSOMA onderskeidelik 63.6% en 65.2% en in TL33 en CDL28 onderskeidelik 39.9% en 46.8%. Net so was die konsentrasie wateroplosbare proteïene van ELSOMA steeds 75.5% en die van GS46 65.4% van hul onderskeie kontrolewaardes by 'n Ψ_L van ca. -2.51 MPa. Dit is in kontras met die ooreenstemmende waardes van 46.6% en 55.3% vir TL33 en CDL28 onderskeidelik.

Die tabakkultivars het ook tót 'n groot mate gedurende die droogtestremming aangepas deur die akkumulering van opgeloste stowwe, met die gevolg dat daar 'n afname in die osmotiese potensiaal by volle turgor ($\Psi_\pi°$), wat meer dramaties in die geval van die droogteverdraagsame kultivars was, plaasgevind het. Die droogtestremming het ook 'n toename in die verhouding gebondewater (B) en die volumetriese elastisiteitsmodulus (ϵ) teweeg gebring, maar het nie die relatiewe waterinhoud waarby aanvangsplasmoliese (RWC°) plaasgevind het, verander nie. Ten spyte van laasgenoemde, weens die laer ε van die droogtesensitiewe kultivars, was die Ψ_L waarby aanvangsplasmoliese ($\Psi_L°$) plaasgevind het egter deurentyd minder negatief in dié kultivars. Verder, weens hul hoër ε (ca. 0.23 MPa in GS46 en 0.28 MPa in ELSOMA) was die drumpel Ψ_L waarby sowel absisiensuur (ABA) en prolien vinnig begin akkumuleer, vroeër deur hierdie kultivars bereik. 'n Aanmerklike toename in beide ABA en prolien is in al vier kultivars waargeneem met 'n afname in Ψ_L , die omvang waarvan, in die geval van die prolien, maar nie noodwendig die geval van ABA nie, positief met die droogteverdraagsaamheid van die vier kultivars gekorreleer het nie. Kwantitatiewe vergelykings van die ABA-konsentrasies in die subsellulêre kompartemente van GS46 voor en tydens droogtestremming, is gemaak met die immunogoud merk prosedure (bees albumien serum - (±) - ABA konjugaat). Dit is bevind dat by 'n Ψ_L van ca. -0.45 MPa daar kwantitatief 'n ooreenstemmende immunogoudmerkpatroon in die chloroplas en apoplas voorkom, terwyl by 'n Ψ_L van ca. -1.55 MPa 'n verdubbeling van die ABA-konsentrasie in die apoplas plaasvind sonder 'n noemenswaardige verandering in die ABA-konsentrasie in die chloroplas relatief tot die van die kontrole.

Resultate word ook aangebied wat die aanpassingswaarde van effektiewe blaarbewegings beklemtoon en ook aandui dat droogtestremming die karotenoidsamestelling spesifiek beïnvloed. 'n Sterk kwantitatiewe korrelasie bestaan tussen die vorming van zeaxanthien en die tipe van fluoresensieblussing, wat dui op nie-uitstralende energieverstrooiing, waarvan laasgenoemde tot 'n mindere mate in GS46 en ELSOMA plaasgevind het. Ultrastrukturele ondersoeke het getoon dat die droogteverdraagsame kultivars hul meer as genoegsame styselreserwe in 'n groter mate gedurende vogstremming mobiliseer. Anatomiese verskille, in terme van die weerstand teen watervloei en persentasie intersellulêre lugruimtes is ook tussen die onderskeie kultivars waargeneem. Beide hierdie eienskappe het positief gekorreleer met die stadiger afname in WUE en vinniger herstel na herbenatting by die droogteverdraagsame kultivars. Dit was ook die geval

by verskeie van die fisiologiese parameters wat gemonitor is.

Uit die resultate wat aangebied is en die uitgebreide literatuurstudie, in hierdie verband, is dit duidelik dat droogteverdraagsaamheid in tabakkultivars die komplekse resultaat van verskeie fisiologiese, biochemiese en morfologiese faktore is. Verder, omdat elkeen van die kenmerke wat gemonitor is deel van 'n geïntegreerde plantreaksie vorm, wil dit voorkom asof planttelers die beste stelle fisiologies-gegronde aanpassingseienskappe vir 'n bepaalde omgewing moet selekteer. In hierdie opsig mag die volgende algemene gevolgtrekkings (waarvan die besonderhede aangaande die potensiële gebruik daarvan as seleksie kriteriums in elke hoofstuk verskaf word) soos dit uit die resultate van hierdie ondersoek geblyk het van waarde wees: Droogteverdraagsaamheid mag

(i) weerstand wees teen (a) stremming-geïnduseerde afname in $\partial A/\partial c_i$, wat mag lei tot die handhawing van 'n hoër A-tempo, wat essensieël is vir vinnige herstel na herbenatting of, (b) -SH oksidasie of -SH/-SS- interomskakeling soos verklaar kan word deur 'n kombinasie van die -SH/-SS- hipotese en die konsep van proteïenomskakeling,

(ii) die gevolg wees van regulerende meganismes soos (a) PSII regulering soos weerspieël deur fluoressensieparameters of, (b) 'n effektiewe anti-oksidantsisteem,

(iii) die vermoë wees om droogtestremming (a) te voorspel (ABA akkumulering), (b) uit te stel (osmoregulering) of, (c) geïnduseerde beskadiging te beperk (prolienakkumulering), en/of moontlik

(iv) die resultaat omgewingsgedrewe anatomiese verskille soos (a) verskille in die lengte en deursnee van vaatelemente, of (b) verskille in die persentasies intersellulêre lugruimtes wees.

CHAPTER ONE

1. General introduction

The aim of this introduction is to provide the reader with an overview of the organization of this thesis; how the various contributing papers are placed in this organisation, and to serve as an overview on the subject of water stress in plants. Because this thesis contains nine papers covering a wide variety of different aspects and approaches to drought stress, in my efforts to organise the thesis, I sought to accomplish two goals. Firstly, I have purposely avoided artificial placement of the contents into distinct subject categories. I am of the opinion, as was argued by Ludlow (1980) that the characteristics and processes involved in drought tolerance tend to occur in combination or in groupings rather than independently and that this apparent coincidence or grouping appears to have mechanistic links. I hope that this approach presents a unique and fruitful departure from the historical view of cataloguing characteristics and processes into a particular dichotomous scheme. However, having just said that, I have superimposed my own organisation on the content of this thesis, the aim of which is merely to guide the reader being not familiar with the subject matter presented. Secondly, because of the potentially overlapping and interrelated nature of the contents of the various papers a general discussion is included in order to provide additional focus and to serve as a synthesis.

On a global basis, drought limits plant growth and crop productivity more than any other single environmental factor (Boyer, 1982). This may, at least in part, explain the recognition within the international community, both in the rich donor countries and in the poor recipients of that aid, that the poor and possibly declining environmental conditions and, more crucially, the amplitude and unpredictability of climatic changes and cronic edaphic conditions, are major threats to the welfare of much of the human race. Therefore, understanding and exploiting the resistance of some plants to an important environmental factor such as drought, must not simply be regarded as a physiological or ecological problem, but must increasingly be viewed as important goals of international economical, political and humanitarian significance.

In this regard it should be stated that during the evolution of the plant kingdom, innumerable modifications in structures and processes have occurred as a result of random mutations and recombinations. Most of these were deleterious and disappeared, but a few were beneficial because they enabled the plants possessing them to survive and reproduce more successfully, and these were preserved by natural selection. As a result plants growing in increasingly dry habitats accumulated various modifications of characters with adaptive values, that increased the probability of their survival. However, the modifications or adaptations that enable plants to survive droughts and live in dry habitats did not originate exclusively for those

purposes. They originated from random mutations and recombinations that probably occurred in plants growing in both moist and dry habitats, but usually were not preserved in moist habitats because they had no survival value. Furthermore, according to Kramer (1980), the fact that a certain kind of modification would be beneficial in a changing environment, has no bearing on whether it will appear. From the above mentioned, it should be clear that although evolutionary forces have played a major role in generating the existing stress-tolerance mechanisms, the requirements of agriculture, with high yields having a higher premium than mere survival mechanisms, indicate that new combinations of characters are required even though they may never have evolved naturally.

As a further complicating factor, it should be realised that plant responses to stress are conditioned not only by the nature and intensity of the environmental factors involved but also by the ecological histories of species, cultivars and genotype (Grime, 1989). It is thus clear that there needs to be a systematic approach in our studies, paying more attention to genetic differences that exist and including observations of what happens when stress is relieved.

Admittedly, yield improvement under drought stress occurred before many of the physiological issues of drought tolerance were understood and resulted partly from the genetic improvement of yield potential and partly from the improvement of stress resistance (Blum, 1989). The development of better varieties for dry conditions conventionally involved extensive selection and testing for yield performance in diverse environments using various biometrical approaches (Hanson & Robinson, 1963). These empirical methods have led to the development of drought-tolerant cultivars of many crops such as wheat, barley, rice, maize, sorghum and soybean. The intricate statistical and biometrical designs necessary for this approach are required for two reasons: Firstly, the inheritance of yield, which is the major selection criterion in such programmes, is "complex" as it is determined by a multitude of physiological, biochemical and metabolic processes, whose genetics are largely unknown. Even their exact association with plant productivity is unclear. Secondly, the various environments in which genotypes are tested and in which their stability is measured, are largely undefined in the biological sense. Breeders are rarely able to identify the main factors that reduce yield in a given environment and their order of importance. Therefore, with the conventional breeding approach the environment is taken as a random occurrence of infintesimal probabilities, fitting a statistical approach. The level of plant stress in a given environment is measured by the main yield of the population grown in it, compared with other environments. The biological effects of the different undefined environments are integrated into one statistical parameter of stability in yield performance over changing environments (Finlay & Wilkinson, 1963). Drought tolerance is evidently one component of this stability and in cases where environments are variable mainly in the water regime, stable genotypes could indeed be physiologically classified as drought tolerant (Blum,

1983; Aggarwal & Sinha, 1984).

The main difficulty in the past approach was, therefore, the use of yield, which is a genetically complex trait, as a principal selection index and the use of biologically invisible environments for selection towards general stability. A further problem is that the heritability of yield is reduced under conditions where yield is reduced, namely under conditions of stress (Rumbaugh *et al.*, 1984). Selected genotypes that appear to yield relatively well under stress in one cycle of selection may therefore not perform as well in the next cycle, because a large fraction of the apparent variation in the population under stress is environmental rather than genetic. Therefore, selection for drought tolerance by the use of yield as a selection index under drought conditions, is inefficient (Blum, 1989). Furthermore, selection for yield under drought stress must be performed in an environment that corresponds to the drought conditions of the target environment. However, large temporal variability is inherent to the target drought environment and this puts the breeder in a dilemma as to what kind of selection environment represents the target environment (Rumbaugh *et al.*, 1984). In order to overcome the low efficiency of such selection programmes, some plant breeders resort to the expensive solution of growing huge populations and performing repeated testing over years and locations to secure results. In such programmes the probability for success becomes a function of investment rather than a function of science.

Although drought-tolerant varieties have been developed by the use of empirical breeding methods that employ yield as a selection index, these methods are too costly and require a long period of testing and evaluation. Further improvements by such methods will entail still greater investment. Therefore, breeding for drought tolerance must depart from the use of yield as the exclusive selection index. A direct reference to some physiological attributes in the selection for drought tolerance would allow us to address the underlying factors of stability. Thus, when physiological attributes are addressed, the genetics involved, should become simpler, as a function of the lower level of plant organisation.

According to Kramer (1980), drought stress can affect plant growth and yield only by afflicting the physiological processes and conditions in a plant, and therefore if a plant breeder produces a higher yielding variety, it is because a more efficient combination of physiological processes has been arrived at for a particular environment. Furthermore, before advocating selection for a trait with potential adaptive value, both the limitations and full potential of such a trait as determined by its practical applicability, should first be assessed for the crop under question. Without the knowledge obtained in this manner, the potential for biotechnological improvement of crop performance cannot be realised until genes and gene products have been identified which are responsible for the desired characteristics of drought tolerance. This in turn,

according to Sharp and Davies (1989), will not occur without a thorough understanding of the biophysical, biochemical and physiological pertubations that are induced by a restricted water supply.

Thus, rather than merely quantifying again the responses of plants to drought stress, this thesis focuses attention on the responses or mechanisms that enable four tobacco cultivars of different but known drought tolerance to maintain differential degrees of functionality during drought stress.

A variety of mechanisms enable plants to tolerate drought, but these vary in terms of cost to the plant as far as productivity is concerned. Stomatal control or a reduction in electron flow will almost certainly significantly reduce productivity and yield. These two mechanisms receive attention in the papers of section one.

The process of light capture and conversion of light energy into chemical energy not only produces the driving force for carbon fixation but can also produce singlet oxygen, superoxide, hydrogen peroxide, and hydroxyl radicals. The appearance of the first cyanobacteria (blue-green algae), approximately 2 x 10^9 years ago (Echelin, 1970), and the subsequent oxygenation of the biosphere imposed a stringent evolutionary pressure on the many organims that, up to then, had lived and had been enclosed in an anaerobic world. While evolving mechanisms for the utilization of oxygen, they had to develop defenses against its potential toxicity. Considering the situation of a common evolutionary pressure applied to a varied biota, it is not surprising that multiple defenses arose and have persisted. In the second (paper five) section of this thesis the extent to which the latter occurred in the four tobacco cultivars was investigated and the efficiency, with which it operates, compared between the four cultivars.

The importance of biochemical mechanisms to dissipate excess energy, was stressed by Farquhar *et al.* (1989) who indicated that leaves in general need to be able to dissipate 60-90% of the quanta at high irradiance to avoid the potentially damaging formation of oxygen radicals from ferredoxin (Asada & Takahashi, 1987). Farguhar *et al.* (1989) also proposed that when plants are under stress which restricts CO_2 assimilation, light will be in excess even at lower irradiances. In the short term (minutes), while fluorescence itself accounts for less than 1% of absorbed light, it offers one way of achieving this, as discussed in the second paper of section one. Over longer periods, energy-dependent quenching may involve a carotenoid cycle (Demmig *et al.*, 1987), as is proposed in the second paper in section two.

As Hsiao (1973) reported, the soluble protein content in wheat leaves was either hardly affected or else markedly reduced when the relative water content was lowered to 60%, and Bewley *et al.* (1983) demonstrated that protein synthesis ceases when maize seedlings were subjected to mild water stress, we also evaluated the sulphydryldisulphide hypothesis of Levitt (1962) and subsequent considerations by Gaff (1966). According to this hypothesis the -SH/-SS- ratio may be regarded both as an index of protein denaturation and as an index of stress resistance (section two, paper six).

Osmotic adjustment as discussed in paper ten of section two, however, provides the potential for maintaining photosynthesis and growth of at least some parts of the plant as drought stress increases. Thus the cost of osmotic adjusment ought to be lower, although the solutes accumulated during osmotic adjustment are not necessarily used elsewhere. Since these compounds, such as proline (dealt with in papers seven and nine) can in some instances account for complete osmotic adjustment, it appears unlikely that new biochemical pathways are involved during osmotic adjustment. It is more probable that the lowering of the osmotic potential to arises from alteration of existing pathways and translocation patterns in the plants.

To minimise the physiological cost in terms of productivity and yield, some adaptive responses are evoked by the onset of drought stress, rather than being present as permanent features. Changes in endogenous hormone content, in particular, may represent such a response, and variation in the concentration of all the major plant hormones during stress, in fact have been recorded. There is sufficient evidence, as given in papers seven and eight of section two, to support realistic speculation regarding the adaptive role for abscisic acid (ABA).

The persistence with relatively little change during the evolution of higher plants of the basic metabolic pathways which evolved more than two billion years ago, suggests to the possibility of a dearth of genetic diversity in the biochemistry of higher plants in the context of adaptation to drought stress and offers little hope for rapid improvement through conventional breeding techniques (Begg, 1980). By contrast, the successful colonization of terrestrial environments by higher plants, largely as a result of the evolution of a wide range of morphological adaptations, indicates that this may provide a more fruitful basis for adapting crops to water stress environments through selection and breeding (Turner, 1979). Thus, we need a better understanding of the various static and dynamic features of plant morphology that may render some plant species better adapted to stress. These features as well as their integration with some physiological aspects are dealt with in papers five and nine of section two. Passioura (1976) stated "It is the control of leaf area and morphology which is often the most powerful means a mesophytic plant has for influencing its fate when subjected to long term water stress in the field".

It may therefore be said that in the several contributing papers of this thesis some of the specific physiological and morphological adaptations that contribute to the tolerance of drought stress was identified and evaluated. The information included may thus be equally valuable to ecologists, crop physiologists and plant breeders, as it could potentially be used by plant breeders in crop improvement programs and by ecologists in explaining the distribution of natural vegetation.

CHAPTER TWO

2. COMPARATIVE ANALYSIS OF DIFFERENTIAL DROUGHT STRESS-INDUCED SUPPRESSIONS OF AND RECOVERY IN CARBON DIOXIDE FIXATION: STOMATAL AND NON-STOMATAL LIMITATION

(VAN RENSBURG, L. & KRüGER, G.H.J. 1993. *JOURNAL OF PLANT PHYSIOLOGY*, 142 : 296-306.)

2.1 Abstract

Observed differences in drought tolerance in C_3 plants have in the past been incorrectly ascribed to differences in water use efficiency (WUE) and low CO_2 compensation points (Γ). When these parameters were applied as screening procedures for cultivars with higher net photosynthetic rates (A) and/or lower photorespiration rates, they have proved to be invalid. To clarify this discrepancy, the stomatal and non-stomatal limitations to photosynthesis were evaluated in four tobacco cultivars of different, but known, drought tolerance. Experiments were carried out under controlled environmental conditions at increasing drought stress and upon rewatering. A drought stress-induced decrease in the carboxylation efficiency ($\partial A/\partial c_i$) A which was less pronouced in the drought-tolerant cultivars, was observed in all four cultivars. This coincided with a slower stress-induced increase in Γ and intercellular CO_2 concentration (c_i) which was due to the maintenance of higher A rates, in the drought-tolerant cultivars. All cultivars showed a decrease in WUE and an increase in the marginal cost in terms of water used to carbon gained ($\partial E/\partial A$). These changes occurred more slowly in the drought-tolerant cultivars, which was due to the maintenance of higher A values, as stomatal conductance (g) decreased more slowly in spite of higher transpiration rates (E). As the relative degree of stomatal limitation (l) did not increase by much, (ca. 35%) nor differ significantly among the cultivars, the increase in c_i and Γ was interpreted as indicating that mesophyllic rather than stomatal factors were responsible for the drought stress-induced decrease in A, because of the decrease in the $\partial A/\partial c_i$ component of the mesophyllic photosynthetic capacity.

Upon rewatering all the parameters monitored recovered to a greater or lesser extent, though the recovery time needed by the drought-sensitive cultivars were much longer. We conclude that in all cultivars, decreasing leaf water potential (Ψ_L) caused simultaneous reductions in g and the biochemical capacity for photosynthesis. The $\partial A/\partial c_i$ component of the biochemical capacity, however, proved to be the most sensitive to drought stress. Thus during drought stress diffusion itself only marginally limits A, a fact not widely appreciated. Drought tolerance may therefore be resistance to stress-induced decreases in $\partial A/\partial c_i$, which in turn will result in the maintenance of higher A rates, which, in addition is vital for fast recovery upon rewatering.

Keywords: *CO_2 compensation point, drought stress, net photosynthetic rate, Nicotiana tabacum* L., *non-stomatal limitation, water use efficiency.*

Abbreviations: A; net photosynthesis; A_{max} = photosynthetic rate when C_i = Ca and stomatal limitation is infinite; $\partial A/\partial c_i$ = carboxylation efficiency; E = transpiration rate; $\partial E/\partial$ A = marginal cost in terms of water loss to carbon gained; c_i = calculated intercellular CO_2 concentration; g = stomatal conductance; l = relative stomatal limitation to photosynthesis; WUE = water use efficiency; Γ = CO_2 compensation point; Ψ_L = leaf water potential.

2.2 Introduction

Numerous reports of phase changes in net photosynthesis (A) and transpiration (E) strongly implicate stomatal control as primary causal factor in decreasing A under drought stress conditions (O'Toole *et al.*, 1976 and literature cited therein). Stomatal movement is the most rapid means which plants possess to adjust to changes in the environment of their photosynthesizing organs. The movements are reversible and are inexpensive in terms of energy used. Because stomata tend to open with environmental changes which promote carbon fixation and close with changes which promote water loss, their operation has the effect of reducing the average rate of water lost relative to the average rate of carbon fixation. This empirical observation gave rise to the theory of optimal variation proposed by Cowan (1977a). But, admittedly, according to Cowan (1982), it would be a gross over-simplification to assume that the only reversible effect of drought stress on plant functioning is stomatal closure and that all drought stress-induced effects are concentrated in a single event, at a particular critical soil water deficit.

A considerable amount of evidence then also exists, which supports the supposition that non-stomatal factors may be responsible (Redshaw & Meidner, 1972) for the decline in A with decreasing leaf water potential (Ψ_L), which may often be linear (Ehleringer, 1983). Although there is evidence that fairly extreme Ψ_L may be required for non-stomatal effects on A (Kaiser, 1987), and that the interpretation of CO_2-response curves (A/c_i) may result in an overestimation of non-stomatal inhibition (Terashima *et al.*, 1988; Cheeseman, 1991), there are many examples of non-extreme Ψ_L having direct effects on the photosynthetic capacity of chloroplasts (Graan & Boyer, 1990).

Attention has been focused on quantitatively assessing the extent to which A is co-limited by the above-mentioned stomatal and non-stomatal components by Farquhar and Sharkey (1982). The different limitations can be analyzed by evaluating what has been called the supply function (stomatal or diffusional component) and the demand function (non-stomatal component) (Ehleringer & Cook, 1984). From A/c_i curves much can be learned about the sequence of events and different sensitivities of different partial photosynthetic reactions. In this regard CO_2-response curves of intact leaves (A/c_i), proved to be a valuable measure for comparing and characterizing the status of the photosynthetic apparatus on the basis of gas exchange (Lange *et al.*, 1987). In spite of the extensive attention that has been paid to the above-mentioned effects of drought stress on A, the events during the recovery from drought stress have, however, received little and infrequent attention in the past (Boyer, 1971; Bielorai & Hopmans, 1975; Collatz *et al.*, 1976; Kirschbaum, 1988). As part of our efforts (Van Rensburg *et al.*, 1993; Van Rensburg & Krüger, 1993) to explain the observed differential drought stress-induced decline in

photosynthesis, and to understand its possible connotation with the known difference in drought tolerance of four tobacco cultivars; we deemed it necessary to evaluate the stomatal and non-stomatal limitation to photosynthesis in four tabacco cultivars when subjected to progressively lower Ψ_L and during the recovery period (over a period of of several days, such as might occur periodically under field conditions), and to relate this to their known different individual drought tolerance.

This was done, as for a quantitative understanding of crop functioning in the absence and presence of drought stress, it is necessary to collect data on photosynthesis and transpiration at the level of the individual leaf (Vos & Oyarzún, 1987). Stomatal and non-stomatal limitations on photosynthesis are discussed in relation to one another at specific Ψ_L, and absolutely in respect to the overall decline in A with decreasing Ψ_L. The resulting photosynthetic behaviour is discussed in relation to plant performance and potential adaptive value.

2.3 Material and methods

Plant material and growth conditions

Four cultivars of tobacco (*Nicotiana tabacum* L.) namely: TL33, CDL28, GS46 and ELSOMA - in sequence of increasing drought tolerance (personal communication Dr. C.J. Steenkamp, TCRI, Rustenburg, RSA), were grown from seed in soil under glasshouse conditions with optimal water application. At the age of ca. 90 days after planting, before the onset of experimentation, plants were moved to computerized growth rooms. The exact controlled environmental conditions which prevailed in the glasshouse and growth rooms have previously been extensively described (Van Rensburg *et al.*, 1993). Drought stress of increasing intensity (ca. 0.095 MPa d^{-1}) was induced by withholding water, in so doing allowing the soil water to be depleted. All measurements were conducted on the sixth leaf from the apex, which constituted fully expanded young leaves.

Leaf water potential measurements

Predawn measurements (four replicates per determination) were conducted with a Scholander pressure chamber (PMS- instrument, Oregon, USA), while assuming that Ψ_L was equal to xylem pressure potential. Due to the size of a tobacco leaf and specific nature of the petiole, Ψ_L determinations was conducted on side veins while at the same time including as much intravenous lamina tissue as possible. Following this procedure the accuracy of the determinations was increased, as not only could the side vein be placed in the chamber and sealed airtight without damaging the conductive tissue, but it also minimized the distance and, therefore, resistance to waterflow from the lamina to the cut surface. The latter is very important, as resistance to water flow in tobacco leaves is high (Begg & Turner, 1970; Hoffman & Splinter, 1968), and even more so when under stress (De Roo, 1970).

Gas exchange measurements

A calibrated A.D.C. LCA-2 infra-red gas analyser (IRGA) coupled to an A.D.C. DL-2 datalogger and an A.D.C. ASUM-2 mass flow regulator (all Analytical Development CO., Hoddeson, England), was used in open circuit for all measurements. Leaves were clamped in a broad leaf chamber (Parkinson PCL-B Analytical Development CO.) on the same position for consecutive measurements at decreasing Ψ_L , to avoid possible overestimation of any factor caused by non-homogenous closure of fields of stomata (Terashima *et al.*, 1988). For the construction of A/c_i curves, different CO_2 concentrations were generated by the stepwise reduction of CO_2 from 700 μmol mol^{-1} to 0 μmol mol^{-1} by passing it through an A.D.C. gas

diluter. After passing through a temperature filter a constant photon flux density (PPFD) of 1100 μmol m^{-2} s^{-1}, was supplied with the aid of a high pressure sodium light source.

Data were downloaded to a computer and results calculated according to the equations described and discussed by Farquhar and Sharkey (1982) and Chaves (1991). Curve fittings were done with the aid of simplex algorithms (Willmott, 1982). By using the latter programme statistically justifiable curves were obtained, as the programme is capable of determining interpolation values to generate the best fit for any number of variables and parameters, and for any function, irrespective of its complexity. The initial slope of the demand function ($\partial A/ \partial c_i$) and marginal cost of water used to carbon gained ($\partial E/\partial A$) was computed by linear regression analysis.

2.4 Results and discussion

Models of CO_2 assimilation in leaves of C_3 plants have related rates of CO_2 assimilation with the kinetic properties of RuBP carboxylase/oxygenase, and with the limitations placed on electron transport and pyridine nucleotide reduction by temperature and photon flux (Farquhar *et al.*, 1980a,b). These models are compatible with experimental data derived from studies of gas exchange in leaves and the conclusion has been drawn that mesophyll resistance to CO_2 diffusion is small (Farquhar & Sharkey, 1982), but not necessarily insignificant (Evans, 1983). Furthermore, both stomatal and non-stomatal components are thought to be responsible for a decrease in rate of net photosynthesis, as drought stress increases (Ehleringer & Cook, 1984).

As Ψ_L decreased with time in the four tobacco cultivars, differential decreases in A, g, WUE and their RuBP carboxylation efficiency ($\partial A/\partial c_i$) were observed while Γ, c_i, E and the unit marginal cost in terms of water lost for carbon gained ($\partial E/\partial A$), differentially increased in accordance with their respective drought tolerance (Table 1). In general, these changes are in accordance with several reports (Comstock & Ehleringer, 1984; Forseth & Ehleringer, 1983; Radin & Ackerson, 1981), in which the investigators have measured the change in the dependence of A and c_i as an estimate of the extent of the change in the non-stomatal components during drought stress. In these studies, as also proved to be the case in the present investigation (Fig. 1a to d), there was a decline of the $\partial A/\partial c_i$ slope at low c_i as well as a decrease in A, and A_{max} at high c_i. According to Ehleringer and Cook (1984) data such as this suggest a coordinated leaf level response to long term soil water depletion. Not all studies of photosynthesis during drought stress have shown a decrease in the $\partial A/\partial c_i$, which has been ascribed to the fact that in these studies stress development was too rapid (Hanson, 1982). This did not constitute a limiting factor in our investigation, as the rate at which the Ψ_L declined was ca. 0.095 MPa d^{-1}, which according to Turner and Jones (1980) is slow enough to allow any osmoregulatory capability to come to the fore.

The occurrence of an increasing Γ with decreasing Ψ_L which was more pronounced in the drought-sensitive cultivars (Table 1), has been interpreted as indirect evidence for the involvement of non-stomatal factors in the response of photosynthesis to drought stress (O'Toole *et al.*, 1976). Several species have shown a similar response to drought stress: wheat (Heath & Meidner, 1961), palm (Meidner, 1961), spinach (Plaut & Bravdo, 1973) and maize, as well as tobacco (Meidner, 1967). An increase in Γ indicates either a decrease in A, or an increase in respiration in the light, or both. The combined conclusions of several of these reports emphasize that moderate drought stress inhibits several biochemical functions within the chloroplast (O'Toole *et al.*, 1976; Van Rensburg & Krüger, 1993).

Table 1: Differential drought stress-induced changes in some stomatal and non-stomatal factors that influence the WUE and $\partial E/\partial A$ of four tobacco cultivars, of differing, but known drought tolerance.

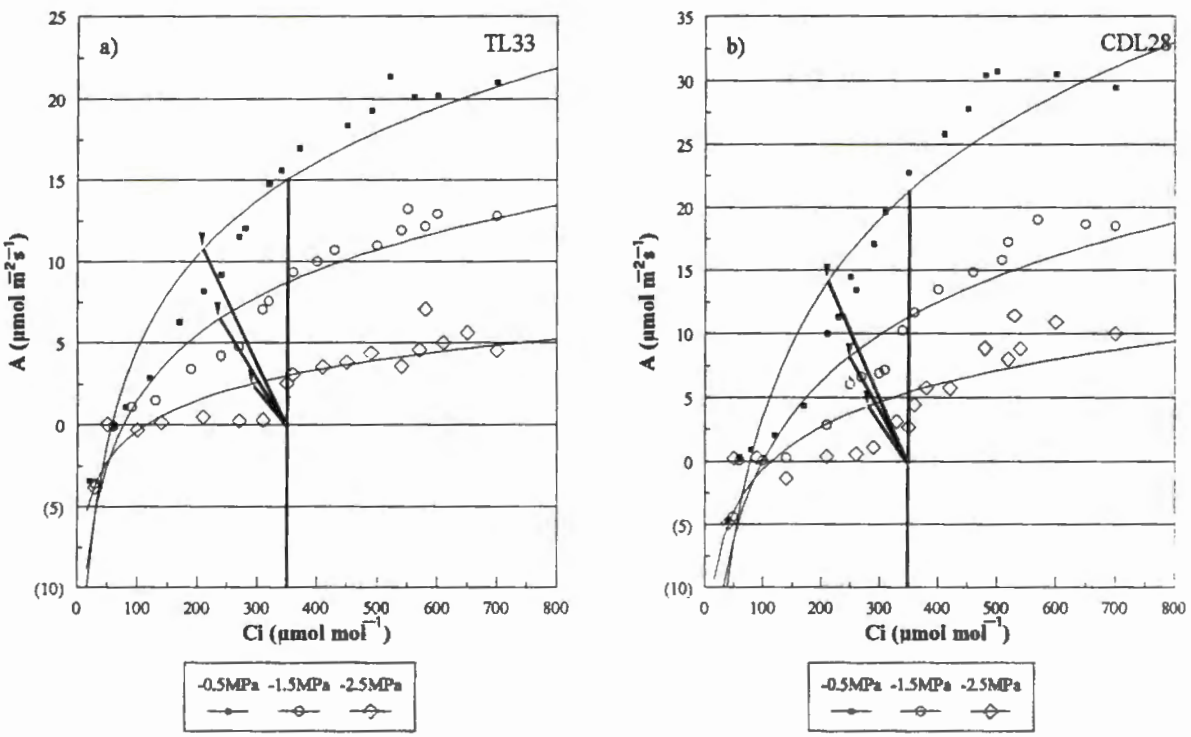
Cultivars	Ψ_L MPa	l %	Γ μmol mol^{-1}	$\partial A/\partial c_i$ mol m^{-2}s^{-1}	WUE μmol mmol^{-1}	$\partial E/\partial A$ mmol μmol^{-1}	c_i μmol mol^{-1}
TL33	-0.5	27.6 ± 3.5	65.30 ± 5.9	0.559 ± 0.2	2.251 ± 0.6	0.444 ± 0.4	228.5 ± 3.7
	-1.5	34.3 ± 2.4	78.26 ± 2.9	0.229 ± 0.2	0.758 ± 0.5	1.325 ± 0.5	281.2 ± 5.9
	-2.5	42.8 ± 2.6	180.00 ± 10.2	0.091 ± 0.2	0.153 ± 0.5	6.556 ± 0.5	303.8 ± 9.6
CDL28	-0.5	21.5 ± 6.4	93.52 ± 7.0	0.710 ± 0.1	2.247 ± 0.4	0.606 ± 0.5	293.2 ± 5.1
	-1.5	34.7 ± 3.6	100.25 ± 3.5	0.395 ± 0.3	0.738 ± 0.5	1.356 ± 0.5	282.5 ± 6.2
	-2.5	48.3 ± 1.8	169.08 ± 10.9	0.109 ± 0.2	0.183 ± 0.4	5.455 ± 0.4	304.9 ± 9.9
GS46	-0.5	26.9 ± 7.9	84.25 ± 8.9	0.889 ± 0.2	2.727 ± 0.7	0.367 ± 0.6	242.7 ± 2.6
	-1.5	34.2 ± 4.1	100.01 ± 4.1	0.401 ± 0.2	1.375 ± 0.8	0.747 ± 0.3	270.3 ± 6.1
	-2.5	37.2 ± 2.4	138.54 ± 10.7	0.171 ± 0.3	0.884 ± 0.6	1.131 ± 0.3	294.9 ± 9.3
ELSOMA	-0.5	28.9 ± 4.5	64.26 ± 7.9	0.779 ± 0.1	2.935 ± 0.8	0.472 ± 0.4	245.6 ± 1.9
	-1.5	37.3 ± 3.6	86.83 ± 4.1	0.410 ± 0.2	2.860 ± 0.5	0.489 ± 0.3	269.2 ± 6.7
	-2.5	40.6 ± 1.9	105.97 ± 14.1	0.149 ± 0.2	0.918 ± 0.6	1.089 ± 0.5	292.7 ± 9.9

* Standard deviations reflect the deviations from the mean of three replicates per leaf (at three different positions) of four different plants.

Furthermore, with the exception of TL33, our results (Table 1) confirm the statement that a significant inverse curvilinear relationship exists between Γ and A per unit of foliage, and that Γ may, therefore, serve as criterion for photosynthetic efficiency (Luukkanen, 1976), but not necessarily of drought tolerance. In this regard it should be noted that the Γ of GS46 and ELSOMA increased by only ca.64 % of their controls, while maintaining higher rates of A i.e. 2.60 and 2.70 μmol m^{-2}s^{-1}, respectively at a Ψ_L of -2.50 MPa. On the other hand the Γ of TL33 and CDL28 increased by ca.175% and ca.81% of their controls respectively, while their A rates decreased from 8.17 to 0.27 μmol m^{-2}s^{-1} and 6.22 to 0.33 μmol m^{-2}s^{-1} respectively, at the severe stress level. However, screening procedures based on survival under CO_2-compensation concentration conditions for obtaining high yield C_3 lines are not advised, as even though the method is simple and has possible large-scale applications, the success rate has in the past been very low (Delgardo *et al.*, 1992).

Intercellular CO_2 concentration (c_i) also provides a means of assessing the dynamics of changes in the relative importance of stomatal and mesophyll processes in limiting A, following a change in the environment. The validity of the calculated c_i has been verified by Sharkey *et al.* (1982). If an increase in stomatal limitation is the dominant cause of a reduction in A, then c_i must decrease; if, on the other hand, as proved to be the case in the four tobacco cultivars (all data not supplied), as well as sunflower, bean and soybean (Lauer & Boyer, 1992), an increase in limitations within the mesophyll dominates the reduction in A, an increase in c_i might be expected (Long & Hallgren, 1985). The c_i observed in the four tobacco cultivars depends on both the

conductance to CO_2 diffusion into the leaf, and the capacity of the leaf to utilize internally available CO_2 for photosynthetic carboxylations. The arrows in Fig. 1a to d (intercept of demand and supply functions), indicate the calculated c_i for all four tobacco cultivars under ambient conditions at decreasing Ψ_L . It is evident that c_i differentially increased in all four cultivars, more so in TL33 and CDL28 than in GS46 and ELSOMA (Fig. 1a to d and Table 1). This is in accordance with the drought stress-induced reduction in g and increased c_i reported for cotton, which were interpreted as indicating a proportionally greater non-stomatal inhibition of photosynthesis in drought stressed plants (Hutmacher & Kieg, 1983). Lauer and Boyer (1992) also concluded that water deficit under growth conditions caused simultaneous reductions in the stomatal apertures and biochemical capacity for photosynthesis, but that the biochemical capacity limited photosynthesis and that stomatal closure did not, because c_i remained essentially the same or increased. Stuhlfauth *et al.* (1988) have gone so far as to say that as c_i does not decrease to the Γ, but remains high even during moderate drought stress, it invalidates the hypothesis that only stomatal closure is responsible for the loss in A.



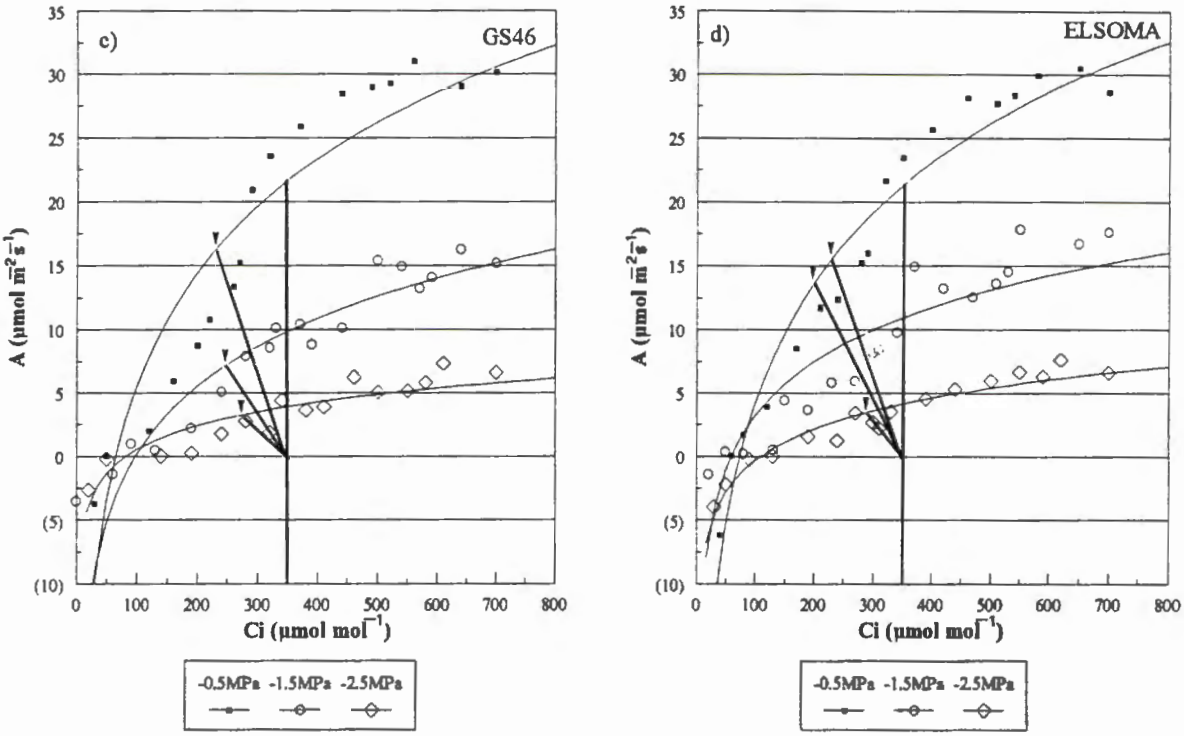


Figure 1. Differential drought stress-induced changes in the A/c_i photosynthetic-relationships of the four tobacco cultivars: (a) TL33 [at a Ψ_L of -0.5 MPa $Y = 7.50*x/(1.51*x)$ with $R^2 = 0.954$; Ψ_L of -1.5 MPa $Y = 1.37*X/(5.82*X)$ with $R^2 = 0.952$ and at a Ψ_L of -2.5 MPa $Y = 7.14*X/(9.15*X)$ with $R^2 = 0.795$], (b) CDL28 [at a Ψ_L of -0.5 MPa $Y = 2.03*X/(3.27*X)$ with $R^2 = 0.922$; Ψ_L of -1.5 MPa $Y=1.09*X/(3.74*X)$ with $R^2 = 0.948$ and at a Ψ_L of -2.5 MPa $Y = 1.39*X/(9.57*X)$ with $R^2 = 0.834$], (c) GS46 [at a Ψ_L of -0.5 MPa $Y = 1.31*X/(1.93*X)$ with $R^2 = 0.916$; Ψ_L of -1.5 MPa $Y = 1.65*X/(3.03*X)$ with $R^2 = 0.916$ and at Ψ_L of -2.5 MPa $Y = 4.96*X/(4,97*X)$ with $R^2 = 0.880$], (d) ELSOMA at a Ψ_L of -0.5 MPa $Y = 1.13X/(1.69*X)$ with $R^2 = 0.958$; Ψ_L of -1.5 MPa $Y = 4.78*X/(1.68*X)$ with $R^2 = 0.921$ and at a Ψ_L of -2.5 MPa $Y = 7.11*X/(6.67*X)$ with $R^2 = 0.900$] if (TL 33, CDL 28 regarded as drought sensitive and GS 46 and ELSOMA as drought tolerant).

Decreases in A and A_{max} are associated with decreases in g. At a Ψ_L of -2.50 MPa, g had decreased by ca.74% in TL33 and ca.68% in CDL28 of their controls respectively, while it had only decreased by ca.59% in GS46 and ca.45% in ELSOMA. For C_3 species such as tobacco, however, the correlation is not simply due to environmental influences on g, which in turn, influences A through effects on the diffusion of CO_2 into leaves (Schulze & Hall, 1982). Values of g and the relation between g and A declined in a curvilinear fashion as Ψ_L decreased (data not shown), which once again indicates that the physiological coupling of these parameters is not as close as has previously been thought. The relationship between A and c_i was determined at different values of Ψ_L for all for cultivars as respresented in the curves in Fig. 1a to d. As Ψ_L became more negative, a decrease in both $\partial A/\partial c_i$ (at low c_i) and A_{max} (at high c_i) was observed. Using linear regressions from the data collected at CO_2 concentrations below ambient CO_2, we calculated that their $\partial A/\partial c_i$ decreased by ca.84%, (TL33), ca.85% (CDL28), ca.81% (GS46) and ca.81 (ELSOMA) respectively, from their control values, as the Ψ_L decreased to -2.50 MPa.

In the present investigation, as was also reported by Sanchez *et al.* (1983), for two maize genotypes, the tobacco cultivars which exhibited a greater drought tolerance were characterized by higher control A rates; 8.17 μmol $m^{-2}s^{-1}$ (TL33), 6.22 μmol $m^{-2}s^{-1}$(CDL28), 13.8 μmol $m^{-2}s^{-1}$ (GS46) and 12.2 μmol $m^{-2}s^{-1}$ (ELSOMA). Higher A rates (ca. 10%) were maintained by GS46 and ELSOMA over the entire drought stress-range (Fig. 1a to d). Plant survival during and after drought stress is partly made possible due to the maintenance of A during drought stress, which allows rapid recovery of the plant after rehydration (Chaves, 1991). At a Ψ_L of -2.50 MPa the drought stress reduced A by ca.97% and ca.95% in TL33 and CDL28 respectively, but by only ca.81% in GS46 and ca.78% in ELSOMA respectively. Unfortunately these higher A rates were as predicted by Hamid *et al.* (1990), accompanied by high E. We express E as the dependence of E on g by using the measure (mmol $m^{-2}s^{-1}$) of conductance introduced by Cowan (1977a), which has several features to recommend it (Hall, 1982). At the severe stress level GS46 and ELSOMA still maintained E rates of ca.41% and ca.55% of their respective control rates, while in TL33 and CDL28 their E rates had already decreased by ca.74% and ca.68% respectively. Cultivar improvement efforts have, however, to the present, sought genotypes with high yield potentials, having high A (Dornhoff & Shibles, 1970), but without a concomitant increase in E. In this regard it should be noted that despite the apparent drought tolerance of some tobacco cultivars, tobacco is still a mesophytic C_3 species, which would seem to fix as much carbon as possible to the detriment of wateruse, when available.

Farquhar and Sharkey (1982) proposed a measure for determining relative stomatal limitation (l), which has been defined as the percentage reduction in A below the rate which would occur if stomatal conductance was infinite. This enabled the quantification of the extent to which stomatal and non-stomatal components are coupled, by determining l as drought stress intensified. As l takes into account the changing nature of the dependence of A on c_i (Ehleringer & Cook, 1984), an increase in l as observed in the present investigation (Table 1) could have been caused by 1) decrease in g, 2) decrease in $\partial A/\partial c_i$, or 3) increase in A_{max} (increase in the RuBP regeneration capacity) (Comstock & Ehleringer, 1984). l increased, as $\partial A/\partial c_i$ decreased relatively more than A_{max} as Ψ_L became more negative. Values obtained for l (Table 1) which increased from 21% to 48%, correlate well with those reported by Farquhar and Sharkey (1982) which they calculated from the data presented by Mooney *et al.* (1977).

If diffusional limitations to CO_2 movement between the intercellular spaces and the sites of carboxylation are small relative to the limitation of photosynthesis imposed by the carboxylation step itself, then $\partial A/\partial c_i$ should be directly proportional to the activity of RuBP carboxylase in the leaf (Von Caemmerer & Farquhar, 1981). The observed decline in $\partial A/\partial c_i$ (Fig. 1a to d and Table 1) can be ascribed to a drought stress-induced decline in the RuBP carboxylase activity, and not to a decrease in the RuBP carboxylase concentration, as not only did

Kawashima and Mitake (1969) find that the activity of the carboxylase decreased more rapidly than did the fraction I protein (crude RuBP carboxylase) in tobacco, but Peterson *et al.* (1973) also proved that RuBP carboxylase was remarkably stable after its synthesis. Furthermore, since photosynthesis no longer responds to light intensity at low c_i when above 1000 μmol $m^{-2}s^{-1}$ (used in this investigation), and as under such conditions Taylor and Terry (1984) found that RuBP carboxylase was nearly 100% activated, the results presented (Fig. 1a to d) clearly indicate that drought stress markedly influenced the carboxylation efficiency of the tobacco cultivars and more so in TL33 and CDL28 than in GS46 and ELSOMA. This is in correspondence with the observations of several authors, to the effect that the activity of RuBP carboxylase isolated from plants sensitive to drought stress, such as cotton (Jones, 1973), spinach (Plaut, 1974), barley (Huffaker *et al.*, 1970) and wheat (Johnson *et al.*, 1974), decreased when Ψ_L became more negative. On the other hand, in sorghum (Shearman *et al.*, 1972) and *Triticum kotschyi*, which is very drought-tolerant, though also decreasing their RuBP carboxylase activity was much more stable during drought stress (Mayoral *et al.*, 1981) as was the case in GS46 and ELSOMA (Fig. 1a to d). Drought stress-induced changes in mesophyll resistance may indicate adaptation or damage of the light and dark reactions of photosynthesis (Björkman & Powles, 1982). According to chlorophyll fluorescence data (Van Rensburg & Krüger, unpublished), we are of the opinion that the former is the case in the drought-tolerant cultivars.

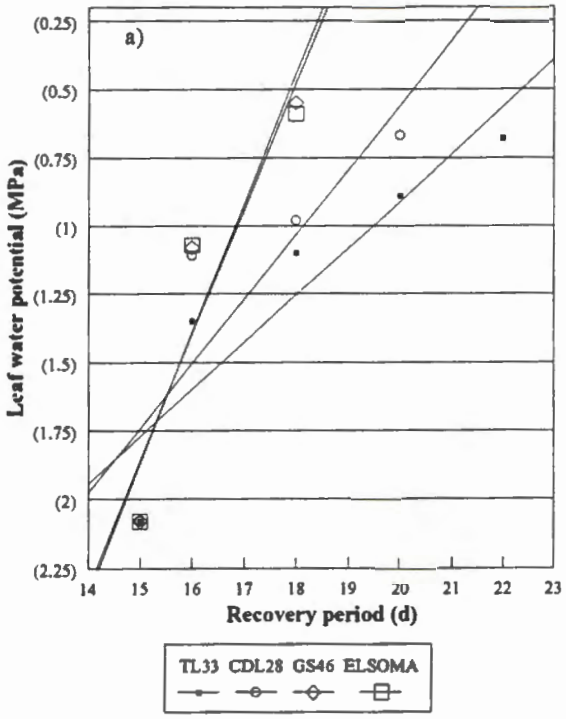
It has been shown mathematically, that in order to gain carbon most economically with respect to water loss, stomata should function in such a way that the marginal cost of assimilating more CO_2 ($\partial E/\partial A$) should remain constant (Cowan, 1982). The first experimental support for the optimization hypothesis (Cowan, 1977b) was presented by Farquhar *et al.* (1980) who showed that $\partial E/\partial A$ remained substantially constant over a range of ambient humidities. Hall and Schulze (1980) who found that $\partial E/\partial A$ also remained constant over a range of temperatures, confirmed the finding of Farquhar *et al.* (1980) in other species. We are of the opinion that due to the close coupling that exists between stomatal functioning, humidity, light intensity and temperature, as indicated by Schulze (1986), the latter results (Farquhar *et al.*, 1980; Hall & Schulze, 1980), could also be interpreted as stomatal control, in order to keep the species within their respective optimum thermal kinetic windows (Radin, 1989; Krieg, 1989; Burke *et al.*, 1989). During drought stress, however, when the emphasis falls on survival rather than optimal functioning, the $\partial E/\partial A$ ratio may not remain constant, as proved to be the case in the present investigation (Table 1), and as stated by Begg and Turner (1976). The drought stress-induced increase in $\partial E/\partial A$ was significantly slower in the drought-tolerant cultivars, as at a Ψ_L of -2.50 MPa, it had increased by ca.1376% in TL33 and ca.800% in CDL28 respectively, while it had only increased by ca.208% and ca.130% in GS46 and ELSOMA respectively. This contention is supported by the observations that c_i was higher after and during drought stress than in the controls (Radin & Ackerson, 1981), despite reductions in g. Stomatal control was obviously of secondary

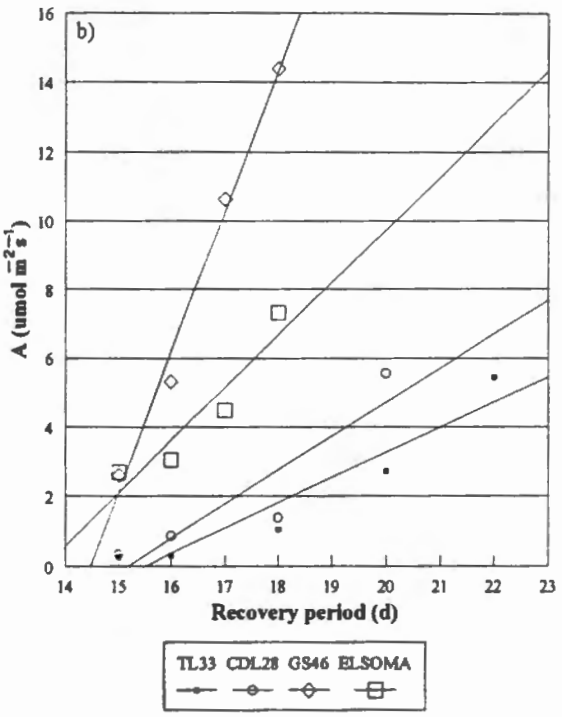
importance in causing the drought stress-induced reduction in A. This was not only indicated by the results presented, which indicate that c_i did not change in the same direction as the change in A [the conditions set to establish primacy of the stomatal response, according to Farquhar and Sharkey (1982)], but as is also evident from the conclusion drawn by Farquhar and Sharkey (1982), that reduced mesophyll capacity was the dominant feature in most cases of drought stress-induced reduction in A.

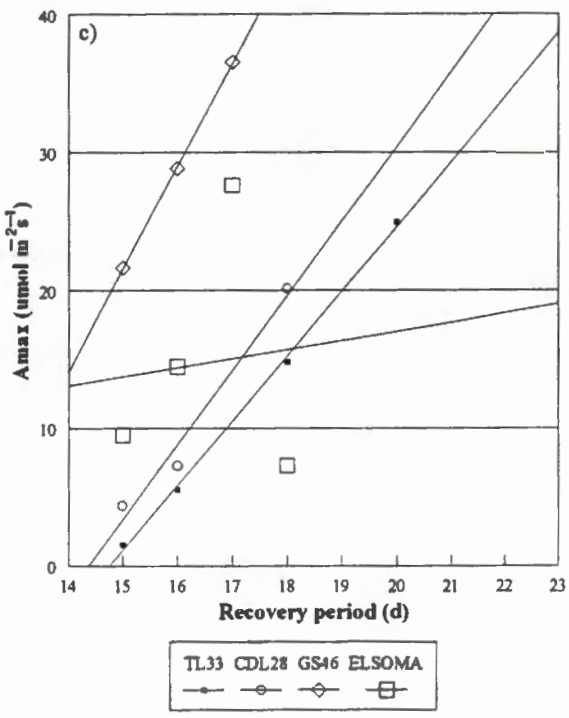
WUE, calculated as the ratio of μmol CO_2 fixed/mmol H_2O transpired, did not increase in any of the four tobacco cultivars with decreasing Ψ_L , but decreased as was the case in tomato (Martin & Thorstenson, 1988). This is contrary to the study with *Encelia fructescens* (Gray) a drought-deciduous leaved subshrub of the Mohave and Sonoran Deserts, of which the WUE increased by 59% as a consequence of a decrease in c_i, caused by a disproportionately large decrease in g compared to $\partial A/\partial c_i$ as its Ψ_L dropped from -1.5 to -4.0 MPa (Comstock & Ehleringer, 1984). Due to the maintenance of higher $\partial A/\partial c_i$, and therefore, A rates, the rate and extent to which the WUE of the drought-tolerant cultivars decreased was less extreme. It is important to note that species differ with regard to the role played by stomatal functioning in assuring success during drought (Chaves, 1991). Increased WUE is observed in well-adapted genotypes (Osmond *et al.*, 1987; Chaves & Rodriques, 1987), where stomatal control represents the major fraction of the total limitation to A. In contrast, in other genotypes which keep their stomata relatvely "open", because they are capable to compensate for water loss, their WUE may remain constant (Suzuki, 1988), or decrease (Table 1 and Martin & Thorstenson, 1988). In the latter types, adaptation to drought stress may be achieved by large and/or deep root systems (Turner, 1986; Parsons, 1979) or alternatively opting for strategies that reduce the amount of radiation intercepted (Begg & Turner, 1976), such as leaf rolling or hanging limp when stressed. This is achieved with a greater efficiency in the drought-tolerant tobacco cultivars (Fig. 3 a to d). This capability is of great adaptive value, as crop evaporation is linearly related to leaf area until complete ground cover is reached (Ritchie, 1974). We are therefore of the opinion that the value of determining WUE of mesophytic crop species during drought stress, for possible use as a selection parameter, is limited, not only because of the reasons mentioned, but also as (unfortunately, but not unexpectedly), instantaneous WUE has been found to be a poor indicator of the season-long WUE of a crop (Martin & Thorstenson, 1988). Furthermore, WUE and drought tolerance have in the past been incorrectly used synonymously, as according to Begg and Turner (1976) they are frequently unrelated.

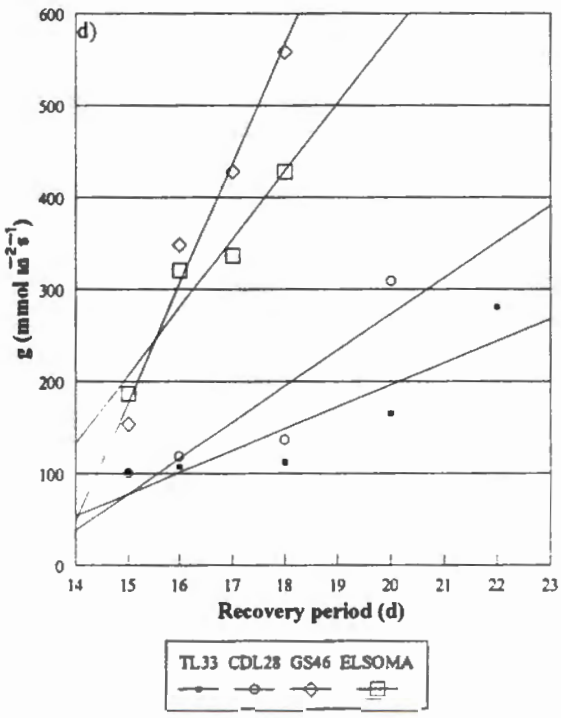
The remarkable resistance of the photosynthetic apparatus to dehydration (Cronic *et al.*, 1989; Quick *et al.*, 1992) is reflected by its capability to recover upon rehydration (Chaves, 1991). Often, however, plants recover only partially from desiccation after they have been rewatered. There is evidence that following a period of desiccation, A and E may remain lower,

resistance to water flow through the plant be larger, and stomata may open less than those of non-stressed control plants (Boyer, 1971). Contrary to the explanation given by Boyer (1971) in explaining the above-mentioned delayed stress effects, our results indicated, as has also been reported by Kirschbaum (1988), that Ψ_L showed no lag and commenced to recover, as soon as the plants were rewatered, to levels present before desiccation (Fig. 2a). As also proved to be the case with A (Fig. 2b), A_{max} (Fig. 2c), g (Fig. 2d) and E (Fig. 2e), the recovery rate, was however, significantly faster in GS46 and ELSOMA. Once recovery commenced, although it involved concurrent increases in g and the $\partial A/\partial c_i$ relationship at low c_i (Table 2), we are of the opinion that this synchrony, rather than indicating a determining role for stomatal functioning, indicates, as stated by Kirschbaum (1988) that both parameters respond similarly to a hormonal balance. This contention is supported by the observation that rather than increasing after rewatering occurred, c_i differentially decreased in the four tobacco cultivars. Furthermore, A_{max} increased in all four cultivars to higher values than in the control plants, and even more so in the drought-tolerant cultivars. This increase in the RuBP carboxylase regeneration capacity, which occurred sooner and was more pronounced in GS46 and ELSOMA (Fig. 2c), *inter alia* depends on the capacity for electron transport, which in turn depends on the absorbed irradiance (Farquhar & Sharkey, 1982), can be explained by the fact that GS46 and ELSOMA reached their control Ψ_L faster (Fig. 2a), which allowed their leaves to regain turgor and expand earlier to absorb more radiant energy. The delayed recovery of g (Fig. 2d) was not caused by an inability of the stomata to open further as they could be induced to do so, as was also found by Kirschbaum (1988). The recovery of A is likely to correspond to active repair of photosynthetic components that may have been damaged during the drought stress or even during rewatering (Nir *et al.*, 1981). Such recovery has previously been shown to be light- and temperature-dependent (Greer *et al.*, 1986), and probably requires chloroplast-encoded protein synthesis (Ohad *et al.*, 1984). By using chlorophyll fluorescence, Demming and Björkman (1987) and Van Rensburg and Krüger, (unpublished data), have indicated that the capacity for repair is an important contributing factor in determining differences in the susceptibility to, and recovery from, photo-inhibition.


a)
Leaf water potential (MPa)
(0.25)
(0.5)
(0.75)
(1)
(1.25)
(1.5)
(1.75)
(2)
(2.25)
14 15 16 17 18 19 20 21 22 23
Recovery period (d)
TL33 CDL28 GS46 ELSOMA


b)
A (umol m-2 s-1)
0 2 4 6 8 10 12 14 16
14 15 16 17 18 19 20 21 22 23
Recovery period (d)
TL33 CDL28 GS46 ELSOMA


c)
Amax (umol m-2 s-1)
0 10 20 30 40
14 15 16 17 18 19 20 21 22 23
Recovery period (d)
TL33 CDL28 GS46 ELSOMA


d)
g (mmol m-2 s-1)
0 100 200 300 400 500 600
14 15 16 17 18 19 20 21 22 23
Recovery period (d)
TL33 CDL28 GS46 ELSOMA

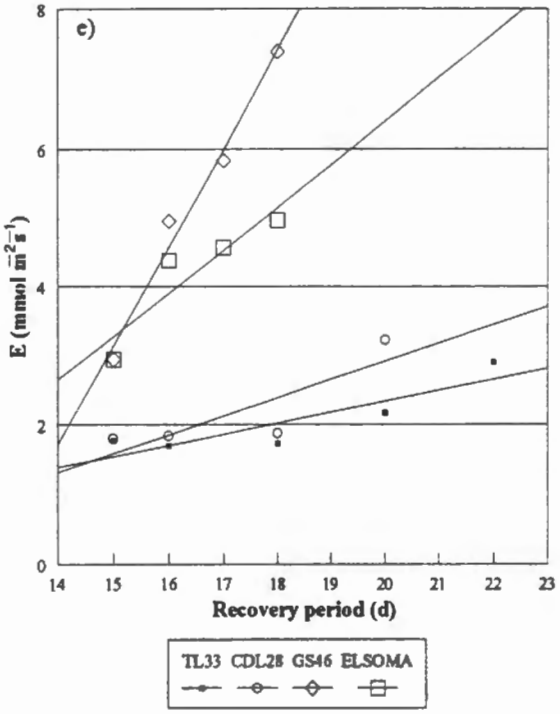


Figure 2. Recovery time course in (a) Ψ_L , (b) A, (c) A_{max} (d) g and (e) E, of four tabacco cultivars of differing drought tolerance, upon being rewatered on day 15 after reaching a drought stress-induced Ψ_L of -2.5 MPa.

Table 2: Differential recovery time course in some stomatal and non-stomatal factors, that influence the WUE and $\partial E/\partial A$ of four tobacco cultivars, of differing, but known drought tolerance, upon rewatering.

Cultivars	Time after rewatering d	Ψ_L MPa	l %	Γ µmol mol^{-1}	$\partial A/\partial c_i$ mol m^{-2}s^{-1}	WUE µmol mmol^{-1}	$\partial E/\partial A$ mmol µmol^{-1}
TL33	1	-1.35	38.39 ± 4.6	121.75 ± 4.6	0.215 ± 0.3	0.247 ± 0.2	4.049 ± 0.5
	3	-1.10	33.96 ± 4.3	99.91 ± 4.3	0.429 ± 0.4	0.607 ± 0.2	1.647 ± 0.4
	5	-0.89	26.90 ± 3.4	78.06 ± 3.4	0.516 ± 0.2	0.738 ± 0.6	1.355 ± 0.3
	7	-0.68	26.22 ± 3.5	63.50 ± 3.7	0.603 ± 0.3	1.495 ± 0.8	0.669 ± 0.4
CDL28	1	-1.11	47.28 ± 4.6	123.77 ± 16.4	0.331 ± 0.1	0.459 ± 0.5	2.179 ± 0.5
	3	-0.98	36.39 ± 3.6	106.15 ± 13.6	0.553 ± 0.3	0.734 ± 0.7	1.362 ± 0.4
	5	-0.67	21.30 ± 3.4	88.53 ± 3.8	0.717 ± 0.2	1.724 ± 0.5	0.580 ± 0.3
GS46	1	-1.08	35.75 ± 4.2	108.52 ± 5.2	0.364 ± 0.3	1.078 ± 0.9	0.928 ± 0.4
	2	-0.79	29.02 ± 4.9	91.43 ± 4.3	0.466 ± 0.2	1.497 ± 0.7	0.668 ± 0.5
	3	-0.55	22.25 ± 4.1	80.64 ± 4.1	0.699 ± 0.4	1.948 ± 0.6	0.513 ± 0.4
ELSOMA	1	-1.07	34.30 ± 4.1	103.82 ± 18.2	0.288 ± 0.3	0.932 ± 0.7	1.073 ± 0.4
	2	-0.83	32.09 ± 5.0	74.42 ± 4.3	0.473 ± 0.3	1.024 ± 0.6	0.874 ± 0.5
	3	-0.59	29.88 ± 4.3	60.90 ± 5.1	0.885 ± 0.2	1.476 ± 0.8	0.678 ± 0.4

Standard deviations reflect the deviation from the mean of three replicates per leaf (at three different positions), of four different plants.

2.5 Conclusion

Linear resistance analysis appears to indicate a large stomatal limitation of A under most natural conditions (Biscoe *et al.*, 1977), as well as suggesting large effects of stomatal closure on photosynthesis during drought stress (O'Toole *et al.*, 1977). If the inherent capacity of the leaf for CO_2 diffusion decreased in a perfectly proportional manner as Ψ_L decreased, then c_i should have remained constant (Farquhar & Sharkey, 1982). Instead of a balanced drought stress-induced decrease in these parameters and a constant c_i, we observed an increase in c_i as Ψ_L became more negative, which resulted as $\partial A/\partial c_i$ decreased (ca.80%) relatively more than g. In spite of the fact that the measured l tends to overestimate stomatal limitations (Farquhar & Sharkey, 1982), our calculated l values generally increased from 21% to 48% during, and decreased 48% to 21% after drought stress, which further proves that in the long term reduced mesophyll capacity was the dominant factor causing a reduction in A during the slowly intensifying drought stress. This is further supported by the observed rise in Γ with decreasing Ψ_L, which has also been interpreted as indirect evidence for the involvement of non-stomatal factors in the response of photosynthesis to drought stress. In the short term, however, we concede the importance of stomatal functioning in keeping plants within their optimum thermal kinetic windows during environmental changes in light intensity, humidity and temperature. But, as $\partial E/\partial A$ did not remain constant in our experiment, further indicates that the ability of stomatal control to keep the plants within their respective thermal kinetic windows at medium to severe stress, was exceeded.

The drought stress-induced decrease of starch observed in all four tobacco cultivars (Van Rensburg *et al.*, 1993), not only confirms the finding that drought stress stimulates starch mobilization (Fox & Geiger, 1984), but also clearly indicates a drought stress-induced limitation of photosynthate production rather than a limitation of utilization. Although evidence is provided which demonstrates the remarkable tolerance of the photosynthetic apparatus to desiccation, however, due to the true mesophytic nature of tobacco, neither $\partial E/\partial A$ nor WUE may be useful as drought tolerance selection criteria. In conclusion, it can be stated that the findings presented not only identified a drought stress-induced decrease in $\partial A/\partial c_i$ as a possible key control point in determining the drought tolerance of a cultivar, but also indicated that GS46 and ELSOMA are more drought tolerant than TL33 and CDL28, because the former cultivars are capable of maintaining a reasonable A during drought stress, which facilitates their recovery upon rewatering.

The known difference in drought tolerance may therefore be the result, though be it only in part, of a capability on the part of the drought-tolerant cultivars to maintain a higher $\partial A/\partial c_i$ during decreasing Ψ_L , and resistance to a drought stress-induced rise in Γ, decrease in WUE,

and specifically a decrease in A. In this regard it has been speculated that differences in the ability to osmoregulate may be responsible for the observed difference in the maintenance of carbon fixation in leaves during drought (Conroy *et al.*, 1988).

2.6 Literature cited

BEGG, J.E. & TURNER, N.C. 1970. Water potential gradients in tobacco. *Plant Physiology*, 46: 343-346.

BEGG, J.E. & TURNER, N.C. 1976. Crop water deficits. *Advances in Agronomy*, 28: 161-217.

BIELORAI, H. & HOPMANS, P.A.M. 1975. Recovery of leaf water potential, transpiration and photosynthesis of cotton during irrigation cycles. *Agronomy Journal*, 67: 629-632.

BISCOE, P.V., INCOLL, L.D., LITTLETON, E.J. & OLLERENSHAW, J.H. 1977. Barley and its environment VII: Relationships between irradiance leaf photosynthetic rate and stomatal conductance. *Journal of Applied Ecology*, 14: 293-302.

BJÖRKMAN, O. & POWLES, S.B. 1982. Inhibition of photosynthetic reactions under water stress: interaction with light level. *Planta*, 161: 490-504.

BOYER, J.S. 1971. Recovery of photosynthesis in sunflower after a period of low leaf water potential. *Plant Physiology*, 47: 816-820.

BURKE, J.J., HATFIELD, J.L. & MAHAN, J.R. 1989. Quantifying thermal stress in cotton through the thermal kinetic window. Proceedings, Beltwide cotton production research conferences. Nashville Tennessee: National Cotton Council of America, 1: 44-45.

CHAVES, M.M. 1991. Effects of water deficits on carbon assimilation. *Journal of Experimental Botany*, 42: 1-16.

CHAVES, M.M. & RODRIGUES, M.L. 1987. Photosynthesis and relations in grapevines: response to environmental factors. (*In* Tenhunen, J.D., Catarino, F.M., Lange, O.L. & Oechl, W.C., *eds.* Plant Response to Stress, Functional Analysis in Mediterranean Ecosystems, pp 379-390. Berlin Heidelberg New York: Springer-Verlag.)

CHEESEMAN, J.M. 1991. PATCHY: simulating and visualizing the effects of stomatal patchiness on photosynthetic CO_2 exchange studies. *Plant Cell Environment*, 14: 593-599.

COLLATZ, G.J., FERRAR, P.J & SLATYER, R.O. 1976. Effects of water stress and differential hardening treatments of photosynthetic characteristics of a xeromorphic shrub, *Eucalyptus socialis*, F. Muell. *Oecologia*, 23: 95-105.

COMSTOCK, J. & EHLERINGER, J.R. 1984. Photosynthetic responses to slowly decreasing leaf water potentials in *Encelia fructescens*. *Oecologia*, 61: 241-248.

CONROY, J.P., VIRGONA, J.M., SMILLIE, R.M. & BARLOW, E.W. 1988. Influence of drought acclimation and CO_2 enrichment on osmotic adjustment and chlorophyll a fluorescence of sunflower during drought. *Plant Physiology*, 86: 1108-1115.

COWAN, I.R. 1977a. Stomatal behavior and environment. *Advanced Botanical Research*, 4: 117-228.

COWAN, I.R. 1977b. Water use in higher plants. (*In* McIntire, A.K., *ed.* Water Planets, Plants and People, Canberra*: Australian Academy of Science*, 182. p.71-107.)

COWAN, I.R. 1982. Regulation of water use in relation to carbon gain in higher plants. (*In* Lange, O.L., Nobel, P.S., Osmond, C.B. & Ziegler, H., *eds.* Encyclopedia of Plant Physiology, Physiological Plant Ecology II: Water Relations and Carbon Assimilation, Berlin Heidelberg New York: Springer-Verlag p. 589-613.)

CRONIC, G., LE GOUALLEC, J.L., BRIANTAIS, J.M. & HODGES, M. 1989. Effect of dehydration and high light on photosynthesis of two C_3 plants (*Phaseolus vulgaris* L. and *Elatostema repens* (Lour). Hall F.) *Planta*, 177: 84-90.

DE ROO, H.C. 1970. Leaf water potentials of tobacco, estimated with the pressure bomb. *Tobacco Science*, 14: 105-106.

DELGARDO, E., AZOÒN-BIETO, J., AVANDO, X., PALAZÓN, J. & MEDIANO, H. 1992. Leaf photosynthesis and respiration of high CO_2-grown tobacco plants selected for survival under CO_2 compensation point conditions. *Plant Physiology*, 98:949-954.

DEMMIG, B. & BJÖRKMAN, O. 1987. Comparison of the effect of excessive light on chlorophyll fluorescence (77K) and photon yield of O_2 evolution in leaves of higher plants. *Planta*, 171: 171-184.

DORNHOFF, G.M. & SHIBLES, R.M. 1970. Varietal differences in net photosynthesis of soybean leaves. *Crop Science*, 10: 42-45.

EHLERINGER, J. 1983. Ecophysiology of *Amaranthus palmeri*, a Sonoran Desert summer annual. *Oecologia*, 57: 107-112.

EHLERINGER, J.R. & COOK, C.S. 1984. Photosynthesis in *Encelia farignosa* Gray in response to decreasing leaf water potential. *Plant Physiology*, 75: 688-693.

EVANS, J.R. 1983. Nitrogen and photosynthesis in the flag leaf of wheat (*Triticum aestivum* L.). *Plant Physiology*, 72: 297-302.

FARQUHAR, G.D., SCHULZE, E.D. & KÜPPERS, M. 1980a. Responses to humidity by stomata of *Nicotiana glauca* L. and *Corylus avellana* L. are consistent with the optimization of carbon dioxide uptake with respect to water loss. *Australian Journal of Plant Physiology*, 7: 315-327.

FARQUHAR, G.F., VON CAEMMERER, S. & BERRY, J.A. 1980b. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta*, 49: 78-90.

FARQUHAR, G.F. & SHARKEY, T.D. 1982. Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology*, 33: 317-345.

FORSETH, I.N. & EHLERINGER, J.R. 1983. Ecophysiology of two solar tracking desert winter annuals. III. Gas exchange responses to light, CO_2 and VPD in relation to long term drought. *Oecologia*, 57: 340-351.

FOX, T.C. & GEIGER, D.R. 1986. Osmotic response of sugar beet leaves at CO_2 compensation point. *Plant Physiology*, 80: 239-241.

GRAAN, T. & BOYER, J.S. 1990. Very high CO_2 partially restores photosynthesis in sunflower at low water potentials. *Planta*, 181: 378-384.

GREER, D., BERRY, J.A. & BJÖRKMAN, O. 1986. Photoinhibition of photosynthesis in intact bean leaves: role of light and temperature and requirement for chloroplast-protein synthesis during recovery. *Planta*, 168: 253-260.

HALL, A.E. 1982. Mathematical models of plant water loss and plant water relations. (*In* Lange, O.C.; Nobel, P.S,; Osmond, C.B., & Ziegler, H., *eds.* Encyclopedia of Plant Physiology,, Physiological Plant Ecology II: Water Relations and Carbon Assimilation. Berlin Heidelberg New York: Springer-Verlag. p. 231-261.)

HALL, A.E. & SCHULZE, E.D. 1980. Stomatal response to environment and a possible interrelation between stomatal effects on transpiration and CO_2 assimilation. *Plant Cell Environment,* 3: 467-474.

HAMID, A., AGATA, W. & KAWAMITSU, Y. 1990. Photosynthesis, transpiration and water use efficiency in four cultivars of mugbean, *Vigna radiata* L. Wilczek. *Photosynthetica,* 24: 96-101.

HANSON, A.D. 1982. Metabolic responses of mesophytes to plant water deficits. *Annual Review of Plant Physiology,* 33: 163-203.

HEATH, O.V.S. & MEIDNER, H. 1961. The influence of water strain as the minimum intercellular space CO_2-concentration, Γ, and stomatal movement in wheat leaves. *Journal of Experimental Botany,* 12: 226-242.

HOFFMAN, G.J. & SPLINTER, W.E. 1968. Water potential measurements of an intact plant- and soil-system. *Agronomy Journal,* 60: 408-413.

HUBER, S.C., ROGERS, H.M. & MOWRY, F.L. 1984. Effects of water stress on photosynthesis and carbon partitioning in soybean (*Glycine max* L. Merr) plants grown in the field at different CO_2 levels. *Plant Physiology,* 76: 244-249.

HUFFAKER, R.C., RADMIN, T., KLEINKOPF, G.E. & COX, E.L. 1970. Effect of mild water stress on enzyme nitrate reductase assimilation and of carboxylation phase of photosynthesis in barley. *Crop Science,* 10: 471-473.

HUTMACHER, R.B., & KRIEG, D.R. 1983. Photosynthetic rate control in cotton: Stomatal and nonstomatal factors. *Plant Physiology,* 73: 658-661.

JOHNSON, R.R., FREY, N.M. & MOSS, D.N. 1974. Effect of water stress on photosynthesis and transpiration of flag leaves and spikes of barley and wheat. *Crop Science,* 14: 728-731.

JONES, H.G. 1973. Moderate term water stresses and associated changes in some photosynthetic parameters in cotton. *New Phytologist,* 72: 1095-1105.

KAISER, W.M. 1987. Effects of water deficit on photosynthetic capacity. *Physiologia Plantarum,* 71: 142-149.

KAWASHIMA, N. & MITAKE, T. 1969. Studies on protein metabolism in higher plants. VI: Changes in ribulose diphosphate carboxylase activity and fraction 1 protein content in tobacco leaves with age. *Agricultural and Biological Chemistry,* 33: 539-543.

KIRSCHBAUM, M.U.F. 1988. Recovery of photosynthesis from water stress in *Eucalyptus pauciflora:* a process in two stages. *Plant Cell Environment,* 11: 685-694.

KRIEG, D.R. 1989. Leaf gas exchange as a function of temperature. Proceedings, Beltwide Cotton Production Research Conferences. Nashville Tennessee: National Cotton Council of America, 1: 49-52.

LANGE, O.L., HARLEY, P.C., BEYSCHLAG, W. & TENHUNEN, J.D. 1987. Gas exchange methods for characterizing the impact of stress on leaves. (*In* Tenhunen, J.D., Catarino, F.M., Lange, O.L. & Oechl, W.C., *eds.* Plant Response to Stress, Functional Analysis in Mediterranean Ecosystems, Berlin Heidelberg New York: Springer-Verlag p. 3-25.)

LAUER, M.J. & BOYER, J.S. 1992. Internal CO_2 measured directly in leaves: Abscisic acid and low leaf water potential cause opposing effects. *Plant Physiology,* 98: 1310-1316.

LONG, S.P. & HÄLLGREN, J.E. 1985. Measurement of CO_2, assimilation by plants in the field and the laboratory. (*In*: Coombs, J., Hall, D.O., Long, S.P. & Scurlock, J.M.O.,*eds.* Techniques in Bioproductivity and Photosynthesis. Oxford: Pergamon Press. p. 62-94.)

LUUKKANEN, O. 1976. Relationship between the CO_2-compensation point and carbon fixation efficiency in trees. (*In* Cannell, M.G.R. & Last, F.T., *eds.* Tree Physiology and Yield Improvement, London, New York: Academic Press. p. 111-118.)

MARTIN, B. & THORSTENSON, Y.R. 1988. Stable carbon isotope composition ($\delta^{13}C$), water use efficiency, and biomass productivity of *Lycopersicon exculentum, Lycopersicon pennellii*, and the F_1 hybrid. *Plant Physiology,* 88: 213-217.

MAYORAL, M.L., ATSMON, D., SHIMISHI, D. & GROMET-ELHANAN, Z. 1981. Effect of water stress on enzyme activities in wheat and related wild species: Carboxylase activity, electron transport and photophosphorylation in isolated chloroplasts. *Australian Journal of Plant Physiology*, 8: 385-393.

MEIDNER, H. 1961. The minimum intercellular space carbon dioxide concentration in leaves of the palm *Pheonix reclinata*. *Journal of Experimental Botany*, 12: 409-413.

MEIDNER, H. 1967. Further observations on the minimum intercellular space carbon dioxide concentration (Γ) of maize leaves and the postulated role of "photorespiration" and glycolate metabolism. *Journal of Experimental Botany*, 18: 177-185.

MOONEY, H.A., BJÖRKMAN, O. & COLLATZ, G.J. 1977. Photosynthetic acclimation to temperature and water stress in the desert shrub *Larrea divaricata*. *Carnegie Institution Washington Year Book*, 76: 328-335.

NIR, I., POLJAKOFF-MAYER, A. & KLEIN, S. 1970. The effect of water stress on the polysome population and the ability to incorporate amino acids in maize root tips. *Israel Journal of Botany*, 19: 451-462.

O'TOOLE, J.C., CROOKSTON, R.K., TREHARNE, K.J., & OZBUN, J.L. 1976. Mesophyll resistance and carboxylase activity: a comparison under water stress conditions. *Plant Physiology*, 57:465-468.

O'TOOLE, J.C., OZBUN, J.L. & WALLACE, D.H. 1977. Photosynthetic response to water stress in *Phaseolus vulgaris*. *Physiologia Plantarum*, 40: 111-114.

OHAD, I., KYLE, D.J. & ARNTZEN, C.J. 1984. Membrane protein damage and repair: removal and replacement of inactivated 32-kilodalton polypeptides in chloroplast membranes. *Journal of Cell Biology*, 99: 481-485.

OSMOND, C.B., AUSTIN, M.P., BERRY, J.A., BILLINGS, W.D., BOYER, J.S., DANCEY, J.W.H., NOBEL, P.S., SMITH, S.D. & WINNER, W.E. 1987. Stress physiology and the distribution of plants. *Bioscience*, 37: 38-48.

PARSONS, L.R. 1979. Breeding for drought resistance: what plant characteristics impart resistance? *Horticultural Science*, 14: 590-593.

PETERSON, L.W., KLEINKOPF, G.E. & HUFFAKER, R.C. 1973. Evidence for lack of turnover of ribulose 1,5-diphosphate carboxylase in barley leaves. *Plant Physiology,* 51: 1042-1045.

PLAUT, Z. 1971. Inhibition of carbon dioxide fixation in isolated spinach chloroplasts exposed to reduced osmotic potential. *Plant Physiology,* 48: 591-595

PLAUT, Z. & BRAVDO, B. 1973. Response of carbon dioxide fixation to water stress. *Plant Physiology,* 52: 28-32.

QUICK, W.P., CHAVES, M.M., WENDLER, R., DAVID, M.M., RODRIQUES, M.L., PASSAHARINHO, J.A., PEREIRA, J.S., LEEGOOD, R.C., & STITT, M. 1992. Effects of water stress on photosynthetic carbon metabolism in four species grown in field conditions. *Plant Cell Environment,* 15: 25-35.

RADIN, J.W. 1989. When is stomatal control of water loss consistent with the thermal kinetic window concept? Proceedings, Beltwide Cotton Production Research Conferences.Nashville Tennessee: National Cotton Council of America, 1: 46-49.

RADIN, J.W. & ACKERSON, R.C. 1981. Water relations of cotton plants under nitrogen deficiency III. Stomatal conductance, photosynthesis, and abscisic acid accumulation during drought. *Plant Physiology,* 67: 115-119.

REDSHAW, A.J. & MEIDNER, H. 1972. Effects of water stress on the resistance to the uptake of carbon dioxide in tobacco. *Journal of Experimental Botany,* 23: 229-240.

RITCHIE, J.T. 1974. Atmospheric and soil water influences on the plant water balance. *Agricultural Meteorology,* 14: 183-198.

SANCHEZ, R.A., HALL, A.J., TRAPANI, N. & HUNAU, R.C. 1983. Effects of water stress on the chlorophyll content, nitrogen level and photosynthesis of leaves of two maize genotypes. *Photosynthesis Research,* 4: 35-47.

SCHULZE, E.D. 1986. Carbon dioxide and water vapor exchange in response to drought in the atmosphere and in the soil. *Annual Review of Plant Physiology,* 37: 247-274.

SCHULZE, E.D. & HALL, A.E. 1982. Stomatal responses, water loss and CO_2 assimilation rates of plants in contrasting environments. (*In* Lange, O.L., Nobel, P.S., Osmond, C.B. & Ziegler, H., *eds.* Encyclopedia of Plant Physiology, Physiological Plant Ecology II: Water Relations and Carbon Assimilation, Berlin Heidelberg, New York: Springer-Verlag. p. 181-230.)

SHARKEY, T.D., IMAI, K., FARQUHAR, G.D. & COWAN, I.R. 1982. A direct confirmation of the standard method of estimating intercellular partial pressure of CO_2. *Plant Physiology*, 69: 657-659.

SHEARMAN, L.L., EASTIN, J.D., SULLIVAN, C.Y. & KINBACHER, E.J. 1972. Carbon dioxide exchange in water stressed sorghum. *Crop Science*, 12: 406-409.

STUHLFAUTH, T., SÜLTEMEYER, D.F., WEINZ, S. & FOCK, H.P. 1988. Fluorescence quenching and gas exchange in a water stressed C_3 plant, *Digitalis lanata*. *Plant Physiology*, 86: 246-250.

SUZUKI, S. 1988. The effects of environmental factors and SADH on photosynthesis, transpiration and water use efficiency in bean plants. Sci. Rep. Fac. Agric., Nagoya (Japan): Meijo University, 24: 1-8.

TAYLOR, S.E. & TERRY, N. 1984. Limiting factors in photosynthesis. V. Photochemical energy supply co-limits photosynthesis at low values of intercellular CO_2 concentration. *Plant Physiology*, 75: 82-86.

TERASHIMNA, I., WONG, S.C., OSMOND, C.B. & FARQUHAR, G.D. 1988. Characterisation of non-uniform photosynthesis induced by abscisic acid in leaves having different mesophyll anatomies. *Plant and Cell Physiology*, 29: 385-394.

TURNER, N.C. 1986. Crop water deficits: a decade of progress. *Advances in Agronomy*, 39: 1-51.

TURNER, N.C. & JONES, M.M. 1980. Turgor maintenance by osmotic adjustment: A review and evaluation. (*In* Turner N.C. & Kramer P.J., *eds.* Adaptation of Plants to Water and High Temperature Stress, New York: Wiley and Sons. p. 87-103.)

VAN RENSBURG, L., KRÜGER, G.H.J., & KRÜGER, H. 1993. Proline accumulation as drought tolerance selection criterion: Its relationship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. *Journal of Plant Physiology*, 141:188-194.

VAN RENSBURG, L & KRÜGER, G.H.J. 1993. Differential inhibition of photosynthesis *in vivo* and *in vitro*, and changes in chlorophyll *a* fluorescence induction kinetics of four tobacco cultivars under drought stress. *Journal of Plant Physiology*, 141:357-365.

VON CAEMMERER, S. & FARQUHAR, G.D. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta*, 153: 376-387.

VOS, J., & OYARZÚN, P.J. 1987. Photosynthesis and stomatal conductance of potato leaves - effects of leaf age, irradiance, and leaf water potential. *Photosynthesis Research*, 11: 253-264.

WILLMOTT, C.J. 1982. Some comments on the evaluation of model performance. *Bulletin of the American Meteriological Society*, 63: 1309-1313.

CHAPTER THREE

3. DIFFERENTIAL INHIBITION OF PHOTOSYNTHESIS (*IN VIVO* AND *IN VITRO*), AND CHANGES IN CHLOROPHYLL *A* FLUORESCENCE INDUCTION KINETICS OF FOUR TOBACCO CULTIVARS UNDER DROUGHT STRESS.

(VAN RENSBURG, L. & KRüGER, G.H.J. 1993. *JOURNAL OF PLANT PHYSIOLOGY*, 141 : 357 - 365.)

3.1 Abstract

This study was conducted in an attempt to explain the observed difference in drought tolerance of four cultivars of *Nicotiana tabacum* L., by monitoring plant water status and several aspects of photosynthesis, including chlorophyll *a* fluorescence under controlled environmental conditions. Drought stress of increasing intensity, ranging from light to severe, was induced over a twelve-day period by withholding water. Photosynthetic oxygen evolution was monitored *in vivo* at a CO_2 concentration exceeding 5% to exclude any stomatal limitation and was expressed as quantum efficiency (Φ). Partial photochemical reactions were determined polarographically, with isolated thylakoids of the drought stressed tissue. From the Kautsky curves the I/P-ratio and ΔF-values were determined at various decreasing leaf water potentials (Ψ_L).

All four cultivars exhibited a drought stress-induced decline in Φ, PSI-, PSII- and PSI + PSII-activity, but the extent and nature differed among the different cultivars. The Φ-values of the drought-tolerant cultivars were characterized by a fast initial decline which stabilized as the Ψ_L declined, whereas the Φ-values of drought-sensitive cultivars did not show a similar response, but declined at a slow continuous rate as the drought stress intensified. The PSII-activity and PSI- + PSII-activity were influenced in much the same way. PSII-activity was found to be more sensitive than PSI-activity and even more so in the case of the drought-sensitive than in the drought-tolerant cultivars. It was concluded that the observed stress-induced decline in Φ and PSI- + PSII-activity could largely be ascribed to an inhibition of PSII-activity. This conclusion is corroborated by both the fluorescence data regarding the I/P-ratio and ΔF-values, of which the former was found to increase earlier and reach higher end-values in the drought-sensitive cultivars, and the latter, which indicated that the drought-tolerant cultivars were characterized by a higher photosynthetic efficiency at low Ψ_L. A mechanism is proposed which might retard or reverse drought stress induced stromal acidification and might explain the apparently higher photosynthetic dehydration tolerance of the drought-tolerant cultivars. The possible involvement of ABA is discussed.

Keywords: *Drought tolerance, Nicotiana tabacum* L., *photochemical activity, quantum efficiency.*

Abbreviations: ABA = abscisic acid; Asc = ascorbate; DCPIP = 2,6-dichlorophenolidophenol; DCMU = 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; FeCy = ferricyanide; MV = methyl viologen; PDox = *p*-phenylenediamine (oxidized form); PQ = plastoquinone; SOD = superoxide dismutase; Φ = quantum efficiency; Ψ_g = gravitational potential; Ψ_L = leaf water potential; Ψ_m = matric potential; Ψ_p = pressure potential; Ψ_Π = osmotic potential.

3.2 Introduction

Damage to crop plants and reduction in yield are often brought about by unfavourable environmental conditions. Looking to the future, it seems that some environmental stresses will inevitably intensify, and that this, coupled with practices such as using land marginally suited for agriculture and growing crop plants in climates for which the plants are ill-adapted, are likely to create an increasing need for new stress tolerant varieties. Unless it is clear which physical, chemical and metabolic problems the cells of the stressed plant must avoid or surmount, the means by which it does so are unlikely to be recognized for what they are.

Stromal acidification has been identified as a mediating mechanism for the drought stress induced inhibition of photosynthesis in isolated chloroplasts (Berkowitz & Gibbs, 1983). Elucidation of the mediating mechanism of water deficit-induced inhibition of the basic biochemical processes facilitating carbon assimilation could thus be significant in terms of understanding and possibly manipulating crop responses to drought, as Farquhar and Sharkey (1982) have indicated that non-stomatal-mediated inhibitions of photosynthesis are a major limitation to crop growth during drought stress. Non-stomatal-mediated water deficit effects on carbon assimilation, therefore, warrant further investigation.

This paper presents our results and observations of the nature and extent of the effects of the intensity and duration of drought stress on photosynthetic oxygen evolution (*in vivo* and *in vitro*), and chlorophyll *a* fluorescence induction kinetics in four cultivars of *Nicotiana tabacum* L., as a function of the genetically-determined capacity of the different cultivars to cope with drought stress. An inhibition site is identified, and the possible causes of the observed drought stress-induced inhibition of oxygen evolution as well as some aspects of photosynthetic dehydration tolerance are discussed.

3.3 Material and methods

Plant material and general growth conditions

In sequence of increasing drought tolerance, the four cultivars of *Nicotiana tabacum* L. under investigation were TL33, CDL28, GS46 and ELSOMA. These cultivars were selected due to their differing performances in the field during drought stress periods (Personal communication: Dr. C.J. Steenkamp, Tobacco and Cotton Research Institute, Rustenburg, R.S.A.). Seed was germinated in soil in pots under glasshouse conditions, with optimal water application (watered once daily to field capacity). Before the onset of experimentation the plants were moved to a growth chamber (photon flux density, 400-600 μmol $m^{-2}s^{-1}$; relative humidity, 35%) and allowed to acclimatize for a period of 96 hours. During the acclimatization period the plants were watered twice daily to avoid their experiencing any drought stress because of the slightly higher growth chamber temperature. The growth chambers were lit for 13 hours at 25°C followed by an 11-hour dark period at 16°C. Experimentation started when the plants were approximately 90 days old and a drought stress of increasing intensity (0.2 - 0.3 MPa d^{-1}) was induced by withholding water. The drought stress lasted for 12 days and ranged from light (-0.52 MPa), to severe (-2.51 MPa). As was the case with all other measurements, Ψ_L determinations were done on alternate days with a Scholander pressure chamber (PMS-instrument, Oregon, USA).

Quantum efficiency measurements, *in vivo*

Steady-state rates of photosynthetic O_2 evolution were measured at CO_2 saturation in a polarographic leaf disc electrode (LD-2 Hansatech Ltd., Kings Lynn, Norfolk) designed by Delieu and Walker (1981, 1983). Saturating CO_2 (±5%) was provided by means of a carbonate/bicarbonate buffer (Delieu & Walker, 1981). Photosynthesis was measured as a function of light intensity by using a fan-cooled 50 Watt quartz lamp (Hansatech LS2). This light source delivered uniform illumination over 8.85 cm^2 of the 10 cm^2 leaf disc and when used in conjunction with different combinations of neutral density filters (Melles Griot, Amsterdam), defined lower photon flux densities were obtained. Usually 7-12 filter combinations (48-2080 μ mol $m^{-2}s^{-1}$) were selected to establish the initial slope of the light response curve, from which the quantum effiency was calculated (after correcting the slope for the particular absorbance of *Nicotiana tabacum* L. namely ca.85%). The exact procedure followed and equations used concur with those used by Delieu and Walker (1981) and Walker and Osmond (1986).

Assay of electron transport

By using the isolation medium and procedure as described by Buschmann and Grumbach (1985), chloroplasts were isolated and thylakoid membranes prepared by homogenizing approximately 0.5g intravenous leaf lamina in a pre-cooled 50-250ml blender (Waring Dynamics Corp., New Hatford, V.S.A.) for 10s at high speed, after making sure that the pre-chilled (2-4°C) isolation medium (pH set at 6.1 with NaOH) totally covered the leaf material. After isolation, the moderately pure sediment of chloroplasts was resuspended in resuspension medium with the same constitution as that of the isolation medium but of which the pH was set at 6.5 with NaOH.

Chlorophyll determinations were done as described by Arnon (1949) and electron transport rates were determined polarographically, by measuring O_2 evolution or consumption with a Clark-type electrode which monitors a continuously stirred and thermostatted (25°C) glass cuvette. PSII-activity (reduction of FeCy by reduced PD), was conducted in a reaction medium which contained 2.5mL 30mM NaCl, 0.1mM PDox, 280 µM FeCy, 5mM Na-Tricine and 50µg chlorophyll mL^{-1} of thylakoid suspension in a final volume of 2.5mL. This reaction medium corresponds well with that used by Nielsen and Smillie (1978). PSI activity (reduction of MV by DCPIP as electron donor), was assayed in a 3.0mL reaction medium which varied slightly from that used by Izawa (1980) and contained 30mM NaCl, 3µM DCMU, 30mM methylamine hydrochloride, 20mM DCPIP, 100µM MV, 2.5mM Asc, 0.4mg SOD, 1mM KCN, 50mM Na-Tricine and thylakoids with a chlorophyll concentration of 22.5µg chlorophyll mL^{-1}. Whole-chain electron transport: $H_20 \rightarrow$ PSII $\rightarrow$ PSI $\rightarrow$ FeCy, was assayed in a reaction mixture modified from that used by Nielsen and Smillie (1978) and contained 30mM NaCl, 30mM methylamine hydrochloride, 80µM FeCy, 5mM Na-Tricine, and thylakoids equivalent to 50µg chlorophyll mL^{-1} in a final volume of 2.5mL.

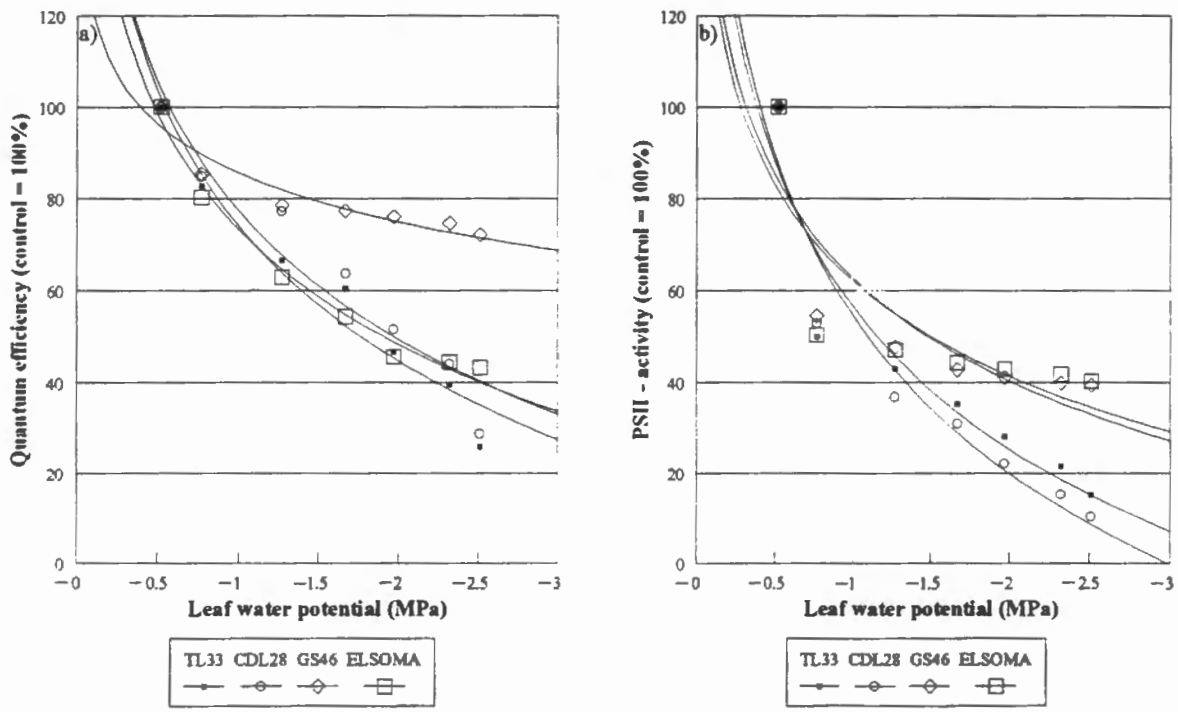
Chlorophyll fluorescence induction determinations

Kinetics of the induced chlorophyll fluorescence (after a dark adaptation period of half an hour) were assayed by using a portable fluorometer (SF-10, Richard Brancker Research Ltd., Ottawa). The measuring sensor of the fluorometer, which was placed directly on the leaf surface (intravenously) of an intact plant, contained both a light-emitting diode to irradiate the leaf surface and a photodiode to detect the induced chlorophyll fluorescence emission. This provided a "monochromatic" illumination at a 670nm maximum and $10Wm^{-2}$ for periods of 3min duration. A Corning CS7-69 cut-off filter behind the LED excluded any stray light, reflected light, and fluorescence at wavelenghts below 710nm while allowing PSII fluorescence to reach the sensor. The output was monitored on a pen recorder (Rikadenki, Kogyo Co., Ltd., Tokyo). From the Kautsky curves obtained for each cultivar the following parameters were determined: the I/P-

ratio as described by Shaw *et al.* (1985) and the ΔF-values determined and calculated according to the method described by Kulandaivelu and Daniell (1980). The I/P-ratio has been defined as the ratio of the height of the inflection point (I) and the height of the peak fluorescence level (P) of a Kautsky curve, and is therefore, a good indicator of photosyntetic inhibition (Shaw *et al.*, 1985). When the electron transport, however, is blocked before plastoquinone by DCMU, the fluorescence level increases; and this increase in level (fluorescence level in the presence of DCMU minus fluoresence in the absence of DCMU, ΔF) reflects the relative photosynthetic efficiency (Samuelsson & Öquist, 1977).

3.4 Results and discussion

In accordance with literature (Mohanty & Boyer, 1976), this investigation also proved that drought stress causes a decline in the quantum efficiency of photosynthesis in intact leaves (Φ) (Fig. 1a). Mooney *et al.* (1977) observed that when the desert shrub *Larrea diveriacata* was exposed to drought stress, the Φ of photosynthesis declined at almost the same rate as its light-saturated photosynthetic rate. Of utmost importance (as possible cause of the known difference in drought tolerance among the four tobacco cultivars), is the observation that the magnitude and rate of their decline in Φ differed as drought stress intensified (Fig. 1a). Furthermore, the calculated Φ -values at different Ψ_L for GS46 and ELSOMA showed a close correlation, but contrasted sharply with those of TL33 and CDL28, which in turn correlate in value (Table 1). The Φ -values of the drought-tolerant cultivars showed a sharp initial decline during a mild drought stress but as the drought stress intensified, the decline in Φ occurred more gradually. In contrast, the Φ -values of TL33 and CDL28 show a more gradual but continuous decline over the total drought stress range (Fig. 1a). To explain the observed decline in Φ during a drought stress, it should be noted that in this investigation, similar to that of Mohanty and Boyer (1976), it was found that the decline in photosynthetic Φ of the intact leaf tissue corresponded with a decline in PSII electron transport in the isolated thylakoid membranes of the drought stressed leaves (Fig. 1a and b). Our study and other studies (Keck & Boyer, 1974) provide strong evidence that the inhibition of total photosynthetic activity (Fig. 1d), which has also been reported for sunflower and soybean plants exposed to drought stress, can be partly attributed to an inactivation of PSII-activity and a corresponding decrease in non-cyclic photophosphorylation.



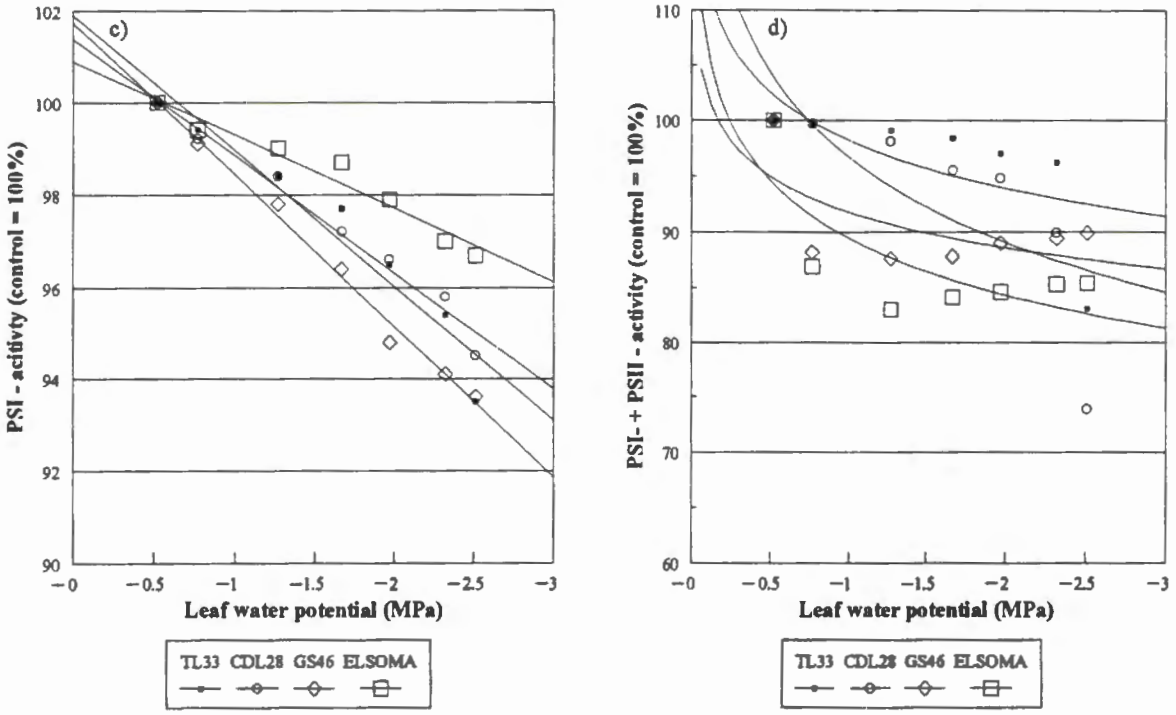


Figure 1. Differential drought stress-induced declines in (a) the quantum efficiency of photosynthesis of intact leaves (Φ); (b) PSII-activity; (c) PSI-activity, and (d) PSI- + PSII-activity in four cultivars of *Nicotiana tabacum* L. (b-d: isolated thylakoids).

The stability of PSI-activity, in contrast with the sensitivity of the PSII-mediated chloroplast reactions observed in this investigation (Table 1; Fig. 1b and 1c), has also been observed in plants reacting to other environmental stresses such as heat and cold (Sharkey & Badger, 1982). It therefore seems quite evident that PSII-activity is more sensitive to a variety of stresses than PSI-activity, and even more so in drought-sensitive cultivars. This statement corresponds with the general conclusion of Witt *et al.* (1965), which is based on the results of experiments conducted with mechanical and chemically-isolated photosystems, namely that PSI-activity is pH independent between 4.0 and 11.0 while PSII-activity shows a sharp maximum at a pH of 6.5. Furthermore, Harnischfeger (1974) observed maximum Hill reaction rates at a pH of 6.3 in the system: $H_20 \rightarrow$ FeCy. When taking the above-mentioned results into account and seeing that the method of Buschmann and Grumbach (1985) was employed in the study, the photochemical activity of all four the cultivars under investigation was conducted within the correct pH range (Sesták *et al.*, 1978), with the result that the observed inhibition of especially the PSII-activity (Fig. 1b) cannot be ascribed to an artificial buffer-induced pH effect.

Table 1: Drought stress-induced changes in the *in vivo* photosynthetic activity (expressed as quantum efficiency) and *in vitro*, partial photochemical reactions, in four cultivars *Nicotiana tabacum* L. of different drought tolerance.

Cultivars	Ψ_L MPa	Φ-efficiency µmol O_2mol quanta^{-1}	%	PSII-activity µmol O_2mg Chl.$^{-1}h^{-1}$	%	PSII-activity µmol O_2mg Chl.$^{-1}h^{-1}$	%	PSII-activity µmol O_2mg Chl.$^{-1}h^{-1}$	%
TL33	-0.52	0.081 ± 0.001	100.0	430 ± 3.7	100.0	1732 ± 13.3	100.0	1566 ± 12.3	100.0
	-0.77	0.003 ± 0.003	82.7	215 ± 9.6	50.0	1723 ± 11.3	99.4	1561 ± 13.9	99.6
	-1.27	0.004 ± 0.004	66.6	185 ± 9.0	43.9	1705 ± 10.2	98.4	1552 ± 10.6	99.1
	-1.67	0.005 ± 0.005	60.4	152 ± 5.9	35.3	1693 ± 16.2	97.7	1541 ± 17.2	98.4
	-1.97	0.005 ± 0.005	46.9	121 ± 7.8	28.1	1673 ± 14.2	96.5	1520 ± 10.5	97.0
	-2.32	0.003 ± 0.003	39.5	93 ± 5.1	21.6	1654 ± 10.9	95.4	1508 ± 95.4	96.2
	-2.51	0.004 ± 0.004	25.9	65 ± 5.7	15.1	1621 ± 16.2	93.5	1302 ± 12.3	83.1
CDL28	-0.52	0.066 ± 0.004	100.0	494 ± 1.7	100.0	1514 ± 13.8	100.0	1380 ± 11.9	100.0
	-0.77	0.056 ± 0.005	84.8	261 ± 6.2	52.8	1502 ± 16.0	99.2	1376 ± 10.0	99.7
	-1.27	0.051 ± 0.005	77.2	182 ± 7.3	36.8	1491 ± 15.5	98.4	1654 ± 14.2	98.1
	-1.67	0.042 ± 0.004	63.6	153 ± 9.6	30.9	1472 ± 17.9	97.2	1319 ± 13.7	95.5
	-1.97	0.034 ± 0.003	51.5	109 ± 7.0	22.1	1463 ± 10.7	96.6	1309 ± 11.9	94.8
	-2.32	0.029 ± 0.001	43.9	75 ± 2.5	15.2	1451 ± 11.4	95.8	1241 ± 12.8	89.9
	-2.51	0.019 ± 0.004	28.7	51 ± 5.7	10.3	1431 ± 13.4	94.5	1020 ± 13.2	73.9
GS46	-0.52	0.075 ± 0.001	100.0	464 ± 1.9	100.0	1808 ± 17.2	100.0	1656 ± 11.8	100.0
	-0.77	0.064 ± 0.002	85.3	253 ± 7.7	54.5	1792 ± 16.5	99.1	1416 ± 15.6	88.2
	-1.27	0.059 ± 0.001	78.6	221 ± 6.9	47.6	1769 ± 10.9	97.8	1452 ± 15.2	87.6
	-1.67	0.058 ± 0.004	77.3	198 ± 3.7	42.7	1744 ± 18.2	96.4	1454 ± 18.5	87.8
	-1.97	0.057 ± 0.002	76.0	191 ± 2.5	41.1	1715 ± 21.4	94.8	1475 ± 17.5	89.0
	-2.32	0.056 ± 0.001	74.6	185 ± 5.5	39.8	1703 ± 22.4	94.1	1482 ± 17.9	89.4
	-2.51	0.054 ± 0.005	72.1	183 ± 3.2	39.4	1694 ± 19.9	93.6	1489 ± 13.9	89.9
ELSOMA	-0.52	0.081 ± 0.003	100.0	512 ± 1.5	100.0	1857 ± 11.9	100.0	1655 ± 12.3	100.0
	-0.77	0.065 ± 0.005	80.2	258 ± 3.0	50.3	1846 ± 23.6	99.4	1439 ± 11.9	86.9
	-1.27	0.051 ± 0.004	62.9	241 ± 6.8	47.1	1839 ± 19.2	99.0	1375 ± 15.6	83.0
	-1.67	0.044 ± 0.005	54.3	227 ± 4.6	44.3	1834 ± 16.9	998.7	1393 ± 17.3	84.1
	-1.97	0.037 ± 0.004	45.6	220 ± 1.5	42.9	1819 ± 10.0	97.9	1401 ± 17.2	84.6
	-2.32	0.036 ± 0.003	44.4	214 ± 5.9	41.7	1802 ± 14.1	97.0	1412 ± 10.9	85.3
	-2.51	0.035 ± 0.002	43.2	211 ± 5.8	40.2	1797 ± 17.8	96.7	1414 ± 11.7	85.4

As far as drought stress is concerned, the results obtained (Tables 1 and 2) provide a clear indication of the difference in sensitivity of the photochemical reactions of the thylakoid membrane systems of the respective cultivars. According to Sundari and Raghavendra (1990), the inability of previous researchers to observe a decline in the photochemical activity of chloroplasts (Plaut & Bravdo, 1973), can possibly be attributed to the progressive inhibition of reactions by osmotic stress caused by a prolonged inhibition period and the specific need for magnesium, which was lacking. The magnesium, content of the soil in our experiment was 430mg kg^{-1} and therefore not a limiting factor. The decline in photochemical activity (Fig. 1d) was noticed, even though the thylakoid preparations which were isolated from leaves with different Ψ_L , were measured in a medium with the same osmotic potential for all treatments (ca -1.0 to -1.1 MPa). If the photochemical reactions, therefore, were an exclusive function of

the osmotic potential of the isolation medium, no differences would have been observed in the photochemical activity at the different drought stress levels of the different cultivars. As differences were observed, these changes or disturbances must have occurred while the thylakoid membranes were still present in the leaf tissue and must have been irreversible or only slowly reversible by nature. Sundari and Raghavendra (1990) who observed corresponding changes, were not able to suggest a mechanism which could explain such drought stress-induced changes, but they suggested that it would appear that the decline in photochemical activity during drought stress was more dependent on the intensity than the duration of drought stress and that the extent of the inhibition was species-specific.

A literature survey, initiated to find an explanation for the observed drought stress-induced decline in Φ and photochemical activities, as well as for the observed variation in these parameters among the different tobacco cultivars investigated (Fig. 1a and d), revealed the following facts: Biological variation might well have influenced the total extent of the drought stress-induced inhibition, but it cannot be used as an explanation for the occurrence of the inhibition itself and neither can it account for the nature of the differences between the different cultivars, as all comparisons of the photosynthetic parameters were based on results obtained from plant material which was almost identical in their development. Furthermore, neither of the following can be regarded as underlying causes: (i) stomatal closure (Björkman & Powles, 1984); (ii) increased leaf temperature (Björkman & Powles, 1984); (iii) chloroplast turgidity *per se* (Boyer & Potter, 1973); (iv)Ψ_π *per se* (Potter & Boyer, 1973); (v) Ψ_g (Berkowitz, 1987); (vi) Ψ_m (Boyer, 1967); (vii) the Gibbs free energy of water (Potter & Boyer, 1973); (viii) measurable activity of various enzymes of the carboxylative phase of photosynthesis (Huffaker *et al.*, 1970; Plaut, 1971); (ix) plastocyanine concentration (Keck & Boyer, 1974); (x) or a limited diffusion of photosynthetic agents apart from CO_2.

It is known, however, that during drought stress the pH of the chloroplast stroma declines sharply. Variations as large as 2 pH units have been reported (Wedan & Heldt, 1972), with the result that the rate of photosynthetic O_2 evolution may be considerably inhibited (Maury *et al.*, 1981). According to Berkowitz and Gibbs (1983) the concentration of protons (H^+), resulting from a decline in cell volume caused by drought stress, is only a contributing factor and not the exclusive reason for stromal acidification. In correlation, the research conducted by Murray *et al.* (1981) on the influence of Mg^{2+} on chloroplasts showed that the rate of acidification caused by the addition of Mg^{2+} which leads to the induction of reversible H^+/K^- fluxes over the chloroplast envelope, are sufficient to explain the decline in O_2 evolution rates. A mechanism which may explain the release of K^+ from chloroplasts during drought stress was proposed by Berkowitz and Gibbs (1983). The latter implies that if the stroma K^+ concentration increases due to a decline in the chloroplast volume, the increased stroma K^+ concentration will

initiate an increased release of K^+, coupled to an import of H^+ via the K^+/H^+ antiport system. The crux of the above-mentioned suggestions is that drought stress causes a decline in stromal pH via ion exchange across the chloroplast envelope. This fact is then suggested as an explanation for the results obtained regarding the drought stress-induced decline in the Φ and photochemical reactions (Table 1). Apart from the fact that a decline in the stroma pH increases the sensitivity of photosynthesis for phosphate, which markedly influences the photosynthetic rate (Huber, 1979), doubt still exists about the exact reason why a decline in stromal pH inhibits photosynthesis. To our minds, the reason for the decline in the Φ and partial photochemical reactions (Fig. 1a to d) during drought stress could possibly be the result of the disturbance of the proton gradient caused by stromal acidification. This is in accordance with results published very recently (Rubenstein *et al.*, 1992).

To substantiate the latter statement, the observed results concerning the calculated chlorophyll *a* fluorescence induction parameters (Table 2) can be presented as evidence, as several components of the initial peak fluorescence curves have been used by many investigators as criteria for analysing PSII electron transport. In this regard the ratio of the inflection point (I) to that of the variable fluorescence peak (P) as determined from a Kautsky curve is, according to Shaw *et al.* (1985), an excellent *in vivo* system for the determination of inhibition of PSII photosynthetic activity.

Substantial increases in the level of I in relation to the level of P give an I/P-ratio of one or close to one, which indicated maximal photosynthetic inhibition (Shaw *et al.*, 1985). High I/P-values occur earlier in the drought-sensitive cultivars (already at a Ψ_L = -0.77 MPa in TL33 and CDL28) than in the drought-tolerant cultivars (Ψ_L = -1.97 MPa in GS46 and ELSOMA). For example, at a Ψ_L of -0.77 MPa the I/P-ratio of the drought-sensitive cultivars differed statistically significantly from their respective controls, while this is only the case at a Ψ_L of -1.77 MPa in GS46 and ELSOMA (Table 2). Furthermore, the maximal I/P-values of the drought-sensitive cultivars tend to reach an I/P-value of 1.0 earlier (already at a Ψ_L of -2.32 MPa in the case of TL33 and at -2.51 MPa in the case of CDL28) and it is higher throughout at any Ψ_L. Thus, for example, the I/P-values of TL33 and CDL28 are 0.959 and 0.941 respectively at a Ψ_L of -1.97 MPa, while they only increase to values of 0.291 and 0.994 in GS46 and ELSOMA respectively at a Ψ_L of -2.51 MPa. To summarize, it can be stated that the drought stress-induced damage to the photosynthetic apparatus occurred earlier in the drought-sensitive cultivars (due to the early attainment of a maximal I/P-ratio) and that the extent of the damage was more severe in the case of these cultivars (due to the steep gradients of the curves describing these cultivars' I/P-ratios), as the drought stress intensified (Fig. 2a).

Table 2: Changes in the chlorophyll *a* fluorescence induction kinetics, of four cultivars of *Nicotiana tabacum* L., during a period of water stress.

Cultivars	Ψ_L MPa	I/P-ratio	%	ΔF-values	%
TL53	-0.52	0.455 ± 0.01	100.0	4.98 ± 0.06	100.0
	-0.77	0.586 ± 0.03	128.7	4.49 ± 0.09	150.4
	-1.27	0.717 ± 0.09	157.5	7.85 ± 0.06	157.6
	-1.67	0.838 ± 0.07	184.2	8.23 ± 0.05	165.2
	-1.97	0.959 ± 0.05	210.8	8.59 ± 0.07	172.4
	-2.32	1.000 ± 0.03	219.8	8.92 ± 0.08	179.1
	-2.51	1.000 ± 0.00	219.8	9.25 ± 0.07	185.7
CDL28	-0.52	0.445 ± 0.01	100.0	4.24 ± 0.09	100.0
	-0.77	0.577 ± 0.03	129.6	6.96 ± 0.07	164.1
	-1.27	0.709 ± 0.07	159.3	7.89 ± 0.09	186.0
	-1.67	0.825 ± 0.06	185.4	8.23 ± 0.08	194.1
	-1.97	0.941 ± 0.08	211.5	8.73 ± 0.07	205.8
	-2.32	0.971 ± 0.04	218.2	9.13 ± 0.06	215.3
	-2.51	1.000 ± 0.00	224.7	9.31 ± 0.09	219.5
GS46	-0.52	0.394 ± 0.02	100.0	4.11 ± 0.07	100.0
	-0.77	0.468 ± 0.03	188.8	6.79 ± 0.09	165.2
	-1.27	0.543 ± 0.05	137.8	7.20 ± 0.05	175.1
	-1.67	0.615 ± 0.03	156.1	7.49 ± 0.03	182.2
	-1.97	0.686 ± 0.09	174.1	7.58 ± 0.09	184.4
	-2.32	0.843 ± 0.06	213.9	7.66 ± 0.08	186.3
	-2.51	0.921 ± 0.04	233.7	7.69 ± 0.04	187.1
ELSOMA	-0.52	0.394 ± 0.02	100.0	5.17 ± 0.06	100.0
	-0.77	0.498 ± 0.03	177.7	7.58 ± 0.03	146.6
	-1.27	0.598 ± 0.02	141.3	7.73 ± 0.06	149.5
	-1.67	0.686 ± 0.07	162.2	7.87 ± 0.09	152.2
	-1.97	0.775 ± 0.06	183.2	7.93 ± 0.05	153.3
	-2.32	0.887 ± 0.07	209.6	7.99 ± 0.08	154.8
	-2.51	0.994 ± 0.05	234.9	8.06 ± 0.07	155.8

* Standard errors reflect the deviation from the mean of four replicates per determination.
* Relative fluorescence units given in millivolt

When the electron flow is blocked before PQ as is done by DCMU, fluorenscence levels increase sharply up to the Fm-level. The increase in the ΔF fluorescence parameter (that is, the DCMU, induced fluorescence level Fm, minus the peak fluorescence level in the absence of DCMU (Fp)), reflects relative photosynthetic efficiency (Samuelsson & Öquist, 1977). Kulandaivelu and Daniell (1980) who evaluated the above-mentioned ΔF method (to determine whether it could also be used for *in vivo* determinations) observed that the O_2 evolution rate of algae, mesophyll cells and chloroplasts correlated closely (within 5%) with their ΔF-values. The calculated ΔF-values of the tobacco cultivars (Fig. 2b) confirm that the initial drought stress-induced inhibition of photochemical activity can largely be ascribed to the disturbance of PSII-associated processes (Lawlor, 1990), as the curves (Fig. 2b) describing the changes in the ΔF-

values resulting from the intensification of drought stress in the respective cultivars, appear almost like mirror images of those that represent the changes in Φ and PSII-activity (Fig. 1a and b). The high ΔF-values of TL33 (9.25) and CDL28 (9.31) at a Ψ_L of -2.51 MPa (Table 2) do not only correspond with the known difference of the drought tolerance of the respective cultivars, but also support the results obtained in connection with the Φ of the four cultivars (Table 1), as they indicate that under intense drought stress, GS46 and ELSOMA (with respective ΔF-values of 7.69 and 8.06 at a Ψ_L of -2.51 MPa), are characterized by a higher photosynthetic efficiency than TL33 and CDL28.

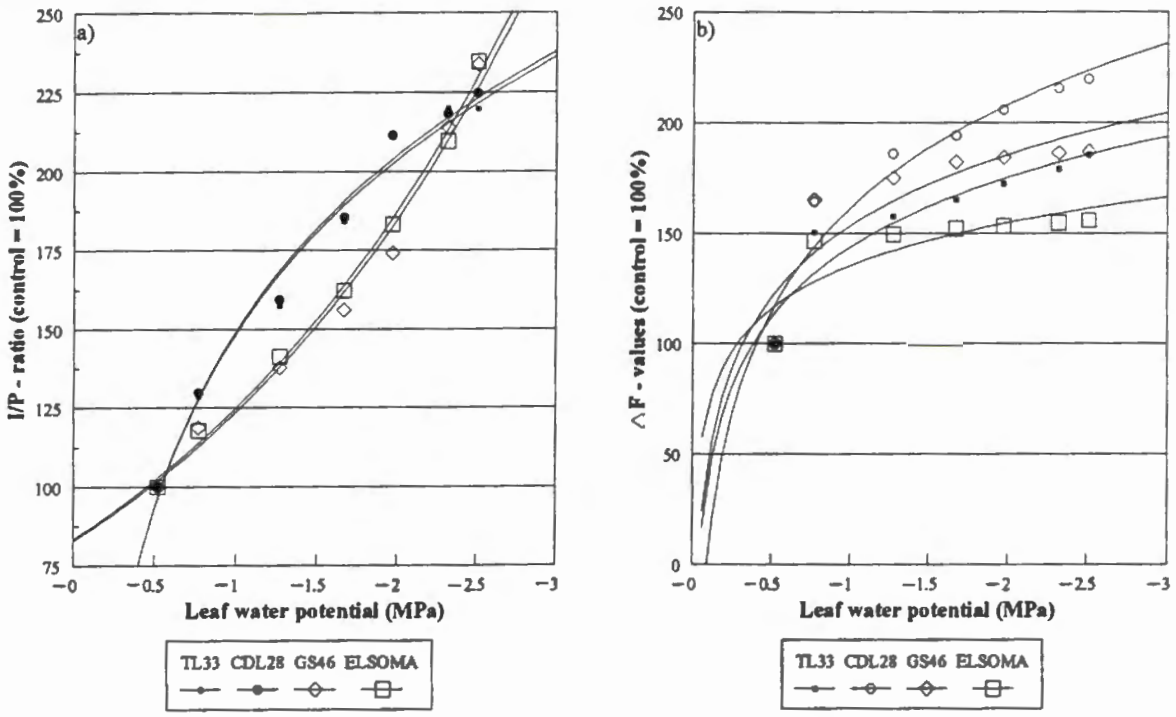


Figure 2. Drought stress-induced deviations from the control chlorophyll *a* fluorescence induction kinetics of (a) the I/P-ratio and (b) the ΔF-values, as calculated from the Kautsky curves of four tabacco cultivars of differing drought tolerance.

Based on the results obtained in this investigation (Tables 1 and 2), and results presented elsewhere (Van Rensburg & Krüger, 1993; Van Rensburg *et al.*, 1993) together with overwhelming evidence in the literature cited, a possible mode of action (absent in the drought-sensitive cultivars or merely less effective), is consequently proposed as an explanation for the results obtained. Such an operative system is reconcilable with the statement of Berkowitz and Gibbs (1983) that the inversion of stromal acidification is of critical importance for the maintenance of the photosynthetic process during drought stress. The proposed mechanism comprises, among others, the partial recovery of the normal stromal pH, or a delay of drought stress-induced stromal acidification, mediated by the stress hormone ABA. According to relevant literature, the distribution pattern of ABA in a leaf is dependent on pH gradients in the cell, the poor acid characteristics of the ABA molecule and the permeability characteristics of the

chloroplast envelope (Heilmann *et al.*, 1980; Taiz & Zeiger, 1992). In the uninhibited photosynthesizing leaf the pH of the chloroplast stroma is normally higher than that of the cytosol, which results in an accumulation of ABA in the chloroplasts. During drought stress, a net transfer of ABA takes place from the plastid to the cytosol and apoplast (Hartung *et al.*, 1988). It is at present generally accepted that a decline in turgidity during drought stress is responsible for the accumulation of ABA (Raschke, 1982). Although the biochemical reaction mechanisms of ABA on a cellular level are still not quite clear, existing evidence indicates that ABA affects membrane-associated processes (Van Steveninck & Van Steveninck, 1983). This is especially applicable to the electrochemical pump which is associated with K^+ efflux. In this way sporadic fluxes are caused in *Vicia faba* through the K+ channels and it has furthermore been indicated that ABA mediates K^+ efflux from unilamellar lipsomes (Harkers *et al.*, 1986).

We are of the opinion that it is highly probable that an interrelationship exists between the drought stress-induced processes of stromal acidification, the inhibition of PSII-activity (Fig. 1b), the decline in Φ (Fig. 1a), and the pH-dependent release of ABA from chloroplasts. It is also suggested that ABA inhibits the detrimental effects of stromal acidification on the process of photosynthesis by influencing the membrane permeability which leads to the accumulation of K^+ in the cytosol. This mechanism not only provides a possible explanation for the observed gradual decline in the Φ of the drought-tolerant cultivars, at the Ψ_L -values when the ABA levels start to increase rapidly (Van Rensburg & Krüger, unpublished), but also for the results of Berkowitz and Gibbs (1983) which indicated that high external K^+ concentrations inhibit the detrimental effects of drought stress on photosynthesis. Additionally, it is interesting to note that Kaiser (1982) indicated that the photosynthetic activity of leaf tissue in plants with a high cytosolic K^+ content shows more resistance to a decline in Ψ_L than leaf tissue with a low cytosolic K^+ content. The validity of the existence of the proposed mechanism is further supported by the fact that in general the stromal K^+ concentration is probably not higher than 25mM (Barber, 1976) and as it has already been calculated that the cytoplasmic K^+ concentration is considerably higher (ca.100mM) (Clarkson & Hanson, 1980). Furthermore, not only have Shimazaki *et al.* (1986) indicated, by using guard cell protoplasms, that ABA inhibited the acidification of the suspension media by 40%, but Lauer and Boyer (1992) have also very recently found that a low $\Psi_L$and a high ABA concentration cause opposing effects from which they concluded that the photosynthetic biochemistry limited photosynthesis at low Ψ_L but not at high ABA concentrations.

There is also evidence in literature (Berkowitz & Gibbs, 1983), that K^+, which is osmotically active and which can normally not diffuse through the chloroplast envelope, has been shown to be exchanged across the chloroplast membranes for free stromal H^+ (possibly stoichiometrically) (Demmig & Gimmler, 1979), cause an alkalization of the stroma (Kaiser *et*

al., 1980), and enhance photosynthetic rates under conditions which induce stromal acidification (Huber & Maury, 1980). It thus seems highly probable likely that the above-mentioned process could be mediated by ABA.

In conclusion, it may therefore be stated that in spite of the sharp initial decline in Φ and PSII-activity (Fig. 1a and b), which is probably caused by drought stress-induced stromal acidification, and the fact that damage to the photosynthetic systems of the drought-sensitive cultivars (TL33 and CDL28) occurred earlier and was much more severe (Fig. 2a and b), an adaptation mechanism (as the one described above), which may bestow some photosynthetic dehydration tolerance on the drought-tolerant cultivars (GS46 and ELSOMA), may exist in these cultivars. If this were to be the case, it would also explain the stabilization observed in Φ-values and PSII-activity at the moderate to severe stress levels.

3.6 Literature cited

ARNON, D.I. 1949. Copper enzymes in isolated chloroplasts: Polyphenol oxidase in *Beta vulgaris*. *Plant Physiology*, 24: 1-15.

BARBER, J. 1976. Ion regulation of intact chloroplasts and its effect on primary photosynthetic processes. (*In* Barber, J., *ed.* The intact chloroplast. New York: Elsevier. p. 89-134.)

BERKOWITZ, G.A. 1987. Chloroplast acclimatization to low osmotic potential. *Plant Cell Reports*, 6: 208-211.

BERKOWITZ, G.A. & GIBBS, M. 1983. Reduced osmotic potential effects on photosynthesis: Identification of stromal acidification as a mediating factor. *Plant Physiology*, 71: 905-911.

BJöRKMAN, O. & POWLES, S.B. 1984. Inhibition of photosynthetic reactions under water stress: Interaction with light level. *Planta*, 161: 490-504.

BOYER, J.S. 1967. Matric potentials of leaves. *Plant Physiology*, 42: 213-217.

BOYER, J.S. 1971. Nonstomatal inhibition of photosynthesis in sunflower at low leaf water potentials and high light intensities. *Plant Physiology*, 48: 532-536.

BOYER, J.S. & POTTER, J.R. 1973. Chloroplast response to low leaf water potentials. I: Effect of turgor. *Plant Physiology*, 51: 989-992.

BUSCHMANN, C. & GRUMBACH, K. 1985. Physiologie der Photosynthese. New York: Springer-Verlag. 245p.

CLARKSON, D.T. & HANSON, J.B. 1980. The mineral nutrition of higher plants. *Annual Review of Plant Physiology*, 31: 239-312.

DELIEU, R.J. & WALKER, D.A. 1981. Polarographic measurement of photosynthetic O_2 evolution by leaf pieces. *New Phytologist*, 89: 165-175.

DELIEU, T. J. & WALKER, D.A. 1983. Simultaneous measurement of oxygen evolution and chlorophyll fluorescence by leaf pieces. *Plant Physiology*, 73: 534-541.

DEMMIG, B. & GIMMLER, H. 1979. Effect of divalent cations on cation fluxes across the chloroplast envelope and on photosynthesis of intact chloroplasts. *Zeitschrift für Naturforschung*, 246: 233- 241.

FARQUHAR, G.D. & SHARKEY, T.D. 1982. Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology*, 33: 317-345.

HARKERS, C., HARTUNG, W. & GIMMLER, H. 1986. Abscisic acid mediated K^+-efflux from large unilamellar liposomes. *Journal of Plant Physiology*, 122: 385-394.

HARNISCHFEGER, G. 1974. Studies on chloroplast degradation *in vivo* II: Effects of aging on Hill activity of plastids from *Cucubita* cotyledons. *Zeitschrift für Pflanzenphysiologie*, 71: 301-312.

HARTUNG, W., RADIN, J.W. & HENDRIX, D.L. 1988. Abscisic acid movement into the apoplastic solution of water stressed cotton leaves: Role of apoplastic pH. *Plant Physiology*, 86: 908-913.

HEILMANN, B., HARTUNG, W. & GIMMLER, H. 1980. The distribution of abscisic acid between chloroplasts and cytoplasm of leaf cells and the permeability of the chloroplast envelope for abscisic acid. *Zeitschrift für Pflanzenphysiologie*, 97: 67-78.

HUBER, S.C. 1979. Effect of pH on chloroplast photosynthesis: Inhibition of O_2 evolution by inorganic phosphate and magnesium. *Biochimica et Biophysica Acta*, 545: 131-140.

HUBER, S.C. & MAURY, W. 1980. Effects of magnesium on intact chloroplasts. I: Evidence for activation of (sodium) potassium/proton exchange across the chloroplast envelope. *Plant Physiology*, 65: 350-354.

HUFFAKER, R.C., RADIN, T., KLEINKOPF, G.E. & COX, E.L. 1970. Effects of mild water stress on enzymes of nitrate assimilation and of the carboxylative phase of photosynthesis in barley. *Crop Science*, 10: 471-474.

IZAWA, S. 1980. Acceptors and donors for chloroplast electron transport. *Methods in Enzymology*, 69: 413-433.

KAISER, W.M., URBACH, W. & GIMMLER, H. 1980. The role of monovalent cations for photosynthesis of isolated intact chloroplasts. *Planta*, 149: 170-175.

KAISER, W.M. 1982. Correlation between the changes in the photosynthetic activity and changes in total protoplast volume in leaf tissue from hygro-, meso-, and xerophytes under osmotic stress. *Planta*, 154: 538-545.

KECK, R.W. & BOYER, J.S. 1974. Chloroplast response to low leaf water potentials. II: Differing inhibition of electron transport and photophosphorylation. *Plant Physiology*, 53: 474-479.

KULANDAIVELU, G. & DANIELL, H. 1980. Dichlorophenyl dimethylurea (DCMU) induced increase in chlorophyll a fluorescence intensity: An index of photosynthetic oxygen evolution in leaves, chloroplasts and algae. *Physiologia Plantarum*, 48: 385-388.

LAUER, M.J. & BOYER, J.S. 1992 Internal CO_2 measured directly in leaves: Abscisic acid and low leaf water potential cause opposing effects. *Plant Physiology*, 98: 1310-1316.

LAWLOR, D.W. 1990. Photosynthese: Stoffwechsel, Kontrolle, Physiologie. Stuttgart, New York: Thieme Verlag, 377p.

MAURY, W.J., HUBER, S.C. & MORELAND, D.E. 1981. Effects of magnesium on intact chloroplasts.II: Cation specificity (sodium) potassium/proton exchange across the envelope. *Plant Physiology*, 68: 1257-1263.

MOHANTY, P. & BOYER, J.S. 1976. Chloroplast response to low leaf water potentials. IV: Quantum yield is reduced. *Plant Physiology*, 57: 704-709.

NIELSEN, N.C. & SMILLIE, R.M. 1978. The effect of p-phenylenediamine and dibromothymoquinone on the photosynthetic electron transport and proton pump activities of isolated barley chloroplasts. *Archives of Biochemistry and Biophysics*, 186: 52-59.

MOONEY, H.A., BJöRKMANN, O. & COLLATZ, G.J. 1977. Photosynthetic acclimation to temperature and water stress in the desert shrub *Larrea divaricata. Carnegie Inststitution Washington Year Book*, 74: 328-335.

PLAUT, Z. 1971. Inhibition of carbon dioxide fixation in isolated spinach chloroplasts exposed to reduced osmotic potentials. *Plant Physiology*, 48:591-595.

PLAUT, Z. & BRAVDO, B. 1973. Responses of carbon dioxide fixation to water stress: Parallel measurements in isolated chloroplasts and intact spinach leaves. *Plant Physiology*, 53: 28-32.

POTTER, J.R. & BOYER, J.S. 1973. Chloroplast response to low leaf water potentials. II: Effect of osmotic potential. *Plant Physiology*, 51: 993-997.

RASCHKE, K. 1982. Involvement of abscisic acid in the regulation of gas exchange: Evidence and inconsistencies. (*In* Wareing, P.F., *ed.* Plant growth substances. New York: Academic Press. p. 581-590.)

RUBINSTEIN, B., STERN, A.I. & CHALMERS, J.D.C. 1992. Relationship between electron transport across the plasmalemma and a pH decrease in the bulk medium. *Plant Physiology*, 98: 988-994.

SAMUELSSON, G. & ÖQUIST, G. 1977. A method for studying photosynthetic capacities of unicellular algae based on *in vivo* chlorophyll fluorescence. *Physiologia Plantarum*, 40: 315-319.

SESTáK, Z., ZIMA, G. & WILHELMOVá, N. 1978. Ontogenetic changes in the internal limitations to bean leaf photosynthesis. IV: Effect of pH of the isolation and/or reaction medium on the activities of photosystems 1 and 2. *Photosynthetica*, 12: 1-6.

SHARKEY, T.D. & BADGER, M.R. 1982. Effects of water stress on photosynthetic electron transport, photophosphorylation and metabolite levels of *Xanthium strumarium* mesophyll cells. *Planta*, 156: 199-206.

SHAW, D.R., PEEPER, T.F. & NOFZINGER, D.L. 1985. Comparison of chlorophyll fluorescence and fresh and dry weight as herbicide bioassay techniques. *Weed Science*, 33: 29-33.

SHIMAZAKI, K., ILNO, M. & ZEIGER, E. 1986. Blue ligth dependent proton extrusion by guard-cell protoplasts of *Vicia faba*. *Nature*, 319: 324-326.

SUNDARI, D.S. & RAGHAVENDRA, A.S. 1990. Sensitivity of photosynthesis by spinach chloroplast membranes to osmotic stress *in vitro*: Rapid inhibition of O_2 evolution in the presence of magnesium. *Photosynthesis Research*, 23: 325-330.

TAIZ, L. & ZEIGER, E. 1991. Stress physiology: Water and drought resistance. (*In* Taiz, L. & Zeiger, E., *eds*: Plant Physiology. New York. Benjamin-Cummings. p. 346-356.)

VAN RENSBURG, L., KRüGER, G.H.J. & KRüGER, H. 1993. Proline accumulation as drought tolerance selection criterion: Its relationship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. J. *Plant Physiology,* 141: 188-194.

VAN STEVENINCK, R.F.M. & D VAN STEVENINCK, M.E. 1983. Abscisic acid and membrane transport. (*In* Addicott, F.T., *ed.* Abscisic acid. New York: Praeger Press.p. 171-236.)

WALKER, D.A. & OSMOND, C.B. 1986. Measurement of photosynthesis *in vivo* with a leaf disc electrode: Correlations between light dependence of steady state photosynthetic O_2 evolution and chlorophyll fluorescence transients. *Proceedings of the Royal Society of London,* 227: 367-280.

WERDAN, K. & HELDT, H.W. 1972. Accumulation of bicarbonate in intact chloroplasts following a pH gradient. *Biochimica et Biophysica Acta,* 283: 430-441.

WITT, H.T., RUMBERG, B., SCHMIDT-MENDE, P., SIGGEL, U., SKERRA, B., VATER, J. & WEIKARD, J. 1965. Über die Analyse der Photosynthese mit Blitzlicht. *Angewandte Chemie,* 77: 821-842.

CHAPTER FOUR

4. EVALUATION OF COMPONENTS OF OXIDATIVE STRESS METABOLISM FOR USE IN SELECTION OF DROUGHT TOLERANT CULTIVARS OF *NICOTIANA TABACUM* L.

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4.1 Abstract

Effect of drought stress on lipid peroxidation, chlorophyll stability and several antioxidant enzyme activities was evaluated under controlled environmental conditions, for possible use as drought tolerance selection criteria, in four tobacco (*Nicotiana tabacum* L.) cultivars of different, but known, drought tolerance. A progressive highly significant ($p < 0.01$), but differential (in correlation with their individual drought tolerance), increase in glutathione reductase (EC 1.6.4.2) activity was detected in all four cultivars as their leaf water potential (Ψ_L) decreased. In spite of an initial lag (up to a Ψ_L of -1.5 MPa), on average, the superoxide dismutase (SOD, EC 1.15.1.1) activity of the drought-tolerant cultivars increased by 244% while that of the drought-sensitive cultivars only increased by 161%. Contrary to the other enzyme activities monitored, only a moderate increase in catalase (EC 1.11.1.6) activity ($p< 0.05$) relative to their respective controls was observed in all four cultivars. Increased ascorbate peroxidase (EC 1.11.1.7) activity was not only observed to be ca. 300-400% higher ($p<0.01$ already at a Ψ_L of -1.5 MPa) in the drought-tolerant cultivars under stress, but was also more pronounced than the increase in catalase activity. This indicated that ascorbate peroxidase rather than catalase may be mainly responsible for scavenging drought stress produced H_2O_2. On reaching a Ψ_L of -2.5 MPa, the glutathione reductase activity of the drought-sensitive cultivars increased by only 159% (TL33) and 187% (CDL28), opposed to the 233% (GS46) and 250% (ELSOMA) in the drought-tolerant cultivars. A differential (significantly higher in the drought-tolerant cultivars at a Ψ_L of ca. -2.5 MPa) drought stress-induced increase in the level of lipid peroxidation occurred in all cultivars which decreased faster in the drought-tolerant cultivars upon rehydration. The latter was also observed for catalase and glutathione reductase activity, but of which the activity returned to levels comparable with that of their respective controls. The levels of SOD and ascorbate peroxidase activity on the other hand remained higher ($p < 0.01$) than their respective controls after rehydration, but did not differ statistically significantly between the respective cultivars. No destruction of chlorophyll, due to photoperoxidation was detected in any cultivar, as reflected by a stable chlorophyll *a/b* ratio. These results are discussed in relation to the potential drought tolerance adaptive advantage of an effective antioxidant system. In this regard of monitoring of the capacity to increase ascorbate peroxidase activity and/or glutathione reductase activity as possible drought tolerance selection criteria in tobacco is advocated.

Key words: *Antioxidant system, ascorbate peroxidase, drought stress, glutathione reductase, hydrogen peroxide, Nicotiana tabacum* L., *selection criteria, tobacco.*

Abbreviations: DHA = dehydroascorbic acid; GSH = reduced glutathione; GSSG = oxidized glutathione; K' = constant (0.049 $\mu L/3mL^{-1}$); NBT = nitro-blue tetrazolium; MDA = malondialdehyde; O_2^- = superoxide anion radical; SOD = superoxide dismutase; TBA = thiobarbituric acid; Ψ_L = leaf water potential; PVP = polyvinylpyrrolidone; PMSF = phenylenemethane-sulphonyl fluoride.

4.2 Introduction

Implicit to the theory of free radical damage during drought stress, is the notion that the cell's defensive mechanisms break down or are overloaded (Stewart, 1989). If this is correct, it follows that plants able to tolerate drought stress, must be able to maintain their defensive mechaisms or amplify them under conditions of increased stress. In this regard definitive mass spectral data have verified the *in vivo* occurrence of an oxygen cycle (Radmer *et al.*, 1978) in green plants which becomes quantitatively greater under the conditions of limiting CO_2 (Radmer & Ollinger, 1980). However, the availability of CO_2 may not be the major limiting factor during drought stress as we have previously indicated (Van Rensburg & Krüger, 1993b), nor may it be in a future atmosphere.

It has also been proposed that a capacity to limit membrane damage to a reparable level, by controling lipid peroxidation, may be an important facet of the drought tolerance of plants (Dhindsa & Matowe, 1981), as during drought stress plants are susceptible to oxidative and free radical damage (Smirnoff & Colombé, 1988). The chloroplast is at particular risk due to its high internal O_2 concentration (Robinson *et al.*, 1980). Furthermore, several chloroplast enzymes of the carbon fixation pathway lose activity upon reaction of essential sulfhydryl groups with activated O_2 species, and the highly unsaturated fatty acids of the thylakoids enhance the possiblity of lipid peroxidation (Burke *et al.*, 1985). Furthermore, there is a considerable amount of evidence (Huq & Palmer, 1978), that drought stress can generate the superoxide anion radical (O_2-) in plant tissues which is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). The increasing level of H_2O_2 in water stressed tissue has been found to be a function of increasing magnitude of water stress (Mukherjee & Choudhuri, 1985). An interaction between O_2- and H_2O_2 may also generate singlet oxygen ($^1O_2$) and hydroxyl free radicals (-OH) (Fridovich, 1978), all of which are potentially cytotoxic.

Because of the existence of a series of detoxification reactions, which may be drought stress-enhanced (Gamble & Burke 1984) such as:

$$O_2 + O_2\text{-} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

$$H_2O_2 + \text{ascorbate} \xrightarrow[peroxidase]{ascorbate} DHA + H_2O$$

$$DHA + GSH \xrightarrow[reductase]{DHA} \text{ascorbate} + GSSG$$

$$GSSG + NADPH \xrightarrow[reductase]{glutathione} GSH + NADP^+$$

it has been speculated (Carrol *et al.*, 1988), that an increase in the activity of one of these enzymes or a rise in the concentration of an intermediary electron carrier (glutathione or ascorbate) essential to this detoxification scheme, could explain tolerance towards a number of environmental stresses and some herbicides.

Therefore, in order to gain more insight in the underlying biochemical mechanisms which may bestow drought tolerance on a tobacco cultivar, and to identify possible drought tolerance selection parameters, we made use of four tobacco cultivars of known but different drought tolerance and examined: (i) the drought stress-induced changes in the degree of membrane degradation through lipid peroxidation - a process well recognized to cause membrane deterioration (Dhindsa *et al.*, 1981); (ii) the activities of SOD, catalase, ascorbate peroxidase and glutathione reductase, since these enzymes are known to catalyze the destruction of O_2- and H_2O_2 (Fridovich, 1975), and (iii) changes in the individual concentrations of both chlorophyll *a* and chlorophyll *b* profiles, during a slowly intensifying drought stress and upon rewatering.

4.3 Material and methods

Plant material, experimental design, cultivation practices and sampling procedures have already been extensively described elsewhere (Van Rensburg & Krüger, 1993a, Van Rensburg *et al.*, 1993). Experimentation was done under controlled environmental conditions in computerized growth rooms, as both light quality and quantity have been shown to influence the level and distribution of glutathione reductase in wheat (Gamble & Burke, 1983). The rooms were lit (photon flux density ca. 600 µmol $m^{-2}s^{-1}$, Philips, SA) for 13 hours at 25°C followed by an 11 hour dark period at 16°C. All chemicals were purchased from Sigma. Data were statistically analysed with the aid of the Dunnet *t*-test as a number of treatment means had to be compared with a single control and one of the several t-statistics was applicable (Miller, 1966).

Determination of lipid peroxidation

This involved measuring the amount of malondialdehyde (MDA), a product of lipid peroxidation, in the tobacco tissue (ca. 0.5g fresh weight) by the thiobarbituric acid (TBA) reaction (Heath & Packer, 1968). The procedure followed concur to that described by Dhindsa and Matowe (1981). The concentration of MDA was calculated using its extinction coefficient of 155mM^{-1} cm^{-1} (Heath & Packer, 1968).

Determination of chlorophylls *a* and *b*

By using the coefficients and equations derived by Lichtenthaler and Wellburn (1983), we were able to determine the individual levels of both chlorophyll *a* (C_a) and chlorophyll *b* (C_b) in µg mL^{-1} of plant extract, spectrophotometrically (Hitachi, Japan). The chlorophyll *a* and *b* ratio was calculated and drought stress-induced changes followed, in order to be able to relate our results to those reported by other workers (Alberte & Thornber, 1977; Dhindsa *et al.*, 1981).

Enzyme extraction and assay procedure

Leaf samples of ca. 0.5g, was homogenized in a pre-chilled Waring Blendor for 30s and 4°C. The grinding medium contained 0.1M acetate buffer (pH 4.0), 20mM ß-mercaptoethanol, and 0.1% (w/v)BSA. Polyvinylpyrrolidone (PVP), 0.2g per mL^{-1} of grind, was added to the samples to scavenge leaf phenolics. Homogenates were filtered through four and then twelve layers of cheesecloth and centrifuged (Heraeus, Sepatech, Germany) at 17000g for 15 minutes. All steps in the preparation of the enzyme extracts were carried out at 0°C to 4°C. All activities were determined at 25°C. Changes in enzyme activity in response to drought stress and upon rehydration can be expressed either per unit leaf area, chlorophyll or protein content as was done

by Gamble and Burke (1984). Seeing, however, that protein turnover occurs in tobacco during drought stress (Van Rensburg & Krüger, 1993c) and because a positive correlation does not necessarily exist between enzyme activity and enzyme concentration (Cooke *et al.*, 1979), we preferred to express all enzyme activities on a protein basis. Protein concentrations were determined according to Bradford (1976) using BSA as standard.

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). The 3mL reaction mixture contained 50mM phosphate buffer (pH 7.8), 13mM methionine, 75µM NBT, 2µM riboflavin and, 0.1mM EDTA. Riboflavin was added last, tubes were shaken and placed 30 cm below a light bank (10 fluorescent lamps). The reaction was initiated by switching on the lights, and then allowed to run for 2 minutes, after which it was terminated by covering the tubes with a black cloth. A non-irradiated reaction mixture served as control and A_{560} was plotted as a function of the volume of enzyme extract used in the reaction mixture (Giannopolitis & Ries, 1977). From the resultant graph, the volume of enzyme extract corresponding to 50% inhibition of the reaction was read and was considered to be equal to one enzyme unit (Beauchamp & Fridovich, 1971). Since at 50% inhibition of the assay reaction the product K'[SOD] equals unity, the SOD activity was determined directly from the V/v ratio according to the equation: SOD units mL^{-1} = [(V/v)-1]·(dilution factor), where V and v represents the rate of the assay reaction in the absence and presence of SOD, respectively.

The ascorbate peroxidase activity was determined as described by Arrigoni *et al.* (1992), using a reaction mixture composed of 50µM ascorbate, 90µM H_2O_2 and 50mM potassium phosphate buffer (pH 6.5). The H_2O_2 -dependent oxidation of ascorbate was followed by means of the decrease in absorbance at 265nm (1 unit = 1nmol ascorbate oxidized min^{-1} mg $protein^{-1}$).

Catalase activity was determined by measuring the rate of decrease in absorbance at 240nm from 0.450 - 0.400 in a solution of 12.5mM H_2O_2 in 50mM potassium phosphate (pH 7.0) as was done in tobacco by Hawir and McHale (1987). One unit was defined as the amount of enzyme catalyzing the decomposition of 1µM H_2O_2 per minute, calculated from the extinction coefficient for H_2O_2 at 240nm of 0,036 $cm^2.\mu M^{-1}$ (Lück, 1963).

Glutathione reductase activity was assayed in 0.5mL reaction mixture which contained 0,1mM NADPH, 0,18M Tricine - NaOH (pH 7.8), and 50-100µL of enzyme extract. The reaction was initiated by the addition of 50mM oxidized glutathione (GSSG) and the rate of NADPH oxidation monitored at 340nm (Burke *et al.*, 1985). Glutathione reductase activity was expressed in nM NADPH oxidized min^{-1} mg $protein^{-1}$.

4.4 Results

Lipid peroxidation

A limited drought stress-induced increase in the MDA concentration, which was higher ($p < 0.05$ at day 12 and $p < 0.01$ at day 14) in the drought-tolerant cultivars (GS46 and ELSOMA) than in the drought-sensitive cultivars (TL33 and CDL28) was observed (Fig. 1). As MDA is a decomposition product of tri-unsaturated fatty acid hydroperoxides (Heath & Packer, 1968), this would seem to indicate that lipid peroxidation occurred to a greater extent in the drought-tolerant cultivars during drought stress. Upon rehydration on day 15, the level of peroxidation decreased much faster in the drought-tolerant cultivars (Fig. 1).

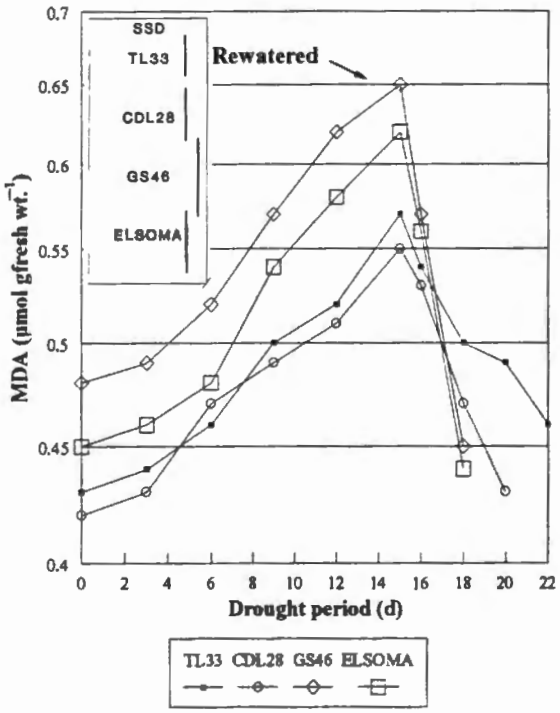


Figure 1. Differential changes in the level of lipid peroxidation (MDA content) in two drought-tolerant (GS46 and ELSOMA) and two drought-sensitive (TL33 and CDL28) tobacco cultivars, during decreasing Ψ_L and upon rehydration on day fifteen. Results represent mean values (n = 3), of samples extracted and assayed independently. The smallest statistically significant deviation (SSD) from their respective control for each cultivar, is indicated.

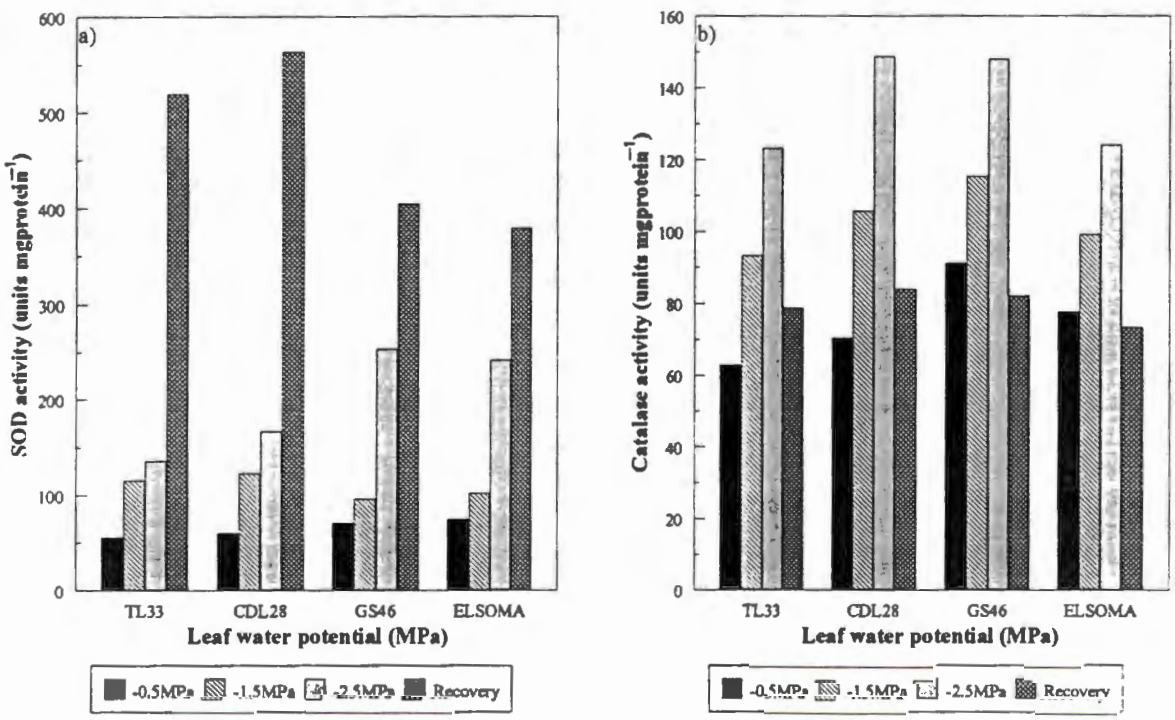
Chlorophyll content

The chlorophyll content (*a*/*b* ratio) did not deviate significantly from their respective controls which was 2.82 ± 0.2 (TL33), 2.88 ± 0.2 (CDL29), 3.01 ± 0.1 (GS46) and in 2.93 ± 0.2 (ELSOMA), in any one of the four tobacco cultivars nor did it differ statistically significantly between the respective cultivars.

Enzyme activities

At Ψ_L of -2.5 MPa the SOD activity of all four the tobacco cultivars showed a significant increase ($p < 0.01$) relative to their controls (Fig. 2a). However, the initial rate of increase (up to a Ψ_L of -1.5 MPa) even though being expressed on a protein basis was slightly lower in the drought-tolerant cultivars. The SOD activity of the latter cultivars was significantly ($p < 0.01$) higher than the drought-sensitive cultivars at a Ψ_L of -2.5 MPa. Upon reaching their respective control CO_2 fixation rates after rehydration, the SOD activity of the drought-sensitive cultivars remained ca. 100 units mg protein^{-1} higher ($p < 0.01$) than that of the drought-tolerant cultivars.

As was the case when two mosses of differing drought tolerance were subjected to drought stress (Dhindsa & Matowe, 1981), but contrary to experiments in which cotton plants were exposed to an O_2 enriched atmosphere (Foster & Hess, 1980) and in *Vigna* seedlings during drought stress (Mukherjee & Choudhuri, 1985), we observed a slight increase ($p < 0.05$), compared to the controls, in the catalase activity of all four tobacco cultivars at a Ψ_L of -2.5 MPa. However, in no instance did the catalase activity differ statistically significantly between the drought-sensitive and drought-tolerant cultivars (Fig. 2b).



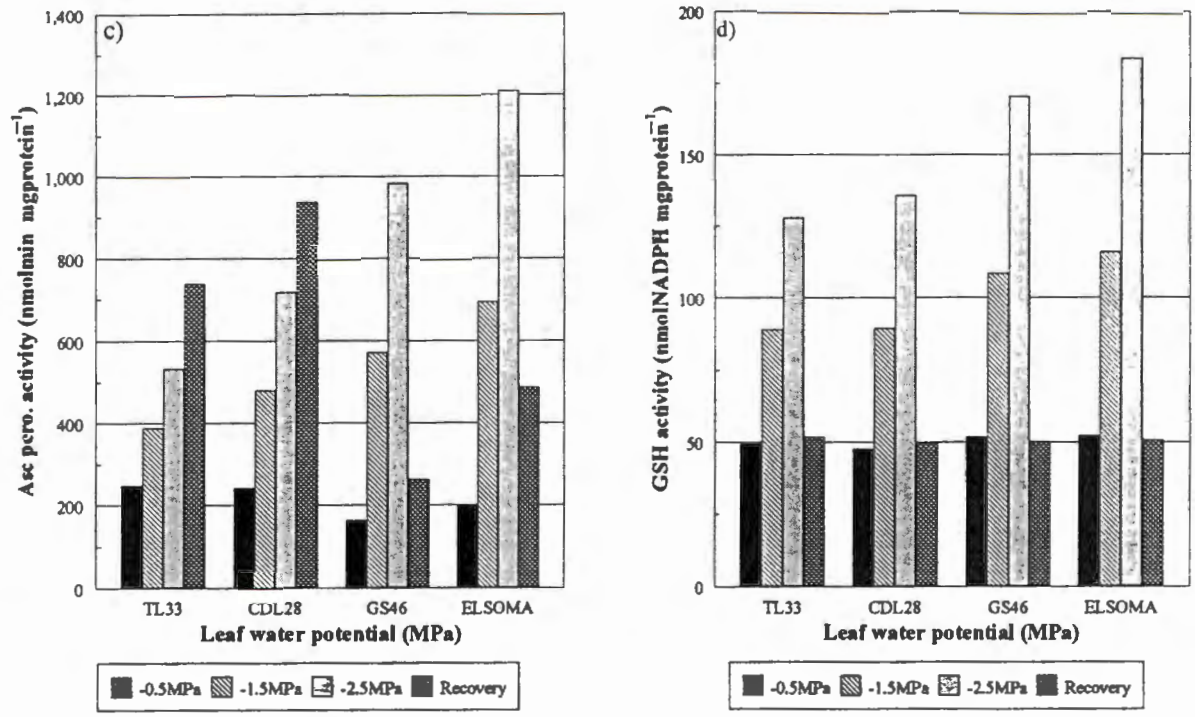


Figure 2. Drought stress-induced changes in some antioxidant enzyme activities in four tobacco cultivars of different drought tolerance, (a) SOD activity; (b) Catalase activity; (c) Asc.-peroxidase activity and (d) GSH-activity. Results represent mean values ± S.D. (n = 3), of samples extracted and assayed independently. Decrease in Ψ_L were continuously monitored in each cultivar until reaching predetermined Ψ_L value means, of which the S.D. were less than 10% and are therefore not indicated. Recovery activities represent determined enzyme activities when the respective cultivars reached their control CO_2 -fixation rates, which was seven days after being rehydrated (on day fifteen) for TL33, five days for CDL28 but only three days for both GS46 and ELSOMA.

A pronounced drought stress-induced increase in ascorbate peroxidase activity (Fig. 2c), which was already significantly higher ($p < 0.01$) than that of the drought-sensitive cultivars at a Ψ_L of -1.5 MPa, and 300-400% higher than in the latter cultivars at a Ψ_L of -2.5 MPa, may constitute an important component of an effective antioxidative sequence of events as can be seen in the discussion of these results. In accordance with the significant increase in peroxidase activity, the drought stress-induced increase in glutathione reductase activity (Fig. 2d) was not only already statistically significant ($p < 0.01$) for all four cultivars relative to their controls at a Ψ_L -1.5 MPa, but was also significantly higher ($p < 0.05$) in the drought-tolerant cultivars opposed to the drought-sensitive cultivars at the severe stress level. During recovery, upon reaching their respective CO_2 fixation rates, the glutathione reductase activity decreased in all four cultivars to levels within ca. 5% in the drought-sensitive and ca.3% in the drought-tolerant cultivars of their respective controls (i.e. it did not differ statistically significantly).

4.5 Discussion

Some authors have considered drought stress to be an oxidative stress (Dhindsa *et al.* , 1981), since lipid peroxidation causes alterations in the membrane similar to those noticed during dehydration. In the present investigation three possible reasons may explain why during the induced drought stress a higher degree of lipid peroxidation occurred in the drought-tolerant cultivars (GS46 and ELSOMA), and why this process was limited to a greater extent in these cultivars upon rewatering (Fig. 1). Firstly, more energy might have been directed towards the maintenance of the protein network, possibly by synthesizing proline which leads to the maintenance of membrane integrity (Van Rensburg *et al.*, 1993). This statement is in accordance with the conclusion drawn by Meyer *et al.* (1992), who suggested upon observing a strong drought stress-induced loss of lipids in two lupin genotypes of differing drought tolerance, that membrane cohesion during drought stress was better insured by a tight protein network than by the lipid matrix. Secondly, the free fatty acids may have been reutilised for the sysnthesis of di- and tri-acyl glycerols (Meyer *et al.*, 1992). Thirdly, and most likely, the higher degree of lipid peroxidation in these cultivars ($p < 0.01$ at Ψ_L of -2.5 MPa) may merely have been the unfortunate consequence of a drought stress-induced increase in the activity of the enzymes of glutathione metabolism, specifically glutathione peroxidase (Dhindsa, 1991). Furthermore, according to Quartacci and Navari-Izzo (1992), the generally accepted mechanism of radical attack involves acyl chain oxidation and as no such a phenomenon has been observed in studies of senescence nor drought stress, it suggests that changes in the unsaturation level might in fact be a relatively minor response to the action of free radicals, and therefore also be of limited value as drought tolerance selection criterion.

During drought stress, contrary to experiments which induced elevated levels of O_2^- and therefore also increased SOD levels, such as O_2 enriched atmospheres (Foster & Hess, 1980) or by subjecting plants to a number of diverse compounds such as paraquat (Harper & Harvey, 1978), we are of the opinion that drought stress-induced increases in SOD activity, *per se*, may not be such an important component in the oxygen detoxification scheme during drought stress as might be expected, since (i) even if O_2^- is produced in the chloroplast, it is sufficiently stable to diffuse through the chloroplast envelope before causing the peroxidative reactions which damage membranes (Farrington *et al.*, 1973), (ii) in spite of SOD catalyzing the disproportionation of the O_2^-, this reaction also proceeds spontaneously and very rapidly in aqueous solutions (Marklund, 1976), and (iii) the stability of the O_2^- increases with increasing pH (Behar *et al.*, 1970), but chloroplast stromal acidification occurs during drought stress (Berkowitz *et al.*, 1983). This contention is supported both by the fact that (i) a marked increase in SOD activity ($p < 0.01$) only occurred in the drought-tolerant cultivars at the severe stress level, possibly because simply less O_2^- may initially have been generated in the latter two cultivars (GS46 and ELSOMA) due to

the maintenance of higher internal CO_2 concentrations, ribulose bisphosphate carboxylase activity and therefore higher CO_2 fixation rates (Van Rensburg & Krüger, 1993b), and (ii) upon reaching their respective CO_2 fixation rates after rehydration the SOD activities of the drought-sensitive cultivars were significantly higher than that of the drought-tolerant cultivars. The latter may be symptomatic, as according to Stewart (1989) dehydrated sensitive membranes loose their functional integrity when rehydrated. From a drought tolerance selection point of view, it would therefore seem as if the SOD activity during and after drought stress may be more indicative of the amount of $O_2^{\cdot-}$ being generated than the specific drought tolerance of a cultivar. We, as was done by Foster & Hess (1982), thus submit that in photosynthetically active leaves high enough constitutive levels of SOD are present, in order to protect the photosynthetic apparatus against actual or potential detrimental effects of drought stress-induced O_2 metabolism, which may be elevated if need be.

The drought stress-induced increase in catalase activity relative to that of their respective controls, though being statistically significant ($p < 0.05$), was small in relation to the other enzyme activities monitored. In the drought-sensitive cultivars in which it occurred to a greater extent, the catalase activity only doubled. This could be due to the fact that, although leaf peroxisomes contain large amounts of catalase, the enzyme may be inefficient in breaking down low concentrations of H_2O_2, because of the kinetic characteristics of the enzyme (Zelitch, 1992). Furthermore, since enhanced catalase activity (Fig. 2b) is probably associated with the major peroxisomal isozyme known as CAT1 (Zelitch *et al.*, 1991) which is mainly responsible for the breakdown of H_2O_2 generated during photorespiration (the latter has been found to decrease during drought stress (Lawlor & Fock, 1975)), it would seem as if the major role of catalase in tobacco may not be to decompose H_2O_2 but rather to catalyse a peroxidation reaction during drought stress (Lück, 1963). In this regard Havir and Mchale (1987) have indicated that *Nicotiana tabacum* L. possess three forms of catalase which show both peroxidative and catalytic activities. Thus, in the absence of effective catalase activity in the chloroplast (Groden & Beck, 1979) and as Arrigoni *et al.* (1992) have concluded that ascorbate peroxidase rather then catalase might be used to efficiently remove H_2O_2, it can be argued that an alternative, more effective mechanism for the removal of H_2O_2 produced during drought stress, must have been operative in the four tobacco cultivars.

The drought stress-induced increases in glutathione reductase (Fig. 2d) and ascorbate peroxidase activities (Fig. 2c) in the four tobacco cultivars (found to be more pronounced ($p < 0.01$) in the drought-tolerant cultivars), may not only constitute two of the important components of such an antioxidant system, but would also seem to be the two most likely candidates to be used as drought tolerance selection criteria amongst the parameters monitored in this investigation. This line of thought is supported by the knowledge that ascorbate peroxidase

catalyses the peroxidation of ascorbate to dehydroascorbate (DHA), and that DHA reductase, in turn, utilizes reduced glutathione (GSH) to maintain the ascorbate pool in the reduced form (Jablonksi & Anderson, 1981). Oxidized glutathione (GSSG) is then in all probability reduced by NADPH via glutathione reductase (Foyer & Halliwell, 1976). In spite of the GSH generated by glutathione reductase therefore functioning indirectly via the ascorbate-dehydroascorbate cycle which is catalysed by ascorbate peroxidase (Groden & Beck, 1979), glutathione reductase has also been said not only to direct electrons away from O_2 and minimize the production of O_2^- during drought stress, but may also serve to ensure the availability of $NADP^+$ to accept electrons (Foster & Hess, 1982).

The increased glutathione reductase activity in the experimental plants may not only have protected the chloroplastic components against oxidation by H_2O_2, but may also have minimized the potential for H_2O_2 induced inactivation of SOD within the chloroplasts (Foster & Hess, 1980). The observation that the chlorophyll content, as was reflected by the chlorophyll *a/b* ratio, was not affected by the drought stress, is in accordance with the findings of Kulshrehtha *et al.* (1987) when two wheat genotypes of differing drought tolerance were subjected to drought stress. In this regard it is interesting to note that it has been proposed (Mayoral *et al.*, 1981), that chlorophyll stability during drought may be characteristic of drought-tolerant plants, and may therefore be used to differentiate between drought-resistant and drought-susceptible plants which would enable the breeder to screen for drought resistance. In our opinion the degree of chlorophyll stability during drought stress may merely reflect the effective operation of, amongst other things, an effective antioxidant system.

As H_2O_2 strongly inhibits CO_2 fixation, possibly by inactivating transketolase (Kaiser, 1976) or by inactivating several Calvin cycle enzymes upon reaction with essential sulfydryl groups (Smirnoff & Colomb, 1988), the above mentioned H_2O_2 scavenging system were most probably operative in the four tobacco cultivars. This would seem to have been characterized by a higher efficiency in the drought-tolerant cultivars, as during drought stress the drought-tolerant cultivars are capable of maitaining higher CO_2 fixation rates (Van Rensburg & Krüger, 1993b). Additionally, because of the increased glutathione reductase activity as the Ψ_L of the tobacco cultivars decreased, a more favourable GSH/GSSG ratio must have been maintained in the drought-tolerant cultivars. This could have lead to the higher availability of GSH which in turn could reduce oxidized sulfhydryl groups (Van Rensburg & Krüger, 1993c) thereby reactivating certain enzymes.

The latter, as well as the results presented by Dhindsa (1991), namely that the GSSG level correlates positively with the level of lipid peroxidation and solute leakage but negatively with the rate of protein synthesis, and Dhindsa (1987) that GSSG is rapidly metabolized upon rehydration, may serve to partly explain the faster recovery rate upon rewatering, and stability in the water soluble protein concentration at Ψ_L of -1.5 to -2.5 MPa of the drought-tolerant cultivars previously observed (Van Rensburg & Krüger, 1993b, 1993c).

4.6 Conclusion

It is clear that the activities of several antioxidant enzymes were elevated during drought stress (i) which confirm that drought affects the antioxidant enzymes and supports the hypothesis that drought increases the potential for oxidative damage in leaves, and (ii) that an ascorbate-dehydroascorbate cycle operated in all four tobacco cultivars, which was more pronounced in the drought-tolerant cultivars. We are, therefore, of the opinion that neither SOD or catalase activity, nor chlorophyll stability is reliable drought tolerance selection criteria in tobacco, but that breeders should rather opt for screening for cultivars which possess a capacity to elevate their ascorbate perioxidase- and/or glutathione reductase activity. It however, should be kept in mind that the above mentioned may constitute only one of several mechanisms geared to eliminate, or at least limit drought stress-induced damage.

4.7 Literature cited

ALBERTE, R.S. & THORNBER, J.P. 1977: Water stress effects on the content and organization of chlorophyll in mesophyll and bundle sheath chloroplasts of maize. *Plant Physiology*, 59: 351-353.

ARRIGONI, O., DE GARA, L., TOMMASI, F. & LISO, R. 1992. Changes in the ascorbate system during seed development of *Vicia faba* L. *Plant Physiology*, 99: 235-238.

BEAUCHAMP, C. & FRIDOVICH, I. 1971. Superoxide dismutase: Improved assay and an assay applicable to acrylamide gels. *Analytical Biochemistry*, 44: 276-287.

BEHAR, D., CZAPSKI, G., RABANI, J., DORFMAN, L.M. & SCHWARZ, H.A. 1970. The acid dissociation constant and decay kinetics of the perhydroxyl radical. *Journal of Physical Chemistry*, 74: 3209-3213.

BERKOWITZ, G.A., CHEN, C. & GIBBS, M. 1983. Stromal acidification mediates *in vivo* water stress inhibition of nonstomatal-controlled photosynthesis. *Plant Physiology*, 72: 1123-1126.

BRADFORD, M.M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254.

BURKE, J.J., GAMBLE, P.E., HATFIELD, J.L. & QUISENBERRY, J.E. 1985. Plant morphological and biochemical responses to field water deficits. I: Responses of glutathione reductase activity and paraquat sensitivity. *Plant Physiology*, 79: 415-419.

CARROL, E.W., SCHWARZ, O.J. & HICKOK, L.G. 1988. Biochemical studies of paraquat-tolerant mutants of the fern *Ceratopteris richardii*. *Plant Physiology*, 87: 651-654.

COOKE, R.J., OLIVER, J. & DAVIES, D.D. 1979. Stress and protein turnover in *Lemna minor*. *Plant Physiology*, 64: 1109-1113.

DHINDSA, R.S. 1987. Glutathione status and protein synthesis during drought and subsequent rehydration in *Tortula ruralis*. *Plant Physiology*, 83: 816-819.

DHINDSA, R.S. & MATOWE, W. 1981. Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation. *Journal of Experimental Botany*, 32: 79-91.

DHINDSA, R.S. PLUMB-DHINDSA, P. & THORPE, T.A. 1981. Leaf senescence: Correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *Journal of Experimental Botany*, 32: 93-101.

DHINDSA, R.S. 1991. Drought stress, enzymes of glutathione metabolism, oxidation injury, and protein synthesis in *Tortula ruralis*. *Plant Physiology*, 95: 648-651.

FARRINGTON, J.A., EBERT, M., LAND, E.J. & FLETCHER, K. 1973. Bipyridylium quaternary salts and related compounds. V: Pulse radiolysis studies of the reaction of the paraquat radical with oxygen. Implications for the mode of action of bipyridylium herbicides. *Biochimica et Biophysica Acta*, 314: 377-381.

FOSTER, J.G. & HESS, J.L. 1982. Oxygen effects on maize leaf superoxide dismutase and glutathione reductase. *Phytochemistry*, 21: 1527-1532.

FOSTER, J.G. & HESS, J.L. 1980. Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere of enriched oxygen. *Plant Physiology*, 66: 482-487.

FOYER, C.H. & HALLIWELL, B. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta*, 133: 21-25.

FRIDOVICH, I. 1975. Superoxide dismutases. *Annual Review of Biochemistry*, 44: 147-159.

FRIDOVICH, I. 1978. The biology of oxygen radicals: The superoxide radical is an agent of oxygen toxicity; superoxide dismutase provide an important defense. *Science N.Y.*, 201: 875-880.

GAMBLE, P.E. & BURKE, J.J. 1983 Glutathione reductase distribution in etiolated and green wheat leaves. (Abstr.) *Plant Physiology*, 72: Suppl. 42.

GAMBLE, P.E. & BURKE, J.J. 1984. Effect of water stress on the chloroplast antioxidant system. I: Alterations in glutathione reductase activity. *Plant Physiology*, 76: 615-621.

GIANNOPOLITIS, C.N. & RIES, S.K. 1977. Superoxide dismutases. I: Occurrence in higher plants. *Plant Physiology*, 59: 309-314.

GRODEN, D & BECK, E. 1979. H_2O_2 destruction by ascorbate-dependent system from chloroplast. *Biochimica et Biophysica Acta*, 546: 426-435.

HARPER, D.B. & HARVEY, B.M.R. 1978. Mechanism of paraquat herbicide tolerance in perennial ryegrass. II: Role of superoxide dismutase, catalyse and peroxidase. *Plant Cell Environment*, 1: 211-215.

HAVIR, E.A. & MCHALE, N.A. 1987. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. *Plant Physiology*, 84: 450-455.

HEATH, R.L. & PACKER, L. 1968. Photoperoxidation in isolated chloroplasts. I: Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, 125: 189-198.

HUQ, S. & PALMER, J.M. 1978. Superoxide and hydrogen peroxide production in cyanide resistant *Arum macilatum* mitochondria. *Plant Science Letters*, 11: 351-358.

JABLONSKI, P.P. & ANDERSON, J.W. 1981. Light dependent reduction of dehydroascorbate by ruptured pea chloroplasts. *Plant Physiology*, 67: 1239-1244.

KAISER, W. 1976. The effect of hydrogen peroxide in CO_2 fixation of isolated intact chloroplasts. *Biochimica et Biophysica Acta*, 440: 476-482.

KULSHRESHTHA, S., MISHRA, D.P. & GUPTA, R.K. 1987. Changes in contents of chlorophyll, proteins and lipids in whole chloroplasts and chloroplast membrane fractions at different leaf water potentials in drought resistant and sensitive genotypes of wheat. *Photosynthetica*, 21: 65-70.

LAWLOR, D.W. & FOCK, H. 1975. Photosynthesis and photorespiratory CO_2 evolution of water stressed sunflower leaves. *Planta*, 126: 247-258.

LICHTENTHALER, H.K. & WELLBURN, A.R. 1983. Determination of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochemical Society Transactions*, 11: 591-592.

LÜCK, H. 1963. Catalase. (*In* Bergmeyer, H.U., *ed.* Methods of enzymatic analysis. Weinheim, New York: Verlag Chemie. p. 995-894.)

MARKLUND, S. 1976. Spectrophotometric study of spontaneous disproportionation of superoxide anion radical and sensitive direct assay for superoxide dismutase. *Journal of Biological Chemistry*, 241: 7504-7507.

MAYORAL, M.L., ATSMON, D., SHIMSHI, D. & GROMET-ELHANAN, Z. 1981. Effect of water stress on enzyme activities in wheat and related wild species. Carboxylase activity, electron transport and photophosphorylation in isolated chloroplasts. *Australian Journal of Plant Physiology*, 8: 385-393.

MILLER, R.G. 1966. Simultaneous statistical inference. New York: McGraw-Hill.

MEYER, S., HUNG, S.P.N., TRéMOLIéRES, A & DE KOUCHKOVSKY, Y. 1992. Energy coupling membrane lipids and structure of thylakoids of Lupin plants submitted to water stress. *Photosynthesis Research*, 32: 95-107.

MUKHERJEE, S.P. & CHOUDHURI, M.A. 1985. Implication of hydrogenperoxide-ascorbate system on membrane permeability of water stressed *Vigna* seedlings. *New Phytologist*, 99: 355-360.

QUATRTACCI, M.F. & NAVARI-IZZO, F. 1992. Water stress and free radical mediated changes in sunflower seedlings. *Journal of Plant Physiology*, 139: 621-625.

RADMER, R.J., KOK, B. & OLLINGER, O. 1976. Photoreduction of O_2 primes and replaces CO_2 assimilation. *Plant Physiology*, 58: 336-340.

RADMER, R.J. & OLLINGER, O. 1980. Light driven uptake of oxygen, carbon dioxide, and bicarbonate by the green alga *Scenedesmus. Plant Physiology*, 65: 723-729.

ROBINSON, J.M., SMITH, M.G. & GIBBS, M.1980. Influence of hydrogen peroxide upon carbon dioxide photoassmilation in the spinach chloroplast. I: Hydrogen peroxide generated by broken chloroplasts in an "intact" chloroplast preparation is a causal agent of the Warburg effect. *Plant Physiology*, 65: 775-759.

SMIRNOFF, N. & COLOMB, S.V. 1988. Drought influences the activity of enzymes of the chloroplast hydrogen peroxide scavenging system. *Journal of Experimental Botany*, 39: 1097-1108.

STEWART, G.R. 1989. Desiccation injury, anhydrobiosis and survival. (*In* Jones, H.G., Flowers, T.J. & Jones, M.B., *eds*. Plants under stress: Biochemistry, physiology and ecology and their application to plant improvement. Cambridge, New York: Cambridge University Press. p. 115-130.)

VAN RENSBURG, L. & KRüGER, G.H.J. 1993a. Differential inhibition of photosynthesis (*in vivo and in vitro)* and changes in chlorophyll *a* fluorescence induction kinetics of four tobacco cultivars under drought stress. *Journal of Plant Physiology*, 141: 357-365.

VAN RENSBURG, L. & KRÜGER, G.H.J. 1993b. Comparative analysis of differential drought stress-induced suppresion of and recovery in carbon dioxide fixation: Stomatal and non-stomatal limitation in *Nicotiana tabacum* L. *Journal of Plant Physiology*, 142: 296-306.

VAN RENSBURG, L. & KRÜGER, G.H.J. 1993c. Protein turnover: possible contributing factor in determining drought tolerance in *Nicotiana tabacum* L. *South African Journal of Plant and Soil*, 10: 119-123.

VAN RENSBURG, L., KRÜGER, G.H.J. & KRÜGER, H. 1993. Proline accumulation as drought tolerance selection criterion: its relationship to membrane integrity and chloroplast ultrastructure. *Journal of Plant Physiology*, 141: 188-194.

ZELITCH, I., HAVIR, E.A., MCGONILE, B., MCHALE, N.A. & NELSON, T.1991. Leaf catalase RNA and catalase-protein levels in a high-catalase tobacco mutant with O_2-resistant photosynthesis. *Plant Physiology*, 97: 1592-1595.

ZELITCH, I. 1992. Factors affecting expression of enhanced catalase activity in a tobacco mutant with O_2 resistant photosynthesis. *Plant Physiology*, 98: 1330-1335.

CHAPTER FIVE

5. DROUGHT STRESS-INDUCED CHANGES IN CHLOROPLASPIGMENT COMPOSITION AND ANATOMY, AS WELL AS IN THE ULTRASTRUCTURE OF THE LEAF OF *NICOTIANA TABACUM* L. (VAN RENSBURG, L., KRüGER, H. & KRüGER, G.H.J. 1994. *CANADIAN JOURNAL OF BOTANY*, 72: IN PRESS.)

5.1 Abstract

As part of an extensive research programme that attemps to explain and quantify the known difference in drought tolerance of four *Nicotiana tabacum* L. cultivars, some aspects which may contribute to the avoidance and tolerance of drought stress were investigated in order to evaluate overall drought resistance. The results presented emphasize the adaptive significance of effective leaf movements in determining drought tolerance, by reducing or preventing damage to the photosynthetic system caused by photo-inhibition and direct heat damage. Leaf movement seemed to be achieved with greater efficiency by the drought-tolerant cultivars (GS46 and ELSOMA). Drought stress specifically influenced the carotenoid composition. A strong quantitative correlation existed between the formation of zeaxanthin in the xanthophyll cycle and the type of fluorescence quenching, which is indicative of non-radiative energy dissipation. The latter occurred to a lesser extent in the drought-tolerant cultivars GS46 and ELSOMA. In addition, this phenomenon indicated that the rate constant for non-radiative energy dissipation (K_D) in tobacco remains relatively small in relation to the rate constant for photochemistry (K_P) during drought stress. Furthermore, irrespective of their drought tolerance, it would seem as if tobacco plants have a capability for starch overproduction, although being less pronounced in the drought-sensitive cultivars (TL33 and CDL28). Due to anatomical differences, resistance to water flow varied between the respective cultivars, as did the percentage intercellular spaces, both of which correlated positively with the slower decrease in WUE and faster recovery upon rehydration of the drought-tolerant cultivars.

Keywords: *Carotenoid composition, chlorophyll a fluorescence, Nicotiana tabacum* L., *mesophyll surface area, leaf movements.*

Abbreviations: A= net photosynthetic rate; A_L = unit leaf area; A_{mes} = mesophyll surface area; E = transpiration rate; F_M = maximum fluorescence yield; F_O = instantaneous fluorescence yield; g = stomatal conductance for H_2O; K_D = rate constant for nonradioactive energy dissipation; K_P = rate constant for photochemistry; PPFD = photosynthetic photon flux density; Ψ_L leaf water potential; WUE = water use efficiency.

5.2 Introduction

The ultimate goal towards which all stress physiologists, in cooperation with geneticists and plant breeders are striving, is to increase the production of crop plants under conditions that are less optimal than those required for maximum production. To achieve the latter Mussel and Staples (1979) recommended increasing our knowledge of both the form and function components of yield, while Levitt (1972) emphasized the importance of measuring both avoidance and tolerance to determine overall resistance.

Regarding drought avoidance, a strategy (apart from stomatal closure for reducing water loss), is to reduce the amount of radiation intercepted by the plant. This can be achieved by active and passive leaf movement, increased pubescence, and/or increased leaf waxiness (Turner, 1979). Leaves of crops frequently roll or hang limp when severely stressed. This passive leaf movement reduces the interception of radiation (Begg & Turner, 1976), thereby counteracting the increase in leaf temperature arising from stomatal closure (Gates, 1968) and preventing further development of leaf water potential (Ψ_L) deficits. Since crop evaporation is linearly related to leaf area until complete ground cover (Ritchie, 1974), any reduction in leaf area will reduce water loss.

Alternatively, in order to maintain high Ψ_L in the leaves, particularly under conditions of high evaporative demand, plants must not only maintain water uptake by their roots, but must also have low resistances to water flow between root and leaf (Turner, 1979). Resistances to water flow do vary between species (Boyer, 1971b), but differences within a species do not seem to have been explored. It has generally been concluded that the major resistance to water flow occurs in the roots, but evidence for resistance to flow in stems and leaves in several species also exists (Boyer, 1971b). A lowering in the resistance to water flow can be achieved by increasing either the diameter of the xylem vessels or their number (Turner, 1979).

If, however, as may happen during drought stress, electron transfer from reaction centres may be blocked, charge recombination could result in the formation of the triplet excited state of chlorophyll, which can safely be dissipated as heat by reactions with carotenoids (Asada & Takahashi, 1987). It is the xanthophylls, in the light harvesting complex, that undergo a reversible light-induced interconversion called the xanthophyll cycle (Hager, 1980). The photophysical reactions preceeding chlorophyll destruction and the mechanism of protection by carotenoids, are well established (Siefermann-Harms, 1987).

Furthermore, the resistance of the photosynthetic apparatus to dehydration (Cronic *et al.*, 1989; Quick *et al.*, 1992) is reflected by its capability to recover upon rehydration (Chaves,

1991). Often, however, plants recover only partially from desiccation upon rewatering: the net photosynthetic rate (A) and transpiration rate (E) may remain lower, resistance to water flow through the plant may be larger, and stomata may open less than those of non-stressed control plants (Boyer, 1971a). Contrary to the explanation given by Boyer (1971a) for these delayed stress effects, we found that Ψ_L showed no lag but commenced to recover to pre-stress levels as soon as the plants were rewatered. This is in accordance with results of Kirschbaum (1988), and observations in a recent study in our laboratory on four tabacco cultivars of differing drought tolerance (Van Rensburg & Krüger, 1993b). As also proved to be the case with A, g and E, the recovery rate was always significantly faster in the drought-tolerant cultivars.

In an attempt to explain the latter observation, this study was done to determine to what extent: (i) anatomical differences may have been involved, (ii) drought-avoidance strategies such as leaf movements may be operative in the respective cultivars, and, (iii) physiological drought tolerance energy dissipation mechanisms, such as changes in pigment composition and in chlorophyll *a* fluorescence, may contribute to an individual cultivar's drought resistance.

5.3 Material and methods

Plant material and general growth conditions:

Plant material, experimental design, cultural practices and sampling procedures have already been extensively described elsewhere (Van Rensburg, *et al.*, 1993; Van Rensburg and Krüger, 1993a).

Anatomical investigation:

Material for light microscopy and transmission electron microscopy was collected at 08:00 on alternate days. Longitudinal sections, 0.5µm thick, parallel to the epidermis, were made of the palisade parenchyma tissue for measurements of palisade cells. The central 5mm of the petiole of mature leaves was macerated in Jeffrey's fluid (Johansen, 1940). The length and diameter of pitted vessel elements, only representing secondary xylem or metaxylem elements, were measured with a micrometer with inference contrast microscopy and the ratio of length to diameter was calculated. Two-way variance analysis was done and these ratios were highly significant ($p < 0.01$). The diameter of 25 palisade cells of each cultivar was measured with a ocular micrometer. The percentage intercellular spaces was determined with a Quantimet 520 Image Analysis System (SMM Instrumentation). The latter percentage correlates well with the mesophyll surface area per unit leaf area (A_{mes}/A_L) as described by Nobel (1991).

Physiological investigation:

Plant water status was estimated by measuring the Ψ_L of the sixth leaf below the apex with a Scholander pressure chamber on alternate days after water stress, which intensified at a rate of ca. 0.01 MPa d^{-1}, was induced by withholding water. After reaching the severe stress level of -2.5 MPa on day fifteen, the plants were rewatered and their recovery time course monitored.

Half a gram (two 10cm^2 leaf discs) of fresh leaf material was collected concurrently with each Ψ_L determination (from three different plants) and immediately frozen in liquid nitrogen. These samples were later homogenized in a mortar containing 5.0ml acetone at -20°C together with a spatula tip of $CaCO_3$ and quartz sand. The homogenate was centrifuged at 2500g at -4°C for 10min after which most of the acetone was removed from the extract by taking it almost to dryness *in vacuo*.

Two ml of diethyl ether was used to take up the remaining extract in the organic phase. The latter 2ml of extract was then used to determine the pigment composition by ascending thin

layer chromatography (TLC), and to spectrophotometrically quantify the pigment concentrations (Hager & Meyer-Bertenrath, 1966; Hager & Meyer-Bertenrath, 1967). For TLC, 20 x 20 cm glass plates were covered with a 0.4mm layer of Kieselgur G and with the exception of a 2cm band, impregnated with a solution containing 35g coconut fat (Palmin) and 35g plant oil (Livio) in 1L petroleum spirit. Chromatograms were developed for 50min in the dark with a mixture of methanol-acetone-water (20:4:3 v/v). The chlorophylls were eluted in acetone, the xantophylls in ethanol and β-carotene in chloroform. The latter elution liquids were used as blanks, when measuring the absorbance spectrum of the carotenoids from 350 to 550nm and for the chlorophylls from 350 to 750nm at 25°C, after centrifugation of the eluate at 2500g (4°C) for 5min. All determinations were done in triplicate.

Gas exchange measurements were done with a calibrated LCA-2 infra-red gas analyser coupled to a LD-2 datalogger and ASUM-2 mass flow regulator (Analytical Development Company, Hoddesdon, England) in open circuit at a CO_2 concentration of 350μmol mol^{-1} and constant photon flux density (PPFD) of 1100μmol $m^{-2}s^{-1}$, supplied by a high pressure sodium light source. Data were downloaded to a computer and results calculated according to the equations described and discussed by Farquhar and Sharkey (1982) and Chaves (1991). Fluorescence measurements were made with a shutterless fluorescence measuring system (Plant Efficiency Analyser, Hansatech, Norfolk), which can accurately determine both F_O and F_M. In order to minimise inconsistencies due to the patchiness of stomatal closure (Terashima *et al.*, 1988), three replications were done per leaf on four different plants in the case of the gas exchange measurements and ten replications per leaf on four different plants in the case of the fluorescence measurements, after a dark adaptation period of 45 minutes in the case of the latter measurements.

5.4 Results and discussion

Leaf anatomy

The tobacco leaf lamina was found to be dorsiventral, amphistomatous and typically mesomorphic. The general leaf anatomy conforms to that described by De Toni and Paoletti (1891), Avery (1933) and Metcalfe and Chalk (1965). The epidermal cells contained few plastids rich in starch. The cell contents of the adaxial epidermal cells often stained with toluidine blue (Fig. 1). The nature of these contents, however, was not determined in this study. The spongy parenchyma cells were lobed and rich in chloroplasts. The palisade parenchyma was loosely arranged and contained large numbers of chloroplasts. Large starch grains were present in all chloroplasts of unstressed leaves of all cultivars (Fig. 2), although material for microscopic study was collected at most one hour after onset of the light period. In terms of drought tolerance, this may be of adaptive value, for it may occur during drought stress that the CO_2 supply is limited or the carboxylation efficiency decreases (Van Rensburg and Krüger, 1993b), which would necessitate dissipation of excess amounts of excitation energy to avoid reactive states of oxygen causing photobleaching. The glycolate pathway offers one way of achieving this; for as reductant is consumed, it generates ATP which may be limiting in stressed leaves, and by regulating the carbon flow between the RPP cycle and mitochondria in combination with energy availability, the varying demands of metabolism may be balanced. To be able to achieve this, large carbon reserves are needed, for they dampen the fluctuations in supply and demand (Foyer *et al.*, 1990). Hence, many plants, of which tobacco seems to be one (Fig. 2), appear to have an excess capacity for carbohydrate production (Lawlor, 1979).

The percentage intercellular spaces in palisade tissue of the unstressed leaf laminas proved to be smaller in GS46 (21%) and ELSOMA (20%) than in both TL33 and CDL28, i.e. 25%. Furthermore, on average the diameter (in mm) of 25 palisade cells measured in longitudinal sections of leaf lamina also proved to be less in GS46 (17.94 ± 1.4) and ELSOMA (15.96 ± 1.4) than in TL33, (23.94 ± 2.1) and CDL28 (23.33 ± 2.6). The possible adaptive significance of the latter two sets of results is only fully realised when seen in conjunction with the data regarding

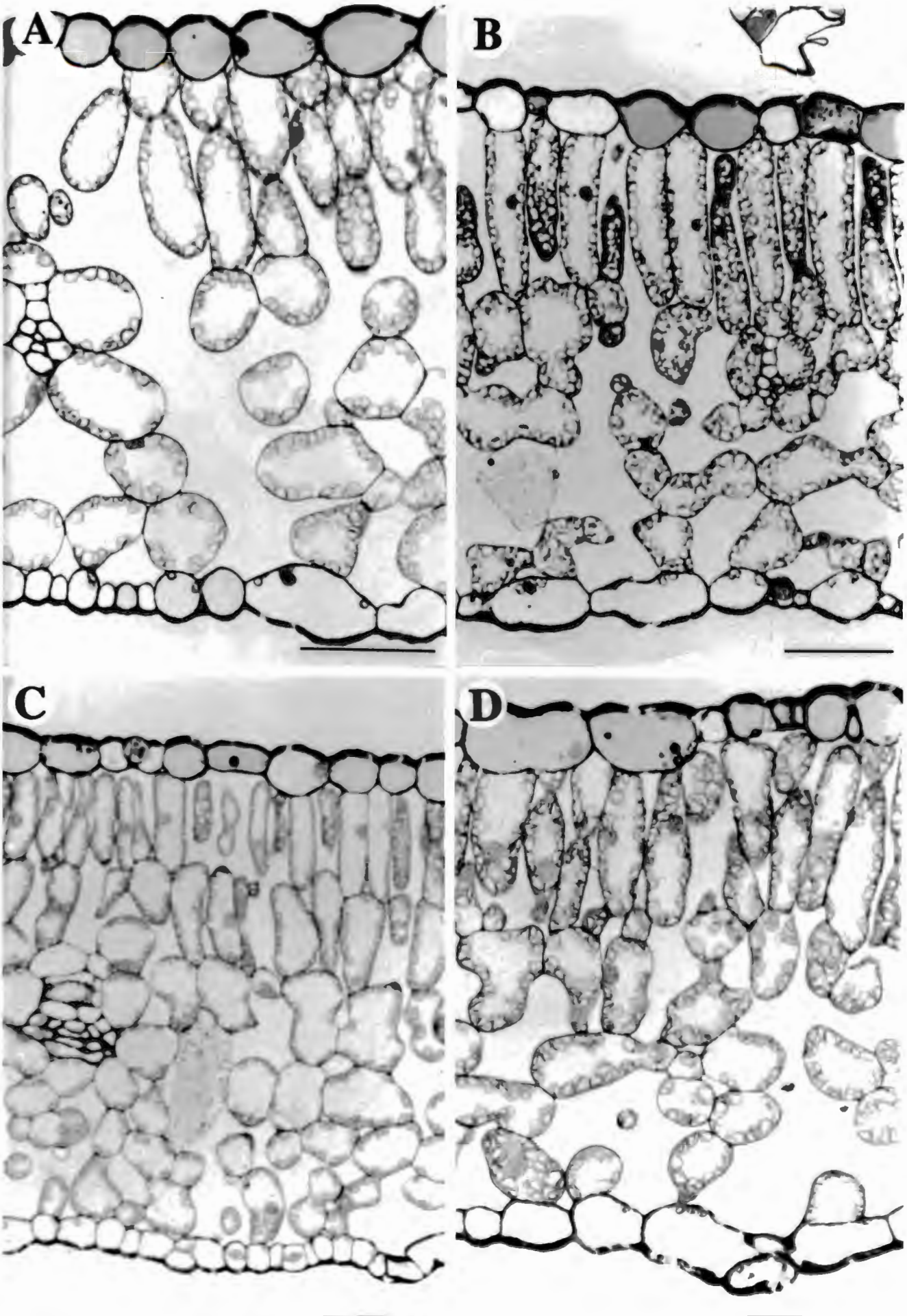


Figure 1. Light micrographs of transverse sections of unstressed leaves, i.e. at a Ψ_L of -0.5 MPa of four tobacco cultivars: (a) TL33; (b) CDL28; (c) GS46 and (d) ELSOMA. Scale bars = 50 μm.

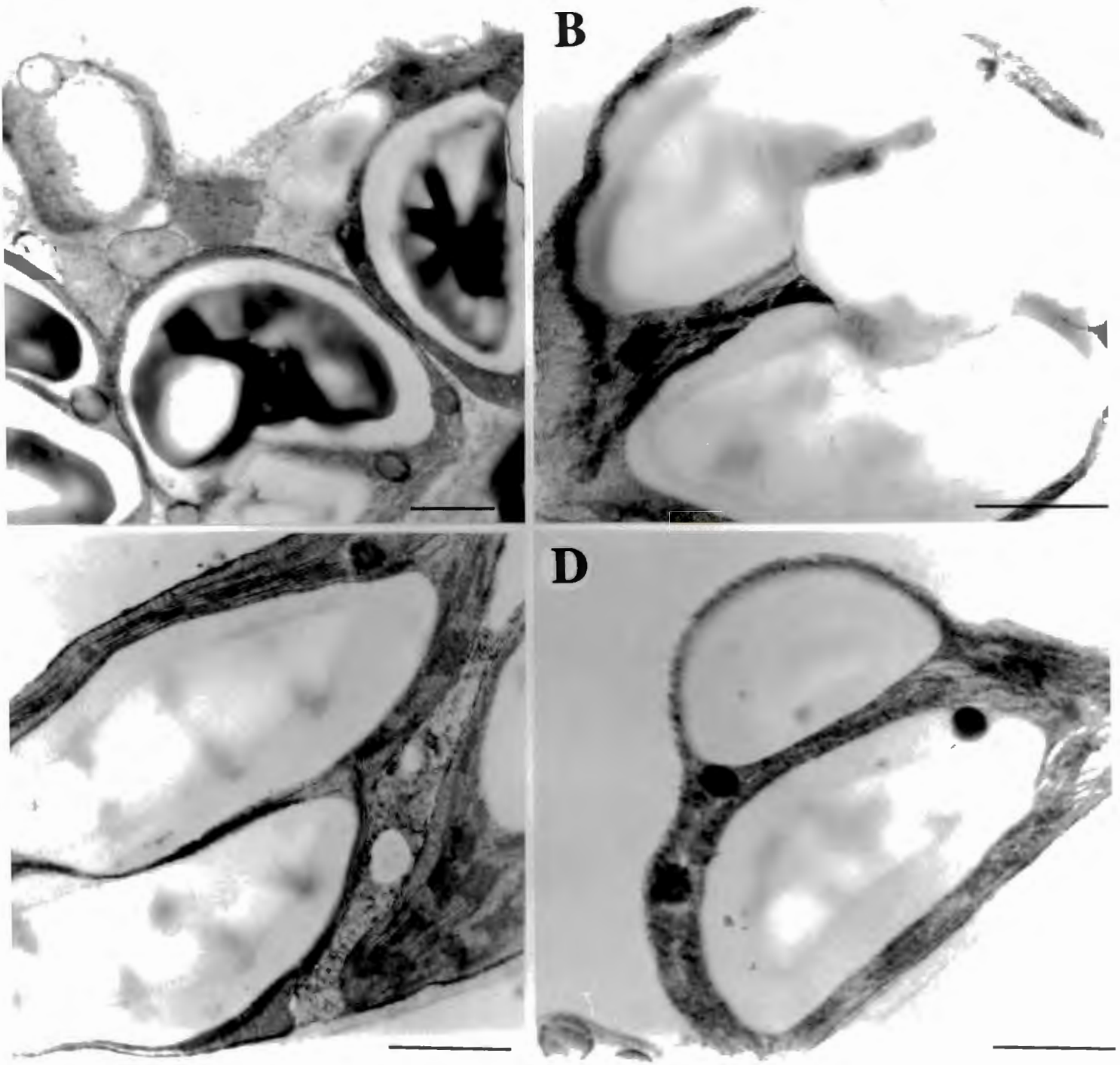


Figure 2. Electron micrographs of chloroplasts in transverse sections of unstressed leaves of four tobacco cultivars: (a) TL33; (b) CDL28; (c) GS46 and (d) ELSOMA. Scale bars = 1 μm.

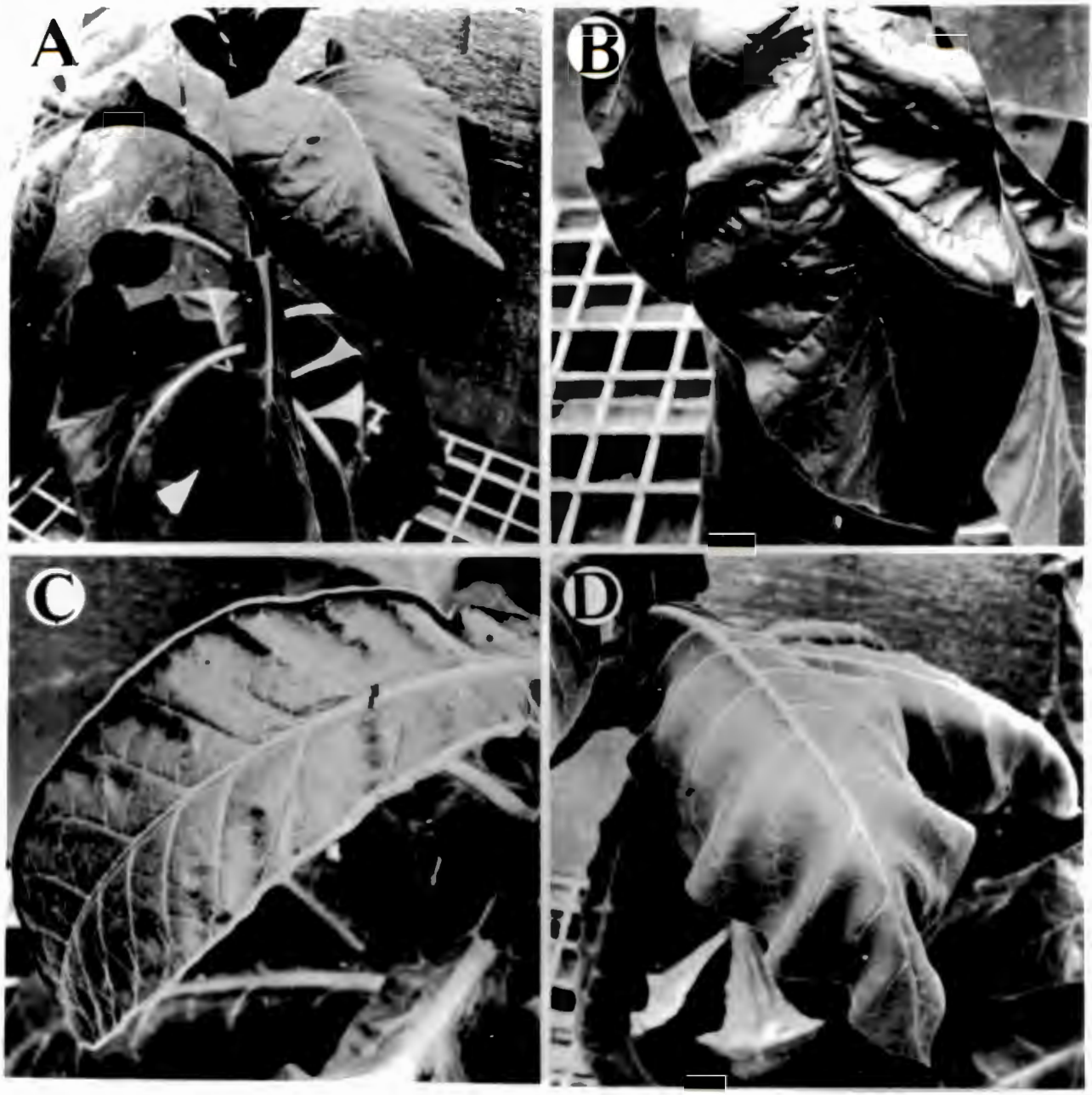


Figure 3. Although the leaves of all four cultivars, i.e. TL33 (a), CDL28 (b), GS46 (c) and ELSOMA (d) already hang limp at a Ψ_L of -1.5 MPa, the drought-tolerant cultivars GS46 (c) and (d) ELSOMA employ leaf rolling with a greater efficiency than the drought-sensitive cultivars to minimise the amount of intercepted radiation.

gas exchange, supplied in Table 1. It may serve to explain why (apart from the maintenance of higher A rates due to the maintenance of higher carboxylation efficiency by GS46 and ELSOMA, Van Rensburg & Krüger, 1993b), the rate of decrease of the water use efficiency (WUE) of the drought-tolerant cultivars (GS46 and ELSOMA) was lower. This, despite the lower control rates of both E and A are more rapid decreasing g of the drought-sensitive cultivars (TL33 and CDL28). The drought-sensitive cultivars may simply have a larger intercellular area from which evaporation takes place. Though only the percentage intercellular spaces at a Ψ_L of -0.5 MPa was calculated, our results confirm the statement of Nobel (1991) that the higher the A_{mes}/A_L-ratio and therefore the smaller the percentage of intercellular spaces (due to a higher number of palisade cells), the higher the WUE of a plant ought to be. The palisade tissue consisted of one layer, except in the leaves of GS46, where indications of a second palisade layer were evident in most of the sections studied (Fig. 1c).

Table 1: Differential drought stress-induced changes in both A and g which determine WUE and E in two drought-sensitive (TL33, CDL28) and two drought-tolerant (GS46, ELSOMA) tobacco cultivars.

Cultivars	Ψ_L* MPa	A $\mu mol\ m^{-2}s^{-1}$	E $mmol\ m^{-2}s^{-1}$	g $mmol\ m^{-2}s^{-1}$	WUE $\mu mol\ mmol^{-1}$
TL33	-0.5	8.2 ± 0.2	3.6 ± 0.4	369.7 ± 92.4	2.25 ± 0.2
	-1.5	1.6 ± 0.2	2.1 ± 0.5	116.6 ± 44.2	0.76 ± 0.2
	-2.5	0.3 ± 0.2	1.8 ± 0.5	101.7 ± 41.7	0.15 ± 0.1
CDL28	-0.5	6.2 ± 0.7	3.8 ± 0.5	310.1 ± 72.1	2.25 ± 0.4
	-1.5	1.8 ± 0.3	2.4 ± 0.4	246.7 ± 56.8	0.74 ± 0.2
	-2.5	0.3 ± 0.2	1.8 ± 0.5	100.2 ± 45.7	0.18 ± 0.3
GS46	-0.5	13.8 ± 0.3	5.1 ± 0.3	373.3 ± 50.1	2.73 ± 0.5
	-1.5	4.4 ± 0.2	3.2 ± 0.3	180.4 ± 28.8	1.38 ± 0.3
	-2.5	2.6 ± 0.3	2.9 ± 0.4	153.3 ± 30.6	0.88 ± 0.2
ELSOMA	-0.5	12.2 ± 0.3	5.8 ± 0.3	336.5 ± 63.9	2.94 ± 0.6
	-1.5	6.8 ± 0.2	3.3 ± 0.5	200.6 ± 44.8	2.09 ± 0.3
	-2.5	2.7 ± 0.2	2.9 ± 0.4	186.3 ± 41.6	0.92 ± 0.4

*Decrease in Ψ_L was continuously monitored in each cultivar until reaching predetermined Ψ_L values at which time measurements were done. Standard deviations of all Ψ_L values' means were less than 10% and are therefore not indicated. Results represent mean values ±SD(n=9).

Pallardy (1989) stated that evidence exists which indicate that the xylem vessels in the petioles of many angiosperms may provide high resistance flow points. The ratio of length to diameter in the petioles of the tobacco cultivars studied (Table 2) may therefore be a direct indication of the situation in the stems. As the tobacco leaf is the product utilized, it was preferred to measure vessel elements from petioles. The diameter of the vessels of all four

cultivars at a Ψ_L of -0.5 MPa falls within the 35- 65 µm class, indicating that they are fairly resistant to blockage by cavitation as well as being capable of recovering after blockage (Salleo & Lo Gullo, 1989). The latter authors regarded xylem conduits of this size as being a compromise between efficiency and safety. Ewers and Fisher (1989) found evidence that vessel length correlates positively with vessel diameter. It may, therefore, be stated that the wide vessels of woody plants are longer than narrow vessels. In our investigation vessel element length was determined and revealed a different correlation between vessel element length and diameter (Table 2). In this regard it is especially interesting to note that in a recent study, Sperry and Sullivan (1992) indicated that the size of the xylem conduit, found to be larger in GS46 and ELSOMA in our study (Tabel 2), may not be indicative of its vulnerability to cavitation during water stress. This is contrary to the widely held assumption that larger conduits cavitate at lower tensions than smaller ones. It must be stressed, however, that *Vitis vinifera*, studied by Salleo and Lo Gullo (1989) is a woody perennial, while *Nicotiana tabacum* L. is a mesophytic annual plant. The faster recovery of the Ψ_L in the case of the drought-tolerant cultivars GS46 and ELSOMA, may, therefore, be partly explained by the fact that on rewatering, water movement could have occurred at a faster rate and, therefore, alleviated the stress sooner.

Table 2: Size of pitted vessel elements (metaxylem or secondary xylem) in the central part of petiole of four tobacco cultivars, of differing drought tolerance.

Cultivars	Average length µm	Average diameter µm	Ratio of length to diameter
TL33	465.0 ± 20.2	38.0 ± 1.5	12.24
CDL28	518.4 ± 22.5	38.5 ± 0.8	13.46
GS46	327.1 ± 15.2	54.5 ± 1.7	7.19
ELSOMA	359.2 ± 16.4	42.0 ± 1.8	8.55

Recovery from water stress however, has also previously been shown to be both light and temperature dependent (Greer *et al.*, 1986) and also probably requires chloroplast-encoded protein synthesis (Ohad *et al.*, 1984).

Leaf movements

The significance of the observation that leaf movements occur in tobacco (Figs. 3a to b) and furthermore, that the efficiency (degree and extent of wilting) with which these movements operate correlates positively with the known drought tolerance of the respective cultivars, must not be underestimated. It has been suggested that, in conjunction with stomatal responses, leaf movements may provide the crop with the capability to optimize production over a greater range of environmental conditions (Shackel & Hall, 1979; Meyer & Walker, 1981). Oosterhuis *et al.* (1985) have proposed, that after extensive field testing these observations with regard to leaf

movements, may serve as early and easily identifiable indicators for the onset of water stress in soybeans, and could therefore possibly be used in irrigation scheduling. The physiological foundation for this line of thought is that leaf movement reduces the amount of incident solar radiation on water-stressed leaves to a large extent and hence minimizes the increase in leaf temperature that cannot be dissipated as heat. Thus, as water stress evidently prediposes the photosynthetic system to photoinhibition, and high leaf temperatures exacerbate this photoinhibitory damage (Ludlow & Björkman, 1984), it seems probable that the cultivars in which leaf movements effectively operate i.e. GS46 and ELSOMA, will have a drought-avoidance adaptive advantage.

Physiological changes: Carotenoid composition and chlorophyll *a* fluorescence

During the drought stress period a drought stress-induced increase in the zeaxanthin ($p < 0.01$) concentration and decrease in the β-carotene ($p < 0.01$) and violaxanthin (not statistically significant) concentrations (Figs. 4a to d) compared to their respective controls were observed. The increase in zeaxanthin and therefore K_D (rate constant for nonradiative energy dissipation) was, however, not as substantial in the drought-tolerant cultivars, as was expected. During periods of high light intensity and low Ψ_L it was shown that both *Nerium oleander* (Demmig *et al.*, 1988) and *Arbutus unedo* (Demmig-Adams *et al.*, 1989) have a strong capacity to increase their zeaxanthin content not only at the expense of violaxanthin and antheraxanthin but also at the expense of β-carotene. Furthermore, this capacity is independent of the growth PPFD and is probably genetically determined (Demmig *et al.*, 1988). It may therefore be expected that the potential for rapid interconversions between β-carotene, and zeaxanthin + antheraxanthin + violaxanthin is related to the resistance of a plant to photoinhibition as drought-tolerant genotypes, water stress has specifically been found to be accompanied by a slowed-down reconversion of zeaxanthin to its precursors (Demmig, 1988). However, the opposite holds true for sensitive species such as *Populus balsamifera*, which undergo photoinhibitory damage (Demmig, 1987). Two possibilities may explain this irregularity. In the first instance, because of the more effective operation of leaf movements in the drought-tolerant cultivars resulting in a smaller total exposed leaf area, there may simply have been a smaller amount of excess excitation energy to dissipate as was the case in Siratro (Ludlow & Björkman, 1984). Secondly in these cultivars thermal deactivation may play only a minor role among the energy-dependent quenching mechanisms.

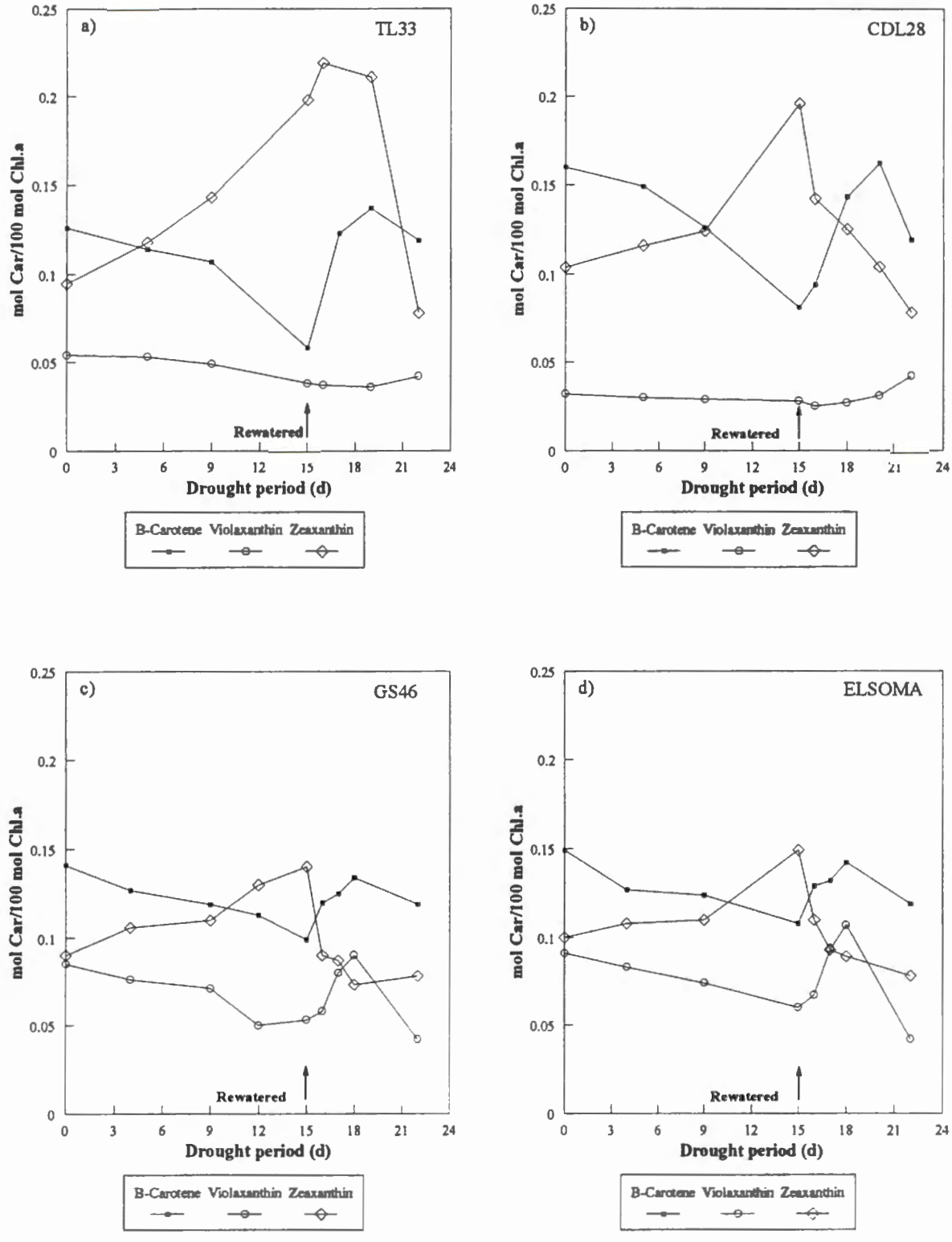


Figure 4. Differential drought stress-induced changes in the pigment composition of the drought-sensitive (a) TL33 and (b) CDL28, and drought-tolerant (c) GS46 and (d) ELSOMA tobacco cultivars, during drought stress and on rewatering.

The latter is most probably the case in tobacco, for in a previous investigation (unpublished data) we found photoinhibtion to occur to a greater extent in the drought-tolerant cultivars. This photoinhibition may result from two different processes acting singly or in combination (Demmig & Björkman, 1987). It may well have an adaptive significance in preventing photo-oxidation (Foyer *et al.*, 1990). To understand the physiological significance of this it should be realized that in the model of Kitajima and Butler (1975), a decrease in the rate constant for photochemistry (K_P), i.e. photoinhibition of the PSII reaction centres, leads to a rise in F_O (Fig. 5a). In contrast, an increase in the rate constant for nonradiative energy dissipation (K_D), leads to a decrease in F_M (Fig. 5b) and a constant though smaller decrease in F_O. The results presented (Figs. 4a to d and Figs. 5a and b), indicate that the carotenoid composition of leaves and specifically the formation of zeaxanthin in the xanthophyll cycle, not only responds to stress, and that a strong quantitative correlation exists between the type of fluorescence quenching which is indicative of nonradiative energy dissipation (Demmig *et al.*, 1987), but also that K_D remains relatively small in relation to K_P during drought stress. This has been implied by Havaux *et al.* (1991). It is therefore understandable that, contrary to the results reported for *Nerium oleander* (Demmig *et al.*, 1988), we did not observe that water stress specifically slowed down the reconversion of zeaxanthin to its precursors (Figs. 4a to d).

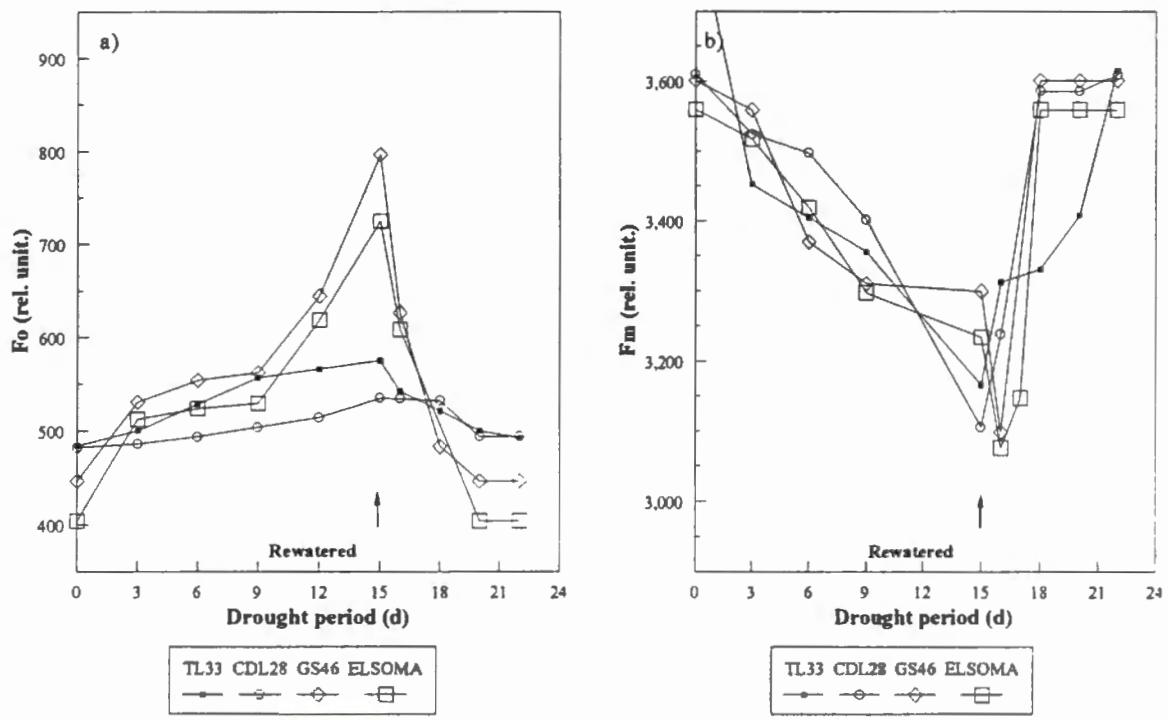


Figure 5. Drought stress-induced increases in (a) F_O and decreases in (b) F_M, which recover at differential rates in the respective cultivars to their individual pre-stress levels upon rewatering on day fifteen.

Thus, it would seem as if the tobacco cultivars studied dissipate more excess excitation energy during drought stress as fluorescence and less by radiationless energy dissipation as

mediated by the carotenoid zeaxanthin. Furthermore, this drought tolerance-mechanism is more effectively used by GS46 and ELSOMA. In this regard it is interesting to tentatively speculate that in general, plants such as *Nerium oleander* which are xeromorphologically well adapted to avoid drought stress, may readily make use of radiationless energy dissipation, whereas the opposite may be true for more mesomorphic plants that have to tolerate drought stress.

5.5 Conclusion

An agricultural production strategy must be based on optimizing plant function in relation to the environment to give high productivity with long term stability. As drought stress is a complex of many morphological, physiological, and biochemical factors, it is doubtful that any one criterion will be adequate for selection of drought-resistant genotypes. However, it has been found that resistance to water flow due to anatomical differences varies between the respective cultivars and that a close correlation exists between a cultivar's WUE and its percentage intercellular spaces. A strong quantitative correlation also exists between the mechanisms of excess energy dissipation and an individual cultivar's drought tolerance (all factors which are genetically determined). Therefore it is postulated that some of the parameters investigated in this work, such as the percentage intercellular spaces and the mechanism of excess energy dissipation as reflected by the F_O fluorescence level, are relatively easy and simple to determine and may be used to differentiate between drought-tolerant and drought-sensitive plants.

5.6 Literature cited

ASADA, K. & TAKAHASHI, M. 1987. Production and scavenging of active oxygen in photosynthesis. (*In* D.J. Kyle, C.B. Osmond & Arntzen, C.J.. *eds*. Photoinhibition. Amsterdam: Elsevier. p. 227-287.)

AVERY, G.S. 1933. Structure and development of the tobacco leaf. *American Journal of Botany*, 20: 565-592.

BEGG, J.E. & TURNER, N.C. 1976. Crop water deficits. *Advances in Agronomy*, 28: 61-217.

BOYER, J.S. 1971a. Recovery of photosynthesis in sunflower after a period of low leaf water potential. *Plant Physiology*, 47: 816-820.

BOYER, J.S. 1971b. Resistances to water transport in soybean, bean and sunflower. *Crop Science*, 11: 403-407.

CHAVES, M.M. 1991. Effects of water deficits on carbon assimilation. *Journal of Experimental Botany*, 42: 1-16.

CRONIC, G., LE GOUALLEC, J.L., BRIANTAIS, J.M. & HODGES, M. 1989. Effect of dehydration and high light on photosynthesis of two C_3 plants (*Phaseolus vulgaris* L. and *Elatostema repens* (Lour.) Hall. f.). *Planta*, 177: 84-90.

DEMMIG-ADAMS, B., ADAMS, W.W., WINTER, K., MEYER, A., SCHREIBER, U., PEREIRA, J.S., KRüGER, A., CZYGAN, F.A. & LANGE, O. 1989. Photochemical efficiency of photosystem II, photon yield of O_2 evolution, photosynthetic capacity, and carotenoid composition during the midday depression of net CO_2 uptake in *Arbutus unedo* growing in Portugal . *Planta*, 177: 377-387.

DEMMIG, B. & BJöRKMAN, O. 1987. Comparison of the effect of excessive light on chlorophyll fluorescence (77K) and photon yield of O_2 evolution in leaves of higher plants. *Planta*, 171: 171-184.

DEMMIG, B., WINTER, K., KRüGER, A., & CZYGAN, F.C. 1987. Photoinhibition and zeaxanthin formation in intact leaves. A possible role of the xanthophyll cycle in the dissipation of excess light energy. *Plant Physiology*, 84: 218-224.

DEMMIG, B., WINTER, K., KRüGER, A. & CZYGAN, F.C. 1988. Zeaxanthin and the heat dissipation of excess light energy in *Nerium oleander* exposed to a combination of high light and water stress. *Plant Physiology*, 87: 17-24.

DE TONI, J.B. & PAOLETTI, J. 1891. Beitrag zur Kenntnis des anatomisches Baues von *Nicotiana tabacum* L. *Berichte der Deutschen botanischen Gesellschaft*, 9: 42-50.

EWERS, F.W. & FISHER, J.B. 1989. Variation in vessel length and diameter in stems of six tropical and subtropical lianas. *American Journal of Botany*, 76: 1452-1459.

FARQUHAR, G.D. & SHARKEY, T.D. 1982. Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology*, 33: 317-345.

FOYER, C., FURBANK, R., HARBINSON, J. & HORTON, P. 1990. The mechanisms contributing to photosynthetic control of electron transport by carbon assimilation in leaves. *Photosynthesis Research*, 25: 83-100.

GATES, D.M. 1968. Transpiration and leaf temperatures. *Annual Review of Plant Physiology*, 19: 211-238.

GREER, D., BERY, J.A. & BJöRKMAN, O. 1986. Photoinhibition of photosynthesis in intact bean leaves: role of light and temperature and requirement for chloroplast-protein synthesis during recovery. *Planta*, 168: 253-260.

HAGER, A. 1980. The reversible, light-induced conversion of xanthophyll in the chloroplast. (*In* Czygan, F.C., *ed.* Pigments in Plants. Stuttgart: Gustav Fischer-Verlag. p. 57-79.)

HAGER, A. & MEYER-BERTENRATH, T. 1966. Die Isolierung und quantitative Bestimmung der Carotinoide und Chlorophylle von Blättern, Algen und isolierten Chloroplasten mit Hilfe dünnschichtchromatographischer Methoden. *Planta*, 69: 198-217.

HAGER, A. & MEYER-BERTENRATH, T. 1967. Die Identifizierung der an Dünnschichten getrennten Carotinoide grüner Blätter und Algen. *Planta*, 76: 149-68.

HAVAUX, M., GREPPIN, H. & STRASSER, R.J. 1991. Functioning of photosystem I and II in pea leaves exposed to heat stress in the presence and absence of light: Analysis of using *in vivo* fluorescence, absorbance, oxygen and photo-acoustic measurements. *Planta*, 186: 88-98.

JOHANSEN, D.A. 1940. Plant microtechnique. New York: McGraw-Hill.

KIRSCHBAUM, M.U.F. 1988. Recovery of photosynthesis from water stress in *Eucalyptus pauciflora*: a process in two stages. *Plant Cell Environment*, 11: 685-694.

KITAJIMA, M., & BUTLER, W.L. 1975. Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. *Biochimica et Biophysica Acta*, 376: 105-115.

LAWLOR, D.W. 1979. Effects of water and heat stress on carbon metabolism of plants with C_3 and C_4 photosynthesis.(*In* Mussell, H. & Staples, R.C., *eds*. Stress physiology in crop plants. New York: Wiley & Sons. p.303-326.)

LEVITT, J.V. 1972. Responses of plants to environmental stress. New York: Academic Press.

LUDLOW, M.M. & BJöRKMAN, O. 1984. Paraheliotropic leaf movement in Siratro as a protective mechanism against drought-induced damage to primary photosynthetic reactions: damage by excessive light and heat. *Planta*, 161: 505-518.

METCALFE, C.R. & CHALK, L. 1965. Anatomy of the dicotyledons. Vol. II. Oxford: Clarendon.

MEYER, W.S. & WALKER, S. 1981. Leaflet orientation in water-stressed soybeans. *Agronomy Journal*, 73: 1071-1074.

MUSSEL, H. & STAPLES, R.C. 1979. Stress physiology in crop plants. New York: Wiley & Sons.

NOBEL, P.S. 1991. Physiochemical and environmental plant physiology. 4 ed. New York: Academic Press.

OHAD, I., KYLE, D.J. & ARNTZEN, C.J. 1984. Membrane protein damage and repair: removal and replacement of inactivated 32-kilodalton polypeptides in chloroplast membranes. *Journal of Cell Biology*, 99: 481-485.

OOSTERHUIS, D.M., WALKER, S. & EASTHAM, J. 1985. Soybean leaflet movements as an indicator of crop water stress. *Crop Science*, 25: 1101-1106.

PALLARDY, S.G. 1989. Hydraulic architecture and conductivity: An overview.(*In* Kreeb, K.H., Richter H. & Hinckley, T.M., *eds.* Structural and functional responses to environmental stresses. The Hague: SPD Academic Publishing. p. 3-19.)

QUICK, W.P., CHAVES, M.M., WENDLER, R., DAVID, M.M. RODRIQUES, M.L., PEREIRA, J.S., LEEGOOD, R.C. & STITT, M. 1992. Effects of water stress on photosynthetic carbon metabolism in four species grown in field conditions. *Plant Cell Environment*, 15: 25-35.

RICHIE, J.T. 1974. Atmospheric and soil water influences on the plant water balance. *Agricural Meteorology*, 14: 183-198.

SALLEO, S. & LO GULLO, M.A. 1989. Xylem cavitation in nodes and internodes of *Vitis vinifera* L. plants subjected to water stress. (*In* Kreeb, K.H., Richter H.& Hinckley, T.M., *eds.* Structural and functional responses to environmental stresses. The Hague: SPD Academic Publishing. p.33-42.)

SHACKEL, K.A. & HALL, A.E. 1979. Reversible leaf movements in relation to drought adaptation of cowpeas, *Vigna unguicalata* (L.) Walp. *Australian Journal of Plant Physiology*, 6: 265-276.

SIEFERMANN-HARMS, D. 1987. The light-harvesting and protective functions of carotenoids in photosynthetic membranes. *Physiologia Plantarum*, 69: 561-568.

SPERRY, J.S. & SULLIVAN, J.E.M. 1992. Xylem embolism in response to freeze-thaw cycles and water stress in ring-porous, diffuse-porous, and conifer species. *Plant Physiology*, 100: 605-613.

TERASHIMA, I., WONG, S.C., OSMOND, C.B. & FARQUHAR, G.D. 1988. Characterisation of non-uniform photosynthesis induced by abscisic acid in leaves having different mesophyll anatomies. *Plant Cell Physiology*, 29: 385-394.

TURNER, N.C. 1979. Drought resistance adaptation to water deficits in crop plants.(*In* Mussell, H. & Staples, R.C., *eds.* Stress physiology in crop plants. New York: Wiley & Sons. p.343-372.)

VAN RENSBURG, L. & KRüGER, G.H.J. 1993a. Differential inhibition of photosynthesis (*in vivo* and *in vitro*), and changes in chlorophyll *a* fluorescence induction kinetics of four tobacco cultivars under drought stress. *Journal of Plant Physiology*, 141: 357-365.

VAN RENSBURG, L., KRüGER, G.H.J. & KRüGER, H. 1993. Proline accumulation as drought tolerance selection criterion: its relationship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. *Journal of Plant Physiology*, 141: 188-194.

VAN RENSBURG, L. & KRüGER, G.H.J. 1993b. Comparative analysis of differential drought stress-induced inhibition of and recovery in, carbon dioxide fixation: Stomatal and non-stomatal limitation in *Nicotiana tabacum* L. *Journal of Plant Physiology*, 142: 296-306.

CHAPTER SIX

6. PROTEIN TURNOVER: PROBABLE DETERMINING FACTOR FOR DROUGHT TOLERANCE IN *NICOTIANA TABACUM* L. (VAN RENSBURG, L. & KRüGER, G.H.J. 1993. *SOUTH AFRICAN JOURNAL OF PLANT AND SOIL*, 10:119-123.)

6.1 Abstract

Drought stress-induced changes in the water-soluble protein concentration and total number of sulphydryl (-SH) groups were monitored under controlled environmental conditions in four tobacco cultivars (*Nicotiana tabacum* L.) with different drought tolerance. The drought stress ranged from light (-0.52 MPa) to severe (-2.5 MPa). A statistically significant decline in both water soluble protein concentration and total number of -SH groups already occurred at a leaf water potential Ψ_L of -0.77 MPa. The initial rate of decline in both the concentration of water-soluble protein and number of -SH groups, as well as the end-values reached by both these parameters, differed significantly among the drought-tolerant (GS46 and ELSOMA) and drought-sensitive (TL33 and CDL28) cultivars. In contrast to the fast initial decline observed in GS46 and ELSOMA at light stress levels, which stabilized and occurred more gradually as the drought stress intensified, the concentration of water soluble protein and number of -SH groups declined at a slower but continuous rate in TL33 and CDL28 as Ψ_L decreased. At a Ψ_L of -2.51 MPa, the number of -SH groups present in GS46 and ELSOMA respectively, was 63.6% and 65.2%, and that of TL33 and CDL28 was 39.9% and 46.8% of their respective control values. Furthermore, the concentration of water-soluble protein of ELSOMA still was 75.5% and that of GS46, 65.4% of their controls, even at a Ψ_L of -2.51 MPa, which contrasts with the corresponding values of 46.6% and 55.3% for TL33 and CDL28, respectively. The steep initial decline in the water-soluble protein concentration of ELSOMA and GS46 is explained on basis of the concept of protein turnover. The known difference in drought tolerance between the four tobacco cultivars was best explained by a combination of the -SH/-SS- hypothesis and the concept of protein turnover.

Keywords: *Drought tolerance, Nicotiana tabacum* L., *protein turnover, sulphydryl groups, concentration of water soluble protein.*

6.2 Introduction

Drought stress constrains almost every aspect of a plant's growth by inducing anatomical, morphological, physiological and biochemical alterations (Tomati & Galli, 1979). Many of these changes are believed to be adaptive responses which make the organism fitter for growth in the altered environment. In many such instances, these changes are complex and difficult to evaluate, thus making it difficult to establish a precise quantitative relationship between the environmental parameter and the induced change.

Furthermore, it is well known that even small reductions in leaf water potential (Ψ_L) not only modify both carbohydrate and nitrogen metabolism, but also cause changes in the structure of proteins and alterations in enzyme action (Gale *et al.*, 1976). Hsiao (1973) reported that the soluble protein content of wheat leaves was markedly reduced when the relative water content (RWC) was lowered to about 60%. When stress is more severe, denaturation of protoplasmic proteins occurs with a consequent loss in enzyme activity (Tomati & Galli, 1979). Levitt (1962) and Gaff (1966) demonstrated that protein disulphide (-SS-) groups increase at the expense of sulphydryl (-SH)- groups during drought stress and that this is associated with protein aggregation. In other words, as protein denaturation occurs, the -SH/-SS- ratio decreases with the consequent alteration of physiological functions and death of the plant tissue when the stress is too severe.

According to Navari-Izzo *et al.* (1990), it, however is not always clear whether a decrease in protein content reflects a retardation of protein synthesis, or an accelerated degradation rate leading to an increase in the free amino acid content (Sivaramakrishnan *et al.*, 1988) and/or to an inhibition of amino acid incorporation into proteins. It has also been indicated that not only protein degradation but also protein synthesis still take place during drought stress, in spite of a general decline in the overall rate of protein synthesis (Maranville & Paulsen, 1972).

To be able to exploit metabolic pathways either as components of adaptation or as indicators of stress, researchers must, according to Navari-Izzo *et al.* (1990), first be able to describe the behaviour of different crop species with respect to their metabolic response to the same sequence of water stress and be able to interpret the physiological significance of these responses. These factors can greatly influence the responses of the plant, and even where data concerning the imposed stress are available, the stress levels are often not clearly defined in terms of the plant water status (Navari-Izzo *et al.*, 1990). This makes comparisons between different species, and even between cultivars, quite difficult. In our investigation, therefore, two parameters that describe a plant's water status are supplied to enable and aid comparisons.

The present comparative investigation was conducted to determine whether a correlation exists between drought stress-induced changes in the soluble protein content and the amount of -SH groups in four tobacco cultivars with different, but known, drought tolerances. This was done to determine whether, in practice, the -SH/-SS- ratio at low Ψ_L may be used as an index of protein denaturation and/or as an index of the drought stress resistance of a tobacco cultivar.

6.3 Material and methods

Plant material and general growth conditions

In sequence of increasing drought tolerance, the four tobacco cultivars under investigation were TL33, CDL28, GS46 and ELSOMA. These cultivars were selected owing to their differing performances in the field during drought stress periods (Dr CJ Steenkamp, Tobacco and Cotton Research Institute, Rustenburg, RSA pers. comm.). Seed was germinated in soil in 24-L pots (from which excess water could freely drain) under glasshouse conditions, with optimal water application. Before the onset of experimentation the plants were moved the a growth chamber (photon flux density, 400-600 $\mu Em^{-2}s^{-1}$, relative humidity, 35%) and allowed to acclimatize for a period of 96 h. During the acclimatization period, the plants were watered twice daily to field capacity to avoid their experiencing any drought stress and possibly hardening (an ability which may differ among the different cultivars) because of the slightly higher growth chamber temperature. The growth chamber was lit for 13h at 25°C, followed by an 11h dark period at 16°C. Experimentation started when the plants were about 90 days old, and a drought stress of increasing intensity (ca. 0.166 MPa d^{-1}) was induced by withholding water. The drought stress lasted for 12 days and ranged from light (-0.52 MPa) to severe (-2.51 MPa). As was the case with all other measurements, Ψ_L determinations were done simultanuously on alternate days with a Scholander pressure chamber (PMS-instrument, Oregon, USA). That is, on a specific day the Ψ_L of all four cultivars was continuously monitored until it reached a certain predetermined Ψ_L, at which time sampling occurred. The rational behind this line of thought was that any stress-responses would only be comparable if there was certainty that all four cultivars experience the same drought stress.

Sampling and assay procedure

Sampling consisted of collecting $10cm^2$ leaf discs intravenously per plant between the secondary vascular intravenously strands, from the sixth leaf from the apex, on alternate days and therefore progressively lower Ψ_L. The exact procedure followed and equation used for determining the relative water content (RWC), concur with the method developed by Weatherly (1951). The soluble protein concentration was determined by the Lowry-method (Lowry *et al.*, 1951), after the phenolic and related compounds known to interfere with this method had been removed by denaturating and precipitating the proteins with trichloroacetic acid (TCA). The number of -SH groups was determined by the method developed by Cavallini *et al.*, (1966). This method entails using sodium borohydride ($NaBH_4$) in 8M urea as reducing

agent and 5,5'-dithio-*bis* (2-nitrobenzoic) acid (DTNB) as a thiol-disulphide exchanger. The extinction coefficient of 12 000 reported by Flavin (1962) was used to determine the number of total sulphydryl groups (N) after reduction, using the formula:

$$N = \frac{MW \cdot A \cdot V}{12000 \cdot m}$$

where MW is the molecular weight of the protein; A, the absorbancy (412nm); V, the volume of the final solution; and m, the weight in mg of the protein sample analyzed.

Statistical analysis

Data were statistically analysed with the aid of the Dunnet *t*-test as a number of treatment means had to be compared with a single control and one of the several *t*-statistics were applicable (Miller, 1966). Curve fittings were done with simplex algorithms (Willmott, 1982), which are capable of calculating interpolation values between the data points to give statistically justifiable curves.

6.4 Results and discussion

The concentration of soluble protein of the controls (at a Ψ_L of -0.52 MPa, see Table 1) of all four cultivars correlated well with the total nitrogen content of tobacco reported by Osmond and Raper (1981) and Raper *et al.* (1976). The observed decline in the concentration of soluble protein on all four cultivars with decline in Ψ_L (Fig. 1), is in accordance with results cited in the literature (Dungey & Davies, 1982). At a Ψ_L of only -0.77 MPa, the concentration of soluble protein of all four cultivars already deviated statistically significantly ($p < 0.05$) from their respective controls. According to the sulphydryl-disulphide hypothesis of Levitt (1962), and subsequent considerations by Gaff (1966), injury to frost and drought stress can be ascribed to irreversible conformational changes in the structural proteins and to oxidation of -SH- groups to -SS- bridges. Thus, resistance to stress could be resistance towards -SH oxidation and -SH/-SS- interchange and, therefore, to formation of intermolecular bonds (Tomati & Galli, 1979). On this basis, it has therefore been stated that the -SH/-SS- ratio may both be regarded both as an index of protein denaturation and as an index of stress resistance.

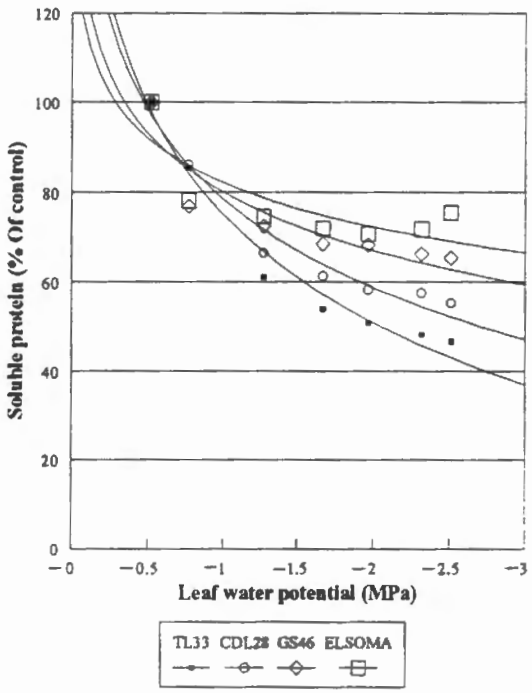


Figure 1. Drought stress-induced changes in the concentration of soluble protein of four tobacco cultivars, which differ with respect to their individual drought tolerance. (Elsoma and GS46 are drought-tolerant).

In spite of the observation that both the soluble protein concentration and number of -SH groups (Fig. 2) in all four cultivars declined over the total drought stress range (i.e. -0.52 MPa to -2.51 MPa), it is important to note that the initial rate at which the soluble proteins concentration and number of -SH groups declined, as well as the end-values reached by both these parameters,

differed significantly between the drought-sensitive (TL33 and CDL28) and drought-tolerant (GS46 and ELSOMA) cultivars. It is obvious (Table 1, Figs. 1 and 2) that both the concentration of soluble protein and number of -SH groups of GS46 and ELSOMA were characterized by a faster rate of decline at the mild to moderate stress levels. However, as the drought stress became more severe, this decline occurred more gradually and later stabilized at round about 69.7% in the case of the concentration of soluble protein and 65.5% in the case of the number of -SH groups (Figs. 1 and 2). The concentration of soluble protein and number of -SH groups of TL33 and CDL28, however, declined at a slower but continious rate over the entire drought stress range. The results obtained (Table 1), with exception of the observed rapid initial decline in the concentration of soluble protein and number of -SH groups in the drought-tolerant cultivars, support the above-mentioned statements of Tomati and Galli (1979). In support of the latter contention, it can be stated that both GS46 and ELSOMA maintained a higher number of -SH groups at a Ψ_L of -2.51 MPa. At this Ψ_L, the number of -SH groups of GS46 and ELSOMA were respectively 63.6% and 65.2% of their respective controls compared with the 39.9% and 46.8% for TL33 and CDL28. The corresponding concentration of protein of ELSOMA only declined to 75.5% and that of GS46 to 65.4% of their respective controls, whereas it declined to 46.6% and 55.3% respectively in TL33 and CDL28.

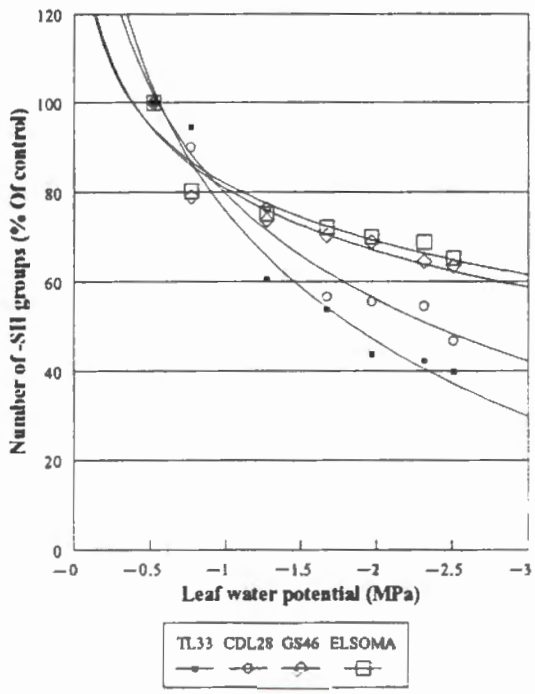


Figure 2. Differential drought stress-induced declines in the number of -SH groups in four tobacco cultivars with different drought tolerances.

Tabel 1: Differential drought stress-induced changes in the concentration of soluble protein and number of -SH groups in four tobacco culitvars, with different drought tolerances. Standard deviations (SE±) reflect the deviation from the mean of four replicates per determination.

Cultivars	Ψ_L MPa	RWC %	-SH groups μmol mg^{-1}	Water-soluble protein mg g^{-1}
TL33	-0.52	88.7 ± 0.1	34.28 ± 0.1	34.2 ± 0.05
	-0.77	84.9 ± 0.1	32.33 ± 0.9	29.2 ± 0.06
	-1.27	81.7 ± 0.7	20.79 ± 0.3	20.0 ± 0.02
	-1.67	69.9 ± 0.9	18.45 ± 0.9	18.6 ± 0.02
	-1.97	62.4 ± 0.7	15.02 ± 0.2	17.4 ± 0.01
	-2.32	56.2 ± 0.4	14.94 ± 0.4	16.5 ± 0.06
	-2.51	54.1 ± 0.9	14.35 ± 0.2	15.9 ± 0.08
CDL28	-0.52	87.7 ± 0.6	27.59 ± 0.1	35.1 ± 0.02
	-0.77	96.1 ± 0.6	24.87 ± 0.5	30.2 ± 0.05
	-1.27	81.3 ± 0.5	21.14 ± 0.3	23.3 ± 0.08
	-1.67	76.5 ± 0.7	18.11 ± 0.7	21.5 ± 0.03
	-1.97	68.6 ± 0.8	16.42 ± 0.6	20.5 ± 0.01
	-2.32	59.9 ± 0.9	16.32 ± 0.8	20.2 ± 0.07
	-2.51	56.2 ± 0.6	14.83 ± 0.4	19.4 ± 0.02
GS46	-0.52	90.1 ± 0.3	31.82 ± 0.1	36.4 ± 0.04
	-0.77	86.7 ± 0.3	25.14 ± 0.1	27.9 ± 0.07
	-1.27	80.4 ± 0.2	23.48 ± 0.3	26.4 ± 0.04
	-1.67	68.2 ± 0.9	23.33 ± 0.5	24.9 ± 0.04
	-1.97	55.9 ± 0.3	21.89 ± 0.3	24.2 ± 0.05
	-2.32	47.5 ± 0.2	17.33 ± 0.2	24.0 ± 0.06
	-2.51	46.5 ± 0.9	10.68 ± 0.9	23.8 ± 0.01
ELSOMA	-0.52	89.2 ± 0.6	33.05 ± 0.1	33.9 ± 0.06
	-0.77	86.2 ± 0.6	26.53 ± 0.6	26.5 ± 0.07
	-1.27	77.5 ± 0.4	24.87 ± 0.4	25.3 ± 0.03
	-1.67	57.2 ± 0.5	24.16 ± 0.8	24.2 ± 0.08
	-1.97	50.7 ± 0.3	23.14 ± 0.3	23.9 ± 0.02
	-2.32	43.9 ± 0.2	19.43 ± 0.2	24.3 ± 0.09
	-2.51	39.0 ± 0.4	16.62 ± 0.7	25.5 ± 0.04

On the basis of results obtained with *Lemma minor* subjected to several types of stress, Cooke *et al.* (1979) identified a possible adaptation mechanism which might explain the initial high rate of decline in the concentration of soluble protein (Fig. 1) and number of -SH groups (Fig. 2) observed in the drought-tolerant tobacco cultivars, as this observation seems to be contradictory to the -SH/-SS- hypothesis. According to the concept of protein turnover, the high initial drought stress-induced protein degradation rate, supply the necessary amino acid precursors needed for the synthesis of an altered enzyme complement, which may be better adapted to maintain growth during the new environmental conditions. This implies that as the plant adapts to the stress, the initially high protein degradation rate will decrease as a better

suited and more stable enzyme complement is synthesized. This is probable the case in the drought-tolerant cultivars (Fig. 1). Data obtained by Cooke *et al.* (1979) support the latter statement, as they indicated that the protein synthesized during stress was more stable than the original cellular protein under stress conditions. From this Cooke *et al.* (1979) concluded that resistance to stress could either be ascribed to changes in the activity of the degradative system or to alterations in the susceptbility of the protein to degradation. It is therefore clear that the observed rapid initial decline in both the concentration of soluble protein and number of -SH groups in GS46 and ELSOMA (Table 1) can pre-eminently be explained by using the concept of protein turnover.

6.5 Conclusion

The observed drought stress-induced changes in concentration of soluble protein (Fig. 1) and number of -SH groups (Figure 2), as well as the known difference in drought tolerance among the four tobacco cultivars investigated, can be explained best by combining the concept of protein turnover and the -SH/-SS- hypothesis. The results obtained (Table 1), corroborate the statement that the -SH/-SS- ratio can be regarded as a measure of the general well-being of a plant, as it confirms a strict dependance of the -SH/-SS- ratio on the Ψ_L and tissue water content. At light to moderate stress levels a high initial protein degradation rate may bestow a drought tolerance ability on a plant, as it would supply the necessary amount of amino acid precursors for the synthesis of an altered enzyme complement, which may be better suited for growth under the new environmental conditions. At the whole plant level, drought stress-induced amino acid accumulation can create a dynamic adjustment in the nitrogen metabolism (Hanson & Hitz, 1982; Stewart & Larher, 1980), or associate with hydrophobic groups to enhance the stability of macromolecules (Schobert & Tsesche, 1978; Van Rensburg & Krüger, 1993). At severe stress levels, tolerant plants may be resistant to -SH oxidation or -SH/-SS- interchange. Thus, at low Ψ_L the -SH/-SS- ratio may be regarded as an index of stress resistance. Furthermore, by manipulating the process of protein turnover and therefore osmoregulation, it may be possible to render some tobacco cultivars more drought-tolerant while on the land.

6.6 Literature cited

CAVALLINI, D., GRAZIANI, M.T. & DUPRé,, S. 1966. Determinnation of disulphide groups in proteins. *Nature*, 212: 294-295.

COOKE, R.J., OLIVIER, J. & DAVIES, D.D. 1979. Stress and protein turnover in *Lemna minor*. *Plant Physiology*, 64: 1109-1113.

DUNGEY, N.O. & DAVIES, D.D. 1982. Protein turnover in the attached leaves of non-stressed and stressed barley seedlings. *Planta*, 154: 435-440.

FLAVIN, M. 1962. Microbal transsulfuration: the mechanisms of an enzymatic disulfide elimination reaction. *Journal of Biological Chemistry*, 237: 768-777.

GAFF, D.F. 1966. The sulphydryl-disulphide hypothesis in relation to desiccation injury of cabbage leaves. *Australian Journal of Biological Science*, 19: 291-299.

GALE, J., KOHL, H.C. & HAGAN, R.M. 1967. Changes in the water balance and photosynthesis of onion, bean and cotton plants under saline conditions. *Physiologia Plantarum*, 20: 408-426.

HANSON, A.D. & HITZ, W.D. 1982. Metabolic responses of mesophytes to plant water deficits. *Annual Review of Plant Physiology*, 33: 162-203.

HSIAO, T.C. 1973. Plant responses to water stress. *Annual Review of Plant Physiology*, 24: 519-570.

LEVITT, J. 1962. A disulphydryl-disulphide hypothesis of frost injury and resistance in plants. *Journal of Theoretical Biology*, 3: 355-391.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A. & RANDALL, R.J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biochemistry*, 139: 265.

MARANVILLE, J.W. & PAULSEN, G.M. 1972. Alteration of protein composition of corn (*Zea mays* L.) seedlings during moisture stress. *Crop Science*, 12: 660-663.

MILLER, R.G., 1966. Simultaneous statistical inference. New York: McGraw-Hill.

NAVARRI-IZZO, F., QUARTACCI, M.F. & IZZO, R. 1990. Water stress-induced changes in protein and free amino acids in field grown maize and sunflower. *Plant Physiology and Biochemistry*, 28: 531-537.

OSMOND, D.L. & RAPER, C.D. Jr. 1981. Growth and nitrogen accumulation in tobacco plants as affected by nitrate concentration, root temperature and aerial temperature. *Agronomy Journal*, 73: 491-496.

RAPER, C.D., WEEKS, W.W. & WANN, M. 1976. Temperatures in early post-transplant growth: influence on carbohydrate and nitrogen utilization and distribution in tobacco. *Crop Science*, 16: 753-757.

SCHOBERT, B. & TSESCHE, H. 1978. Unusual solution properties of proline and its interaction with proteins. *Biochimica et Biophysica Acta*, 541: 270-277.

SIVARAMAKRISHNAN, S., PATELL, V.Z., FLOWERS, D.J. & PEACOCK, J.M. 1988. Proline accumulation and nitrate reductase activety in contrasting sorghum lines during mid-season drought stress. *Physiologia Plantarum*, 74: 418-426.

STEWART, G.R. & LARHER, F. 1980. Accumulation of amino acids and related compounds in relation to environmental stress. (*In* Miflin, B.J., *ed.* The biochemistry of plants. A comprehensive treatise. New York: Academic Press.)

TOMATI, U. & GALLI, E. 1979. Water stress and -SH- dependant physiological activities in young maize plants. *Journal of Experimental Botany*, 30: 557-563.

VAN RENSBURG, L., KRüGER, G.H.J. & KRüGER, H. 1993. Proline accumulation as drought tolerance selection criterion: its relationship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. *Journal of Plant Physiology*, 141, 188-194.

WETHERLEY, P.E. 1951. Studies on the water relations of the cotton plant. II. Diurnal and seasonal fluctuation and environmental factors. *New Phytologist*, 50: 36-51.

WILLMOTT, C.J., 1982. Some comments on the evaluation of model performance. *Bulletin of the American Meteorological Society*, 63, 1309-1313.

CHAPTER SEVEN

7. APPLICABILITY OF ABCISIC ACID AND/OR PROLINE ACCUMULATION AS SELECTION PARAMETER(S) FOR DEPICTING DROUGHT TOLERANCE IN *NICOTIANA TABACUM* L. (VAN RENSBURG, L., KRüGER, H. & KRüGER, G.H.J. 1994. *CANADIAN JOURNAL OF BOTANY*, IN PRESS.)

7.1 Abstract

The efficacy of various aspects of ABA and proline accumulation as potential selection parameters for drought tolerance in tobacco were evaluated under controlled conditions. The results indicated that ABA and proline accumulate rapidly after a distinct threshold leaf water potential value has been reached. Probably on account of their higher cell-wall elasticity at 0.23 MPa and 0.28 MPa for the drought-tolerant cultivars, GS46 and ELSOMA respectively, as opposed to 0.39 MPa and 0.31 MPa for the drought-sensitive cultivars, TL33 and CDL28 respectively, these values are reached sooner in drought-tolerant cultivars (GS46 and ELSOMA). However, rapid ABA accumulation preceeds proline accumulation in both the drought-tolerant and drought-sensitive cultivars (TL33 and CDL28). Proline concentrations increased sharply at a leaf water potential of -1.27 MPa in the drought-tolerant cultivars and at a leaf water potential of -1.5 MPa in the drought-sensitive cultivars. In contrast, at a leaf water potential of -0.77 MPa the ABA concentrations of all four cultivars were already significantly higher than their respective controls and was greater in the drought-tolerant cultivars. A possible explanation is given as to why, in this and other studies, the accumulated ABA end-concentrations do not necessarily show a positive correlation with the known drought tolerance of plants. The leaf water potential value at which ABA and proline start accumulating rapidly, and the accumulated proline end-concentrations, are recommended as selection parameters for drought tolerance in tobacco.

Keywords: *Abscisic acid accumulation, cell-wall elasticity, drought stress, Nicotiana tabacum* L., *proline accumulation, selection parameters.*

Abbreviations: ABA = abscisic acid; ϵ = elasticity modulus; Ψ_L = leaf water potential; RWC = relative water content; Ψ_π = osmotic potential.

7.2 Introduction

One of the most remarkable results of drought stress research has been the observation that ABA accumulates rapidly in most mesophytic plants during drought stress. Milborrow and Noddle (1970) observed a marked increase in ABA levels in tobacco plants when they were exposed to high saline concentrations. It would seem that only a mild to moderate stress condition is sufficient to induce an increase in ABA concentration. The determination of the role of ABA during drought stress is complicated by observations which indicate that the roots of plants exposed to drought stress also show an increase in ABA concentration (Quarrie & Jones, 1977), and that these increases are independent of transport from the stem (Walton *et al.*, 1976). A pronounced increase of free-proline content (10 to 100 fold) in the leaf tissue of certain mesophytic flowering plants, occurs within hours or days after a moderate to intense period of exposure to drought stress. Various experimental methods which induce drought stress can elicit the accumulation of proline in young leaf tissue (Haung & Cavalieri, 1979). It is also known that proline accumulates in the mature leaves still attached to older plants when they suffer a long term water deficit. Accumulation of proline has also been observed in the field (Rjagopal *et al.*, 1977) as well as in leaves of large potted plants (Jones *et al.*, 1980).

The accumulation of certain compounds, such as ABA and proline, is often regarded as an adaptation mechanism to specific stress conditions. However, Kramer (1983) warns that the mere correlation between the accumulation of a compound and the development of a stress condition does not provide sufficient evidence that the specific compound has any adaptive advantage with regard to the extension or alleviation of the stress condition. The main aim in evaluating metabolic characteristics which may possess potential adaptive value is primarily to identify metabolic adaptabilities which could be applied in plant breeding programmes of mesophytic agricultural crops in drought-prone areas. According to Kramer (1983) two of the three proposed metabolic adaptive reactions include ABA and proline accumulation.

To determine whether plants which accumulate ABA and/or proline possess an adaptive advantage, drought stress-induced changes in ABA and proline concentrations in drought-tolerant and drought-sensitive cultivars, grown under controlled conditions, were correlated with previous field observations, which indicated that the tobacco cultivars GS46 and ELSOMA were more drought-tolerant than TL33 and CDL28. This approach is in accordance with the method proposed by Hanson and Hitz (1982). It could therefore, be determined whether reaction to stress was the result of damage due to drought stress or whether it presents an adaptive advantage.

In brief, the aim was, therefore, to observe whether the ABA and/or proline accumulation occurred at the right time, whether its accumulation was extensive enough to be applied as a selection parameter(s), and/or whether the drought stress-induced changes in the concentrations of these compounds, correlated with their known drought tolerance.

7.3 Material and methods

Seeds of four different tobacco cultivars were allowed to germinate in containers with soil. The four cultivars investigated were: TL33, CDL28, GS46 and ELSOMA (of which the first two can be described as drought-sensitive and the other two as being drought-tolerant). They were selected on the basis of their performance during drought conditions in the field (Personal communication - Dr CJ Steenkamp, Tobacco and Cotton Research Institute, Rustenburg, RSA). The seedlings were grown in a glasshouse under an optimal moisture regime, and then transferred to controlled growth chambers and allowed to adapt for 96 hours before the onset of the experiment. For more details concerning the exact growth conditions which prevailed in the glasshouse and growth chamber, see Van Rensburg *et al.* (1993). The experiment commenced by inducing drought stress of increasing intensity (ca. 0.2 to 0.3 MPa d^{-1}) by witholding water from the experimental plants. During the course of the experimental period which lasted 12 days, the control plants were watered daily to field capacity. Sampling was done on alternate days and comprised of taking a sample (10cm^2 leaf disc) from four different plants for each cultivar per variable monitored. Consecutive sampling was done on the same leaf of each plant previously sampled from. Stomatal closure was monitored with an infra red gas analyser (A.D.C. LCA-2, Hoddeson, England). Furthermore, two groups of plants from different age groups were used, namely, group A: plants approximately 150 days old and group B: plants 90 days old.

Water status measurements

The drought stress experienced by the different cultivars were monitored by measuring the leaf water potential (Ψ_L) of all four the experimental cultivars and their respective controls on alternate days (Scholander pressure chamber, PMS-Instrument, Oregon, USA). Concurrently with each Ψ_L -determination, two 10cm^2 leaf discs were collected from the sixth leaf from the apex (one from a control plant and the other from a plant under drought stress) and the relative water content (RWC) was determined and calculated according to the methods and formulae proposed by Weatherly (1951). Pressure-volume curves (PV-curves) were constructed according to the method of Hinckley *et al.* (1980) and Richter and Wagner (1983). As suggested by Kubiske and Abrams (1990), a PV-curve represents the reciprocal of the leaf water potential ($1/\Psi_L$) as related to the RWC of a specific cultivar. From the PV-curve of each cultivar the modulus of elasticity ($\in$) of the cell walls was determined at incipient plasmolysis, by using the formula $\in = RWC \cdot \tan a$, where **a** represents the regression of the linear part of the PV-curve (Wilson *et al.*, 1980). Zur *et al.* (1981) provide an alternative formula which can be used to calculate $\in$ in drought stress studies.

ABA and proline determinations

The extraction procedure for the ABA determinations corresponds to that described by Robertson *et al.* (1985). Analyses were, however, made by HPLC (Waters 600 delivery system; Waters 490 multi-wave-length detector, Varian 4290 integrator). 50µl of the extract was injected into an reverse-phase C18 phase column (Nova-pack, Radial-Pack 8mm X 10cm, 4µm; Millipore). The HPLC conditions at a flow rate of 2 ml min^{-1}, were as follows:

Programme gradient:

Time (min)	A%	B%	Curve
Starting	30	70	*
2	30	70	6
20	69	31	6
25	90	10	6
30	100	0	1
35	30	70	1

Solvents: A = methanol; B = 1% glacial acetic acid.

Under the above-mentioned conditions the retention time for ABA was 12 to 13 minutes, as detected at a wavelenght of 262nm. Proline extraction and determination were conducted by means of a fast colorimetric method developed for plant tissue Bates *et al.* (1973).

Statistical analysis

The Dunnet *t*-test was used for statistically analysing the data. This is a complex statistical technique, used when a number of treatment-means have to be compared to a single type of control, and one of the *t*-statistics is enforceable (Miller, 1966). Curve fittings were done with "simplex algorithms" (Willmott, 1982). This program is capable of calculating interpolation values in order to obtain the best fit through a set of data points for any number of variables. Thus, an equation was calculated of which the d-statistic and R^2 were known, and statistically justifiable deductions could be made regarding the trend described in each figure (data supplied in Table 1). In spite of the fact that an average rate at which the drought stress intensified, was ca. 0.2-0.3 MPa d^{-1}, it did differ between the respective cultivars and between the plants of group A and that of group B. In order to overcome this, the above mentioned statistical technique was

used to generate the data as supplied in Table 2, for in order to be able to compare and evaluate physiological responses, we have to be sure that we are comparing a drought stress-induced response between respective cultivars when they experienced the same drought stress.

Table 1: Substitution values and statistic of the equation $Y = C*\exp(-(x-A)^2/2*B^2))$ that best describe the drought stress-induced trend of proline accumulation in four tabacco cultivars of differing drought tolerance.

Cultivar	Substitution values			d-index	R^2 value
	A	B	C		
Group A					
TL33	4.536	1.199	136730	0.994	0.976
CDL28	2.552	0.308	102170	0.999	0.999
GS46	2.889	0.672	157040	0.999	0.997
ELSOMA	2.625	0.485	99708	0.987	0.951
Group B					
TL33	4.407	0.840	31937	0.994	0.980
CDL28	4.054	0.790	25572	0.990	0.974
GS46	3.193	0.706	38936	0.999	0.999
ELSOMA	1.811	0.659	14172	0.547	0.527

7.4 Results

ABA accumulation in the four tobacco cultivars (Table 2) showed a considerable increase during the induced drought stress (Fig. 1a and b), as also reported earlier (Terry *et al.*, 1988). In addition the ABA concentration of the drought-tolerant cultivars, in group A and group B, showed a rapid increase earlier (Ψ_L = -0.77 MPa) than those of the drought-sensitive cultivars (only at a Ψ_L of -1.27 MPa). Contrary to expectation, the rate of ABA accumulation in all four the cultivars did not stabilise nor decline after stomatal closure [usually at about -1.3 MPa in tobacco, according to Turner (1974)] had occurred. All four cultivars continued to accumulate ABA, even up to a Ψ_L of -2.5 MPa, when no more *in vivo* photosynthesis activity was observable in any of the cultivars (Van Rensburg & Krüger, 1993). This observation is in agreement with Robertson *et al.* (1985) and Jones (1978) who suggested that, apart from stomatal closure during drought stress, ABA could play an important additional role in determining a plant's drought tolerance.

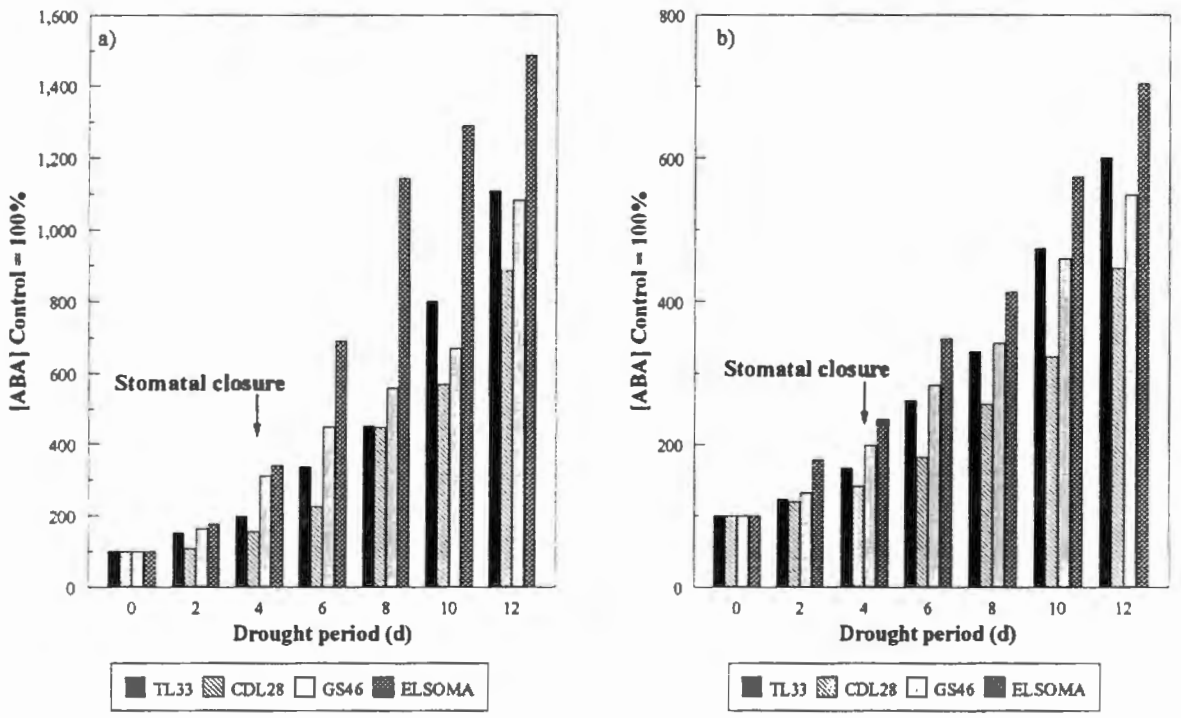


Figure 1. Drought stress-induced ABA accumulation in four cultivars of *Nicotiana tabacum* L., of two different ages: (a) ca 150 days old; (b) ca. 90 days old. The trend observed in both these figures, was best described by the function, Y = A * exp (B * X). In the case of (a) being $y = 49.31*^{(1.233*x)}$ with $R^2 = 0.95$ and the d-index = 0.97 for TL33, and being $Y = 46.27*^{(1.101*x)}$ with $R^2 = 0.96$ and the d-index = 0.98 for CDL28. For the drought tolerant culticars GS46 and ELSOMA, however, $Y = 61.11*^{(1.123*x)}$ with $R^2 = 0.92$ and the d-index = 0.99,and $Y = 58.68*^{(1.373*x)}$ with $R^2 = 0.92$ and the d-index = 0.97 respectively. Additionaly in the case of (b) $Y = 58.03*^{(0.961*x)}$ with $R^2 = 0.95$ and the d-index = 0.99 and $Y = 64.39*^{(0.709*x)}$ with $R^2 = 0.95$ and the d-index = 0.95 for the two drought sensitive cultivars TL33 and CDL28, respectively, while $Y = 58.42*^{(0.874*x)}$ with R^2 0.98 and the d-index = 0.974 for GS46 and $Y = 73.86*^{(0.895*x)}$ with $R^2 = 0.99$ and the d-index = 0.99 for ELSOMA.

The calculated ϵ -values of the drought-tolerant cultivars (GS46 and ELSOMA) were 0.23 MPa and 0.28 MPa respectively, while these values of the two drought-sensitive cultivars were significantly ($p < 0.05$) higher (TL33 = 0.39 MPa and CDL28 = 0.31 MPa). According to Zur *et al.* (1981) a low calculated ϵ -value is indicative of more elastic cell walls, which render the plant less sensitive to changes in cell volume during drought stress. Their more elastic cell walls may therefore enable the drought-tolerant cultivars to respond sooner, than the drought-sensitive cultivars, to drought stress-induced changes in turgidity.

Accumulation of proline (Table 2 and Fig. 2a and b) was observed during the induced drought stress in all four the cultivars of both groups A and B which is in agreement with earlier studies (Handa *et al.*, 1986). As in the case of ABA accumulation [Fig. 1a and b and also observed by Zabadal (1974)], a clearly distinguishable threshold occurred at a specific Ψ_L value, after which a considerable increase in the rate at which proline accumulation occurred (Fig. 2a and b). See Table 1 for the substitution values and statistics of the equation that best describes the drought stress-induced trend of proline accumulation. In the case of plants of group B, it was noticeable that the proline concentration of all four cultivars already differed statistically ($p < 0.05$) at a Ψ_L of -0.77 MPa from their respective controls. No similar tendency could be identified in group A The base-line proline concentration of GS46 and ELSOMA was lower than that of TL33 and CDL28, in both the older and younger plants. This slightly higher, base-line proline concentration of the older plants (Table 2) could be attributed to a degree of senescence that could already have occurred in the sixth leaf from the apex at the age of 150 days. This statement is based on the fact that the physiological symptoms of senescence, especially with regard to amino acid accumulation, are closely related to those observed in a plant subjected to drought stress (Brady *et al.*, 1974). Due to the specific method used for determining proline concentration, it is possible that an increased glutamine content could also be responsible for the higher base-line proline concentrations (Bates *et al.*, 1973). The observation that both ABA and proline accumulate in plants during the normal process of ageing, (Boroghov *et al.*, 1976), could also serve to explain the finding that the final proline concentrations were almost five times higher and the ABA concentrations almost three times higher in the older than in the younger plants.

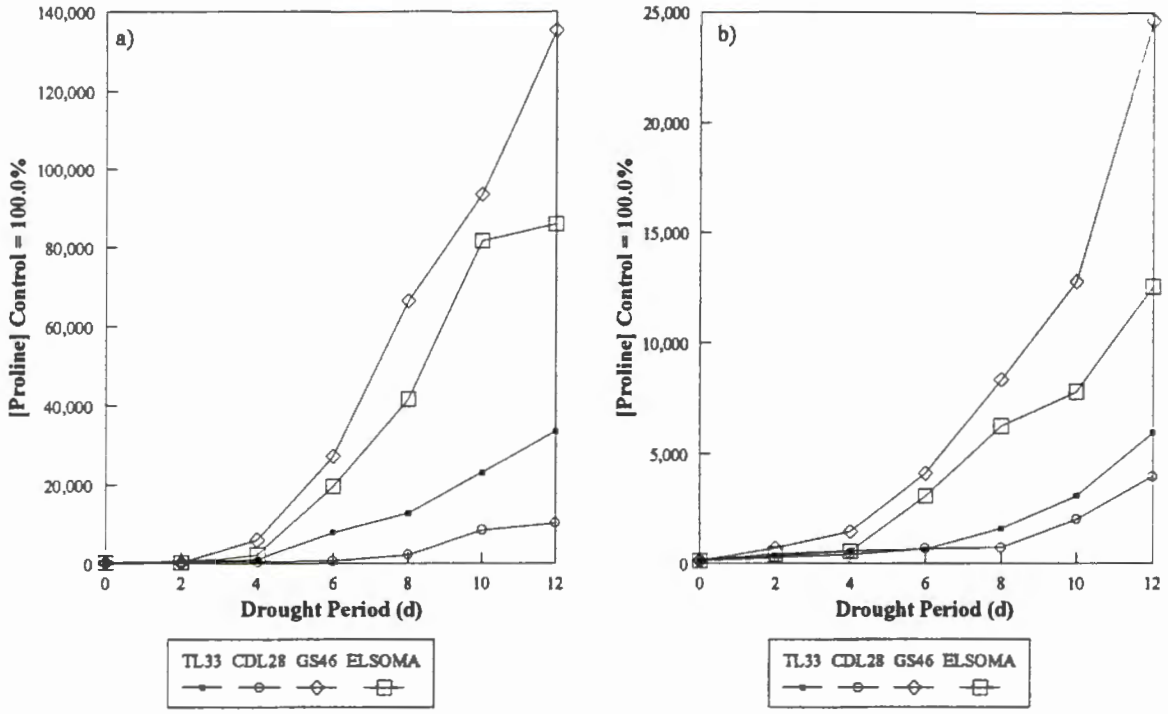


Figure 2. Drought stress-induced proline accumulation in four cultivars of *Nicotiana tabacum* L., of two different ages: (a) ca 150 days old; (b) ca 90 days old, of which TL33 and CDL28 are drought sensitive and GS46 and ELSOMA are drought tolerant. The trend observed in both the latter figures was best described by the function, $y = c * \exp(-(x - a)^2/(2 * b^2))$. Table 1 for the respective substitution values and statistics.

Table 2: Drought stress induced ABA and proline accumulation in four cultivars of *Nicotiana tabacum* L. of two different ages and differing in their individual drought tolerance.

Cultivars	Ψ_L MPa	RWC %	Group A		Group B	
			[ABA] $\mu g\ g^{-1}$ (dry wt.)	[Proline] $\mu mol\ g^{-1}$ (dry wt.)	[ABA] $\mu g\ g^{-1}$ (dry wt.)	[Proline] $\mu mol\ g^{-1}$ (dry wt.)
TL33	-0.52 ± 0.01	88.7 ± 0.1	0.3.9 ± 29.6	0.61 ± 0.04	84.5 ± 19.9	0.60 ± 0.01
	-0.77 ± 0.01	84.9 ± 0.1	157.3 ± 38.7	3.15 ± 0.7	104.4 ± 24.6	1.91 ± 0.04
	-1.27 ± 0.01	81.7 ± 0.7	204.9 ± 44.0	5.71 ± 0.4	139.9 ± 36.5	3.40 ± 0.9
	-1.67 ± 0.01	69.9 ± 0.9	347.5 ± 55.9	70.55 ± 4.0	220.4 ± 47.5	3.72 ± 0.4
	-1.97 ± 0.02	62.4 ± 0.7	465.7 ± 97.8	78.08 ± 5.0	281.0 ± 58.9	9.50 ± 0.3
	-2.32 ± 0.02	56.2 ± 0.4	912.1 ± 151.9	141.70 ± 6.0	423.1 ± 97.1	18.55 ± 0.3
	-2.51 ± 0.02	54.1 ± 0.9	1415.5 ± 277.1	20.542 ± 5.0	526.6 ± 89.7	35.66 ± 0.7
CDL28	-0.52 ± 0.01	87.7 ± 0.6	239.5 ± 28.7	3.57 ± 0.03	65.9 ± 18.2	0.81 ± 0.02
	-0.77 ± 0.01	86.1 ± 0.6	260.0 ± 35.6	9.46 ± 0.80	78.1 ± 27.5	1.99 ± 0.01
	-1.27 ± 0.01	81.3 ± 0.5	370.6 ± 47.2	15.34 ± 0.2	92.4 ± 35.4	3.30 ± 0.03
	-1.67 ± 0.01	76.5 ± 0.7	541.7 ± 56.3	20.61 ± 0.9	118.4 ± 49.7	5.45 ± 0.04
	-1.97 ± 0.02	68.6 ± 0.8	1069.9 ± 98.6	74.37 ± 4.0	167.3 ± 57.9	5.82 ± 0.9
	-2.32 ± 0.02	59.9 ± 0.4	1359.7 ± 149.0	303.70 ± 6.0	210.6 ± 88.6	16.26 ± 0.4
	-2.51 ± 0.02	56.2 ± 0.6	2118.2 ± 254.5	365.29 ± 2.0	290.9 ± 83.1	23.22 ± 0.3
GS46	-0.52 ± 0.01	90.1 ± 0.3	95.5 ± 29.7	0.23 ± 0.02	54.1 ± 19.3	0.17 ± 0.01
	-0.77 ± 0.01	86.7 ± 0.3	156.7 ± 35.7	0.31 ± 0.02	72.6 ± 26.4	1.19 ± 0.04
	-1.27 ± 0.01	80.4 ± 0.2	296.6 ± 44.9	13.65 ± 0.1	95.5 ± 35.9	2.12 ± 0.03
	-1.67 ± 0.01	68.2 ± 0.9	426.0 ± 56.4	62.79 ± 0.6	153.3 ± 47.3	6.83 ± 0.5
	-1.97 ± 0.02	55.9 ± 0.1	531.3 ± 99.7	152.99 ± 2.0	185.7 ± 55.8	14.16 ± 0.2
	-2.32 ± 0.02	47.5 ± 0.5	636.2 ± 159.5	243.20 ± 6.0	248.3 ± 79.9	30.20 ± 0.8
	-2.52 ± 0.02	46.5 ± 0.6	1028.2 ± 265.6	311.32 ± 8.0	296.9 ± 74.4	41.90 ± 0.2
ELSOMA	-0.52 ± 0.01	89.2 ± 0.6	72.0 ± 29.8	0.54 ± 0.01	59.7 ± 17.8	0.38 ± 0.01
	-0.77 ± 0.01	86.2 ± 0.6	124.5 ± 34.5	0.96 ± 0.06	70.6 ± 26.6	1.59 ± 0.02
	-1.27 ± 0.01	77.5 ± 0.4	245.1 ± 49.1	1.25 ± 0.2	133.1 ± 36.9	2.05 ± 0.9
	-1.67 ± 0.01	57.2 ± 0.5	498.3 ± 57.2	12.20 ± 0.8	188.8 ± 47.6	1.71 ± 0.3
	-1.97 ± 0.02	50.7 ± 0.3	823.1 ± 99.6	297.12 ± 5.0	243.2 ± 59.1	23.72 ± 0.2
	-2.32 ± 0.02	43.9 ± 0.2	928.3 ± 176.5	441.95 ± 9.0	338.9 ± 86.3	44.78 ± 0.5
	-2.52 ± 0.02	39.0 ± 0.4	1070.6 ± 289.3	464.77 ± 9.9	415.8 ± 91.6	47.62 ± 0.7

Note: Standard deviations reflect the deviation from the mean of four replicates per determination.

7.5 Discussion

The results (Fig. 1a and b), are similar to that obtained by Mizrahi *et al.* (1971), in which the ABA content showed almost no change until a particular threshold Ψ_L value was reached, after which ABA rapidly accumulated with a further decline in Ψ_L . Bradford and Hsiao (1982) suggested that a loss in leaf turgidity served to trigger (although not necessarily through a direct decline in Ψ_L) the increase in ABA concentration during drought stress. This, seen in the context of the pH-dependent distribution model of ABA (Heilmann *et al.*, 1980) and the presently accepted fact that drought stress induces chloroplast acidification (Berkowitz *et al.*, 1983; Taiz & Zeiger, 1991), underlines the importance of the observation that the cell walls of drought-tolerant cultivars are more elastic than those of the drought-sensitive cultivars. The importance of the low $\in$ -values, which are characteristic of the drought-tolerant cultivars, is not primarily due to in their direct effect on the Ψ_L during drought stress [as Turner (1979) clearly indicated], but in its possible role as a trigger for initiation of possible adaptive reactions in drought-tolerant cultivars at an earlier stage. Furthermore, in higher plants, Ψ_P at a specific Ψ_L, is, *inter alia*, influenced by a combination of Ψ_π and $\in$ (Turner, 1979).

Although certain control points of proline metabolism have already been identified, two questions are to be answered from a metabolic regulatory point of view, namely: can a change in enzyme activity or gene expression be related to the accumulation of proline, and secondly, is/are the effector(s) chemical or physical in nature? The relevance of these two questions becomes clear when the four curves in Figure 2a and b are studied. These indicate that, as in the case of ABA accumulation, a clearly distinguishable Ψ_L occurs after which proline accumulation increases more rapidly with the intensification of drought stress. Like ABA accumulation, this apparent threshold value occurs earlier (that is, at a higher Ψ_L) in the drought-tolerant cultivars (Fig. 2a and b). The advantages which will be bestowed on plants if they were able to recognise moisture shortage at an earlier stage, and modify the necessary associated metabolic survival processes to conform to the available water content, are numerous. The results obtained by Handa *et al.* (1986) imply that the trigger action for a change in cellular proline levels cannot simply be ascribed to changes in turgidity as Chu *et al.* (1976) observed in barley leaves exposed to high NaCl concentrations. In addition it may neither simply be associated, as Hanson and Hitz (1982) suggested, with a change in the Ψ_L of the cell, but it would seem that proline accumulation could be correlated with a change in the concentration of a specific solute. In accordance with the results of Göring and Thien (1979), and the more recent results obtained by Pesci and Beffagna (1985), it was proposed by Handa *et al.* (1986) that proline accumulation could be associated with a change in cytoplasmic pH.

From the results obtained in this investigation (Table 2), and taking the above-mentioned core of knowledge into account, it is our belief that all the above-mentioned proposals (Chu *et al.*, 1976; Handa *et al.*, 1986; Hanson & Hitz, 1982; Pesci & Beffagna, 1985) contain true elements. This led us to propose that ABA can act as *in vivo* effector of one or even more metabolic events and thus give rise to proline accumulation during drought stress. This proposal is supported by the knowledge that: (i) at physiologically responsible doses (in barley leaves), ABA stimulates the accumulation of proline (Rajagopal & Andersen, 1978; Stewart & Voetberg, 1985) and the conversion of [C^{14}] glutamate to proline (Stewart, 1980) and (ii) ABA levels increase before dramatic proline accumulation occurs (Aspinall, 1980), as also proved to be the case in this investigation and (iii) that drought stress-induced stromal acidification leads to the redistribution of ABA within the cell (Taiz & Zeiger, 1991).

The idea that slight changes in the endogenous ABA concentration might be of physiological importance, often serves to explain kinetic contradictions between increases in leaf ABA concentration and stomatal conduction. The most important inconsistency is in the fact that the onset of the changes in ABA concentration and in stomatal conduction do not necessarily overlap. In general, an increase in ABA levels in leaves is only measurable after 20 minutes to one hour after the onset of drought stress (Pierce & Raschke, 1980), while stomatal closure occurs much earlier, often within five minutes after a decline in Ψ_L (Henson,1981). In addition, the guard cells represent only about 1% of the total leaf volume (Raschke, 1983) which makes the ABA content of the total leaf tissue an imperfect indicator of the ABA concentration at the stomata. The extraction of ABA from total leaf tissue is, however (as in the case of this investigation), used by most researchers (Boroghov *et al.*, 1976; Raschke, 1976).

Taking the latter statement into account, and seeing that controversial results exist with regard to the potential of ABA accumulation as a parameter in selecting for drought tolerance, as well as to explain why the ABA end-concentration does not necessarily correlate positively with the known drought tolerance of some cultivars (GS46 in Fig. 1a and b), the following is proposed: It has only recently been determined that the ABA responsible for stomatal closure and the ABA which possibly is related to some other aspects of drought tolerance, are synthesized in different parts of the plant, although they have more or less the same destination. The ABA, which is primarily associated with additional drought tolerance reactions, is synthesized in the leaves and released in reaction to fluctuations in pH gradients (Taiz & Zeiger, 1991). The ABA associated with stomatal closure is synthesized in the roots in reaction to drought stress and is translocated via the transpirational flow to the leaves (Loske & Raschke, 1988; Neales & Mcleod, 1991). An experimental problem encountered in the present and most other drought tolerance studies, where ABA accumulation was determined as a reaction to drought stress by using a complete leaf sample, is that no distinction can be made between the

portion of the ABA content allocated for stomatal closure and that which is available for possible additional drought tolerance reactions. The above-mentioned consideration is very important consideration in our study, as a tobacco leaf is amphistomatal and contains ca. 4000 stomata per cm^2 on the adaxial and ca. 8000 stomata per cm^2 on the abaxial leaf surface which open and close simultaneously (Zelitch, 1974). If the potential of a plant or specific cultivar to accumulate ABA during drought stress were to be associated with its drought tolerance, a distinction would have to be made indicating the part of the total accumulated ABA content that could be associated with the non-stomatal reaction to drought stress. Thus the authors suggest that $[ABA_{total}] = [ABA_{stomatal}] + [ABA_{non\text{-}stomatal}]$ and that the latter may be positively correlated with a plant's drought tolerance. This implies that $[ABA_{non\text{-}stomatal}]$ ought to be higher in ELSOMA and GS46 than in TL33 and CDL28. According to our results (Table 2), with the exception of TL33, this statement holds true, for when the percentage of $[ABA_{stomatal}]$ accumulate at a Ψ_L of -1.27 MPa [that is the Ψ_L at which stomata closure occurs in tobacco (Turner, 1974) and as determined by us] was substracted from the percentage $[ABA_{total}]$ accumulated at a Ψ_L of -2.52 MPa, the resulting percentage was found to be higher in the drought-tolerant cultivars of both group A and B. This aspect is at present being investigated.

A survey of existing literature on the potential value of proline accumulation during stress, irrespective of whether the stress resulted from drought or from high NaCl concentrations, reveals a collection of controversial evidence. Some researchers argue that proline accumulation is advantageous for a plant as far as stress tolerance is concerned (Singh *et al.*, 1973; Singh *et al.*, 1972), while others suggest the opposite to be true, i.e, that proline accumulation is an indication of the damage suffered by the plant during stress periods (Ferreira *et al.*, 1979; Hanson *et al.*, 1979). A possible explanation for these discrepancies could be that in some intact plants, an additional mechanism, simpler or more complex, may be operative, which could determine their drought tolerance. A second, and probably more important reason, could be the uncertainty as to what triggers the change in enzyme activity or gene expression. The exact nature and mechanism (Boggess *et al.*, 1978) whereby these compounds exert their influence are still largely unknown. The latter has given rise to various hypotheses concerning the possible functions of these compounds which accumulate during drought stress. According to Schobert and Tsesche (1978), most of the hypotheses about the role of proline will be proven incorrect in the course of time. The protective effects of proline during drought stress are ascribed to the fact that: (i) it might act as compatible osmoticum (Shevyakova, 1984); (ii) it might serve as a stabilizer or solvent for proteins during a shortage of cell water (Schobert & Tsesche, 1978); (iii) it might act as a reservoir of nitrogen and carbon to facilitate plant recovery during periods of sufficient moisture (Singh *et al.*, 1973); (iv) it stabilizes macro-molecules and membranes during drought stress (Treichel, 1975), and (v) it detoxifies free radicals by forming long-living adducts with them (Floyed & Nz-Nagy, 1984). Still, considering the idea that the accumulated proline might be

advantageous to a plant under drought stress, it is interesting to note in conclusion, that the ability of ten barley cultivars to accumulate proline during stress has also been positively correlated with their drought resistance ratings (Singh *et al.*, 1972).

From the results of the present investigation (Table 2) it is clear that, for the reasons presented, the end-concentration to which ABA accumulates did not correlate positively with the known drought tolerance of all the respective cultivars. This aspect of ABA accumulation would thus not be applicable as a selection criterion, until what has been referred to as [$ABA_{non\text{-}stomatal}$] has been defined and can be differentiated from the [ABA_{total}]. Due to the positive correlation which exists between the cell wall elasticity of the four respective cultivars, the time or Ψ_L at which a cultivar rapidly begins to accumulate ABA, and their known drought tolerance, the Ψ_L (the higher the better) at which a cultivar starts to rapidly accumulate ABA, is recommended as a selection parameter. Due to the interaction between ABA and proline accumulation, the latter would also be applicable to proline. As the final accumulated proline concentrations of the respective cultivars correlate positively with their drought tolerance (Fig. 2a and b) and despite the fact that the precise mechanism by which it is responsible for the plant's protection against stress is still unknown, it is considered that these aspects of proline accumulation are the best potential selection parameters (among those investigated) for drought tolerance in tobacco, as it would seem that the accumulated proline positively increases a cultivar's drought tolerance (Van Rensburg *et al.*, 1993).

7.6 Literature cited

ASPINALL, D. 1980. Role of abscisic acid and other hormones in adaptation to water stress. P.J. (*In* Turner, T.C. & Kramer, P.J., *eds.* Adaptation of plants to water and high temperature stress. New York: Wiley. p. 155-172.)

BATES, L.S., WALDREN, R.P. & TEARE, I.C. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil,* 39: 205-207.

BERKOWITZ, G.A., CHEN, C. & GIBBS, M. 1983. Stromal acidification mediates *in vivo* water stress inhibition of nonstomatal-controlled photosynthesis. *Plant Physiology,* 72: 1123-1126.

BOGGESS, S.F., KOEPPE, D.E. & STEWART, C.R. 1978. Oxidation of proline by plant mitochondria. *Plant Physiology,* 62: 22-25.

BOROGHOV, A., TIROSH, T. & HALEVY, A.H. 1976. Abscisic acid content of senescing petals on cut rose flowers: As affected by sucrose and water stress. *Plant Physiology,* 58: 175-178.

BRADFORD, K.J. & HSIAU, T.C. 1982. Physiological responses to moderate water stress. (*In* Lange, O.P., Nobel, P.S., Osmond, C.B. & Ziegler, H., *eds.* Physiological plant ecology II: Water relations and carbon assimilation. New York: Springer-Verlag. p. 264-324.)

BRADY, C.J., SCOTT, N.S. & MUNNS, R. 1974. The interaction of water stress with the senescence pattern of leaves. (*In* Bellesk, R.L., Ferguson, A.R. & Cresswell, M.M., *eds.* Mechanics of regulation of plant growth. Wellington:The Royal Society of New Zealand. p. 403-409.)

CHU, T.M., ASPINALL, D. & PALEG, L.B. 1976. Stress metabolism. VII: Salinity and proline accumulation in barley. *Australian Journal of Plant Physiology,* 3: 219-228.

FERREIRA, L.G.R., DESOUSA, J.G. & PRISCO, J.T. 1979. Effects of water deficit on proline accumulation and growth of two cotton genotypes of different drought resistance. *Zeitschrift für Pflanzenphysiologie,* 93: 189-199.

FLOYED, R.A. & NZ-NAGY. 1984. Formation of long lived hydroxyl free radical adducts of proline and hydroxyproline in a Fenton reaction. *Biochimica et Biophysica Acta,* 790: 94-97.

GöRING, H. & THIEN, B. 1979. Influence of nutrient deficiency on proline accumulation in the cytoplasm of *Zea mays* L. *Biochemie und Physiologie der Pflanzen,* 174: 9-16.

GRAZIANA, Y. & LIVNE, A. 1971. Dehydration water fluxes, and permeability of tobacco leaf tissue. *Plant Physiology,* 48: 575-579.

HANDA, S., HANDA, A.K., HASEGAWA, P.M. & BRESSAN, R.A. 1986. Proline accumulation and the adaptation of cultured plant cells to water stress. *Plant Physiology,* 80: 938-945.

HANSON, A.D., NELSEN, C.E., PEDERSEN, A.R. & EVERSON, E.H. 1979. Capacity for proline accumulation during water stress in barley and its implications in breeding for drought resistance. *Crop Science,* 19: 489-493.

HANSON, A.D. & HITZ, W.D. 1982. Metabolic responses of mesophytes to plant water deficits. *Annual Review of Plant Physiology,* 33: 162-203.

HEILMANN, B., HARTUNG, W. & GIMMLER, H. 1980. The distribution of abscisic acid between chloroplasts and cytoplasm of leaf cells and the permeability of the chloroplast envelope for abscisic acid. *Zeitschrift für Pflanzenphysiologie,* 97: 67-78.

HENSON, I.E. 1981. Changes in abscisic acid content during stomatal closing in pearl millet (*Pennisetum americanum* (L.) Leeke). *Plant Science Letters,* 21: 121-127.

HINCKLEY, T.M., DUHME, F., HINCKLEY, A.R. & RICHTER, H. 1980. Water relations of drought hardy shrubs: osmotic potential and stomatal reactivity. *Plant Cell Environment,* 3: 131-140.

HUANG, A.H.C. & CAVALIERI, A.J. 1979. Proline oxidase and water stress induced proline accumulation in spinach leaves. *Plant Physiology,* 63: 531-535.

JONES H.G. 1978. How plants respond to stress. *Nature,* 271, 610.

JONES, M.M., OSMOND, C.B. & TURNER, N.C. 1980. Accumulation of solutes in leaves of sorghum and sunflower in response to water deficits. *Australian Journal of Plant Physiology*, 7: 193-205.

KRAMER, P.J. 1983. Water relations of plants. New York: Academic Press.

KUBISKE, M.E. & ABRAMS, M.D. 1990. Pressure-volume relationships in non-rehydrated tissue at various water deficits. *Plant Cell Environment*, 13: 995-1000.

LAHR, W. & RASCHKE, I. 1988. Abscisic-acid contents and concentrations in protoplasts from guard cells and mesophyll cells of *Vicia faba* L. *Planta*, 173: 528-531.

LOSKE, D. & RASCHKE, K. 1988. Determination of carbon reduction-cycle intermediates in leaves of *Arbutus unedo* L. suffering depressions in photosynthesis after application of abscisic acid or exposure to dry air. *Planta*, 173: 275-281.

MILLBORROW, B.V. & NODDLE, R.C. 1970. Conversion of the 5-(1,2 epoxy-2,6,6-trimethylcyclohexyl)-3-methylpenta-cis-2-trans-4- dienoic acid into abscisic acid in plants. *Biochemical Journal*, 119: 727-734.

MILLER, R.G. 1966. Simultaneous statistical inference. New York: McGraw-Hill.

MIZRAHI, Y., BLUMENFELF, A. & RICHMOND, A.E. 1971. Abscisic acid and cytokinin contents of leaves in relation to salinity and relative humidity. *Plant Physiology*, 48: 752-755.

NEALES, T.F. & McLEOD. A.L. 1991. Do leaves contribute to the abscisic acid present in the xylem sap of "droughted" sunflower plants? *Plant Cell Environment*, 14: 979-986.

PESCI, P. & BEFFAGNA, N. 1985. Effects of weak acids on proline accumulation. *Plant Cell Environment*, 8: 129-133.

PIERCE, M. & RASCHKE, K. 1980. Correlation between loss of turgor and accumulation of abscisic acid in detached leaves. *Planta*, 148: 174-182.

QUARRIE, S.A. & JONES, H.G. 1977. Effects of abscisic acid and water stress on development and morphology of wheat. *Journal of Experimental Botany*, 28: 192-203.

RAJAGOPAL, V. & ANDERSEN, A.S. 1978. Does abscisic acid influence proline accumulation in stressed leaves? *Planta*, 43: 85-88.

RAJAGOPAL, V., BALASUBRAMANIAN, V. & SINHA, S.K. 1977. Diurnal fluctuations in relative water content, nitrate reductase and proline content in water stressed and non-water stressed wheat. *Physiologia Plantarum*, 40: 69-71.

RASCHKE, K. 1979. Movement of stomata. (*In* Haupt, W. & Feinlieb, M.E., *eds*. Physiology of movements. Encyclopedia of Plant Physiology. New York: Springer-Verlag. p. 381-441.)

RICHTER, H. & WAGNER, S.B. 1983. Water stress resistance of photosynthesis: some aspects of osmotic relations. (*In* Marcelle, R., Clijsters, H. & Van Pouke, M., *eds*. Effects of stress on photosynthesis. London: Nijhoff-Junk. p. 45-53.)

ROBERTSON, J.M., PHARIS, R.P., HUANG, Y.Y., REID, D.M. & YEUNG, E.L. 1985. Drought-induced increases in abscisic acid levels in the root apex of sunflower. *Plant Physiology*, 79: 1086-1089.

SCHOBERT, B. & TSESCHE, H. 1978. Unusual solution properties of proline and its interaction with proteins. *Biochimica et Biophysica Acta*, 541: 270-277.

SHEVYAKOVA, N.I. 1984. Metabolism and physiological role of proline in plants under conditions of water and salt stress. *Soviet Plant Physiology*, 31: 597-608.

SINGH, T.N., ASPINALL, D., PALEG, L.G. & BOGGESS, S.F. 1973. Stress metabolism. II: Changes in proline concentration in excised plant tissues. *Australian Journal of Biological Sciences*, 26: 57-63.

SINGH, T.N., ASPINALL, D. & PALEG, L.G. 1972. Proline accumulation and varietal adaptability to drought in barley: a potential metabolic measure of drought resistance. *Nature New Biology*, 236: 188-190.

STEWART, C.R. & VOETBERG, G. 1985. Relationship between stress-induced ABA and proline accumulations and ABA-induced proline accumulation in excised barley leaves. *Plant Physiology*, 79: 24-27.

STEWART, C.R. 1980. The mechanism of abscisic acid-induced proline accumulation in barley leaves. *Plant Physiology*, 6: 230-233.

TAIZ, L. & ZEIGER, E. 1991. Stress physiology: Water and drought resistance. (*In* Taiz, L. & Zeiger, E., *eds*. Plant Physiology. New York: Benjamin-Cummings. p. 346-356.)

TERRY, P.H., KRIZEK, D.T. & MIRECKI, R.M. 1988. Genotypic variation in Coleus in the ability to accumulate abscisic acid in response to water deficit. *Physiologia Plantarum*, 72: 441-449.

TREICHEL, S. 1975. The effect of NaCl on the concentration of proline in different halophytes. *Zeitschrift für Pflanzenphysiologie*, 76: 56-68.

TURNER, N.C. 1974. Stomatal behaviour and water status of maize, sorghum, and tobacco under field conditions. II. At low soil water potential. *Plant Physiology*, 53: 360-365.

TURNER, N.C. 1979. Drought resistance and adaptation to water deficits in corn plants. (*In* Mussel, H. & Staples, R.C., *eds*. Stress physiology in crop plants. New York: Wiley. p. 343-373.)

WALTON, D. C., HARRISON, M. A. & COTE, P. 1976. Effect of water stress on abscisic acid levels and metabolism in roots of *Phaseolus vulgaris* L. and other plants. *Planta*, 131: 141-144.

WEATHERLEY, P.E. 1951. Studies on the water relations of the cotton plant. II: Diurnal and seasonal fluctuation and environmental factors. *New Phytologist*, 50: 36-51.

WILLMOTT C.J. 1982. Some comments on the evaluation of model performance. *Bulletin of the American Meteorological Society*, 63: 1309-1313.

WILSON, J.R., LUDLOW, M.M., FISHER, M.J. & SCHULZE, E.D. 1980. Adaptation to water stress of the leaf water relations of four tropical forage species. *Australian Journal of Plant Physiology*, 7: 207-220.

VAN RENSBURG, L. & KRüGER, G.H.J. 1993. Differential inhibition of photosynthesis (*in vivo* and *in vitro*), and changes in chlorophyll *a* fluorescence induction kinetics of four tobacco cultivars under drought stress. *Journal of Plant Physiology*, 141: 357-365.

VAN RENSBURG, L., KRüGER, G.H.J. & KRüGER, H. 1993. Proline accumulation as drought tolerance selection criterion: its relationship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. *Journal of Plant Physiology,* 14: 188-194.

ZABADAL, T.J. 1974. A water potential threshold for the increase of abscisic acid in leaves. *Plant Physiology,* 53: 125-127.

ZELITCH, I. 1974. Effect of biochemical inhibitors on stomatal opening. (*In* San Pietro, A., *ed.* experimental Plant Physiology. Saint Louis: Mosby. p. 161-163.)

ZUR, B., BOOTE, K.J. & JONES, J.W. 1981. Changes in internal water relations and osmotic properties of leaves in maturing soybean plants. *Journal of Experimental Botany,* 32: 1181-1191.

CHAPTER EIGHT

8. IMMUNOGOLD LOCALIZATION AND QUANTIFICATION OF CELLULAR AND SUB-CELLULAR AND ABA CONCENTRATIONS, PRIOR TO, AND DURING DROUGHT STRESS.

8.1 Abstract

An immunogold labelling procedure and experimental data are presented, which demonstrate that antibodies produced against a bovine serum albumin -(±)- ABA conjugate can not only be used to characterize the cellular and sub-cellular localization of abscisic acid (ABA), but can also permit quantitative comparisons of this hormone in the sub-cellular compartments, prior to, and when plants were drought stressed. At the control leaf water potential (ca. - 0.45 MPa) a quantitatively similar positive labelling pattern was observed in the chloroplasts and apoplast. A twofold drought stress-induced increase in the apoplastic ABA concentration was observed in the drought stressed leaf tissue (i.e. at a leaf water potential of ca. - 1.55 MPa) while the ABA concentration in the chloroplasts did not differ from that of their controls. Three histochemical controls and the physiological observations validated the specificity of the procedure. Based on the above mentioned labelling patterns observed and literature cited, the validity of the hypothesis that drought stress induces a release of chloroplastic ABA is questioned. The results however, were interpreted as providing indirect evidence for a drought stress-induced root sourced origin for the increased apoplastic ABA concentrations.

Key words: *Abscisic acid (localization), drought stress, immunogold electron microscopy, Nicotiana tabacum* L.

8.2 Introduction

When recently giving their perspective on abscisic acid (ABA) research in the 1990's, specifically areas of limited progress, Jones and Davies (1992), as did several authors before them (Walton, 1980; Zeevaart & Creelman, 1988), emphasized the importance of developing a technique for the localization of ABA, both at tissue level and more importantly at the organelle level. In this regard it is interesting to note that, as was suggested by Jones and Davies (1992), Sossountzov *et al.* (1988) cited several authors to provide evidence that specific antibodies constitute very sensitive probes which could be useful for hormone localization within plant tissues and cells by immunocytochemical techniques. Furthermore, Sotta *et al.* (1985) provided initial physiologically verified immunohistological evidence to this effect.

La Rosa *et al.* (1985) stated that the generally held view that ABA enhances adaptation to stresses, is based largely on indirect evidence. Similarly we are of the opinion that the now generally accepted (Taiz & Zeiger, 1991) hypothesis that permeability changes in the chloroplast membrane during drought stress, as first proposed by Mansfield *et al.* (1978) and Milborrow (1979) supposedly allow ABA to leak out, is based largely on the theoretical physiochemical description of ABA distribution in the cell (Heilmann *et al.*, 1980; Cowan *et al.*, 1982) and indirect experimental evidence (Hartung *et al.*, 1983; Kaizer *et al.*, 1985). This contention, though not questioning the validity of the pH dependent ABA distribution model (Heilman *et al.*, 1980; Kaiser *et al.*, 1985), nor questioning the proposed redistribution of ABA in the dark (Cowan *et al.*, 1982), was definitely true up to 1980, when Walton (1980) in a review article concluded that "the hypothesis that permeability changes in the chloroplast membrane during water stress, allow ABA to leak out, remains to be demonstrated", and in our opinion still is in question, in spite of the elegant work done by Hartung and co-workers (Hartung *et al.*, 1988; Hartung & Slovik, 1991).

The object of the present investigation was, firstly to demonstrate that by modifying current immunohistochemical techniques (Sotta *et al.*, 1985; Sossountzov *et al.*, 1986) as proposed, antibodies produced against a bovine serum albumin -(±)- ABA conjugate, can be utilized in an immunogold assay not only to characterize the distribution of ABA, but also to permit the direct quantitative comparison in the sub-cellular compartments, prior to and during drought stress, and secondly to determine whether a drought stress-induced redistribution of ABA from the chloroplast occurs.

8.3 Material and methods

Plant material

Tobacco plants (*Nicotiana tabacum* L. cv Groot Swazi 46) were grown under controlled environmental conditions with an optimal water regime as previously described (Van Rensburg *et al.*, 1993). This oriental tobacco cultivar was used because of its comparatively high drought tolerance (Van Rensburg & Krüger, 1993) and ability to accumulate ABA during drought stress (Van Rensburg & Krüger - unpublished). Prior to tissue sampling, the plants were placed in a growth chamber for 72 hours to acclimatize and then placed in the dark for 15 hours at a relative humidity of 40% and 25°C. This was done to minimize the amount of starch present in the tissue (Van Rensburg *et al.*, 1993). As a measure of the water status of the plants, leaf water potential (Ψ_L) determinations were made with a Scholander pressure chamber (PMS-instrument, Oregon, USA). Drought stress was induced by withholding water and leaf water potentials were monitored daily until they reached the stress level of ca.-1.55 MPa, at which time samples were taken.

Tissue processing

Samples of 1cm x 1cm were cut from the centre of the lamina, lateral to the midrib, from the sixth leaf below the apex for reasons previously described (Van Rensburg *et al.*, 1993). These samples were placed in a freshly prepared 2% aqueous solution of water-soluble 1-(3-dimethylaminopropyl)3-ethyl carbodiimide (EDC), which allows fixation of ABA on cellular proteins *in situ* (Sotta *et al.*, 1985), which is an important prerequisite according to Jones and Davies (1992). While immersed in EDC, 1mm x 2mm smaller sub-samples were cut and infiltrated for 30 min under light vacuum. These samples were then rinsed three times for 5 minutes each in 0.075M phosphate-buffer (pH 7.5) and postfixed for 60 min in 4% paraformaldehyde in the same buffer. After extensive rinsing (three times for 5 min each) in the phosphate buffer, the samples were dehydrated in a graded series of ethanol. Following a 100% ethanol rinse the samples were infiltrated with ethanol/LR white (50:50) for 60 min, and then with neat hydrophylic acrylic resin LR White (Craig & Miller, 1984). Ultrathin sections were picked up on 200-mesh nickel grids and due to the generally experienced difficulty of cutting (Craig & Goodchild, 1982) we, as proposed by Sossountzov *et al.* (1986), mounted only entire gold or silver sections.

Preparation of anti-ABA antisera

(±)-ABA was coupled to bovine serum albumin (BSA) by dropwise adding 132mg (±)-ABA, dissolved in 3ml H_2O/dimethyl formamide (1:2), to a solution of 250mg BSA in water (pH 8.5). As described by Weiler (1979), 210mg of EDC was then added in four portions over a period of 90 min and the solution stirred in the dark for 19 hours at 4°C. The solution was then dialyzed against tap water for four days. Antisera were raised in six seven-month-old rabbits by using the method of Sotta *et al.* (1985).

Immunocytochemical labelling

All immunoelectron-microscopy staining procedures, were performed at room temperature. The grids were floated with the section facing downwards on a drop of phosphate buffered saline (0.01M-PBS, pH 7.4), containing 0.5% BSA, 0.5% gelatine for 30 min. The inclusion of Triton x-100 in the latter solution is not recommended, in spite of it possibly preventing nonspecific binding of antibodies to the tissue (Sossountzov *et al.*, 1988) and facilitating the penetration of antibodies while minimizing background (Sossountzov *et al.*, 1986). For some unknown reason, it seemed to loosen the sections from the grids in our trial experiments. Without rinsing, all the grids were then transferred to drops of the primary anti-ABA serum solution, diluted (1:50) with the PBS solution, for 90 min. To prevent non-specific binding of the primary antiserum to the film on the grid (Bertrand *et al.*, 1992) the grids were then carefully rinsed in the PBS solution of which the salinity was adjusted to 0.5 M with NaCl, by floating it for 3 min each on six different drops. The grids were then transferred to drops of gold-labled (10nm) goat-anti-rabbit Immunoglobulin G (IgG)/gold 10nm solution diluted (1:50) with the original PSB solution for 60 min. In both instances the sections were rinsed three times, for 3 min each, in distilled water. The immunogold stained sections were subsequently counterstained in 4% aqueous uranyl acetate for 3 min (Pease, 1964), washed in distilled water, and then transferred to drops of lead citrate (Reynolds, 1963) for 1 min. Counterstaining in this manner do not mask the electron dense gold particles, but partially compensates for the omission of OsO_4 during the fixation step (Sossountzov *et al.*, 1986). Grids were then finally thoroughly washed in distilled water. To serve as controls and to establish the specificity of the immunogold procedure, the primary anti-ABA-serum was replaced by naive control serum or serum which was heat denatured prior to use. The specificity was assessed by incubation with anti-ABA IgG preabsorbed for 4 hours at 4°C with an excess of ABA. Data were quantitatively analysed as was done by Sossountzov *et al.* (1986) and Bertrand *et al.* (1992).

8.4 Results and discussion

The results presented (Fig. 1 and Table 1) confirm the findings of Sotta *et al.* (1985) and Sossountzov *et al.* (1986) that endogenous ABA can effectively be fixed by EDC on the cellular protein network, and subsequently be detected by an immuno-histochemical technique at the tissue level. Admittedly, as the antiserum used in this investigation was raised against (±)-ABA, and due to the identical reactivities of (±)-ABA and its tetraacetyl glucose ester, it therefore may have recognised both free ABA and esterified ABA. This, however does not constitute a major problem in drought stress-studies for not only does the neutral conjugate accumulate at very slow rates during drought stress (Zeevaart, 1983), but it also appears to be restricted to the vacuole (Bray & Zeevaart, 1985) and does not enter the cell without first being hydrolyzed (Zeevaart & Boyer, 1984). Furthermore, there is no evidence to support the idea that conjugated ABA is a source of stress-induced free ABA (Zeevaart & Creelman, 1988).

Table 1: Immunogold labelling density in the chloroplast and apoplast of *Nicotiana tabacum* L.*

	Chloroplast	Apoplast
Control (control serum)	-	-
Control (denaturated)	-	-
Pre-stress	54.6 ± 10.74 (n = 10)	48.8 ± 4.21 (n = 15)
Stressed	43.6 ± 8.71 (n = 10)	89.6 ± 7.11 (n = 15)

* Values are expressed as the mean number of gold particles per 1μm^2 ± standard deviation.
n = the number of areas over which the mean values were obtained.

By using the acrylic resin LR-White as proposed by Sossountzov *et al.* (1986) a large number of inadequacies in structural preservation indicated by the latter author and co-workers when using epoxy resins, for instance distinguising the chloroplast envelope from the adjacent cytoplasm, were overcome (Fig. 1). Not withstanding the omission of OsO_4 postfixation and mild fixation with paraformaldehyde following the necessary carbodiimide-prefixation step, subcellular details were remarkably well delineated (Fig. 1.) The specificity of the procedures employed, was clearly validated in the first place by the control sections (Fig. 1A) which gave appropriate negative reactions, i.e. all the controls revealed none or fewer than one gold particle per μm^2. Secondly where labelling did occur, gold particles were more often seen isolated, rarely in pairs,

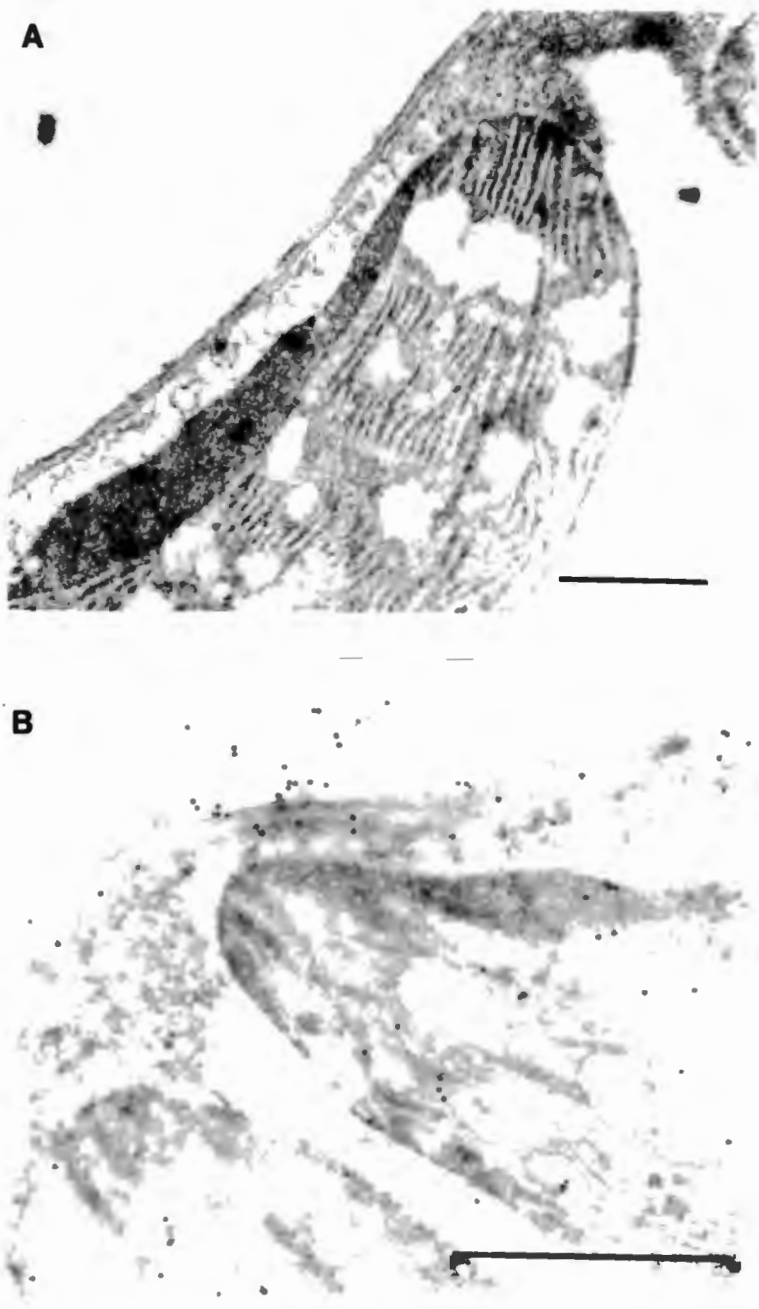
but never as clusters which unambiguously demonstrates that the observed labelling was not an artifact arising from the non-specific binding of antibodies to the tissue sections (Sossountzov *et al.*, 1986).

A similar labelling pattern, with respect to the localization of ABA in the chloroplasts, was observed by us (Fig. 1B) and Sossountzov *et al.* (1986) in unstressed, and Sotta *et al.* (1985) in both unstressed and drought stressed plant tissue (Fig. 1B,C). This was also previously used as validation for the immunolabelling technique (Sotta *et al.*, 1985) as it is consistent with the idea that the chloroplasts act as alkaline anion traps into which ABA may be sequestered (Heilmann *et al.*, 1980). Labelling was, however, not limited to the chloroplasts of the unstressed leaf tissue (i.e. at a leaf water potential of ca -0.45 MPa) (Fig. 1B) but occurred to such an extent in both the apoplast and the chloroplast (Table 1) that there was no statistically significant difference. This was not unexpected for according to Slovik and Hartung (1992a, b), the total ABA content per leaf area and the ABA concentration in most lamina compartments are linear functions of the apoplastic equilibrium concentration of ABA. In this regard, it is interesting to note that according to the latter authors, there may only be two compartments where ABA degradation could occur, i.e. the mesophyllic cytoplasm or in the apoplast (or both).

Compared to the chloroplastic and apoplastic labelling at the control leaf water potential i.e. -0.45 MPa, a twofold drought stress-induced increase in the apoplastic ABA concentration was observed at the -1.55 MPa drought stress level (Table 1). This observation in itself is once again consistent with current literature, for example Trejo and Davies (1991) have reported a two- to threefold increase in the ABA concentration in the xylem sap of unwatered *Phaseolus vulgaris* plants and similar increases were noted by Hartung and Radin (1989) for *Phaseolus coccineus*. However, it is vitally important to note that the drought stress-induced increase in apoplastic ABA was not accompanied by a decrease in the ABA concentration in the chloroplast (Table 1 and Fig. 1B,C). This observation casts doubt over the supposition of Taiz and Zeiger (1991), that drought stress-induced a redistribution of ABA from the chloroplast to the apoplast. This statement as well as the above mentioned observations are corroborated in broad terms by the knowledge that when Walton (1980) reinterpreted the results presented by Loveys (1977), he concluded that although ABA is localized in the chloroplast, it would seem to be both synthesized and accumulated outside the chloroplast during drought stress. Furthermore, as there is no evidence that these organelles are the sites of ABA synthesis or catabolism (Zeevaart & Creelman, 1988). It also has been stated, in agreement with the results presented, that apoplastic ABA is transferred directly from the cytosol (Hartung *et al.*, 1988) and this release of ABA from the cytoplasm depends only on an intracellular pH gradient. This has recently been found to remain nearly unaffected by dehydration (Hartung & Slovik, 1991). It is therefore, unlikely that the chloroplasts could have been the origin of the observed drought stress-induced

increase in apoplastic ABA (Fig. 1C and Table 1).

In our opinion, though it has till recently also been controversial (Munns & King, 1988; Davies & Zang 1991), a more likely source for the increased apoplastic ABA (Table 1), could either have been a drought stress-induced increase in synthesis or release of ABA from the roots (Zang & Davies 1990), or alternatively as proposed by Schurr *et al.* (1992), and Gowing *et al.* (1993) during the drought stress the catabolism of ABA did not match the pace of import.



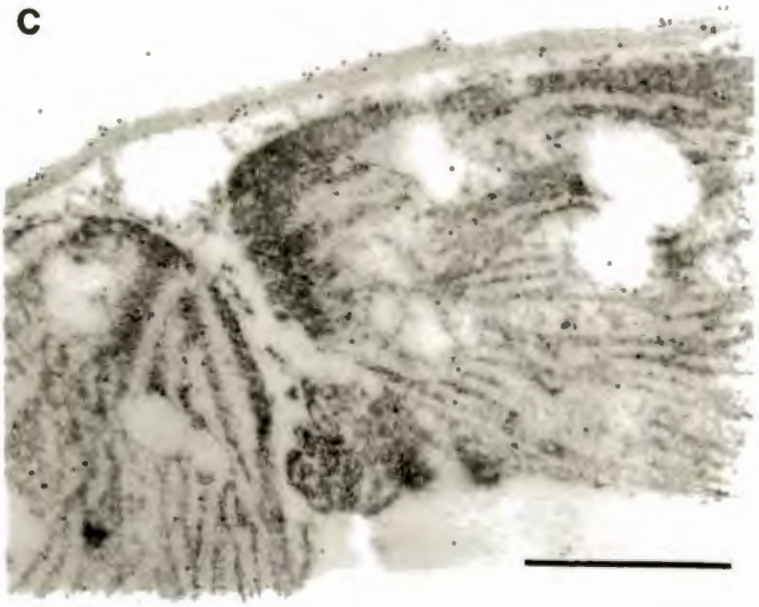


Figure 1. Comparative immunogold labelling (goat-anti-rabbit IgG 10 nm gold) for abscisic acid of ultrathin sections of tobacco leaf mesophyll cells. (A) Control treated with naive control serum (B) Unstressed leaves i.e. Ψ_L ca. -0.45 MPa, and (C) stressed leaves i.e. Ψ_L ca. -1.55 MPa, treated with anti-ABA serum. Scale bar = 0.5 μm.

8.5 Conclusion

The results presented clearly indicate the following: Firstly contrary to some previous immunogold methods that were used to localize ABA in plant tissue, this is the first report of the use of LR-White resin as embedding medium. This procedure preserved the tissue satisfactory and allowed subsequent specific immunostaining. Secondly, the primary antiserum used in this study, was specific enough to localize ABA at the sub-cellular level while the immunohistochemical controls gave appropriate negative reactions. Thirdly, the twofold drought stress-induced increase in apoplastic ABA was not accompanied by a decrease in chloroplastic ABA. However, it is possible that the antiserum used in this study could have detected free and esterified ABA. If the latter was detected it would not alter the above conclusions for the conjugated ABA would be restricted to the vacuoles.

8.6 Literature cited

BERTRAND, S., BENHANOU, N., NADEAU, P., DOSTALER, D. & GOSSELIN, A. 1992. Immunogold localization of free abscisic acid in tomato root cells. *Canadian Journal of Botany,* 70: 1001-1011.

BRAY, E.A. & ZEEVAART, J.A.D. 1985. The compartementation of abscisic acid and β-D-glucopyronosyl abscisate in mesophyll cells. *Plant Physiology,* 79: 719-722.

COWAN, I.R., RAVEN, J.A., HARTUNG, W. & FARQUHAR, G.D. 1982. A possible role for abscisic acid in coupling stomatal conductance and photosynthetic carbon metabolism in leaves. *Australian Journal of Plant Physiology,* 9: 489-498.

CRAIG, S. & GOODCHILD, D.J. 1982. Post-embedding immunolabelling. Some effects of tissue preparation on the antigenicity of plant proteins. *European Journal of Cell Biology,* 28: 251-256.

CRAIG, S. & MILLER, C. 1984. LR white resin and improved ongrid immunogold detection of Vicilin, a pea seed storage protein. *Cell Biology International Reports,* 8: 879-886.

DAVIES, W.J. & ZANG, J. 1991. Root signals and the regulation of growth and development of plants in drying soil. *Annual Review of Plant Physiology and Plant Molecular Biology,* 42: 55-76.

GOWING, D.J.G., JONES, H.G. & DAVIES, W.J. 1993. Xylem-transported abscisic acid: the relative importance of its mass and its concentration in the control of stomatal aperture. *Plant Cell Environment,* 16: 453-459.

HARTUNG, W., KAUZER, W.M. & BURSCHKA, C. 1983. Release of abscisic acid from leaf strips under osmotic stress. *Zeitschrift für Pflanzenphysiologie,* 112: 131-138.

HARTUNG, W., RADIN, J.W. & HENDRIX, D.L. 1988. Abscisic acid movement into the apoplastic solution of water stressed cotton leaves. Role of apoplastic pH. *Plant Physiology,* 86: 908-913.

HARTUNG, W. & RADIN, J.W. 1989. Abscisic acid in the mesophyll apoplast and in the root xylem sap of stressed plants. *Current Topics in Plant Biochemistry and Physiology,* 8: 110-124.

HARTUNG, W. & SLOVIK, S. 1991. Physiochemical properties of plant growth regulators and plant tissues determine their distribution and redistribution. *New Phytologist,* 119: 361-382.

HEILMANN, B., HARTUNG, W. & GIMMLER, H. 1980. Distribution of abscisic acid between chloroplasts and cytoplasm of leaf cells and the permeability of the chloroplast envelope for abscisic acid. *Zeitschrift für Pflanzenphysiologie,* 97: 67-78.

JONES, H.G. & DAVIES, W.J. 1992. A perspective on ABA research in the 1990s (*In*: Davies, W.J. & Jones, H.G., *eds*. Abscisic acid, physiology and biochemistry. Oxford: Bios Scientific Publishers. p 1-4.)

KAIZER, G., WEILER, E.W. & HARTUNG, W. 1985. The intracellular distribution of abscisic acid in mesophyll cells - the role of the vacuole. *Journal of Plant Physiology,* 119: 237-245.

LA ROSA, P.C. HANDA, A.K., HASEGAWA, P.M. & BRESSAN, R.A. 1985. Abscisic acid accelarates adaptation of cultured tobacco cells to salt. *Plant Physiology,* 79: 138-142.

LOVEYS, B.R. 1977. The intracellular location of abscisic acid in tomato plants, and evidence for a novel abscisic acid metabolite. *Physiologia Plantarum,* 46: 79-84.

MANSFIELD, T.A., WELLBURN, A.R. & MOREIRA, T.J.S. 1978. The role of abscisic acid and farnesol in the alluviation of water stress. *Philosophical Transactions of the Royal Society of London.* B284: 471-482.

MILBORROW, B.V. 1979. Antitranspirants and the regulation of abscisic acid content. *Australian Journal of Plant Physiology,* 6: 249-254.

MUNNS, R. & KING, R.W. 1988. Abscisic acid is not the only stomatal inhibitor in the transpiration stream. *Plant Physiology,* 88: 703-708.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology,* 17: 208-212.

SCHURR, U., GALLAN, T. & SCHULZE, E-D. 1992. Stomatal response to drying soil in relation to changes in the xylem sap composition of *Helianthus annuus* II. Stomatal sensitivity to abscisic acid imported from the xylem sap. *Plant Cell Environment,* 15: 561-567.

SLOVIK, S. & HARTUNG, W. 1992a. Compartemental distribution and redistribution of abscisic acid in leaves. II. Model analysis. *Planta,* 187: 26-36.

SLOVIK, S. & HARTUNG, W. 1992a. Compartemental distribution and redistribution of abscisic acid in leaves. III. Analysis of the stress-signal chain. *Planta,* 187: 37-47.

SOSSOUNTZOV, L., MALDENEY, R., SOTTA, B., SABBAGH, I., HABRICOT, Y., BONNET, M. & MIGINIAC, E. 1988. Immunocytochemical localization of cytokinins in Craigella tomato and a sideshootless mutant. *Planta,* 175: 291-304.

SOSSOUNTZOV, L., SOTTA, B., MALDENEY, R., SABBACH, I. & MIGINIAC, E. 1986. Immunoelectron-microscopy localization of abscisic acid with colloidal gold on Lowicryl-embedded tissues of *Chenopodium polyspermum* L. *Planta,* 168: 471-481.

SOTTA, B., SOSSOUNTZOV, L., MALDINEY, R., SABBACH, I., TACHON, P. & MIGINIAC, E. 1985. Abscisic acid localization by light microscopic immunohistochemistry in *Chenopodium polyspernum* L. *Journal of Histochemistry and Cytochemistry,* 33: 201-208.

TAIZ, L. & ZEIGER, E. 1991. Stress physiology: Water deficit and drought resistance. (*In* Taiz, L. & Zeiger, E., *eds.*. Plant physiology, California, U.S.A.: Benjamin/Cummings. p. 346-356).

TREJO, C. & DAVIES, W.J. 1991. Drought-induced closure of *Phaseolus vulgaris* stomata precedes leaf water deficit and any increase in xylem ABA concentration. *Journal of Experimental Botany,* 42: 1507-1515.

VAN RENSBURG, L. & KRüGER, G.H.J. 1993. Comparative analysis of differential drought stress-induced suppression of and recovery in carbon dioxide fixation: Stomatal and non-stomatal limitation in *Nicotiana tabacum* L. *Journal of Plant Physiology,* 142: 296-306

VAN RENSBURG, L., KRüGER, G.H.J. & KRüGER, H. 1993. Proline accumulation as drought tolerance selection criterion: Its relationship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. *Journal of Plant Physiology,* 141: 188-194.

WALTON, D.C. 1980. Biochemistry and physiology of abscisic acid. *Annual Review of Plant Physiology,* 31: 453-489.

WEILER, E.W. 1979. Radioimmunoassay for the determination of free and conjugated abscisic acid. *Planta,* 144: 255-263.

ZANG, J. & DAVIES, W.J. 1990. Changes in the concentration of ABA in the xylem sap as a function of changing soil water status can account for changes in leaf conductance and growth. *Plant Cell Environment,* 13: 271-285.

ZEEVAART, J.A.D. 1983. Metabolism of abscisic acid and its regulation in *Xanthium* leaves during and after water stress. *Plant Physiology,* 71: 477-481.

ZEEVAART, J.A.D. & BOYER, G.L. 1984. Accumulation and transport of abscisic acid and its metabolites in *Ricinus* and *Xanthium. Plant Physiology,* 74: 934-939.

ZEEVAART, J.A.D. & CREELMAN, R.A. 1988. Metabolism and physiology of abscisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology,* 39: 439-473.

CHAPTER NINE

9. PROLINE ACCUMULATION AS DROUGHT TOLERANCE SELECTION CRITERION: ITS RELATIONSHIP TO MEMBRANE INTEGRITY AND CHLOROPLAST ULTRASTRUCTURE IN *NICOTIANA TABACUM* L.

(VAN RENSBURG, L., KRüGER, G.H.J. & KRüGER, H. 1993. *JOURNAL OF PLANT PHYSIOLOGY*, 141 : 188-194.)

9.1 Abstract

The value of proline accumulation as criterion in selecting for drought tolerance, was evaluated in four tobacco cultivars of differing drought tolerance. Proline determination, measurement and calculation of a membrane integrity index (MII), and ultrastructural observations were conducted simultaneously under controlled environmental conditions during the stress period. Water stress of increasing severity (0.2 - 0.3 MPa d^{-1}) that ranged from light (-0.52 MPa) to severe (-2.51 MPa) was induced by withholding water. A substantial accumulation of proline was observed in all four cultivars, the extent of which correlated positively with their individual drought tolerance. Ultrastructural observation indicated that the drought-tolerant cultivars mobilized the more than adequate store of starch to a greater extent than the drought-sensitive cultivars during stress. Water stress-induced membrane damage occurred earlier and was much more severe in the drought-sensitive cultivars, as their MII-values already rose to between 20.4% and 23.4% at a Ψ_L of -1.27 MPa, while those of the drought-tolerant cultivars only reached values of 18.5% and 20.9% at a Ψ_L of -1.67 MPa. The Ψ_L at which proline levels start to increase dramatically (the shorter the response time the better) and the end concentrations of proline accumulated, are advocated as selection criteria to be used in selecting for drought-tolerant tobacco genotypes as early as the F_1- or F_2-generations.

Keywords: *Nicotiana tabacum* L., *membrance integrity, proline accumulation, selection criterion, starch content, ultrastructure, water stress.*

Abbreviations: MII = membrane integrity index.

9.2 Introduction

Within the leaves of many plants subjected to moderate or severe water stress, one striking change in nitrogen metabolism is the accumulation of free proline as a result of net *de novo* synthesis from glutamic acid (Barnett & Naylor, 1966; Boggess *et al.*, 1976). Water stress-induced proline accumulation can account for as much as 1% of the dry leaf matter in many species; this, however, only takes place if there are adequate carbohydrates present in the tissue (Hsiao, 1973). Proline has been shown to ameliorate the deleterious effects of heat, pH, salt, chemicals and dehydration on enzyme activity and organelle systems (Ahmad *et al.*, 1982; Bowlus & Somero, 1979; Nash *et al.*, 1982; Paleg *et al.*, 1984; Yancey & Somero, 1979). The mechanism whereby these "compatible" solutes (Schobert & Tschesche, 1978) exert their influence has, however, not yet been elucidated (Stewart, 1989), nor are the molecular mechanisms underlying these effects completely understood. Proline action has been suggested to involve effects on the hydration layer surrounding phospholipids and possibly also its intercalation between phospholipid head groups (Rudolph *et al.*, 1989). Furthermore, it is striking that many of the solutes which accumulate in stressed plants and which have protective properties are also reported to reduce free radical activity. In this regard it has been indicated that proline can also detoxify free radicals by forming long-lived adducts with them (Floyd & Zs-Nagy, 1984). Just as little is known about the changes which the tonoplast and plasmalemma undergo during desiccation, although leakage studies indicate that some changes do occur (Bewley & Krochko, 1982). Many of these studies have been reviewed by Simon (1974, 1978). What is known, however, is that when enzymes, structural proteins, macromolecular complexes, etc., are desiccated in their native state, the integrity of the molecules and structures can be retained if some water remains associated with them, which prevents the formation of unfavourable conformations (Todd, 1972) or fragmentation (Darbyshire & Steer, 1973).

As stated above, it has been suggested for some time that the often observed accumulation of proline in plant tissues during water stress is an adaptive response (Handa *et al.*, 1986). It can be argued, however, as has been done, that the mere correlation between accumulation and development of stress is not enough proof that the substance has any adaptive value in postponing stress or increasing stress-tolerance and that the accumulation occurs because of disturbance of normal nitrogen metabolism with the result that any beneficial effects, if such exist, are merely coincidental (Kramer, 1983).

As it is very difficult to prove that these compounds have important adaptive value, this study was conducted as an attempt to gain some evidence, albeit indirectly, whether the ability to accumulate proline in four tobacco cultivars, can be positively correlated with their drought tolerance, because of its protective membrane stabilizing effects. To achieve the latter, it was

deemed necessary to find answers to the following questions: Firstly, does a positive correlation exist between proline accumulation, membrane integrity and the known difference in drought tolerance of the four tobacco cultivars? Secondly, do the four cultivars contain enough carbohydrates to allow proline accumulation and does the extent to which starch is mobilized during water stress differ among the four cultivars? Thirdly, how extensive are the water stress-induced ultrastructural changes and do they differ in the four cultivars? Finally, which, if any, aspect(s) of proline accumulation may be useful as selection criteria in selecting for drought tolerance?

9.3 Material and methods

In sequence of increasing drought tolerance the four cultivars of *Nicotiana tabacum* L. under investigation, were TL33, CDL28, GS46 and ELSOMA. These cultivars were selected due to their differing performances in the field during water stress periods (Personal communication: Dr. C.J. Steenkamp, Tobacco and Cotton Research Institute, Rustenburg, RSA). Seed was allowed to germinate in soil in pots. The young seedlings developed under glasshouse conditions with optimal water application (watered once daily to field capacity). Before the onset of experimentation the plants were moved to a growth room (photon flux density, 400-600 μ mole photons m^{-2} s^{-1}; relative humidity, 35%) and allowed to acclimatize for a period of 96 hours. During the acclimatization period the plants were watered twice daily to avoid their experiencing any water stress because of the slightly higher growth chamber temperatures. The growth chambers were lit for 13 hours at 25°C followed by an 11 hour dark period at 16°C. Experimentation started when the plants were approximately 90 days old and a water stress of increasing intensity (0.2 - 0.3 MPa d^{-1}) was induced by withholding water. The water stress period lasted for 12 days and ranged from light (-0.52 MPa) to severe (-2.51 MPa). Leaf water potential determinations were done every second day with a Scholander pressure chamber (PMS-instrument, Oregon, USA). Membrane integrity, measured as ion leakage, was carried out as described by Sullivan and Ross (1979) and modified by Blum and Ebercon (1981). Proline concentration was determined by means of a rapid colorimetric method, developed for plant tissue by Bates *et al.* (1973). All leaf samples were taken at 08:00. The sixth youngest leaf was used in all instances, as this represented a mature, nearly fully expanded leaf, before the onset of senescence.

For ultrastructural investigation samples of $1mm^3$ were cut from the centre of the lamina, lateral to the midrib, while immersed in fixative, and these were immediately transferred to fixative (2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4). After evacuation of 15 minutes, fixation was continued for a further 2 hours. The material was postfixed for 1 hour in 0.5% aqueous osmium tetroxide, dehydrated in acetone and infiltrated with resin (Spurr, 1969). For light microscopy semi-thin sections were made, stained with toluidine blue O (Trump *et al.*, 1961) or toluidine blue O and neo-fuchsin (Botha *et al.*, 1982). For electron microscopy, thin sections were contrasted with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963).

9.4 Results and discussion

The calculated MII-values (Table 1) of the four cultivars clearly indicate that the membranes of the drought-sensitive cultivars (TL33 and CDL28) were damaged earlier than those of the drought-tolerant cultivars (GS46 and ELSOMA). This is evident from the fact that the MII-values of TL33 and CDL28 already increased to 20.4% and 23.4% respectively at a Ψ_L of -1.27 MPa whilst the MII-values of GS46 and ELSOMA only rose to similar levels i.e. 20.9% and 18.5% respectively at a Ψ_L of -1.67 MPa. Furthermore, at the severe stress level (-2.51 MPa) the water stress-induced membrane damage was found to be approximately 10% more in the drought-sensitive cultivars. The value of the above-mentioned results is only fully realized when seen in conjunction with the statement of Sullivan and Ross (1979). These authors have been concerned with the relationships between electrolyte leakage following a shock treatment and general ability of plants to tolerate stress for several years. According to them, the evidence they obtained indicated that the degree of membrane stability to stress (as evaluated by ion leakage) correlates well with the tolerance of other plant processes to stress, even if the stress is at levels lower than that needed to measure appreciable ion leakage. These include soluble protein and enzyme resistance to denaturation, maintenance of photosynthesis by intact tissue and other responses to stress (Sullivan & Kinbacher, 1967; Sullivan & Ross, 1979; Kinbacher *et al.*, 1967). In the light of our results (Table 1 and Fig. 1) regarding membrane integrity of the test plants, and because it is the rate of water stress-induced membrane injury that is estimated through the measurement of electrolyte leakage from cells (Blum & Ebercon, 1981), it may be said that not only the membrane systems of the drought-sensitive cultivars, but also their physiological processes in general were damaged earlier. Furthermore, the extent of the water stress-induced damage, as reflected by the steeper slope of the MII-curves (Fig. 1), was much more severe in these cultivars. In keeping with the idea that the water stress-induced membrane damage was much more severe in the drought-sensitive cultivars, it should also be noted that at a Ψ_L of -2.51 MPa, membrane damage in these cultivars was irreversible. The latter statement is supported by the fact that the MII-values represent the percentage of membrane injury due to desiccation (Blum & Ebercon, 1981) and these values of the drought-sensitive cultivars exceeded 50% at a Ψ_L of -2.51 MPa (Table 1).

Table 1: Water stress-induced proline accumulation and changes in the membrane integrity index of four cultivars of *Nicotiana tabacum* L., which differ with respect to their drought tolerance.

Cultivar	Ψ_L MPa	Proline conc., mmol kg^{-1}dry mass	% of control	MII % of control
TL33	-0.52	0.60 ± 0.01	100.0	0.0 ± 0.0
	-0.77	1.91 ± 0.04	318.5	3.8 ± 0.6
	-1.27	3.40 ± 0.9	566.7	20.4 ± 0.7
	-1.67	3.72 ± 0.4	620.0	22.4 ± 0.5
	-1.97	9.50 ± 0.3	1583.3	30.4 ± 0.4
	-2.32	18.55 ± 0.3	3019.7	38.5 ± 0.8
	-2.51	35.66 ± 0.7	5934.3	51.1 ± 0.9
CDL28	-0.52	0.81 ± 0.02	100.0	0.0 ± 0.0
	-0.77	1.99 ± 0.01	245.7	4.5 ± 0.3
	-1.27	3.30 ± 0.3	407.4	23.4 ± 0.8
	-1.67	5.45 ± 0.4	672.8	28.4 ± 0.9
	-1.97	5.82 ± 0.9	718.5	32.3 ± 0.9
	-2.32	16.26 ± 0.4	2007.4	63.1 ± 0.5
	-2.51	32.22 ± 0.3	3977.7	52.8 ± 0.6
GS46	-0.52	0.17 ± 0.01	100.0	0.0 ± 0.0
	-0.77	1.19 ± 0.04	700.0	6.2 ± 0.1
	-1.27	2.12 ± 0.03	1247.1	7.4 ± 0.8
	-1.67	6.83 ± 0.5	4017.6	18.5 ± 0.7
	-1.97	14.16 ± 0.2	8329.4	29.4 ± 0.2
	-2.32	30.20 ± 0.8	7764.7	40.6 ± 0.7
	-2.51	41.90 ± 0.2	4647.1	42.9 ± 0.4
ELSOMA	-0.52	0.38 ± 0.01	100.0	0.0 ± 0.0
	-0.77	1.59 ± 0.02	418.0	6.8 ± 0.9
	-1.27	2.05 ± 0.9	539.5	8.9 ± 0.5
	-1.67	11.71 ± 0.3	3081.6	20.9 ± 0.3
	-1.97	23.75 ± 0.2	6242.1	26.7 ± 0.6
	-2.32	44.78 ± 0.5	1784.2	32.5 ± 0.4
	-2.51	47.62 ± 0.7	2531.6	41.1 ± 0.5

MII = $1-[1-T_1/T_2)/1-(C_1/C_2)] \times 100$ where according to Blum and Ebercon (1981), T and C refer to mean of treatment and controls, respectively, and subscripts 1 and 2 refer to initial and final conductivities, respectively.

According to Bewley and Krochko (1982) direct evidence that species specific differences in membrane composition or protein structure contribute to the tolerance of plants is generally lacking. This view is shared by Schwab and Heber (1974) who concluded that drought tolerance can not be explained by a particular membrane structure which makes the membrane

insensitive to water stress, but must rather, though it may only be in part, be attributed to the composition of the membrane surroundings, with special reference to membrane compatable solutes. In this regard it is interesting to note that Rudolph *et al.* (1986) found that in the presence of trehalose, sucrose, proline and glycine betaine, ion leakage is prevented.

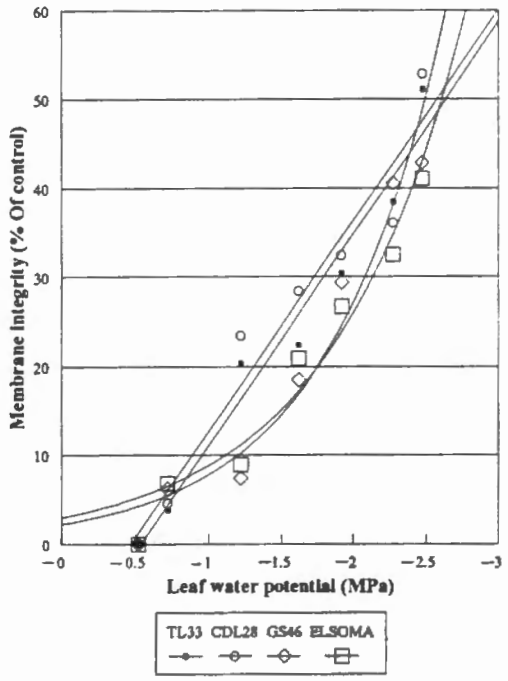


Figure 1: Effect of water stress on the membrane integrity of four cultivars of *Nicotiana tabacum* L. of differing drought tolerance.

In accordance with the results obtained by several authors (Bogges & Stewart, 1980; Handa *et al.*, 1986) in several species, our results showed a dramatic increase in the free proline levels of all four the tobacco cultivars (Fig. 2). At a Ψ_L of -0.77 MPa the proline levels of all four the tobacco cultivars already deviated significantly from the control values. The proline concentrations of the drought-sensitive cultivars were 1.91 and 1.99µmoles gram^{-1} respectively, which is more than double the proline concentration in the unstressed tissue, while the proline concentration in the drought-tolerant cultivars had already risen to 1.19 and 1.59 µ moles gram^{-1}, which is four times more than the proline concentration in the unstressed tissue. The base proline levels of the drought-tolerant cultivars proved to be lower than those of the drought-sensitive cultivars (Table 1). Of greater importance, however, is the fact that the drought-tolerant cultivars were able to accumulate proline earlier and to much higher end concentrations. At a Ψ_L of -1.67 MPa the proline concentrations of both drought-sensitive cultivars were just a little more than six times those of the controls, while that of GS46 was 40 times higher and that of ELSOMA, 30 times higher than their respective controls. At the severe stress level of -2.51 MPa the amount of proline accumulated by ELSOMA was more than three times higher than that of both drought-sensitive cultivars and that of GS46 more than four times higher than both the drought-sensitive

cultivars (Table 1). With reference to the latter two sets of results, it should be noted that Hsiao (1973) stated that sketchy indications are that the level of proline may be insensitive to mild stress and that accumulation only takes place if there are adequate carbohydrates in the tissue. The accumulated proline apparently came from *de novo* synthesis (Thompson *et al.*, 1966) with glutamate as precursor (Morris *et al.*, 1969) and there is evidence to indicate that carbohydrates are the ultimate source of the skeleton (Stewart *et al.*, 1966).

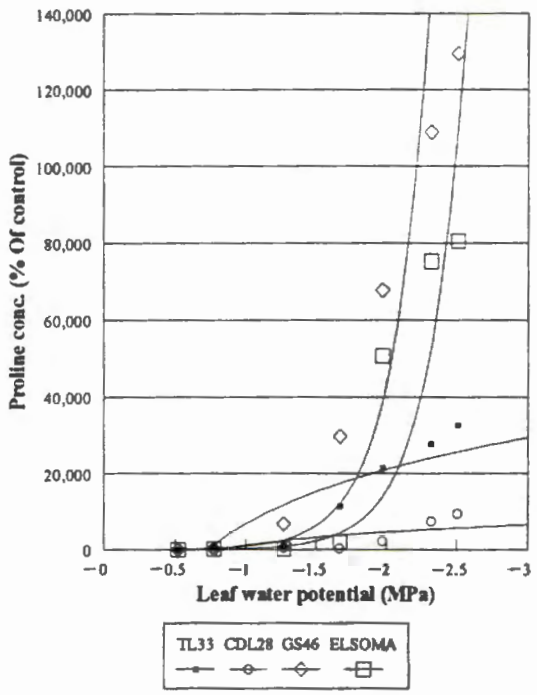


Figure 2: Water stress-induced proline accumulation in four cultivars of *Nicotiana tabacum* L. which differ with respect to their drought tolerance.

With regard to carbohydrate reserves of the test plants the following observations relating to starch grains in the chloroplasts were made. Special attention was, however, paid to the state of the chloroplast envelope membranes as well as to that of the inner thylakoids in this investigation as according to Ferrari-Iliou *et al.* (1982) chloroplasts can be regarded as the most fragile organelles in the cell. In the unstressed leaves of all four cultivars the presence of large starch grains was striking, although the leaves were sampled after only 2-3 hours of exposure to light (Figs. 3a and 3b). The grana were found to be clearly defined, with parallel thylakoids and little dilatation of the intergranal spaces. Osmiophilic globules, some appearing more osmiophilic than others, occurred in most of the chloroplasts studied.

After water had been withheld for 8 days (Ψ_L = -1.97 MPa), the number and size of the starch grains of the chloroplasts of leaves of cultivars TL33 and CDL28 (Fig. 3c), showed little change from the original situation observed in the unstressed leaves. The chloroplast envelope

membranes in some cells of cultivar TL33 were not clearly defined, while in others they were still intact. Large lipidic globules were present in some of the chloroplasts. Granal and stromal thylakoids were severly disrupted in some chloroplasts. The chloroplast membranes in leaves of CDL28 were still intact and the position of the plasmalemma with regard to the cell wall showed little change, although signs of shrinking of cytoplasm were discernible in some cells. Few vesicular structures were present in the cells. Stromal thylakoids showed some signs of dilatation in some of the cells, but the grana were still intact. The size of starch grains decreased in many chloroplasts in the leaves of cultivars ELSOMA and GS46 (Fig. 3d). This was especially apparent in the case of GS46, where many of the chloroplasts lost their original bulging appearance due to large starch grains observed in all unstressed leaves. In leaves of cultivar ELSOMA, vesicular structures were present in some of the vacuoles and signs of plasmolysis could be observed. The granal and stromal thylakoids were still intact as were the chloroplast membranes (Fig. 3d). Cells with granular vacuolar content were observed. Chloroplast membranes in leaves of GS46 were still intact and the thylakoids appeared normal in most of the cells investigated. Vesicular structures were observed in many of the cells. One of the most noticeable features of these cells was the granular or fibrillar material which collected in some of the vacuoles (Fig. 4). This was also discernible in the sections prepared for light microscopy and was not observed in any of the other cultivars at this stage.

After water had been withheld for 12 days (Ψ_L = -2.51 MPa), large lipidic globules, some more osmiophilic than others, appeared in chloroplasts of all the cultivars investigated (Fig. 3e). Leaves of cultivar TL33 still contained starch grains in most of the chloroplasts (Fig. 3e). The starch grains were smaller than those present in unstressed leaves. Cytosolic damage was very severe in some cells where vesicular material collected in large parts of the cell. Large osmiophilic accretions appeared in the degenerated cells. In severely damaged chloroplasts signs of bulging of the outer chloroplast membrane were observed. Some of the chloroplasts assumed a curved shape (Fig. 3e) and the cells showed signs of plasmolysis. Leaves of cultivar CDL28 still contained cells with intact thylakoids. Some of the chloroplasts contained starch grains smaller than those observed in unstressed leaves, while other chloroplasts were almost or completely depleted of starch. This could also be observed by light microscopy. Chloroplast membranes were still intact. Fibrillar material collected in the vacuoles of some of the cells, where the tonoplasts appeared to be poorly defined or damaged. Vesicular structures were present in the vacuoles of some of the cells. Few signs of severe plasmolysis were detected. Leaves of cultivar ELSOMA contained chloroplasts with smaller starch grains (Fig. 3f). Fibrillar or granular material collected in the vacuoles while few vesicular structures were observed in the vacuoles. Chloroplast envelope membranes were still intact, although the first signs of granal and especially stromal thylakoid dilation were apparent in some of the cells. Signs of plasmolysis and curved chloroplasts were observed in some of the cells (Fig. 3f). In leaves of cultivar GS46, the stromal

and granal thylakoids appeared somewhat dilated but all membranes investigated were still intact. Small starch grains were present in the chloroplasts. Fibrillar or granular material was present in the vacuoles (Fig. 4). These cells showed little sign of plasmolysis.

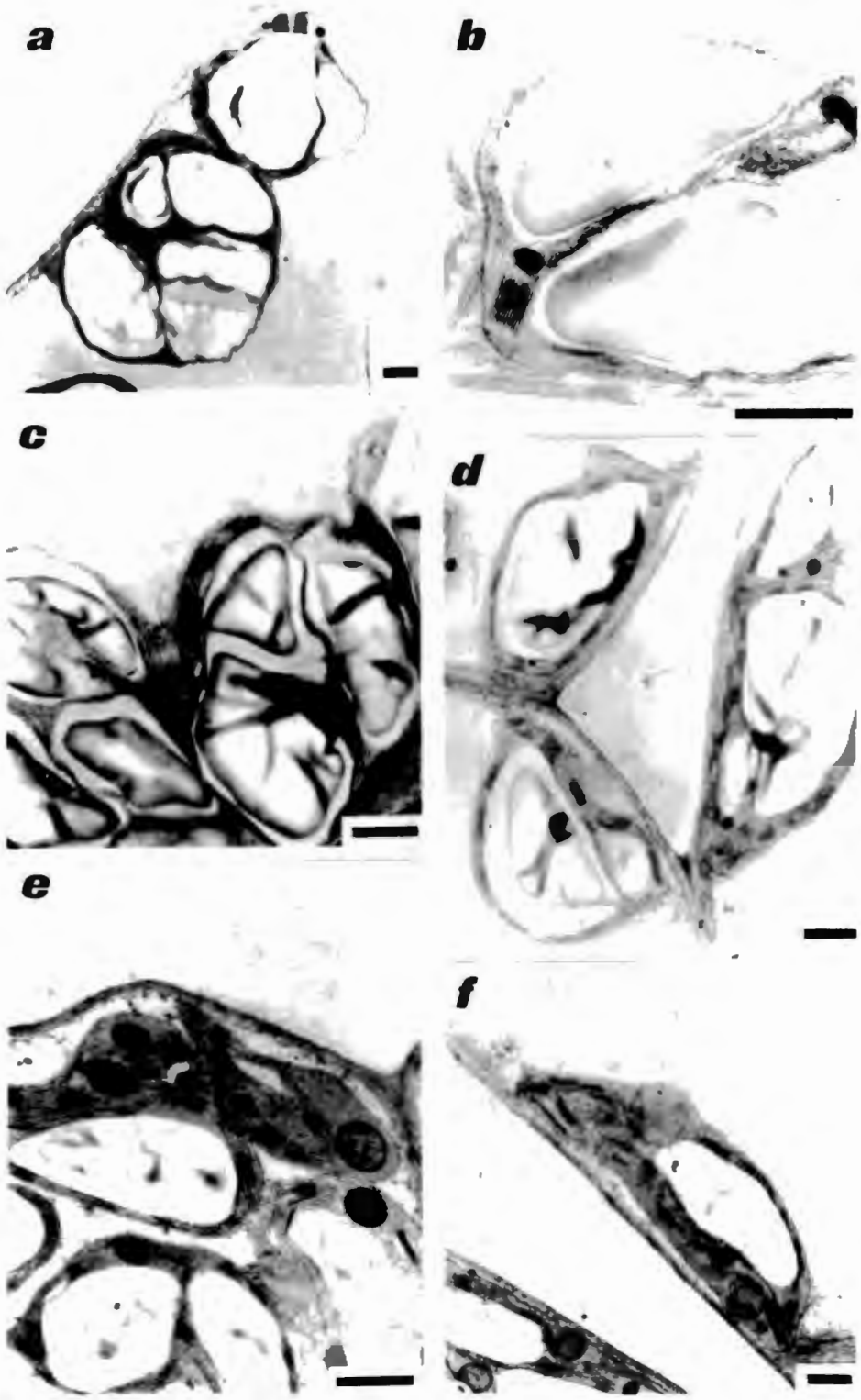


Figure 3: Differential water stress-induced ultrastructural changes in four tobacco cultivars of differing drought tolerance: (a) CDL28 (Ψ_L = -0.52 MPa); (b) ELSOMA (Ψ_L = -0.52 MPa); (c) CDL28 (Ψ_L = -1.97 MPa); (d) ELSOMA (Ψ_L = -1.97 MPa); (e) TL33 (Ψ_L = -2.51 MPa); (f) ELSOMA (Ψ_L = -2.51 MPa). Bar = 1 μm.

Figure 4: Distinctive water stress-induced appearance of granular or fibrilar material in the vacuole of cultivar GS46 after a Ψ_L of -1.97 MPa was reached. Bar = 1μm.

Thus, although no complete loss of starch was observed at any stage during water stress, in contrast to the situation reported by Giles *et al.* (1976) in water stressed leaf tissue of *Sorghum bicolor*, a decline in the starch content was observed in the chloroplasts of all four the cultivars as the water stress intensified. The significance of these results lies not merely in the fact that a decline in the starch content occurred, as increases in free sugars and loss of starch commonly occur in plants during water stress (Baskin & Baskin, 1974), but in the fact that the rate (as observed visually) at which the starch content declined, was higher in the drought-tolerant cultivars (GS46 and ELSOMA).

A synthesis of the overwhelming amount of circumstantial evidence obtained in this study which indicates the existence of a positive correlation between the ability to accumulate proline and drought tolerance may be summarized as follows: The membrane systems of the drought-tolerant cultivars were not damaged as early, nor was the extent of the water stress-induced damage as severe as in the drought-sensitive cultivars. The latter correlates positively with the drought-tolerant cultivars' ability to accumulate proline earlier and to higher end concentrations. Furthermore, although all the cultivars initially contained enough starch to allow proline accumulation, the rate at which the amount of starch declined was higher in the drought-tolerant cultivars. Finally, on the ultrastructural level, the general degree of the water stress-induced disruption was not as severe in the drought-tolerant cultivars. From the results discussed and the

latter seen in conjunction with the literature cited, it seems as if more than a mere casual relationship exists between the ability to accumulate proline and the drought tolerance of a tobacco cultivar. Although the exact mechanism by which proline exerts its protective effect(s), is still unknown (Stewart, 1989), the ability to accumulate proline would possibly confer some adaptive ability on a cultivar. In keeping with the idea that proline accumulation may be beneficial to the plant under stress, it is interesting to note that the ability of ten barley varieties to accumulate proline under severe stress has been positively correlated with their drought resistance ratings (Singh *et al.*, 1972).

According to Blum and Ebercon (1981), a valid and functional drought tolerance test should relate to integrated plant responses at a low plant organizational level (i.e. tissue growth), or a single attribute related to the basic facets of cellular or tissue responses to stress, as drought tolerance is process-specific in that different physiological processes may show tolerance or susceptibility within a genotype. Due to the fact that a positive correlation exists between the known drought tolerance of the tobacco cultivars and the ability to accumulate proline and the convincing nature of other results given and literature cited, its use as selection criterion is advocated; specifically the Ψ_L at which proline levels start to increase dramatically (the less negative the better) and the end concentrations of proline accumulated.

9.6 Literature cited

AHMAD, I., LARHER, F. & STEWART, G.R. 1982. Sorbitol A compatible osmotic solute in *Plantago maritima*. *New Phytologist*, 82: 671-678.

BARNETT, N.M. & NAYLOR, A.W. 1966. Amino acid and protein metabolism in Bermuda grass during water stress. *Plant Physiology*, 41: 1222-1230.

BASKIN, C.C. & BASKIN, J.M. 1974. Responses of *Astragalus tennesseensis* to drought. Changes in free amino acids and amides during water stress and possible ecological significance. *Oecologia*, 17: 11-16

BATES, L.S., WALDREN, R.P. & TEARE, I.C. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil*, 39: 205-207.

BEWLEY, J.D. & KROCHKO, J.E. 1982. Desiccation-tolerance. (*In* Lange, O.L., Nobel, P.S., Osmond, C.B. & Ziegler,H., *eds*. Physiological plant ecology II, Water relations and carbon assimilation, Vol. 123, Berlin: Springer-Verlag. p. 325-378.)

BLUM, A. & EBERCON, A. 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat. *Crop Science*, 21: 43-47.

BOGGESS, S.F., STEWART, C.R., ASPINALL, D. & PALEG, L.G. 1976. Effect of water stress on proline synthesis from radioactive precursors. *Plant Physiology*, 58: 398-401.

BOGGESS, S.F. & STEWART, C.R. 1976. The relationship between water stress-induced proline accumulation and inhibition of protein synthesis in tobacco leaves. *Plant Science Letters*, 17: 245-252.

BOTHA, C.E.J., EVERT, R.F., CROSS, R.H.M. & MARSHALL, D.J. 1982. Comparative anatomy of mature *Themeda triandra* Forsk. Leaf blades: A correlated light and electron microscope study. *South African Journal of Botany*, 48: 311-328.

BOWLUS, R.D. & SOMERO, G.N. 1979. Solute compatibility with enzyme function and structure: rationales for the selection of osmotic agents and end products of anaerobic metabolism in marine invertebrates. *Journal of Experimental Zoology*, 208: 137-152.

DARBYSHIRE, B. & STEER, B.T. 1973. Dehydration of macromolecules. I. Effect of dehydration-rehydration on indoleacetic acid oxidase, ribonuclease, ribulosediphosphate carboxylase, and ketose-1-phosphate aldolase. *Australian Journal of Biological Sciences,* 26: 591-604.

FERRARI-ILIOU, R., PHAM, T., ANH, T. & VIERA DA SILVA, J. 1982. Effect of water stress on the lipid and fatty acid composition of cotton (*Gossypium hirsutum*) chloroplasts. *Physiologia Plantarum,* 62: 219-224.

FLOYD, R.A. & ZS-NAGY 1984. Formation of long lived hydroxyl free radical adducts of proline and hydroxyproline in a Fenton reaction. *Biochimica et Biophysica Acta,* 790: 94-97.

GILES, K.L., COHEN, D. & BEARSELL, M.F. 1976. Effect of water stress on the ultrastructure of leaf cells of *Sorghum bicolor*. *Plant Physiology,* 57: 11-14.

HANDA, S., HANDA, A.K. HASEGAWA, P.M. & BRESSAN, R.A. 1986. Proline accumulation and the adaptation of cultured plant cells to water stress. *Plant Physiology,* 80, 938-945.

HSIAO, T.C. 1973. Plant responses to water stress. *Annual Review of Plant Physiology,* 24: 519-570.

KINBACHER, E.J., SULLIVAN, C.Y. & KNULL, H.R. 1967. Thermal stability of malic dehydrogenase from heat-hardened *Phaseolus actifolius* "Tepary Buff". *Crop Science,*7: 148-151.

KRAMER, P.J. 1983. Water relations of plants. New York: Academic Press.

MORRIS, C.J., THOMPSON, J.F. & JOHNSON, C.M. 1969. Metabolism of glutamic acid and N-acetylglutamic acid in leaf discs and cell-free extracts of higher plants. *Plant Physiology,* 51: 817-824.

NASH, D., PALEG, L.G. & WISKICH, J.T. 1982. The effect of proline, betaine and some other solutes on the heat stability of mitochondrial enzymes. *Australian Journal of Plant Physiology,* 75: 974-978.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron opaque stain on electron microscopy. *Journal Cell Biology,* 17: 208-213.

RUDOLPH, A.S., CORWE, J.H. & CORWE, L.M. 1986. Effects of three stabilizing agents - proline, betaine and trehalose on membrane phospholipids. *Archives of Biochemistry and Biophysics,* 245: 134-143.

SCHOBERT, B. & TSCHESCHE, H. 1978. Unusual solution properties of proline and its interaction with proteins. *Biochimica et Biophysica Acta,* 541: 270-277.

SCHWAB, K.B. & HEBER, U. 1984. Thylakoid membrane stability in drought-tolerant and drought-sensitive plants. *Planta,* 161: 37-45.

SIMON, E.W. 1974. Phospholipids and plant membrane permeability. *New Phytologist,* 73: 377-420.

SIMON, E.W. 1978. Membranes in dry imbibing seeds.(*In* Dry biological systems. New York: Academic Press. p. 205-224.)

SINGH, T.N., ASPINALL, D. & PALEG, L.G. 1972. Proline accumulation and varietal adaptability to drought in barley: a potential metabolic measure of drought resistance. *Nature New Biology,* 236: 188-190.

STEWART, C.R., MORRIS, J.C. & THOMPSON, J.F. 1966. Changes in amino acid content of excised leaves during incubation. II: Role of sugar in the accumulation of proline in wilted leaves. *Plant Physiology,* 41: 1585-1590.

STEWART, G.R. 1989. Desiccation injury, anhydrobiosis and survival.(*In* Jones, H.G. Flowers, T.J. & Jones, M.B., *eds.* Plants under stress-Biochemistry, physiology and ecology and their application to plant improvement. New York: Cambridge University Press. p. 115-130.)

SULLIVAN, C.Y. & KINBACHER, E.J. 1967. Thermal stability of fraction protein from heat hardened *Phaseolus actifolius* Gray, "Tepary Buff". *Crop Science,* 7: 241-244.

SULLIVAN, C.Y. & ROSS, W.M. 1979. Selecting for drought and heat resistance in grain sorghum. (*In* Mussel, H. & Staples, R.C., *eds.* Stress physiology in crop plants. New York: Wiley and Sons. p. 263-281.)

SPURR, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research,* 26: 31-43.

THOMPSON, J.F., STEWART, C.R. & MORRIS, C.J. 1966. Changes in amino acid content of excised leaves during incubation. 1: The effect of water content of leaves at atmospheric oxygen level. *Plant Physiology,* 41: 1578-1584.

TODD, G.W. 1972. Water deficits and enzymatic activity. (*In* Kozlowski, T.T., *ed.* Water deficits and plant growth. Vol. 3, New York: Academic Press. p.177-216.)

TRUMP, B.F., SCHMUCKLER, F.H. & BENDITT, E.P. 1961. A method for staining epoxy sections for light microscopy. *Journal of Ultrastructure Research,* 5: 343-348.

YANCEY, R.N. & SOMERO, G.N. 1979. Counteraction of urea destabilization of protein structure by methylamine osmoregulatory compounds of elasmobranch fishes. *Biochemical Journal,* 183: 317-323.

CHAPTER TEN

10. OSMOREGULATION, MONITORED BY PRESSURE-VOLUME TISSUE WATER COMPONENT ANALYSIS, IN CULTIVARS OF *NICOTIANA TABACUM* L. OF DIFFERENT DROUGHT TOLERANCE

(VAN RENSBURG, L. & KRüGER, G.H.J. 1994. *SOUTH AFRICAN JOURNAL OF BOTANY*, 60: 139-144.)

10.1 Abstract

The extent of osmoregulation was determined by means of pressure-volume analysis, which was also used to determine tissue water components and to estimate relative water contents (RWC), of the leaves of four *Nicotiana tabacum* L. cultivars of different but known drought tolerance. Data were obtained during two drought stress periods induced in computerised controlled growth rooms. The tobacco leaves adjusted largely to drought stress by the accumulation of solutes, leading to a decrease of ca. 3% (TL33), 6% (CDL28), 12% (GS46) and 16% (ELSOMA) in the osmotic potential at full turgor (Ψ_π^{100}), which was more pronounced in the drought-tolerant cultivars (GS46 & ELSOMA). Drought stress induced an increase in the proportion of bound water (B) and the volumetric bulk modulus of elasticity ($\in$), more so in the drought-tolerant cultivars, but did not alter the relative water content at which incipient plasmolysis (RWC°) was reached. In spite of the stability of RWC°, in all four tobacco cultivars which was ca. 79%, 76% for TL33 and CDL28 and 70% for GS46 and 73% for ELSOMA respectively, the lower $\in$ of the drought-sensitive cultivars (TL33 & CDL28) which occurred during both drought stress periods caused the leaf water potential (Ψ_L) at which incipient plasmolysis (Ψ°_L) occurred, to be consistently less negative in these cultivars. Firstly, the results presented, corroborate the existence of differential degrees of osmoregulation in the four tobacco cultivars, which may be part of an integrated plant response. Secondly it emphasise the drought tolerance adaptive advantage of being characterized by a high $\in$, and thirdly, seem to indicate that as both the latter parameters may be genetically determined, it may be used in drought tolerance selection programmes.

Keywords: *Nicotiana tabacum* L., *osmoregulation, pressure-volume analysis, plant tissue water-relations, volumetric bulk modulus of elasticity.*

10.2 Introduction

Empirically, the physical response of plant tissue to drought stress may be divided into two processes: Firstly, the initial instantaneous volume change observed in cells immediately after the onset of osmoregulation which was demonstrated to be almost exclusively due to water flow from the cell (Matsuda & Riazi, 1981). Secondly, the number of particles regulated in the second phase of osmoregulation, in which the cell volume is reversed to its original value by means of metabolic turnover and controlled uptake of osmotically active particles. The rigidity of the cell wall thus requires that turgor pressure ought to be the dominating macroscopic parameter which is to be regulated during the opposite biphasic repsonse to drought stress (Zimmermann, 1978).

Turgor potential (Ψ_p) at a particular leaf water potential (Ψ_L) depends on both the osmotic potential (Ψ_π) and the elastic properties of the cell walls, of which the latter can be described by the so-called volumetric elastic modulus (ϵ), which has been defined as: ϵ = RWC • $\Delta \Psi_p / \Delta$ RWC (Morgan, 1980a). Osmotic potential and elasticity, in turn, are influenced by other factors such as solute accumulation, cell size, RWC and cell wall thickness (Steudle *et al.*, 1977). The relationship between Ψ_L, ϵ, Ψ_π and RWC may be described by the equation: $(1/RWC^o)(\Delta RWC/\Delta \Psi_L) = 1/(\epsilon + \Psi_\pi^o)$, where the superscript 0 indicates the value at incipient plasmolysis (Morgan, 1980b).

From the latter relationships it should be clear that substantial differences may exist in the response of both Ψ_π and RWC to drought stress-induced changes in Ψ_L, due to the degree and presence or absence of osmotic adjustment in the four *Nicotiana tabacum* L. cultivars which are known to differ in respect of their individual drought tolerances. The extent to which these differences reflect divergencies in solute accumulation in the symplasm, opposed to differences in cell wall extensibility, was explored by comparing the observed response with that expected from an ideal osmometer in which the amount of solute does not change but in which Ψ_π changes by concentration of solutes only. The latter response may be described by: $\Psi_\pi = \Psi_\pi^\circ$ V^o/V which may be approximated by: $\Psi_\pi = \Psi_\pi^\circ$ RWC^o/RWC, where V is the osmotic volume and the superscript **0** again signifies a reference state such as incipient plasmolysis (Morgan, 1980a). A convenient linear form of this equation is, $\log \Psi_\pi^\circ = \log (\Psi_\pi^\circ - RWC^o) - \log RWC$, where the intercept is $\log (\Psi_\pi^\circ RWC^o)$ and the slope is one. Thus, by evaluating the slope of the response of $\log \Psi_\pi$ to log RWC it was possible to quantify the degree to which osmoregulation occurred by differentiating between changes that were due to concentration effects and those that may be attributed to changes in the amount of solute in the protoplasm (Morgan, 1984).

Pressure-volume (P-V) tissue water component analysis was used to investigate the nature and magnitude of the above mentioned drought stress-induced responses and the manner

in which they may vary among the four tobacco cultivars. Additionally, though not being the primary objective of the present investigation, it gave us an opportunity to evaluate statements to the effect that, (i) their work and recent data indicate quite unequivocally that higher plants can adjust osmotically through solute accumulation to maintain positive turgor albeit to a limited extent, as they are subjected to increasing drought stress (Wilson *et al.*, 1980) and (ii) because of its adaptive characteristic with a low physiological cost, i.e. of osmoregulation, as it does not impair productivity during and after stress (Ludlow & Machow, 1990). It is statements such as these which fuel hope that the ability to osmoregulate may be the genetical, manipulatible, physiological trait that could enable agriculturists and plant breeders to maintain or increase crop yield during sub-optimal conditions.

10.3 Material and methods

Tobacco, *Nicotiana tabacum* L. cv. TL33, CDL28, GS46 and ELSOMA (in order of increasing drought tolerance) were cultivated in soil in pots under optimal glasshouse conditions for a period of 90 days. Before the onset of experimentation, plants were moved to computerized growth rooms and allowed to acclimatise for 96 hours. For more exact detail regarding growth conditions in the glasshouse and growth rooms, see Van Rensburg & Krüger (1993) and Van Rensburg *et al.* (1993). After the induction of an initial preconditioning drought stress period, which ranged from -0.52 MPa to -2.51 MPa and intensified at a rate of ca. 0.3 MPa d^{-1} by withholding water, the plants were rewatered daily to field capacity for 12 days, and a second drought stress period was then again induced by withholding water. To quantify the drought stress experienced by the plants, predawn leaf water potentials (Zur *et al.*, 1981) were determined with a Scholander pressure chamber (PMS-instrument, Oregon, USA) as De Roo (1969) indicated that the rapid and simple determinations of xylem sap pressure can justifiably be used in estimating absolute values of Ψ_L without accounting for the Ψ_π of the xylem sap. However, due to the size of a tobacco leaf and nature of its petiole, Ψ_L were done on a piece of a leaf. This was done by removing a sidevein with as much as possible intraveneous lamina tissue (from the sixth leaf below the apex) of which the Ψ_L was then determined. This method had two distinct advantages: Firstly the sidevein could be placed in the chamber and sealed airtight within seconds without damaging the conductive tissue. Secondly, it improved accuracy as it reduced the distance between the lamina and cut surface which is important because several authors (Begg & Turner, 1997; Hoffman & Splinter, 1968) have indicated that this resistance to water flow in tobacco is high, and even more so, if the latter is under stress (De Roo, 1970).

Concurrently with each Ψ_L determination (four replications, n = 5) two $10cm^2$ leaf discs per culitvar were sampled from the same leaf (and two from the control) and RWC determined by using the formula RWC = (fresh weight - dry weight)/(turgid weight - dry weight) · 100 and the method as described by Turner (1981). Pressure-Volume (P-V) curves were constructed for each cultivar as described by Hinckley *et al.* (1980) and Richter & Wagner (1983) which represented the inverse of Ψ_L i.e. $(1/\Psi_L)$ versus RWC (Kubiske & Abrams, 1990). From the latter curves the osmotic potential at full turgor (Ψ_π^{100}) (Joly & Zaerr, 1987), the leaf water potential (Ψ_L°) and relative water content at incipient plasmolysis (RWC°) (Turner, 1988), $\in$ from the relationship between Ψ_p and RWC by assuming an approximate linear relation (Wilson *et al.*, 1979; Morgan, 1980a), and Ψ_π (from the relationship between Ψ_π and RWC assuming an approximate linear decrease from Ψ_π^{100} to Ψ_π°), were calculated. The extent of osmotic adjustment was determined as described by Morgan (1980a) where Ψ_p was taken to be the difference between the response line and the line of equality. P-V tissue water component values, calculated from two P-V curves (i.e. constructed from three replicates per leaf of two different

plants), were averaged and standard errors (SD±) calculated for each drought stress period (Tables 1 & 2). Gaussian analytical functions were fitted to the four data sets of Ψ_π and linear regressions to the data sets describing the drought stress-induced decrease in Ψ_π during the second drought stress period, when plotted as functions of RWC (Figs. 2 & 3). Admittedly, the techniques used in characterising Ψ_L together with the components thereof when describing the drought stress-induced chances in the bulk plantwater relations, were to a large extent based on assumptions, approximations and mathematical relations, which may evoke critisism when seen in light of the comments made by Passioura (1988). Although we recognise the validity of some suggested sources of error in the pressure-bomb technique (Talbot *et al.*, 1975), application of this technique can be defended by referring to some of the statements made by Kramer (1988), Schulze *et al.* (1988) and Cosgrove (1988). Furthermore, O'Toole and Moya (1981) have clearly stated that the calibration of the pressure chamber against a standard psychrometric technique was not necessary if relative or comparative estimates of plant water stress were the objective. Thus, since this technique was used in a comparative study, sources of error would have been the same for all four cultivars, indicating that the latter may therefore not serve to explain why differential drought stress-induced changes were observed in the plant water relations of the respective cultivars. As curve fittings were done with simplex algorithms and the smallest square technique (Willmott, 1982), statistical justifiable curves were obtained. This enabled us to quantify the degree of accuracy of deductions and calculations made from the curves supplied.

10.4 Results and discussion

Components of Ψ_L for the leaves of all four tobacco cultivars during the preconditioning drought stress treatment, prior to rehydration on day 12, are shown in Table 1. The similarity between the control Ψ_π^{100} values of the tobacco cultivars confirm the statement of Morgan *et al.* (1991) that in spite of the fact that Ψ_π were used as a measure of drought tolerance in the past (Keim & Kronstad, 1981) when measured alone, it has no value in identifying lines with high osmoregulation. The higher (though not being statistically significant) initial Ψ_π^{100} of the drought-sensitive cultivars (TL33 & CDL28) may merely indicate cultivar differences in the biochemical processes involved in regulating cell energy supply, cell wall extensibility, etc. The adaptive significance of the higher control ϵ of the drought-tolerant cultivars (GS46 & ELSOMA) should be emphasized for they might shorten their response time in identifying a certain shortage of water earlier as being potentially stressfull, before initiating osmoregulatory responses to maintain Ψ_p (Zimmermann, 1978). Furthermore, the lower ϵ values characterising the drought-tolerant cultivars (Table 1) indicate that in these cultivars the maintenance of Ψ_p is to a lesser extent dependent on drought stress-induced volume changes in the leaf cells (Hirton & Zur, 1990), which may in part serve to explain the lower RWC^o values of these cultivars (Table 1) as was predicted by Turner and Jones (1980). Ultimately, however, the adaptive advantage of lower ϵ values with regard to drought tolerance, does not lie in its direct drought stress-induced effect on Ψ_L (as also indicated by Turner and Jones (1980)), but in its possible role as trigger action in initiating additional drought tolerance reactions such as abscisic acid (ABA) accumulation (Van Rensburg & Krüger, unpublished).

Table 1: Calculated P-V tissue water component values during a preconditioning drought stress period of four tobacco cultivars differing in drought tolerance.

Cultivars	Ψ_π^{100} (MPa)	Ψ_π^o (MPa)	RWC^o (%)	ϵ (MPa)	B (%)
TL33	-1.02 ± 0.07	-1.30 ± 0.03	79.3 ± 0.7	0.39 ± 0.07	23.0 ± 0.5
CDL28	-1.03 ± 0.05	-1.51 ± 0.09	75.2 ± 0.6	0.30 ± 0.03	17.5 ± 0.4
GS46	-1.01 ± 0.04	-1.70 ± 0.01	73.4 ± 0.9	0.23 ± 0.06	12.1 ± 0.8
ELSOMA	-0.99 ± 0.02	-1.64 ± 0.03	69.5 ± 0.8	0.25 ± 0.09	11.9 ± 0.9

Since we, as was done by Morgan *et al.* (1984), accepted that Ψ_π = nRT/V, (where n is the number of solute modecules, R the gas constant, T the absolute temperature, and V the osmotic volume which may be approximated by RWC), the drought stress-induced decreases in the Ψ_π values due to increases in n (Fig 1), was differentiated from those due to decreases in RWC causing solute concentration. In the four tobacco cultivars the observed osmotic adjustment during the second drought stress period may largely be attributed to a drought stress-induced increase in n as evidenced by initial slopes of less than one in the relationship log Ψ_π *versus* the

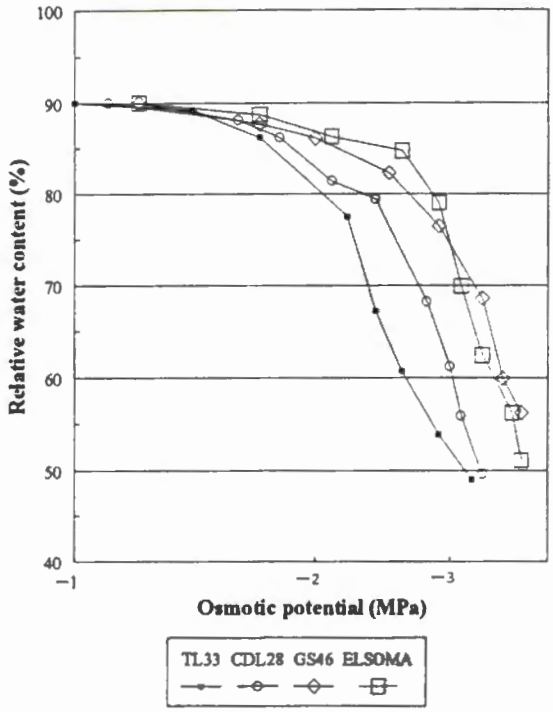


Figure 1. Logarithmic plots of RWC *versus* Ψ_π reflecting differences in the extent and duration of RWC maintenance, in four tobacco cultivars of differing drought tolerance, due to differential drought stress-induced changes in Ψ_π. The Gaussian analytical function which best described the trends observed was $y = 90.77 \times \exp(-(x - 1.02)^2/(2 \times 1.87^2))$ and $y = 89.84 \times \exp(-(x - 1.38)^2/(2 \times 1.82^2))$ for TL33 and CDL28 respectively opposed to $y = 89.52 \times \exp(-(x - 1.52)^2/(2 \times 2.30^2))$ and $y = 91.23 \times \exp(-(x\ 1.72)^2/(2 \times 1.89^2))$ for the two drought-tolerant tobacco cultivars, respectively. R^2 was equal to 0.98 for all four curves.

dependent variable, log RWC (Fig 1). As was also observed by Morgan (1977), during drought stress in wheat genotypes, the tissue water relations of the tobacco cultivars changed in two phases. The initial phase usually involved full or partial maintenance of RWC and a subsequent second phase in which RWC decreased rapidly. The duration of this initial phase differed between the tobacco cultivars, ranging from Ψ_π values of between ca. -1.2 MPa at a RWC of ca. 88% to ca. = -2.6 MPa at a RWC of ca. 84% in the drought-tolerant cultivars, and from ca. -1.1 MPa at a RWC of ca. 86% to ca. -2.2 MPa at a RWC of ca. 78% in the drought-sensitive cultivars. In terms of osmoregulation, this would seem to indicate that in combination with other factors, the -1.4 MPa decrease in Ψ_π enabled the drought-tolerant cultivars to limit the drought stress-induced decrease in RWC to ca. 4% upon reaching a Ψ_L of ca. -1.0 MPa, opposed to the ca. 9% decrease in RWC allowed by the ca. -1.1 MPa difference in Ψ_π in the drought-sensitive cultivars. The latter difference may to a large extent be explained by the differential drought stress-induced increase in n which was more pronounced in GS46 ($y = -0.38 + 3.07$ x, $R^2 = 0.84$) and ELSOMA ($y = -0.49 + 3.36$ x, $R^2 = 0.81$) than that observed for TL33 ($y = -0.4 + 2.21$ x, $R^2 = 0.93$) and CDL28 ($y = -0.07 + 2.43$ x, $R^2 = 0.79$), as is clear from the steeper slopes of the linear regressions which describe the relationship of Ψ_π versus Ψ_L (Fig 2). Though not being as pronounced, this tendency was maintained during the second phase as the drought stress

intensified, with the result that the response of TL33 and CDL28 were characterised by the linear regressions $y = -0.88 + 1.68\ x$, $R^2 = 8.9$ and $y = -0.73 + 1.72\ x$, $R^2 = 0.77$ respectively, and those of GS46 and ELSOMA by $y = -1.22 + 2.09\ x$, $R^2 = 0.84$ and $y = -1.27 + 2.17\ x$, $R^2 = 0.82$, respectively. It is interesting to note that at no stage during the second drought stress period did

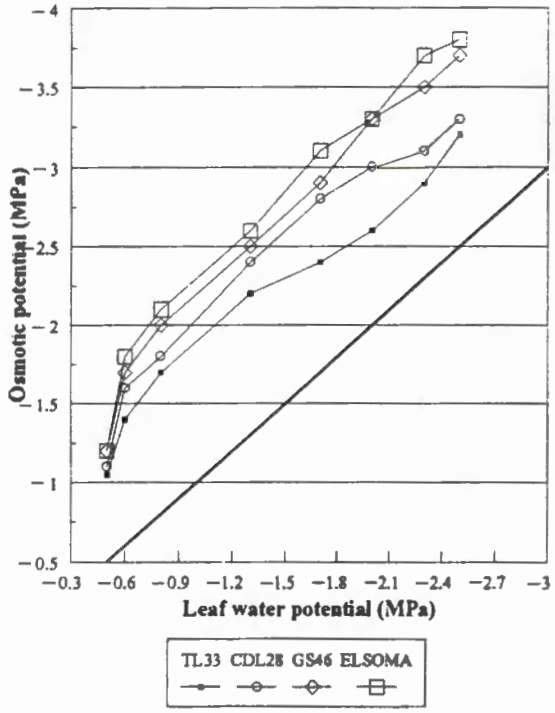


Figure 2. Biphasic relationship between drought stress-induced changes in Ψ_L and Ψ_π in two drought-sensitive (TL33 & CDL28) and two drought-tolerant (GS46 & ELSOMA) *Nicotiana tabacum* L. cultivars during a second drought stress period. The straight line represents the line of equality i.e. the response of a perfect osmometer, deviations from which are indicative of osmoregulation due to the solute accumulation.

the slope of any of the curves depicting the relationship Ψ_π versus Ψ_L (Fig 2) cross the line of equality. This is in accordance with previous results which indicated a capability on the part of all four tobacco cultivars to accumulate both ABA and proline up to a Ψ_L of -2.5 MPa (Van Rensburg & Krüger, unpublished).

As was the case in some chickpea lines (Morgan *et al.*, 1991), after the onset of the drought stress period, Ψ_p was not maintained but decreased at differential rates in the four tobacco cultivars (Fig 3). The rates at which Ψ_p decreased was less pronounced in the drought-tolerant cultivars, as evidenced by the lower slopes of the linear regressions which characterise this drought stress-induced response. Three possible reasons may serve to explain why the decrease in Ψ_p occurred at a higher rate in the drought-sensitive cultivars: i.e. the lower $\in$ that characterises these cultivars (Table 2), the less pronounced accumulation of solutes during drought stress (Fig 2), and the higher rate at which their membrane permeability increases during drought stress (Van Rensburg *et al.*, 1993). The validity of the latter statement is supported by the research done by Kyriakopoulos and Richter (1981) which indicated that the use of Ψ_p

against 1/RWC may be a useful way of demonstrating partial membrane damage arising from drought stress-induced injury. RWC maintenance during drought stress, such as that observed in the present investigation (Fig 1), which occurred mainly due to solute accumulation, must involve maintenance of hydration, though the more specific relationship between these factors are also dependent on the elasticity of the cell walls (Wenkert *et al.*, 1978). The physiological adaptive advantage of effective turgor maintenance by osmoregulation has been exhaustively described by several authors (Zimmermann, 1978; Turner & Jones, 1980; Morgan, 1980b; Morgan, 1984).

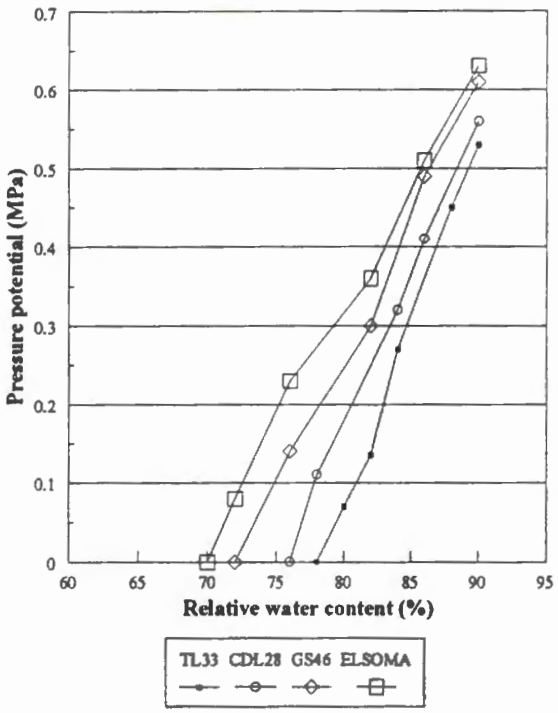


Figure 3. Differential degrees of calculated Ψ_p "maintenance" as indicated by the slopes of the curves which describe the relationship Ψ_p *versus* RWC in TL33 (y = -3.72 + 0.05 x) and CDL28 (y = -2.83 + 0.04 x) the two drought-sensitive, and GS46 (y = -2.49 + 0.04 x) and ELSOMA (y = -2.06 + 0.03 x) the two drought-tolerant tobacco cultivars. R^2 for the four curves was ca. 0.97.

Table 2: Calculated P-V tissue water component parameters during a second drought stress period of four *Nicotiana tabacum* L. cultivars differing in drought tolerance.

Cultivars	Ψ_π^{100} (MPa)	Ψ_π^{o} (MPa)	RWC^{o} (%)	ϵ (MPa)	B (%)
TL33	-1.05 ± 0.03*	-1.27 ± 0.04	78.7 ± 0.1	0.41 ± 0.02	23.3 ± 0.8
CDL28	-1.07 ± 0.09	-1.46 ± 0.06	76.5 ± 0.5	0.35 ± 0.04*	18.1 ± 0.7
GS46	-1.13 ± 0.01**	-1.67 ± 0.09	72.3 ± 0.5	0.33 ± 0.09**	15.7 ± 0.5**
ELSOMA	-1.15 ± 0.05	-1.63 ± 0.07	70.5 ± 0.8	0.36 ± 0.09**	14.9 ± 0.2**

Values of water relation parameters that deviated significantly (* i.e. $p < 0.05$) or highly significantly (** i.e. $p < 0.01$) during the second drought stress period, from that determined during the preconditioning drought stress period as supplied in Table 1.

Lecoeur *et al.* (1992) have said that since they used adult organs, as did we, drought stress-induced variations in ϵ can be neglected and then postulated that the mere stability of RWC (see Fig 1) may be used as a presumption of Ψ_p maintenance by osmoregulation. The ϵ of all four tobacco culitvars decreased, and the ϵ values were statistically significantly ($p < 0.01$) higher in the drought-tolerant cultivars during the second drought stress period. This observation is in accordance with that made by Jones & Turner (1978) in drought stressed sorghum leaves, but according to Wilson *et al.* (1980) it is debatable whether higher ϵ values (Table 2) represent a real change in rigidity of the cell walls. We think that in spite of the consistency of RWC° (Tables 1 & 2) during the second drought stress period, the drought stress-induced decrease in ϵ (TL33, 5%; CDL28, 3%; GS46, 40% and ELSOMA, 30%) was real, but transient, and not merely a mathematical consequence of higher Ψ_π^{100} and greater maximum Ψ_p (Table 2 *versus* Table 1). In explaining the latter statement it should be realised that the B values of all four the tobacco cultivars were consistently higher during the second drought stress period (TL33, 1%; CDL28, 3%; GS46, 30% and ELSOMA, 25%), differences being statistically ($p < 0.01$) highly significant in the drought-tolerant cultivars. As the B value has been associated with the volume of cell wall material (Boyer, 1967), it would seem to indicate an increase in the total cell wall content; more so in the drought-tolerant cultivars, from that of their respective controls (Table 1) during the second drought stress period (Table 2). The increase in ϵ may however be transient, as it has been found to completely dissipate after 15 days of rehydration in rice (Morgan, 1984). On average, the drought stress-induced contribution of both ϵ and B was ca. 32% in the drought-tolerant and ca. 3% in the drought-sensitive cultivars, from which it may be deduced that their combined response accounted for less than ca. 0.45 MPa and ca. 0.03 MPa of the achieved osmoregulation (as reflected by the drought stress-induced change in Ψ_π when $\Psi_L = 1.0$ MPa), respectively. In light of the research done by Morgan (1980a) which indicated that the capacity for osmoregulation, may remain unchanged or increase with age, and as we found that ϵ did change in response to drought stress, we cannot entirely agree with the above mentioned statement made by Lecoeur *et al.* (1992).

The drought stress induced decrease in ϵ due to an increase in B (Table 1 & Table 2) may be only a consequence of an integrated plant response, as the plants attempt to maintain their transpiration rates, for it is most likely that transpirational water largely moves intercellularly and that much of the water in the xylem may never exchange with water present in the bulk of the mesophyll cells (Matsuda & Raizi, 1981). The latter contention is supported by research that showed that genotypes with high levels of osmotic adjustment are characterized by higher photosynthetic rates due to lower leaf resistances at low Ψ_L (Blum, 1974; Oosterhuis & Wullschleger, 1987) in order to gain as much carbon as possible, which may render them better adapted for growth in an altered environment (Wilson *et al.*, 1980; Van Rensburg & Krüger, 1993). Thus, even after turgor is lost, i.e. at RWC°, the drought-tolerant cultivars with higher

osmoregulation will have a higher water content at low Ψ_L, which would seem to enable survival at lower Ψ_L values (Morgan *et al.*, 1984).

10.5 Conclusion

Although the initial volume of leaf water loss needed to reduce Ψ_p to zero, remained unchanged in osmotic adjusted leaves, it would seem that when examined in terms of drought stress-induced changes in Ψ_L and RWC, osmotic adjustment may be effective in combating a drought stress-induced decrease in Ψ_p (more so in drought-tolerant tobacco than drought-sensitive tobacco cultivars). The observed changes in bulk Ψ_π of all tobacco cultivars could contribute to increased leaf-soil water potential gradients and therefore to an improved ability to absorb the decreasing available soil water. Since the differences in osmoregulation were observed under controlled environmental conditions in this study and several others (Morgan, 1984 and others cited by him), it woud suggest that differences in the ability to osmoregulate may be genetically and not environmentally based, and therefore be directly applicable in drought tolerance selection.

10.6 Literature cited

BEGG, J.E. & TURNER, N.C. 1970. Water potential gradients in tobacco. *Plant Physiology,* 46: 343-346.

BLUM, A. 1974. Genotipic responses in sorghum to drought stress. II: Leaf tissue water relations. *Crop Science,* 14: 691-692

BOYER, J.S. 1967. Matric potentials of leaves. *Plant Physiology,* 42: 213-217.

COSGROVE, D.J. 1988. In defence of the cell volumetric elastic modulus. *Plant Cell Environment,* 11:67-69.

DE ROO, H.C. 1969. Water stress gradients in plants in soil-root systems. *Agronomy Journal,* 16: 511-515.

DE ROO, H.C. 1970. Leaf water potentials of tobacco, estimated with the pressure bomb. *Tobacco Science,* 14: 105-106.

HINCKLEY, T.M. DUHME, F. HINCKLEY, A.R. & RICHTER, H. 1980. Water relations of drought hardy shrubs: osmotic potential and stomatal reactivity. *Plant Cell Environment,* 3: 131-140.

HIRTON, O. & ZUR, B. 1990. Differences in stomatal response within a cotton canopy. *Biotronics,* 19: 39-48.

HOFFMAN, G.J. & SPLINTER, W.E. 1968. Water potential measurements of an intact plant-soil system. *Agronomy Journal,* 60: 408-413.

JOLY, R.J. & ZAERR, J.B. 1987. Alteration of cell-wall water content and elasticity in Douglas-Fir during periods of water deficit. *Plant Physiology,* 83: 418-422.

JONES, M.M. & TURNER, N.C. 1978. Osmotic adjustment in leaves of sorghum in response to water deficits. *Plant Physiology,* 61: 122-126.

KEIM, D.L. & KRONSTAD, W.E. 1981. Drought response of winter wheat cultivars grown under field stress conditions. *Crop Science,* 21: 11-15.

KRAMER, P.J. 1988. Changing concepts regarding plant water relations. *Plant Cell Environment*, 11: 565-568.

KUBISKE, M.E. & ABRAMS, M.D. 1990. Pressure-volume relationships in non-rehydrated tissue at various water deficits. *Plant Cell Environment*, 13: 995-1000.

KYRIAKOPOULOS, E. & RICHTER, H. 1981. Pressure-volume curves and drought injury. *Physiologia Plantarum*, 52: 124-128.

LECOEUR, J., WERY, J. & TURC, O. 1992. Osmotic adjustment as a mechanism of dehydration postponement in chickpea (*Cicer arietinum* L.) leaves. *Plant and Soil*, 144: 177-189.

LUDLOW, M.N. & MUCHOW, R. 1990. A critical evaluation of traits for improving crop yields in water-limited environments. *Advances in Agronomy*, 43: 107-153.

MATSUDA, K. & RIAZI, A. 1981. Stress-induced osmotic adjustment in growing regions of barley. *Plant Physiology*, 68: 571-576.

MORGAN, J.M., RODRIGUEZ-MARIBONA, B. & KNIGHTS, E.J. 1991. Adaptations to water-deficit in chickpea breeding lines by osmoregulation: relationship to grain yield in the field. *Crop Research*, 27: 61-70.

MORGAN, J.M. 1977. Differences in osmoregulation between wheat genotypes. *Nature*, 270: 234-235.

MORGAN, J.M. 1980a. Osmotic adjustment in the spikelets and leaves of wheat. *Journal of Experimental Botany*, 31: 655-665.

MORGAN, J.M. 1980b. Differences in adaptation to water stress within crop species. (*In* Turner, N.C. & Kramer, P.J., *eds.* Adaptation of plants to water and high temperature stress, New York: Wiley & Sons. p.369-382.)

MORGAN, J.M. 1984. Osmoregulation and water stress in higher plants. *Annual Review of Plant Physiology*, 35: 299-319.

O'TOOLE, J.C. & MOYA, T.B. 1981. Comparison of pressure chamber and psychrometer estimates of leaf water potential in rice. *Plant and Soil*, 62: 313-317.

OOSTERHUIS, D.M. & WULLSCHLEGER, S.D. 1987. Osmotic adjustment in cotton (*Gossypium hirsutum* L.). Leaves and roots in response to water stress. *Plant Physiology,* 84: 1154-1157.

PASSIOURA, J.B. 1988. Response to Dr. P.J. Kramer's article, 'Changing concepts regarding plant water relations,' Volume 11, Number 7, pp. 565-568. *Plant Cell Environment,* 11: 569-571.

SCHULZE, E.-D., STEUDLE, E., GOLLAN, T. & SCHURR, U. 1988. Response to Dr. P.J. Kramer's article, 'Changing concepts regarding plant water relations', Volume 11, Number 7, pp. 565-568. *Plant Cell Environment,* 11:573-576.

STEUDLE, E., ZIMMERMANN, U. & LüTTGE, U. 1977. Effect of turgor pressure and cell size on the wall elasticity of plant cells. *Plant Physiology,* 59: 285-289.

TALBOT, A.J.B., TYREE, M.T. & DAINTY, J. 1975. Some notes concerning the measurement of water potentials of leaf tissue with specific reference to *Tsuga canadensis* and *Picea abies*. *Canadian Journal of Botany,* 53: 784-788.

TURNER, N.C. 1981. Techniques and experimental approaches for the measurement of plant water status. *Plant and Soil,* 58: 339-366.

TURNER, N.C. 1988. Measurement of plant water status by the pressure chamber technique. *Irrigation Science,* 9: 289-308.

TURNER, N.E. & JONES, M.M. 1980. Turgor maintenance by osmotic adjustment.: A review and evaluation. (*In* Turner, N.C. & Kramer, P.J., *eds.* Adaptation of plants to water and high temperature stress, Ch. 7, New York: Wiley & Sons. p.87-103.)

VAN RENSBURG, L. & KRüGER, G.H.J. 1993. Comparative analysis of differential drought stress-induced suppression of and recovery in carbon dioxide fixation: stomatal and non-stomatal limitation in *Nicotiana tabacum* L. *Journal of Plant Physiology,* 142: 269-306.

VAN RENSBURG, L., KRüGER, G.H.J. & KRüGER, H. 1993. Proline accumulation as drought tolerance selection criterion: its relationship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. *Journal of Plant Physiology,* 141: 188-194.

WENKERT, W., LEMON, E.R. & SINCLAIR, T.R. 1978. Water content-potential relationship in soybean: Changes in component potentials for mature and immature leaves under field conditions. *Annals of Botany*, 42: 295-307.

WILLMOTT, C.J. 1982. Some comments on the evaluation of model performance. *Bulletin of the American Meteorological Society*, 63: 1309-1313.

WILSON, J.R., LUDLOW, M.M., FISHER, M.J. & SCHULZE, E.-D. 1980. Adaptation to water stress of the leaf water relations of four tropical forage species. *Australian Journal of Plant Physiology*, 7: 207-220.

WILSON, J.R. FISHER, M.J., SCHULZE, E.-D., DOLBY, G.R. & LUDLOW, M.M. 1979. Comparison between pressure-volume and dewpoint-hygrometry techniques for determining the water relations characteristics of grass and legume leaves. *Oecologia (Berl.)*, 41: 77-88.

ZIMMERMANN, U. 1978. Physics of turgor and osmoregulation. *Annual Review of Plant Physiology*, 29: 121-148.

ZUR, B., BOOTE,K.J. & JONES, J.W., 1981. Changes in internal water relations and osmotic properties of leaves in maturing soybean plants. *Journal of Experimental Botany*, 32: 1181-1191.

CHAPTER ELEVEN

11 General discussion

In recent years considerable advances were made in our understanding of plant responses to drought stress (see general reviews by Hsiao, 1973; Bradford & Hsiao, 1982; Schulze, 1986; Turner, 1986). In this regard I am of the opinion, as was Chaves (1991), that the impact of a given type of stress on plant performance and, therefore, functionality during drought stress, is the result of the interaction between the plant, functioning as a controlled system according to its genetic potential and information, and the stress, imposing limitations of variable intensity depending on its severity and duration. It may therefore be assumed, as did Chaves (1991), that many of the responses of plants under drought stress are of a regulatory nature rather than drought stress-induced damage. Any such response as the above mentioned should however, be subjected to intense scrutiny for though one may be tempted to assume that if a modification in a character survives, it must be beneficial (i) Taylor *et. al.* (1983) warned that the presence of a character, and, (ii) Hanson and Hitz (1982) stated that the mere correlation of the accumulation of a substance and the onset of drought stress, is not enough proof that it is essential or even beneficial to survival of the plant in the altered environment.

Furthermore, when assessing adaptive responses, it is pertinent to remember that there can be conflict between the requirements for survival which was important in the evolution of natural vegetation and the requirements for production necessary in agricultural crops (Fischer & Turner, 1978). It is axiomatic that survival is an important component of production but the reverse does not always apply (Ludlow, 1980). It also should be emphazed that the ability to maintain an acceptable degree of functionality rarely depends on posession of a single adaptive character. According to Turner (1979), fitness of adaptation of a plant to an environment depends on possession of an optimum combination of characters that minimizes the deleterious effects and maximizes the advantageous effects.

From the foregoing sections of this thesis, it is possible to make a few very important general observations over and above that drawn at the end of each paper, relevant to the selection for drought tolerance in *Nicotiana tabacum* L. plants. Firstly, drought tolerance is the result of numerous anatomical, physiological and biochemical characteristics, both constitutive and inducible, which interact to allow the maintenance of differential degrees of functionality during drought stress. Although the value of individual mechanisms in the overall system of the plant should be considered, it would seem as if dramatic examples of a single mechanism being responsible for increased drought tolerance can only be cited for extremely xerophytic species (Steponkus *et al.*, 1979). The drought tolerance of crops species such as tobacco should be

considered to be a complex of interacting mechanisms and adaptive responses, all contributing to the observed plant performance. Secondly, drought tolerance is the result of both long-term constitutive and short-term facultative adaptations. The latter inducible mechanisms, which in turn depend on phylogenetic adaptation *a priori*, may allow for both amelioration of external factors, which influence the internal plant water status and amelioration of the plant's tolerance of internal water deficits.

The ability to adjust to the environment is one of the most important but difficult to study attributes of plant behaviour. It is vitally important because it has immense practical significance as argued in the general introduction. In this regard it is supportive to note that Yeo and Flowers (1989) concluded, that selection on the basis of physiological characters has several advantages in breeding for complex whole plant responses such as drought tolerance. Where tolerance depends on the summation of several characters, each may be phenotypically cryptic because it is not alone sufficient to confer tolerance, but still needs to be recognized in their own right. It is difficult to study these characteristics because of the plasticity and variability of plant response, which does not only make it difficult to identify responses having adaptive value with a high degree of certainty but also makes it difficult to obtain values with acceptable standard deviations. To be a scientist studying drought stress and adaptation, is an exciting but humbling experience. In this thesis I have attempted to identify the various physiological, anatomical and biochemiscally related mechanisms of adaptation to drought stress and to evaluate their importance in the light of survival, and secondely productivity. The latter led me to the third general conclusion, that for future research in this field, in addition to the physiolocigal and interdisciplinary approach, genetic analysis will be essential in the establishment of a causal relationship between the induction of the adaptive response reported on in this thesis and the establishment of the plant's tolerance to the stress condition.

In conclusion, it is postulated that in practice, when attempting to breed crop plants, such as tobacco, with more drought tolerance adaptive characteristics which has to fit into agricultural systems, will require more coordination than is usually possible among individual scientists working on different species at diverse locations. Establishment of interdisciplinary research teams with a long term mandate to discover new principles and to develop new techniques, should be able to expedite this task. The physiological dissection of the plants, such as that for tobacco presented in this thesis, should be particularly useful in clarifying how certain physiological, biochemical and anatomical traits might be associated and how they could be selected from the broad base of known mechanisms.

In this regard, I am of the opinion that selection of a combination of several individual traits will improve the likelihood of isolating superior genotypes. This initial population shoud, however, also be agronomically adapted, for then it would require much less refinement after selection than a population that has been changed by the introduction of widely divergent genoplasm (Heichel, 1983).

12 Literature cited in chapter one and chapter eleven

AGGARWAL, P.K. & SINHA, S.K. 1984. Effects of water stress on grain growth and assimilate partitioning in two cultivars of wheat contrasting in their yield stability in a drought-environment. *Annals of Botany*, 53: 329-340.

ASADA, K. & TAKAHASHI, M. 1987. Production and scavenging of active oxygen in photosynthesis. (*In* Kyle, D.J., Osmond, C.B. & Arntzen, C.J., *eds*. Photoinhibition. Amsterdam: Elsevier. p.227-287.)

BEGG, J.E. 1980 Morphological adaptations of leaves to water stress (*In* Turner, N.C. & Kramer, P.J., *eds*. Adaptations of plants ot water and high temperature stress. New York: John Wiley and Sons. p.7-20.)

BEWLEY, D.J., LARSEN, K.M. & PAPP, J.E.T. 1983. Water stress-induced changes in the pattern of protein synthesis in maize seedling mesocotyls: a comparison with the effect of heat shock. *Journal of Experimental Botany*, 34: 1126-1133.

BLUM, A. 1983. Genetic and physiological relationships in plant breeding for drought resistance. (*In* Stone, J.F. & Wills, W.O., *eds*. Plant production and management under drought conditions. Amsterdam: Elsevier Science Publishers. p. 195-205.)

BLUM, A. 1989. Breeding methods for drought resistance (*In* Jones, H.G., Flowers, T.J. & Jones, M.B., *eds*. Plants under stress: Biochemistry, physiology and ecology and their application to plant improvement. Cambridge: Cambridge University Press. p.197-216.)

BOYER, J.S. 1982. Plant productivity and environment. *Science*, 219: 443-448.

BRADFORD, K.H., & HSIAO, T.C. 1982. Physiological responses to moderate water stress. (*In* Lange, O.L., Nobel, P.S., Osmond, C.B., & Ziegler, H., *eds*. Physiological plant ecology II. Water relations and carbon assimilation. New York: Springer-Verlag. p. 263-324.)

CHAVES, M.M. 1991. Effects of water deficits on carbon assimilation. *Journal of Experimental Botany*, 42: 1-16.

DEMMIG, B., WINTER, K., KRüGER, A & CZYGAN, F.C. 1987. Photoinhibition and zeaxanthin formation in intact leaves. A possible role of the xanthophyll cycle in the dissipation of excess light energy. *Plant Physiology*, 84: 218-224.

ECHECIN, P. 1970. The origins of plants. (*In* Harbone, J.B., *ed.* Phytochemical phylogeny. New York: Academic Press. p. 1-17.)

FARQUHAR, G.D., WONG, S.C., EVANS, J.R. & HUBICK, K.T. 1989. Photosynthesis and gas exchange. (*In* Jones, H.G., Flowers, T.J. & Jones. M.B., *eds.* Plants under stress: Biochemistry, physiology and ecology and their application to plant improvement. Cambridge: Cambridge University Press. p.47-69.)

FINLEY, K.C. & WILKINSON, G.N. 1963. The analysis of adaptation in plant breeding programme. *Australian Journal of Agricultural Research,* 14: 742-761.

FISCHER, R.A., & TURNER, N.C. 1978. Plant productivity in the arid and semi-arid zones. *Annual Review of Plant Physiology,* 29:227.

GAFF, D.F. 1966. The sulphydryl-disulphide hypothesis in relation to dessication injury of cabbage leaves. *Australian Journal of Biological Sciences,* 19: 291-299.

GRIME, J.P. 1989. Whole-plant responses to stress in natural and agricultural systems. (*In* Jones, H.G., Flowers, T.J. & Jones, M.B. *eds.* Plants under stress: Biochemistry, physiology and ecology and their application to plant improvement. Cambridge: Cambridge University Press. p. 31-46.)

HANSON, A.D., & HITZ, W.D. 1982. Metabolic responses of mesophytes to plant water deficits. *Annual Review of Plant Physiology,* 33: 163-203.

HANSON, W.D. & ROBINSON, H.F. 1963. Statistical genetics and plant breeding. Washington, DC. National Academy of Sciences: National Research Council.

HEICHEL, G.H. 1983. Crop manipulation for efficient use of water: Inadvertent and intended manipulation of crop water use (*In* Taylor, H.M., Jordan, W.R., & Sinclair, T.R., *eds.* Limitations to efficient water use in crop production. Wisconsin, U.S.A.: American Society of Agronomy. p.375-380.)

HSIAO, T.C., 1973. Plant responses to water stress. *Annual Review of Plant Physiology*, 24: 519-570.

KRAMER, P.J. 1980. Drought stress, and the origin of adaptations (*In* Turner, N.C. & Kramer, P.J., *eds*. Adaptations of plants to water and high temperature stress. New York: John Wiley and Sons. p. 7-20.)

LEVITT, J. 1962. A sulphydryl-disulphide hypothesis of frost injury and resistance in plants. *Journal of Theoretical Biology*, 3: 344-391.

LUDLOW, M.M. 1980. Adaptive significance of stomatal responses to water stress. (*In* Turner, N.C. & Kramer, P.J., *eds*. Adaptation of plants to water and high temperature stress. New York: John Wiley and Sons. p. 123-138.)

PASSIOURA, J.B. 1976. The control of water movement through plants. (*In* Wardlaw, I.F. & Passioura, J.B., *eds*. Transport and transfer processes in plants. New York: Academic Press. p. 373-380.)

RUMBAUGH, M.D., ASAY, K.H. & JOHNSON, D.A. 1984. Influence of drought stress on genetic variances of alfalfa and wheat grass seedlings. *Crop Science*, 24: 297-302.

SCHULZE, E.D. 1986. Carbon dioxide and water vapour exchange in response to drought in the atmosphere and in the soil. *Annual Review of Plant Physiology*, 37: 247-274.

SHARP, R.E., & DAVIES, W.J. 1989. Regulation of growth and development of plants growing with a restricted water supply. (*In* Jones, H.G., Flowers, T.J. & Jones, M.B. *eds*. Plants under stress: Biochemistry, physiology and ecology and their application to plant improvement. Cambridge: Cambridge University Press. p. 71-94.)

STEPONKUS, P.L., CUTLER, J.M., & O'TOOLE, J.C. 1979. Adaptation to water stress in rice. (*In* Turner, N.C. & Kramer, P.J., *eds*. Adaptation of plants to water and high temperature stress. New York: John Wiley and Sons. p. 123-138.)

TAYLOR, H.M., JORDAN, W.R., & SINCLAIR, T.R. 1983. Limitations to efficient water use in crop production. United States of America: *American Society of Agronomy*, 538p.

TURNER, N.C. 1979. Drought resistance and adaptation to water deficits in srop plants. (*In* Mussel, H. & Staples, R.C., *eds*. Stress physiology in crop plants. New York: John Wiley and Sons. p. 343-372.)

TURNER, N.C. 1986. Crop water deficits: a decade of progress. *Advances in Agronomy,* 39: 1-51.

YEO, A.R., & FLOWERS, T.I. 1989. Selection for physiological characters - examples from breeding for salt tolerance. (*In* Jones, H.G., Flowers, T.J. & Jones, M.B., *eds.* Plants under stress: Biochemistry, physiology and ecology and their application to plant improvement. Cambridge: Cambridge University Press. p. 217-234.)