THE EFFECTS OF ONTOGENY AND ENVIRONMENTAL OSCILLATIONS ON PLANT RESPONSES TO OXYGEN DEPRIVATION

Fabio Rubio Scarano

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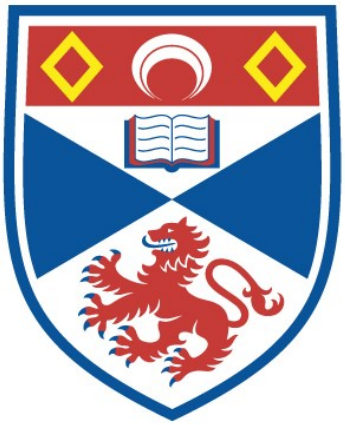
University of St Andrews



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THE EFFECTS OF ONTOGENY AND ENVIRONMENTAL OSCILLATIONS ON PLANT RESPONSES TO OXYGEN DEPRIVATION

by

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Thesis submitted in
application for the degree
of Doctor of Philosophy

JULY 1992

I, Fábio Rubio Scarano, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Fábio Rubio Scarano
St.Andrews, July 1992

I was admitted to the Faculty of Science of the University of St.Andrews as a candidate for the degree of Ph.D. in October 1988.

Fábio Rubio Scarano
St.Andrews, July 1992

I hereby certify that the Candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

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Similarity but not truth; apparent freedom but not freedom: it is because of these two fruits that the Tree of Science does not run the risk of being mistaken for the Tree of Life.

****

Semelhança mas não verdade; aparência de liberdade mas não liberdade: por causa desses dois frutos a Árvore da Ciência não corre o risco de ser confundida com a Árvore da Vida.

(F. Nietzsche)

ABSTRACT

The effects of ontogeny and environmental oscillations on plant responses to oxygen deprivation were investigated for a wide spectrum of species, ranging from agricultural to forestry crops, and from temperate to tropical plants. The extent to which hypoxia- or anoxia-tolerance were affected by ontogeny and environmental oscillations, was assessed mainly through changes in survival and growth and physiological parameters such as respiratory activity, ethanol production and carbohydrate depletion.

Anoxia-tolerance of germinating seeds of chickpea (***Cicer arietinum*** L.) was found to vary, even within the earlier stages of germination, according to the length of the aerobic imbibition period previous to the anoxic shock. The notable fact was that 6 minutes of seed aerobic imbibition prior to anoxic treatment was sufficient to significantly increase post-anoxic survival after 4 days anoxia, compared to seeds not allowed to previously imbibe aerobically. These survival results were mirrored by the significant increase in the oxygen uptake by the embryos of seeds which were allowed to imbibe aerobically for 2 hours prior to anoxia, compared to the embryos of anaerobically imbibed seeds.

Germination stage also affected the response of barley (***Hordeum vulgare*** L.) to washing under anoxia, a treatment used to investigate membrane stability and other factors associated with anoxic injury. Temperature, frequency of washing and nutrients present in the washing solution also affect the post-anoxic responses of barley. Daily washing of seedlings under anoxia was often detrimental to post-anoxic survival. One washing only, at the end of the anoxic period, often enhanced survival. This positive effect seemed to be more linked to protection against plasmamembrane leakage due

to calcium ions present in the washing solution than to removal of anaerobically-produced potentially toxic volatiles.

Two Brazilian tree species were also studied. The seeds of ***Parkia pendula* (Willd.) Benth. ex. Walp.**, a species typically present in unflooded areas in the Amazon, were still able to germinate after seven months submergence. ***P.pendula*** one-month old seedlings, however, did not survive longer than one month flooding, which can be a considerable disadvantage in the ca. six-month long flooding period of the Amazon floodplains. However, adult trees can still be found, although rarely, in flooded areas. The possible strategies involved in an eventual establishment of ***P.pendula*** individuals in flooded areas of the Brazilian Amazon are discussed. A contrast is drawn between the responses to flooding of this species and flood-tolerant ***Parkia discolor***.

***Enterolobium contortisiliquum* (Vell.) Morong**, is a tree species which is present in both the dry soils of the *cerrados* (neotropical savannas) in Central Brazil and in the flood-prone Gallery Forests. This species showed considerable tolerance to flooding and drought, as reflected by the various morphological and metabolic adaptations observed in response to these stresses. The role of the xylopodium, a rigid wood tuber, in such tolerance to flood and drought stresses is discussed.

Water-stressed roots of some crop species studied presented higher ethanol levels than control plants. Additionally, these same drought-treated roots showed a considerable amount of shrinkage compared to control roots, as measured by root diameter. It is argued that drought causes root shrinkage, which possibly reduces ability of such roots to capture oxygen and results in hypoxia in the tissues and

consequent increase in ethanol production. This hypothesis of drought-induced hypoxia is compared with several recent findings in the literature, and is discussed as a possible factor which allows drought, under specific circumstances, to acclimatise plants to a subsequent flooding. From preliminary experiments with alternation of flooding and drought in ***Eucalyptus*** species, it appeared that a previous stress affects a plant's response to a subsequent stress. This effect was not always negative, and in ***E.regnans*** a five-week drought allowed a subsequent 3-fold increase in flooding survival. This experiment, however, needs to be repeated in order to confirm these results.

A common cause for anaerobic injury seems to be unlikely for the diverse plants studied, and anoxia survival often seemed to be related to a combination of morphological and metabolic adaptations.

A critical reflection on the risks of labelling plants as tolerant or sensitive to oxygen deprivation is provided, as well as a discussion on the perspectives of applied research which may further the development of ecophysiological theory.

RESUMO

Os efeitos de ontogenia e oscilações ambientais sobre as respostas de plantas à deprivação de oxigênio foram estudados para um amplo espectro de espécies, incluindo plantas agrícolas e florestais, tropicais e temperadas. O grau de impacto de tais efeitos na tolerância à deficiência de oxigênio das diversas espécies, foi avaliado com base em observações de sobrevivência, crescimento e sintomas morfológicos, e de parâmetros fisiológicos como atividade respiratória, produção de etanol (produto potencialmente tóxico gerado por acelerada atividade glicolítica) e consumo de carboidratos.

Demonstrou-se que a tolerância à anoxia de sementes de grão-de-bico (***Cicer arietinum*** L.) em processo de germinação varia, mesmo durante os momentos iniciais da germinação, em função da duração do período de embebição de água antecedendo o choque anóxico. Notavelmente, 6 minutos de embebição aeróbica das sementes, anteriormente ao choque anóxico de 4 dias, foram suficientes para aumentar de forma significativa a sobrevivência pós-anóxica das plântulas, comparando com sementes que não tiveram acesso à embebição aeróbica prévia. Tal resposta positiva em termos de sobrevivência parece estar relacionada com uma significativa vantagem em termos de consumo de oxigênio na fase pós-anóxica apresentada por embriões de sementes que tiveram acesso a duas horas de embebição aeróbica anteriormente ao choque anóxico, comparados com embriões de sementes embebidas sob anaerobiose.

A fase germinativa de sementes também influenciou a resposta de cevada (***Hordeum vulgare*** L.) à "lavagem" sob anoxia, tratamento usado para investigar estabilidade de membrana e outros fatores associados com injúria anóxica. Temperatura,

freqüência de lavagem e nutrientes presentes na solução usada para lavagem também afetaram as respostas pós-anóxicas de plântulas de cevada. Lavagem diária de plântulas sob anoxia foi um tratamento prejudicial à sobrevivência pós-anóxica. Porém, a efetuação de apenas uma lavagem, ao final do período anóxico, prolongou a sobrevivência pós-anóxica de plântulas de cevada. Este efeito positivo pareceu estar mais relacionado à proteção à membrana plasmática causada por ions de cálcio presentes na solução de lavagem, do que à remoção de produtos potencialmente tóxicos produzidos pelas plântulas sob anoxia.

Duas espécies florestais brasileiras também foram estudadas. Sementes de ***Parkia pendula*** **(Willd.) Benth. ex Walp.**, espécie caracteristicamente presente em áreas não-alagáveis na Amazônia, conseguem germinar após sete meses de submersão. Plântulas com um mês de idade, no entanto, nao sobrevivem mais que um mês sob essas mesmas circunstâncias, o que possivelmente representa uma considerável desvantagem nas áreas alagáveis da Amazônia, em vista das anuais cheias com duração mínima de seis meses. Contudo, árvores adultas desta espécie são encontradas, embora raramente, em áreas alagáveis na Amazônia. As possíveis estratégias envolvidas no eventual estabelecimento de indivíduos de ***P.pendula*** em áreas alagáveis na Amazônia brasileira são discutidas. Uma comparação é feita entre as respostas à submersão desta espécie e as de ***Parkia discolor***, espécie tolerante à alagamentos presente em igapós.

Enterolobium contortisiliquum **(Vell.) Morong**, espécie arbórea presente tanto nos solos secos dos cerrados como em áreas alagáveis nas matas de galerias, apresentou considerável tolerância ao alagamento e à seca, conforme refletiram as observações morfológicas feitas e medições fisiológicas tomadas. O possível papel do xilopódio na tolerância a tais estresses é discutido.

Raízes de plântulas de algumas espécies agrícolas estudadas, apresentaram alta concentração de etanol quando submetidas à seca, em comparação com controles não-estressados. Além disso, estas mesmas raízes quando expostas à seca, sofreram uma considerável compactação de seus tecidos, conforme indicado por medições de diâmetro de raízes. Diante destes fatos, propõe-se que seca causa compactação de raízes, que possivelmente reduz a capacidade de tais raízes capturarem oxigênio, resultando na geração de um estado de hipoxia nos tecidos, o que teria como consequência um aumento na produção de etanol destes mesmos tecidos. Esta hipótese de hipoxia induzida por seca é comparada com diversos recentes achados, citados na literatura, e é discutida como possível fator que permite, sob específicas circunstâncias, que um prévio período de seca aclimatize plantas a um subseqüente período de alagamento. Estudos preliminares sobre os efeitos de alternância de alagamento e seca em mudas de cinco espécies de eucalipto, sugeriram que a resposta a um determinado estresse é afetada caso a planta seja previamente exposta a outro tipo de estresse. Este efeito nem sempre foi negativo, e para ***Eucalyptus regnans*** um período de cinco semanas de seca favoreceu um subsequente surpreendente prolongamento na sobrevivência ao alagamento na ordem de três vezes. Este experimento, no entanto, precisa ser repetido para que esses resultados se confirmem.

Não foi encontrada uma causa comum de injúria anaeróbica às plantas estudadas, e sobrevivência à anoxia freqüentemente pareceu estar relacionada a uma associação de adaptações morfológicas e metabólicas.

Os resultados obtidos favorecem uma reflexão crítica sobre os riscos de se *rotular* plantas como tolerantes ou sensíveis à deprivação de oxigênio, assim como proporcionam margem a uma discussão sobre as perspectivas da pesquisa aplicada colaborar no desenvolvimento da teoria ecofisiológica.

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PREAMBLE

The world's wetlands sustain several distinct forms of terrestrial vegetation able to survive periodic or permanent flooding or submergence. Covering approximately 6% of the earth's land surface, the wetlands present some of the most productive (eg. some marshes) as well as some of the most biologically diverse (eg. the tropical floodplains) ecosystems in the world. Nevertheless, they are amongst the planet's most threatened ecosystems **(Maltby, 1991)**. The increasing pressure on wetland areas is converting these long recognised carbon dioxide sinks into a potentially dangerous carbon dioxide source **(De La Cruz, 1986)**.

Ecological, physiological and agricultural studies on wetlands and their plant species throughout this century, have built up an extensive *corpus* of biological knowledge for several of these ecosystems, which, nevertheless, does not prevent the present rate of destruction of these areas. Associated with the urgent need for measures to prevent further ecological and economic losses, a new approach to the study of wetland vegetation has become necessary. This new approach is currently provided by plant ecophysiology which represents a fusion of disciplines, relating the plants' physiological responses to environmental factors, often resulting in knowledge applicable in agricultural and forestry practices.

The present thesis uses an ecophysiological approach to investigate mechanisms of adaptation to flooding in several plant species. Not all the species studied, however, occur in wetland areas. This work aims to detect situations where wetland plants could be sensitive to flooding and where dryland plants could be tolerant to flooding. These situations, when they occur, can be created either by

environmental oscillations existing before, during or after flooding; or by ontogenetic factors, *i.e.* the developmental stage of the plant at the moment when oxygen stress takes place.

Assessing how ontogeny and environmental oscillations act on plant responses to oxygen deprivation, provides a critical reflection on three controversial points: *i*) is oxygen shortage by itself sufficient to kill plants, and is there a common cause of anaerobic injury?; *ii*) how misleading can be the labelling of plants as tolerant or sensitive to oxygen deprivation?; *iii*) can applied research aid the development of ecophysiological theory?

In order to be able to discuss these issues, several trees and agricultural crops of both economic and ecological interest, from tropical and temperate countries, had seeds, roots and whole-plant responses to oxygen stress examined.

The thesis is divided into nine Chapters grouped into four Sections. Each of the Chapters contained in Sections I ("Ontogeny and Anoxia-Tolerance") and II ("Environmental Oscillations and Anoxia-Tolerance") includes an Introduction, as well as a description of Methods, Results and a Discussion. The most relevant results in these two Sections are in most cases shown in the form of graphs. However, if the reader is interested in the actual numerical values obtained in each experiment, these can be found in the Annexe at the end of each Chapter. In each figure a reference is made to an annexe table where the numerical values, used to draw the figure in question, are listed. In the Annexe of each Chapter there are also tables that will not have a respective figure in the Results section. These tables, although often not as relevant to the discussion as the tables from which graphs were produced, are provided in

order to allow the reader to have access to additional information collected during the development of this thesis.

The Chapters in Section III ("Case Studies") display the results in a less rigid structure than those in the two previous Sections, in order to dedicate most of the content to theoretical considerations. The Final Section ("Discussion") consists of three essays which attempt to discuss the bulk of the results of the thesis in the light of the three points mentioned above: *i*) oxygen deprivation damage; *ii*) stress classification; and *iii*) ecophysiological theory. Every Section is briefly introduced by a "Foreword" and the most important findings are listed in the "Conclusions". The following "Material and Methods" describes only the techniques commonly used for all the experiments, leaving particularities to be dealt with within each Chapter.

MATERIAL AND METHODS

Despite the diversity of experiments, tissues studied and types of plants involved (Table 0.1), several of the investigations described in Sections I, II and III were conducted according to similar procedures.

Material and methods shared by all the following Chapters are described here, particularly those involving the physiological measurements of respiration rate, ethanol production and carbohydrate content. Each Chapter will present in its "Methodology" only the procedures that were exclusive to it.

1) SEED GERMINATION AND SEEDLING GROWTH

1.1 *Agricultural crops*

Chickpea and barley seeds were surface sterilised with a 6% sodium hypochlorite solution for 6 minutes with vigorous shaking. Subsequently, seeds (in most cases 20) were washed in tap water for as long as the smell of chlorine remained, and finally rinsed in sterile distilled water before being placed in plastic Petri dishes with *Whatman* no.3 filter paper moistened with 5.0 ml distilled water. Germination took place in an incubator at 20°C, in the dark, until the stage required by the given experiment. The method used to grow the five agricultural crop species used in Chapter Five, is described in that Chapter.

1.2 Trees

Trees of ***Enterolobium contortisiliquum***, ***Parkia pendula*** and 2 out of 5 ***Eucalyptus*** provenances used, were grown from seed.

The seeds, with the exception of those of the eucalypts, were surface sterilised as conducted for the agricultural crops, and subsequently mechanically scarified. The seeds of all species, including the ***Eucalyptus*** provenances, were placed in Petri dishes with moistened filter paper as carried out for the chickpea and barley seeds, and distilled water was added when necessary during the germination period. Germination took place at 25°C, with 12 hours of light per day (40 μmol.m^{-2}.s^{-1}).

After protrusion of the radicle and emergence of the coleoptile (which normally occurred between 5 and 7 days for all the species used), the seedlings were individually placed in small pots filled with a 1:1:1 mixture of professional compost (Levington M2), pure sand and fine gravel, being gradually transferred to bigger pots as they grew. They were grown in a constant temperature room at 25°C with 12 hours of light per day (40 μmol. m^{-2}.s^{-1}).

2) GROWTH MEASUREMENTS AND SURVIVAL

2.1 *Agricultural crops: chickpea and barley*

After anoxic treatment and/or physiological measurements, the germinating seeds were planted in trays filled with washed sand and were allowed to grow for 20 days, at 20°C, with 12 hours of light per day (150 μmol.m^{-2}.s^{-1}). At the end of the growth period, survival was scored and shoot fresh weight and, sometimes, root fresh weight were measured on a *Mettler PC180* precision balance. When necessary, root and shoot dry weight were also measured by oven-drying the material at 80°C.

Growth was not measured for the agricultural crops used in Chapter Five.

2.2 Trees: *Enterolobium contortisiliquum*, *Parkia pendula* and *Eucalyptus* spp.

In most experiments, individuals of these species, except the ***Parkia pendula*** seedlings, were grown in transparent acrylic tubes kept in light-proof boxes. The transparency of the tubes allowed visual observation and non-destructive measurements of root extension (fig.0.1; see also fig.4.0 in Chapter Four), when the boxes were opened for weekly brief inspection periods. Shoot extension was also monitored weekly. For experiments carried out in pots, only shoot extension was measured or alternatively leaf emergence, as in the case of ***Parkia pendula***, as an indication of growth.

Tubes and pots were filled with a 1:1:1 mixture of professional compost (Levington M2), sand and fine gravel.

3) FLOODING, DROUGHT AND ANOXIA

3.1 *Flooding*

Trees were submitted to flooding by filling the soil inside the bottom sealed tubes or pots with tap water up to the desired level. The water level was frequently monitored and water was added when necessary.

3.2 *Drought*

Trees were submitted to drought simply by not watering the soil filling their tubes or pots. Drought was estimated in terms of field capacity for the plants in the tubes by measuring over two weeks the weight of soil samples extracted from additional ***Eucalyptus*** trees not used in the experiments involving root and shoot measurements. Two hours after filling the soil in the tubes with tap water, the

Fig.0.1. Detail of the acrylic tubes used for planting and growing several tree species. The possibility of observing the roots allowed monitoring of symptoms and measurements of root growth without damaging the plant.

rubber stoppers in the bottom of the tubes were removed to allow drainage. After 12 hours, the first soil samples extracted and measured were considered to be at 100% field capacity. Fresh weight and dry weight of extracted soil samples (samples were oven-dried for 24 hours at 80°C) were also measured at the subsequent days 1, 2, 7 and 14, allowing an estimation of percentage of field capacity at each of those moments, as in **Nikolskii (1964)**. The soil samples were extracted as an intact core by using a graduated perspex tube.

3.3 *Anoxia*

An anoxic environment was provided for seeds and root sections by a *Forma Scientific Anaerobic System, model 1024* (Ohio, USA), which uses a 90% nitrogen, 10% hydrogen atmosphere which is constantly circulated over a palladium catalyst. In order to have more control over temperature conditions, plant material subject to long anoxic periods was placed inside anaerobe jars (*Gas Pak*, USA) which were gas-filled inside the anaerobic chamber and kept there for 30 minutes in order to make sure that no oxygen was still available. Subsequently, the jars were sealed and transferred to appropriate temperature-controlled incubators. The jars contained catalyst pellets to aid oxygen removal and the absence of oxygen inside the jars was monitored by *Gas Pak* disposable methylene blue anaerobic indicators.

4) PHYSIOLOGICAL MEASUREMENTS

4.1 *Respiration rate*

Seeds or root sections were placed in 30 ml glass phials with *Whatman* no.3 filter paper moistened with 2.0 ml

distilled water. The rate of carbon dioxide emission was measured by connecting the phials with a closed loop to an *ADC-225 Mk3* infra-red gas analyser. The loop used was a translucent vinyl tubing (3.0 mm internal diameter), which had two of its ends connected by sterile needles (19G 1½ inch) to the serum cap stoppers (*Suba Seal*) sealing the glass phials.

After connecting the phial containing the plant material to the equipment, air (in the case of aerobic respiration readings) or nitrogen (in the case of anaerobic respiration readings) would be purged through the system. In order to zero the equipment, a change in the connection of the tubing was necessary to take out the CO_2 from the system through a soda lime column. In order to accomplish this change, the pump would be switched off and the connection of the tubing would be rapidly changed manually from one outlet to another, followed immediately by switching the pump on again. After finally bringing the needle down to zero, the pump is again switched off, another rapid change in connection is carried out (now in order to pass the sample gas through the analysis cell), and the pump is switched on again as before, which allows the readings of the carbon dioxide emission of the material analysed to be taken. The procedure of rapidly switching off the pump before changing connections manually, and then immediately switching them on again, avoids gas leakage. Fig.0.2. shows a diagram representing the different steps taken in the analysis of carbon dioxide output by the plant tissues used.

The flow rate of the gas through both the referential and analysis tubes was kept at a range of 200-250 $ml.min^{-1}$.

Standardising was done by using a cylinder with known concentration of CO_2 (400 ppm), and calibration was periodically checked. The total volume of the closed system

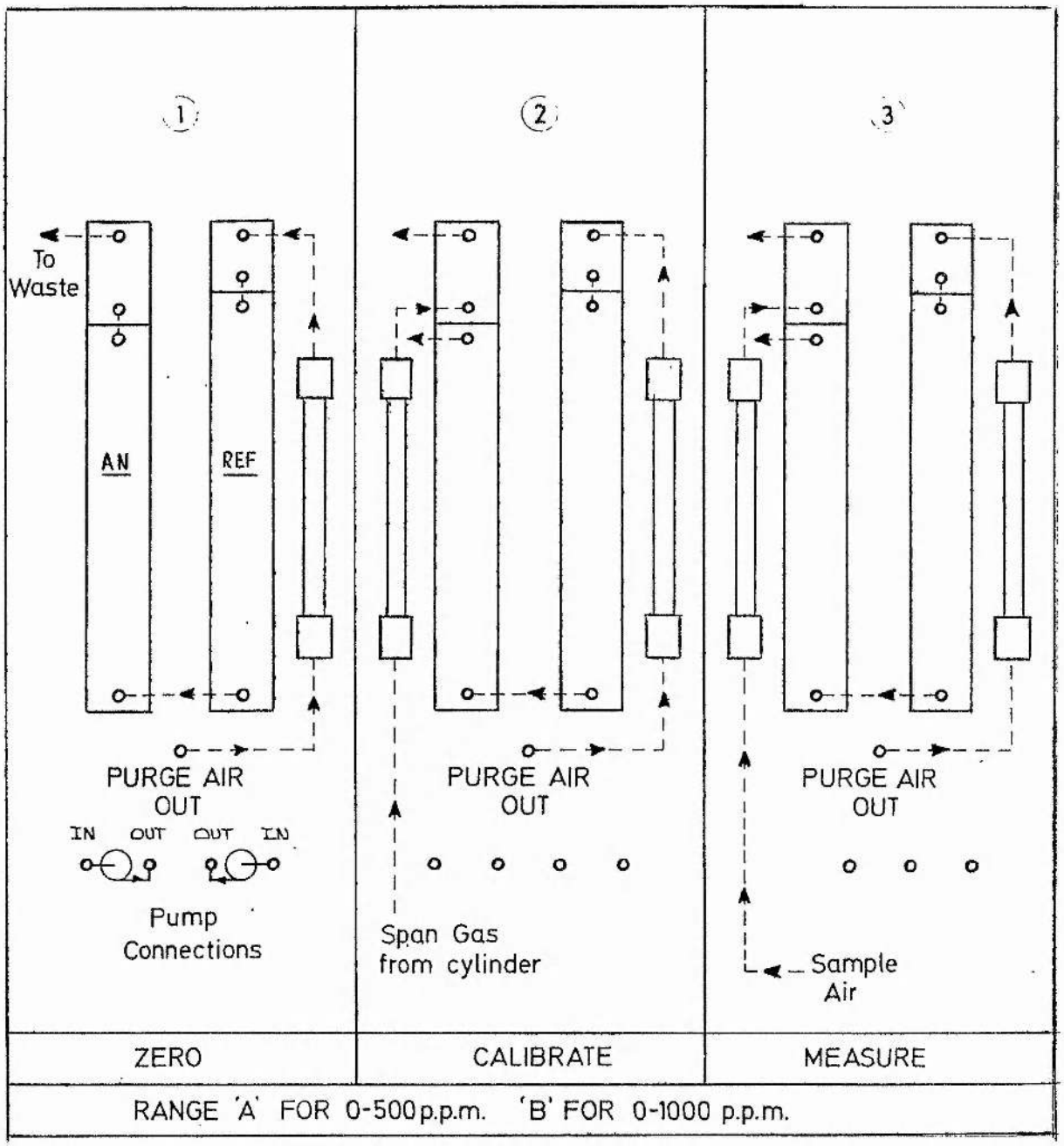

Fig.0.2. Schematic representation of the tubing connections and the gas flow in the infra-red gas analyser at the different steps taken during the readings of plant tissue carbon dioxide output. Flow rate ranged from 200-250 ml/min. Measurements taken in the absolute mode. Diagram adapted from the equipment instruction manual **(ADC, 1980)**.

was approximately 111 ml, which was calculated by injecting in the analysis tube a known amount of pure CO_2 (100 μl) and measuring the consequent change in concentration. All readings were registered in a *Gallenkamp* chart recorder connected to the ADC.

The temperature of the phials was always kept at 20°C in a water bath and readings were taken at a room light intensity of ca. 30 $\mu mol.m^{-2}.s^{-1}$. Aerobic respiration was measured first. Subsequently the materials were submitted to anoxia and anaerobic respiration was measured after 1 hour. During the anaerobic respiration readings, oxygen-free nitrogen was purged through the system to ensure that the atmosphere around the tissues was anoxic during readings. The procedures referring to the handling of plant material are described in each Chapter's "Methodology" section.

In the aerobic respiration process, a molecule of glucose in the presence of six molecules of oxygen produces six molecules of carbon dioxide and six molecules of water. If oxygen is not available, in the anaerobic respiration process a molecule of glucose should produce two molecules of carbon dioxide. When the anaerobic emission rates of carbon dioxide do not fall to a third of their aerobic levels, it characterises the so-called Pasteur effect, which is an indication of acceleration of glycolysis. Rapid carbohydrate depletion and high production of ethanol and other potentially toxic volatiles induced by the Pasteur effect may cause metabolic dysfunctions. The term Pasteur effect will be constantly used in this thesis when referring to the phenomenon here described.

4.2 *Ethanol production*

The following Chapters will present measurements in two different manners for estimating ethanol production and

content. Ethanol production was estimated by the headspace method and ethanol content was estimated by tissue extraction. These tests were carried out after submitting the plant material to differing treatments.

For headspace measurements, seeds and roots after subjection to a stress treatment, were submitted to anoxia or air for a required period of time while placed in 30 ml glass phials (75 mm depth x 24 mm diameter) lined with moist filter paper and closed with serum cap stoppers (*Suba Seal*). For measurements of ethanol production under anoxia, the glass phials were placed in sealed anaerobe jars.

The headspace samples were taken with a gas syringe (1.0 ml) pre-heated to 70°C in an oven to avoid condensation of water and loss of ethanol vapour by solubilisation. Subsequently the samples were injected in a *Philips Pye Unicam 4500* gas liquid chromatograph using a 12.5 m-long wall-coated vitreous silica capillary column. The film thickness of the column was 0.25 microns and the internal diameter 0.22 mm. The carrier gas used was nitrogen, burnt in a hydrogen/air mixture. The flow rate of the carrier gas was 2 $ml.min^{-1}$, the hydrogen flow rate was approximately 10% higher than that of the nitrogen, and the air flow rate was approximately 10 times that of the hydrogen. These flow rates allowed the flame of the Flame Ionisation Detector (F.I.D.) to be lit, before injecting the gas samples. The operating principles of the F.I.D. are described in detail by **Chapman (1986)**. Readings for all seed and root material were taken at a sensitivity range of 32 x 1. Before readings the temperature in the injector zone, detector zone and column oven were brought up to 200°C, 200°C and 150°C respectively. Recording of the data and calculation of peak area were performed by a P3105 *Philips* Microcomputer.

For measurements of ethanol content in roots, after a stress treatment, ethanol would be extracted from the tissues in ice-cold 6% perchloric acid and neutralised in 6 M potassium carbonate. The liquid samples (extracts) were taken with a 1.0 μl liquid syringe and injected in a *Pye Unicam* series 104 model 64 gas liquid chromatograph using a 1.75 m-long glass column, with 4 mm internal diameter. The column was filled with *Porapak* type *Q* (an ethylvinyl-benzene-Divinyl benzene co-polymer), mesh size 100-120, in order to perform analysis of ethanol in liquid samples. Nitrogen was again used as carrier gas, at a flow rate of 35 $ml.min^{-1}$. The proportional flow rates for hydrogen and air were the same as the ones described for the previous equipment. This machine was also equipped with a F.I.D.. Before readings, the temperature in the detector zone was brought up to 160°C and in the column oven to 150°C. Since, this equipment is an older model than the previous one, although it had an injector heater, there was no separate temperature sensor for the injector zone, which however must have been approximately the same as that of the column oven. The sensitivity range of the readings was of $2x10^2$. Recording of data and calculation of peak areas were carried out on a *3390 A Hewlett Packard* Integrator.

The injection of similar gas and liquid samples taken from standard solutions of 0.05, 0.1 and 0.3% ethanol (*Sigma Diagnostics*) provided regression equations for calibration. The injection of standards was carried out at two-hour intervals during measurements of tissue samples.

Each standard solution and plant tissue gas or extract was sampled twice both in the headspace and liquid methods. The mean value of the two was used for the calculations.

4.3 *Carbohydrate content*

Carbohydrate contents were estimated only for underground structures, mainly roots and xylopodia. The material used was deep-frozen in liquid nitrogen and subsequently freeze-dried at -45°C in an *Edwards Modulyo* freeze-drier. Afterwards, the freeze-dried roots were stored at -20°C. Enzymatic analysis was used to determine starch and D-glucose content in the samples. The underground organs were reduced to a fine powder (25-50 mg of powder per sample in the case of root sections and 100-300 mg of powder per sample in the case of xylopodium sections) which was weighed and treated with 1.3 ml hydrochloric acid / 5.0 ml dimethylsulphoxide to solubilise the starch and the D-glucose present. Subsequently the solutions were left for 30 minutes in a 60°C water bath, before being filtered through sterilised muslin. The pH of the extracts was adjusted to 4.5 and the total volume increased to 25 ml by addition of distilled water. *Boehringer-Mannheim* kits for biochemical analysis of starch were used to perform the enzymatic analysis in a *Pye Unicam* ultra-violet spectrophotometer.

Three glass cuvettes (1 cm light path) were used for each sample:

1) *control cuvette*: all solutions and suspensions mentioned below were added in that cuvette, which however had no sample solution. One absorbance reading was taken before the addition of the enzyme suspension and one after. The difference between these two readings (δA_C) was always non-existent or minimal, as expected;

2) *starch cuvette*: amyloglucosidase would be added to the sample solution inside the cuvette in order to hydrolise starch to D-glucose. After incubating the cuvette in a 55-60°C water bath, distilled water and a mixture consisting of

triethanolamine buffer (pH 7.6), NADP, ATP, magnesium sulphate and stabilisers were added to the resulting solution. After gently mixing the contents inside the cuvette with a glass stirring rod, the absorbance (A_1) of the solution was read in the UV spectrophotometer. This step was followed by the addition of an enzyme suspension, consisting of hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH). In the presence of HK, the D-glucose resulted from the hydrolisation of the starch is phosphorylated to glucose-6-phosphate (G6P) by ATP, with the simultaneous formation of ADP. In the presence of G6P-DH, G6P is oxidised by NADP to gluconate-6-phosphate with the formation of NADPH. The amount of NADPH formed is stoichiometric with the amount of D-glucose formed by hydrolysis of the starch. NADPH was determined by means of its absorbance at 340 nm wavelength, after allowing the sample solution to react, in the dark, at room temperature, with the enzyme suspension. The completion of the reaction was often achieved after 10-15 min, and was determined by taking several absorbance readings until the readings stabilised (A_2). Subtracting A_1 from A_2 resulted in the δA_S due to the addition of the enzyme suspension. Subtracting δA_C from δA_S corrected the δA for the starch cuvette, resulting in what is here called δA for starch.

Standardisation of the essays was achieved by monitoring NADPH and NADH concentrations at 340 nm.

3) *D-glucose cuvette*: differs from the starch cuvette as amyloglucosidase is not added, therefore starch is not hydrolised to D-glucose. The remaining procedures are as those taken for the starch cuvette. Subtracting the δA found for this cuvette from the δA found for starch, results in the δA for D-glucose.

Each of these cuvettes always had a final volume of 2.32 ml. In order to obtain maximum sensitivity, the amount of sample solution to be used in the cuvette for a particular type of material was previously determined by testing the response in terms of absorbance for a range of different quantities, namely 0.1, 0.5 and 1.0 ml of sample solution. In the case of root sections, often 0.5 or 1.0 ml of sample solution would be necessary in order to get absorbance readings. In the case of xylopodium, 0.1 ml of sample solution was the ideal amount. In order to keep the final volume of the cuvette at 2.32 ml, the amount of distilled water added varied according to the volume of sample solution used.

In all enzymatic tests the accuracy of the essay was checked by ensuring that a doubling of the aliquot used in the test produced a corresponding doubling of optical absorption change.

The final figures for carbohydrate content were calculated in terms of mg of starch per gram dry weight of material sampled and mg of glucose per gram dry weight of material sampled. The addition of these two figures provided a value for total carbohydrate detected per gram dry weight of material measured.

Further details of the methodology are described in the instruction leaflet which accompanies the kits **(Boehringer & Mannheim, 1987).**

Table 0.1: Plant organs studied, morphological and physiological measurements taken and external observations made for each of the species investigated.

SPECIES	ORGANS			PHYSIOLOGICAL MEASUREMENTS			MORPHOLOGICAL OBSERVATIONS		
	se	r	w	rp	e	c	su	g	s
Cicer arietinum	X	X	-	X	X	-	X	X	X
Daucus carota	-	X	-	-	X	-	-	-	X
E.contortisiliquum[1]	-	X	-	X	X	X	X	X	X
***Eucalyptus* spp.**	-	X	-	X	X	-	X	X	X
Hordeum vulgare	X	X	-	X	X	-	X	X	X
Parkia pendula	X	-	X	-	-	-	X	X	X
Phaseolus vulgaris	-	X	-	-	X	-	-	-	X
Zea mays	-	X	-	-	X	-	-	-	X

se=seed; r=root; w=whole-plant; rp=respiration rate; e=ethanol production; c=carbohydrate content; su=survival; g=growth; s=symptoms.

[1] The xylopodium of ***Enterolobium contortisiliquum*** was also studied.

SECTION I

ONTOGENY AND ANOXIA-TOLERANCE

* FOREWORD *

From the beginning of the life cycle as germinating seeds until the end of it as mature individuals, plants undergo countless metabolic, anatomical and morphological changes. These changes are likely to induce plants to respond differently to stress at different moments in their lives.

The accounts on how ontogeny affects plant response to flooding or anoxia are few if compared with the wide range of works concerned with the simple division of plants as stress-tolerant or stress-sensitive. Among those few reports, the majority describe how age affects flood- or anoxia-tolerance **(Hook & Scholtens, 1978; Kozlowski, 1984).**

This Section examines how ontogeny affects plant response to flooding or anoxia by investigating seeds at distinct germination phases and a tropical tree from seed to maturity.

CHAPTER ONE studies the responses of chickpea and barley germinating seeds to anoxia during different moments in the early stages of imbibition, before and after radicle protrusion. The anoxia-sensitivity of seeds during imbibition is assessed in terms of post-anoxic survival and shoot fresh weight, and aerobic and anaerobic respiration rate and ethanol production. The power of imbibition of seeds during anoxia is also examined.

CHAPTER TWO provides a discussion on why the Amazonian tree species ***Parkia pendula*** does not occur in floodplains in

spite of having seeds adapted to long-term submergence. By comparing waterlogging and submergence tolerance of seeds and young seedlings of ***Parkia pendula*** with flood-tolerant ***Parkia discolor***, this Chapter analyses the ecological advantages and disadvantages of both species strategies.

* CHAPTER ONE *
SEED SENSITIVITY TO ANOXIA DURING IMBIBITION

1) INTRODUCTION

Germination of seeds is influenced by many environmental factors, including water, gases, light, temperature, soil pH, salinity and biotic factors **(Fitter & Hay, 1981; Frankland, Bartley & Spence, 1987; Kinzel, 1983; Mayer & Poljakoff-Mayber, 1978; Ponnamperuma, 1984; Wainwright, 1984)**. Since flooding can cause dramatic changes to all these factors, seed responses under such situations will be consequently affected. These responses will vary according to a number of factors from depth and duration of flooding **(Hook, 1984)** to type of seed involved **(Crawford, 1989)**.

Flood-tolerance of seeds and seedlings therefore is very important in determining species occurrence on wet sites **(Kozlowski, 1984)** and competitive advantages can be achieved by germination under water **(Hook, 1984)** or by withstanding prolonged periods of submersion **(Coutinho & Struffaldi, 1971)**. Metabolic adaptations **(Vartapetian, Snkhchian & Generozova, 1987)** and depletion of ethanol from storage organs on return to an aerobic environment **(Cossins, 1978)** are often associated to seed and/or seedling survival under anoxia.

However, there is a great degree of diversity in the response of germinating seeds to oxygen deprivation. Some species are highly tolerant of oxygen shortage and can germinate with shoot expansion in total absence of oxygen, for instance ***Oryza sativa*** **(Taylor, 1942)**, ***Zizania aquatica*** **(Campinaron & Koukari, 1977)**, and various species of ***Echinochloa*** **(Rumpho and Kennedy, 1981)**. Species such as ***Parkia pendula*** (see Chapter Two), despite a remarkable seed

tolerance to long-term submergence, need oxygen to germinate and to establish seedlings. Other species can proceed only as far as rupturing the testa and protruding the radicle unless oxygen becomes available. There are also species such as ***Pisum sativum*** **(Matthews & Whitbread, 1968)** which can have germination impeded by as little as 24-hour soaking.

The absence of studies concerned with examining the exact moment at which the germinating seed becomes sensitive to anoxic injury accounts for the limited understanding of the reasons for such interspecific variation. This lack of precision in defining the moment of sensitivity to oxygen shortage is in marked contrast to the detailed studies conducted on desiccation tolerance in the imbibing seed. **Koller & Hadas (1982)** have divided the germination process into three phases which characterise the ability of the seed to survive redesiccation, namely: *i*) the initial stage of germination, in which water uptake is mainly non-biological; *ii*) a transition phase, with low metabolic activity and low respiration rate; *iii*) a growth phase, with protrusion of the radicle, cell extension and high metabolic activity. Indeed, **Opik & Simon (1963)** studying seeds of ***Phaseolus vulgaris*** showed that respiratory activity could be controlled at will by regulating water supply and redesiccation during a first phase of germination lasting 10-16 hours. In a second phase lasting 3-8 hours, there was a pause in both water uptake and increase in respiration rate. In a third phase, respiration rate accelerated.

Thus, in face of such distinctions in the metabolic activity of the seed at different germination phases, the exact moment of the germination process that the seed is passing through when subjected to anaerobic conditions may affect plant post-anoxic survival. This Chapter uses the above approach of categorising the varying stages of germination

in three phases as a basis for comparing changes in anoxia-tolerance of germinating seeds of chickpea and barley.

Apart from environmentally-imposed oxygen deprivation, seed coat impermeability to oxygen of some species, by itself, may submit embryos to a period of natural anaerobiosis. **Crawford (1989)** asserts that the early stages of germination of such species (usually large seeds with rapid germination as found in some leguminous plants) are often accompanied by hypoxic conditions within the seed, particularly before the rupture of the testa. After imbibition, there is a gradual acceleration of metabolism and the resulting demand for oxygen can exceed the rate of replacement until radicle protrusion and swelling of cotyledons rupture the testa. **Sherwin & Simon (1969)** found that in french bean (***Phaseolus vulgaris***) lactate and, to a lesser extent, ethanol accumulate during the initial stages of germination, whereas **Aldasoro & Nicolas (1980)** suggest that ethanol is the main glycolytic product to accumulate in chickpea.

The endurance of this period of natural anaerobiosis, although showing much interspecific variation, is strictly limited and when it is prolonged by excessive burial, flooding or soil compactation, emergence can be greatly reduced **(Crawford, 1977, 1992; Reisman-Berman, Kigel and Rubin, 1989)**. In this Chapter, the presence or absence of this period of natural anaerobiosis within seeds is estimated for chickpea and barley seeds by comparing the amounts of aerobically- with anaerobically-produced ethanol.

2) METHODOLOGY

2.1 *Post-anoxic survival and shoot fresh weight*

Chickpea (***Cicer arietinum*** L.) from a commercial source of unknown provenance (80-100% viability) and barley (***Hordeum vulgare*** **L.var. Golden Promise**) (95-100% viability) were surface sterilised, washed in sterile water and placed on moist filter paper (as in the Material and Methods section). Seeds were allowed from 0 to 96 hours aerobic imbibition before being subjected to either 2 or 4 days of total anoxia. The length of the imbibition period allowed a separation of the time of imposition of the anoxic shock into the different phases of germination as defined by **Koller & Hadas (1982)**, *i.e.*: *i*) the initial phase, in which the seeds first come into contact with water (0-0.1 hours aerobic imbibition); *ii*) the transition phase, before there are any visible signs of growth (0.1-12 hours aerobic imbibition); *iii*) the growth phase, established as soon as radicle protrusion is visible (18-96 hours aerobic imbibition) (Table 1.1).

For both species 60 seeds were used for each treatment (3 replicates with 20 seeds each). For the seeds which were allowed no aerobic imbibition, surface sterilisation and rinsing took place inside the anaerobic incubator. The distilled water used in this case was previously bubbled with nitrogen to make sure no air was available for the seeds. Similarly, during the anoxic period the filter papers were moistened with distilled water bubbled with nitrogen.

The aerobic imbibition of 0.1 hours represents the 6-minute surface sterilisation carried out in air, which was followed by immediately transferring the seeds to the anaerobic incubator.

Table 1.1: Summary of aerobic imbibition treatments in relation to germination stages following the system of **Koller & Hadas (1982)**.

Germination phase	Hours of Aerobic Imbibition	Developmental stage when subjected to anoxia	
		CHICKPEA	BARLEY
GROWTH	96	radicle +hypocotyl	seminal roots+ 2 cm coleopt.
	72	2 cm radicle	seminal roots+ 1 cm coleopt.
	48	1 cm radicle	seminal roots
	24	radicle present	
	18	start of radicle protrusion	
TRANSITION	12		
	6	no signs of growth	
	0.1		
INITIAL	0	no signs of growth	

The sealed anaerobe jars containing the seeds were placed in a 20°C, dark, incubator. At the end of the anoxic period, seeds were grown for 20 days before survival and shoot fresh weight were finally recorded. Significant differences were tested at $p<0.05$ by chisquare for the survival experiments.

2.2 *Ethanol production and carbon dioxide output*

Seed sterilisation and germination were conducted as for the survival and shoot fresh weight experiments. The ethanol production was sampled by headspace analysis, after 24 hours of dark anaerobic incubation at 20°C. Controls were measured after 24 hours dark aerobic incubation at 20°C. In order to check the reliability of headspace analysis under the conditions of these experiments, the measurements were initially repeated using different numbers of seeds. At the end of the 24-hour anaerobic incubation, headspace concentration was directly correlated with the fresh weight of the seeds in the phials (Table 1.2). After analysis, the seeds of each replicate were weighed and then planted and allowed to grow for 20 days, when survival and shoot fresh weight were monitored.

Measurements of carbon dioxide emission were done as in the Material and Methods section. Aerobic respiration was measured directly after the seeds reached the germination stage required. Anaerobic respiration was measured after 1-hour anoxia and after 24-hour anoxia. Both respiration and ethanol experiments consisted of five treatments containing seeds at different stages of germination ranging from 0.1 to 24 hours of aerobic imbibition. Each treatment had 5 replicates with 3 seeds each. The course of the experiments indicated a necessity for measurements of embryo oxygen uptake, which are described below.

Table 1.2: Test of reliability of headspace method for ethanol analysis in barley and chickpea seeds (percentage of ethanol). Relationship between number of seeds used, fresh weight of the sample (mg; *x*=mean±st.error) and percentage of ethanol produced after 24-hour anoxia (*x*=mean±st.error).

no. of seeds	rep.	CHICKPEA fw(mg)	CHICKPEA % eth	BARLEY fw(mg)	BARLEY % eth
1	1	801.0	0.126	84.0	0.040
	2	851.0	0.188	45.0	0.027
	3	869.0	0.269	63.0	0.028
	x	840.3±20.3	0.194±0.041	64.3±11.3	0.032±0.004
2	1	1585.0	0.260	143.0	0.035
	2	1552.0	0.249	131.0	0.032
	3	1661.0	0.272	157.0	0.047
	x	1599.3±32.3	0.259±0.008	143.7±7.5	0.038±0.005
4	1	2868.0	0.450	250.0	0.065
	2	3408.0	0.401	286.0	0.085
	3	3248.0	0.301	304.0	0.078
	x	3175.0±160.0	0.384±0.044	280.0±15.9	0.076±0.006
8	1	6326.0	0.409	563.0	0.147
	2	6371.0	0.431	563.0	0.128
	3	5767.0	0.426	544.0	0.148
	x	6155.0±199.4	0.422±0.006	556.7±6.3	0.141±0.007

2.3 *Oxygen uptake*

The oxygen uptake of excised embryos of chickpea seeds was measured, after submitting the whole seed to different imbibition/anoxia treatments. Previously, it was found that 50 non-sterilised chickpea seeds of the same batch of seeds used for the present embryo oxygen uptake experiments showed 100% germination, which resulted in the decision of not sterilising the seeds used in these experiments in order to reach total precision in terms of length of imbibition. Groups of 30 seeds were laid with their flat face in plastic Petri dishes lined with filter paper, and were given 15 ml of distilled water. The seeds were submitted to five distinct treatments: *i*) 12 hours imbibition under anoxia; *ii*) 12 hours imbibition under anoxia followed by 2 hours aerobic imbibition; *iii*) 2 hours aerobic imbibition; *iv*) 2 hours aerobic imbibition followed by 12 hours imbibition under anoxia; *v*) 12 hours aerobic imbibition. Treatments *iii* and *v* were used as controls. Seed dry weight before imbibition, and fresh weight after imbibition were measured. After imbibition, embryos were carefully excised from each of the 180 seeds used per treatment (6 repetitions with 30 seeds each). Each group of 30 embryos was weighed after excision.

Oxygen uptake was measured with a Warburg Constant Volume Respirometer (*B.Braun*). This apparatus consists of a detachable flask attached to a manometer. The 30 embryos of each repetition were placed inside this flasks, on a moist filter paper. The centre of the flasks contained a well where 0.5 ml of KOH would be placed in order to absorb any liberated carbon dioxide, thus keeping the carbon dioxide pressure zero. Therefore, the changes noted in pressure were due exclusively to the oxygen uptake. The flasks were kept in a water bath at 25°C throughout the measurements. Readings were taken every 20 minutes and between readings

the system was shaken to promote a rapid gas exchange between the fluid and the gas phase. The measurements used in the embryo oxygen uptake calculations were carried out during a period of time when the readings in the manometer were stabilised, which often took place between the readings at 40 and 80 minutes. The oxygen uptake of each repetition was finally calculated per gram fresh weight of embryo by multiplying the difference in pressure read in the manometer in a known amount of time by the constant attributed to each respective flask. Additional details about the Warburg apparatus can be found in **Umbreit, Burris & Stauffer (1957)**.

Significant difference between treatments was tested by a t-test.

2.4 *Imbibition power*

The ability of barley and chickpea desiccated seeds to imbibe water was assessed by individually weighing the seeds before and after different imbibition treatments. As in 2.3, sterilisation was proved to be unnecessary and was, therefore, not conducted for the seeds used. The treatments were: *i*) 2-hour imbibition under anoxia without previous aerobic imbibition; *ii*) 2-hour aerobic imbibition followed by 2-hour imbibition under anoxia; *iii*) 2-hour aerobic imbibition followed by 4-hour imbibition under anoxia; *iv*) 2-hour aerobic imbibition followed by 10-hour imbibition under anoxia. For chickpeas, two additional treatments were tested: *v*) 12-hour aerobic imbibition followed by 2-hour imbibition under anoxia; *vi*) 10 days under anoxia (no imbibition), before being allowed 4-hour imbibition under anoxia. Each of the treatments had an aerobic control, respectively 2, 4, 6, 12 hours of aerobic imbibition, and 10 days in air before 4-hour aerobic imbibition. Each treatment had 10 replicates consisting of 10 seeds symetrically distributed in a Petri dish, on filter paper. The barley

seeds were soaked in 5 ml of distilled water, whereas the chickpeas were soaked in 10 ml distilled water. The experiments were conducted at 20°C.

3) RESULTS

3.1 *Post-anoxic survival and shoot fresh weight*

Two experiments examined the effects of anoxia at different phases of germination on post-anoxic survival and shoot fresh weight of chickpea and barley. In the first experiment, 2 and 4 days anoxic treatments were given beginning with the first access of the seeds to water, after surface sterilisation, and then at 6-hour intervals up to 24 hours, in order to have a detailed view of the effects of anoxia when imposed early in the germination process. These results are shown in figs.1.1 and 1.2, where a marked difference is found between chickpea and barley. In the latter species, tolerance of anoxia is high and unchanged irrespective of when it is imposed in the first 24 hours of germination. However, in chickpea there is a difference in the post-anoxic survival of different germination stages, with the early stage of 6-hour aerobic imbibition being the most tolerant to 4 days of anoxia. The viability of the chickpea seeds used in this experiment was 80%.

In the second experiment, 2 and 4 days anoxia were first given before any aerobic contact of the seeds with water (for these seeds, sterilisation was carried out inside the anaerobic chamber; see "Methodology"), then to seeds which have had only their first access to water (through the six-minute sterilisation in aerobic conditions), and subsequently at intervals of 1-day aerobic imbibition up to 4 days. For this reason, in this experiment the germination stages prior to the anoxic treatment presented noticeably different morphological development from each other, *i.e.* some were in the initial stage (no external sign of development), while others had radicle protruded, others had coleoptile expanded, *etc.* (see Table 1.1). After 4 days of anoxia, decline in viability was marked for chickpea seeds

given more than 24 hours of aerobic imbibition prior to anoxia, and for barley allowed more than 48 hours aerobic imbibition prior to anoxia (figs.1.3; 1.5). Chickpea seedlings also showed the somewhat surprising result that 6 minutes of aerobic imbibition prior to 4-day anoxia resulted in a significant increase in post-anoxic survival and seedling shoot fresh weight when compared with seeds allowed no previous aerobic imbibition (fig.1.3, 1.5). The chickpea seeds used in this experiment were 100% viable seeds, which is a higher percentage than the batch of seeds used in the first experiment described above (80%). This accounts for the higher percentage survival results seen in fig.1.3 compared with those in fig.1.2.

Subsequent shoot fresh weight of surviving individuals after the 20-day post-anoxic period shows a similar pattern to that observed for survival in barley, not showing considerable variation in response among the various germination stages tested earlier than 24-hour imbibition (fig.1.4). The chickpea results indicate less of a variation for growth (fig.1.4) than that observed for survival (fig.1.2). This could indicate that those seedlings that were able to survive during post-anoxia, irrespective of the phase they were when submitted to anoxia, do not have their shoot growth impaired, as indicated by the fresh weight measurements. However, the cost of this shoot growth in this conditions, could be of an impaired root growth which would have future negative consequences. Root growth, unfortunately, was not assessed in the present work.

Another similarity for barley between the survival and shoot fresh weight results, which is also true for chickpea (fig.1.5) is that if the longer anoxic treatment (4 days) is begun during the earliest stages of germination, it can be less damaging than when imposed later.

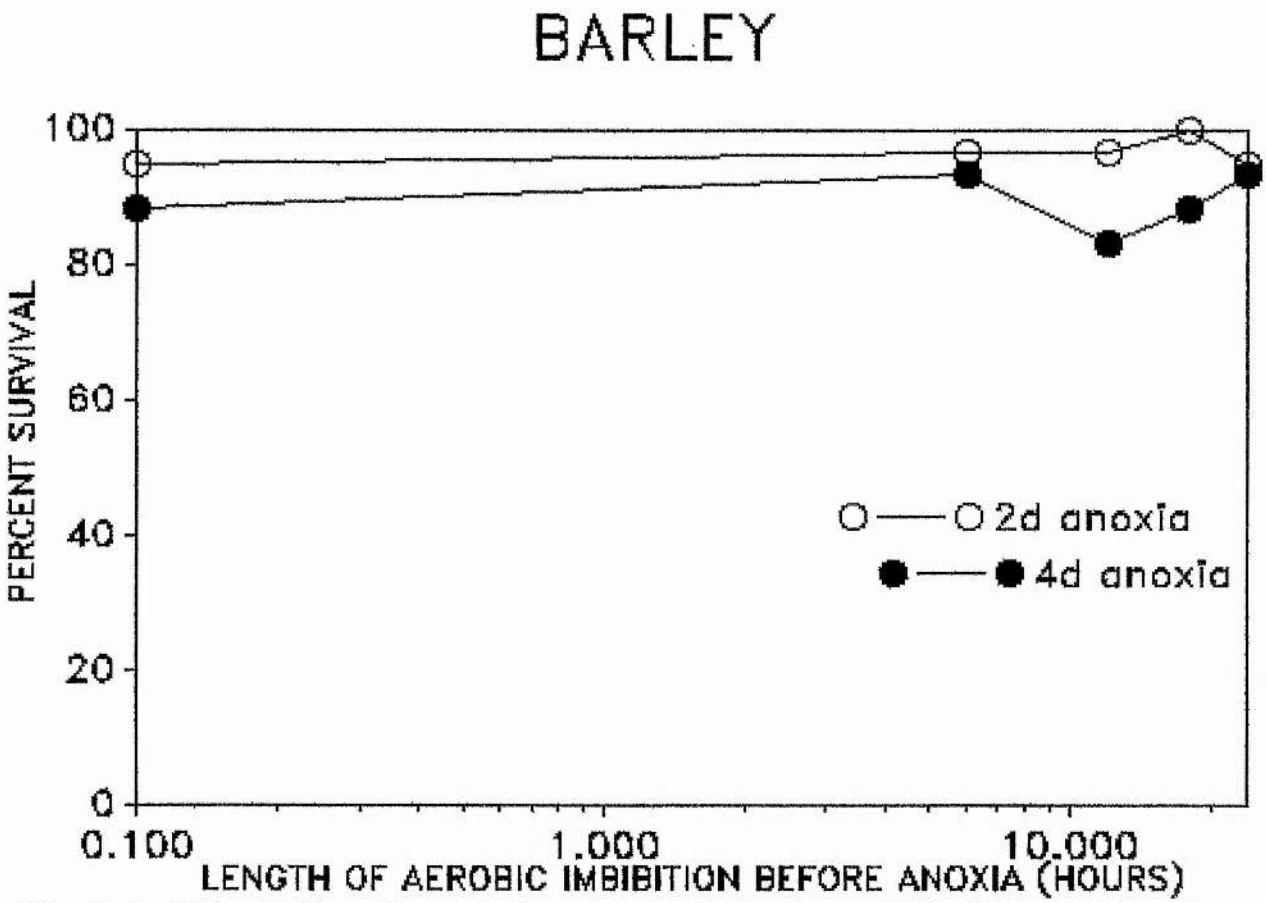

Fig.1.1. Effect of early anoxia on survival of germinating seeds of barley (n=60). Figures are displayed in the annexe Table 1.5a. There was no significant difference between treatments (chisquare).

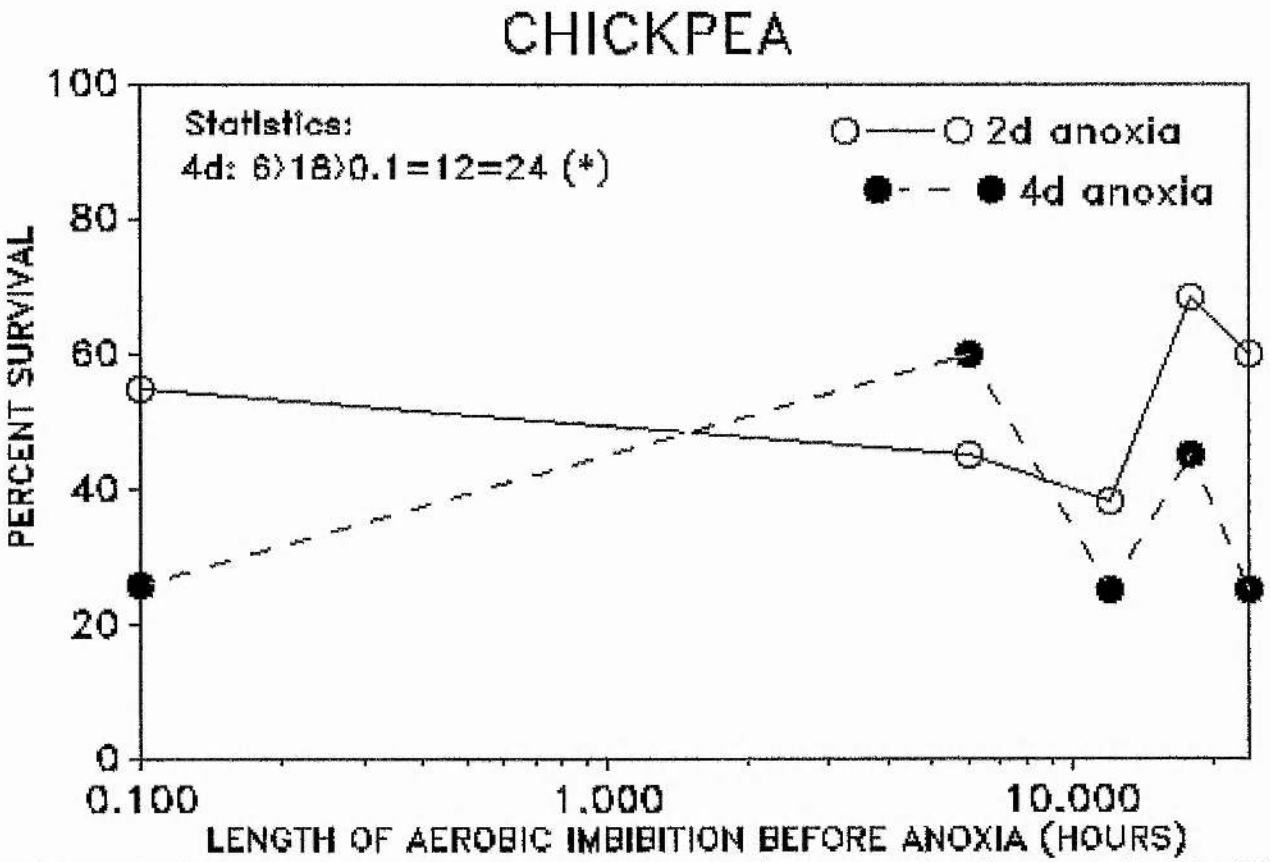

Fig.1.2. Effect of early anoxia on survival of germinating seeds of chickpea (n=60). Figures are displayed in the annexe, Table 1.5a. Significant differences (*) were tested by chisquare ($p<0.05$).

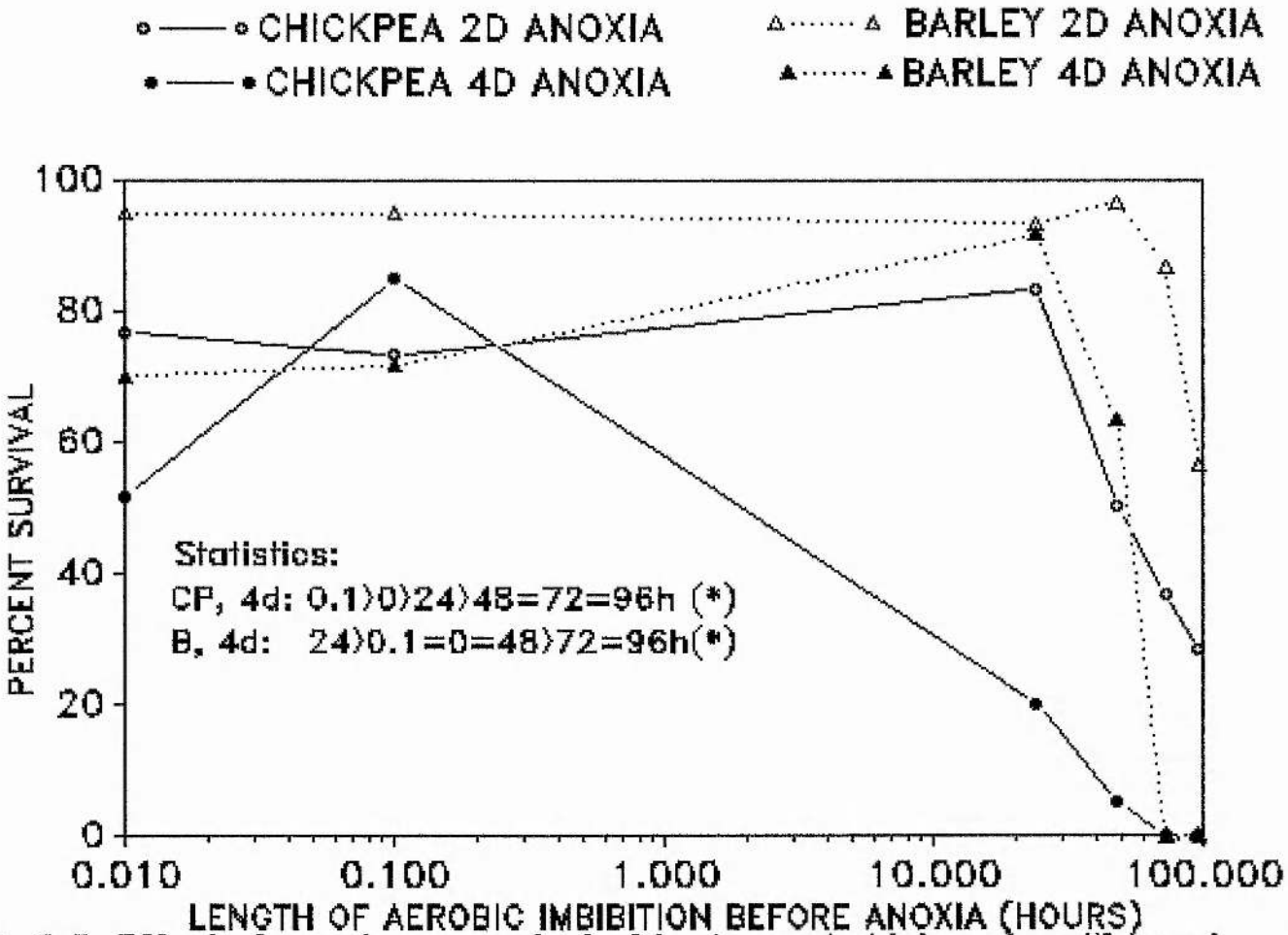

Fig.1.3. Effect of anoxia on survival of barley and chickpea seedlings at different germination stages (n=60). Figures are displayed in the annexe, Table 1.6a4. 0.01(log scale) represents 0h imbibition. Significant difference (*) were tested by chisquare (p<0.05).

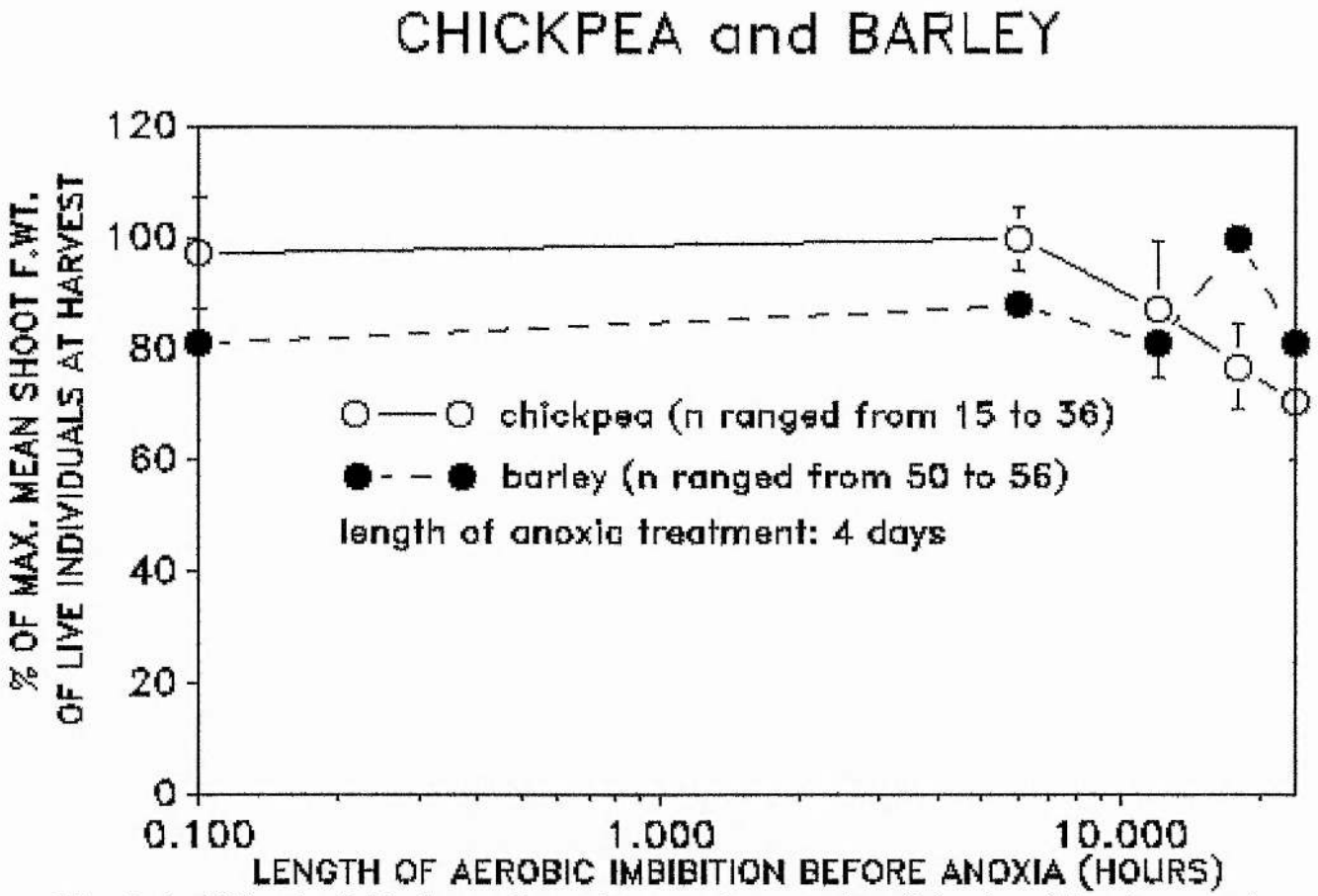

Fig.1.4. Effect of timing of early anoxia on shoot f.wt. of barley and chickpea seedlings. Figures are displayed in the annexe, Table 1.5a*ii*. Shoot f.wt. was measured 20 days after planting, for live individuals, thus the variation in n. Bars indicate standard error.

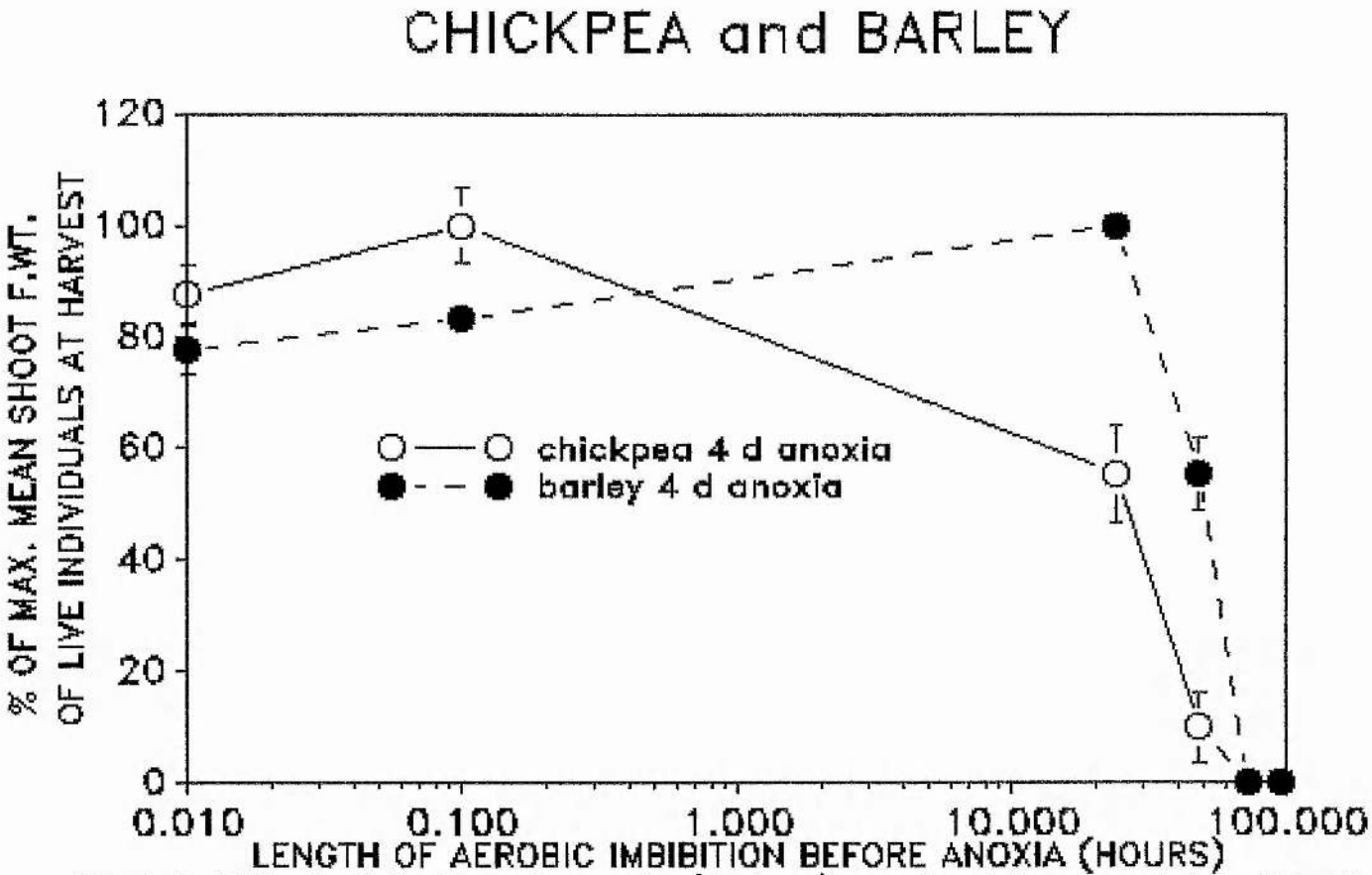

Fig.1.5. Effect of timing of anoxia (4 days) on shoot fresh weight of barley and chickpea seedlings. Figures are shown in the annexe, Table 1.6a,b. 0.01 (log scale) represents 0h imbibition. Shoot f.wt. was measured 20 days after planting, for live individuals. Thus, n ranged from 0 to 55. Bars indicate standard error.

3.2 *Carbon dioxide output*

The course of change in respiratory activity of the two species throughout the first 96 hours of germination is shown in figs.1.6 and 1.7, together with the effects of either 1 or 24 hours of anoxia. The aerobic controls of chickpea germinating seeds show little change, irrespective of germination stage. Barley is more active during the first hours of imbibition and then declines during the second pause or transition phase and then accelerates in the third phase. Despite a small acceleration in carbon dioxide output after 1-hour anoxia for some of the stages tested, after 24 hours of anoxia anaerobic respiration is reduced in barley, irrespective of stage. However, seeds at the stages 12- and 18-hour aerobic imbibition still showed a small Pasteur effect. In chickpea, by contrast there is an acceleration in carbon dioxide output which starts after 1-hour anoxia and is even more marked after 24 hours, characterising a high Pasteur effect. The exception were the chickpea seeds at the early stage of 6-hour aerobic imbibition, which after an initial rise following 1 hour of anoxia, show a marked reduction after 24 hours. This stage, as seen in the survival results above (fig.1.2), is the most anoxia-tolerant for chickpeas.

3.3. *Ethanol Production*

As mentioned before, many seeds undergo a period of natural anaerobiosis during the initial stages of germination. It is therefore important to ascertain whether the imposition of an anoxic treatment augments significantly the natural production of ethanol that can be found in the tissues at this time. Fig.1.8 shows that in chickpea it is only after 18-24 hours of imbibition that an anoxic stress makes any significant addition to the amount of ethanol produced in the germinating seedling, which matches the effect found for

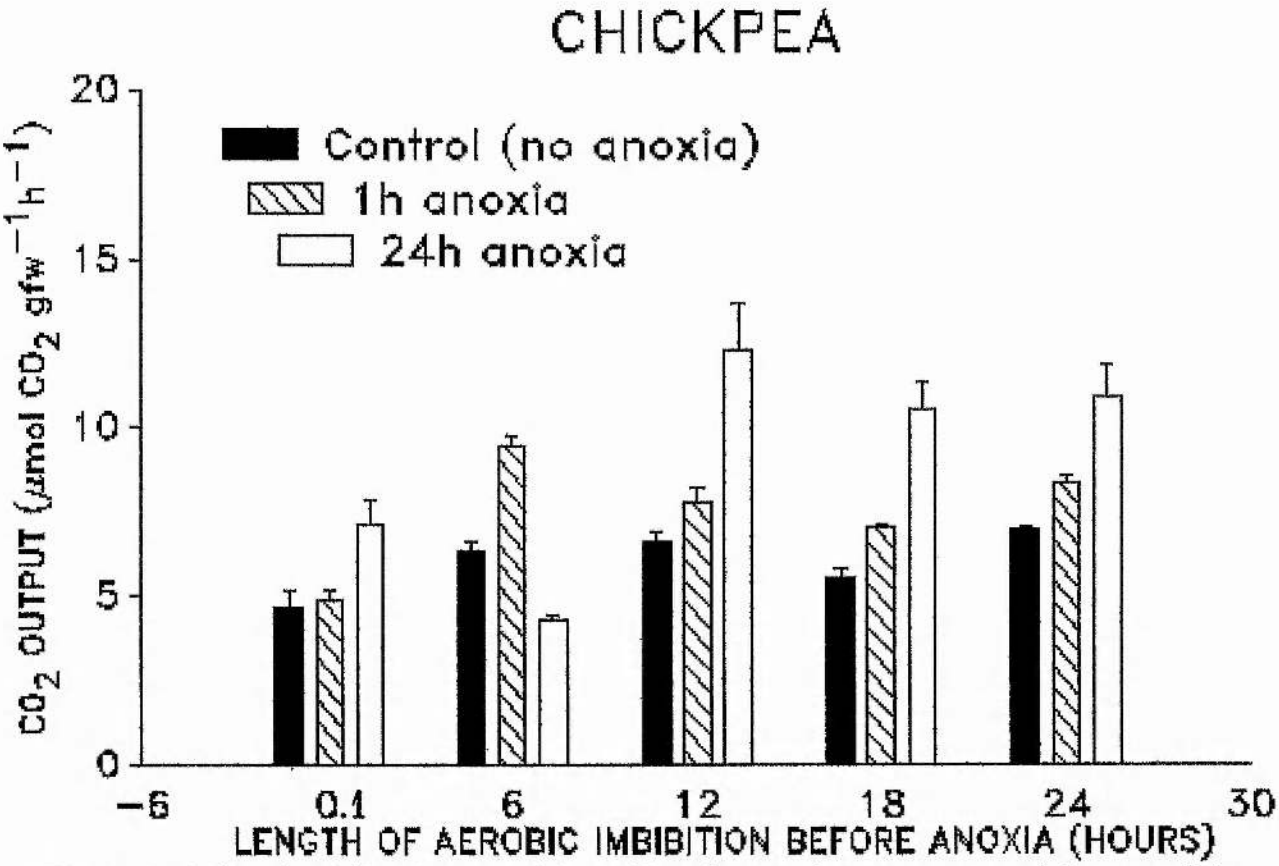

Fig.1.6. Effect of timing and length of anoxia, compared to aerobic controls, on the carbon dioxide output of chickpea germinating seeds. Bars indicate standard error (n=5; 3 seeds per replicate). Figures are displayed in the annexe, Table 1.7a.

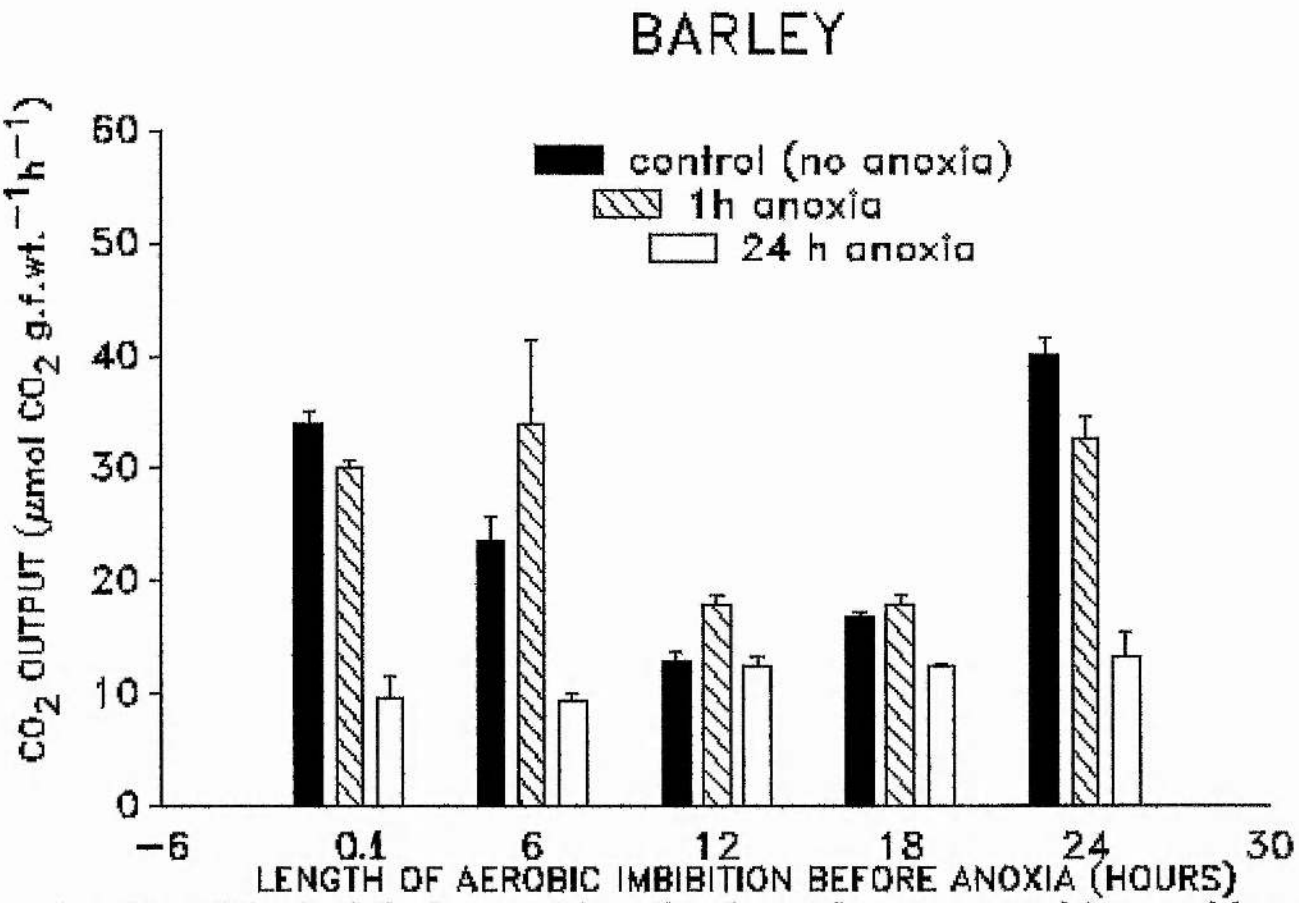

Fig.1.7. Effect of timing and length of anoxia, compared to aerobic control on the carbon dioxide output of barley germinating seeds. Bars indicate standard error (n=5; 3 seeds per replicate). Figures are displayed in the annexe, Table 1.8a.

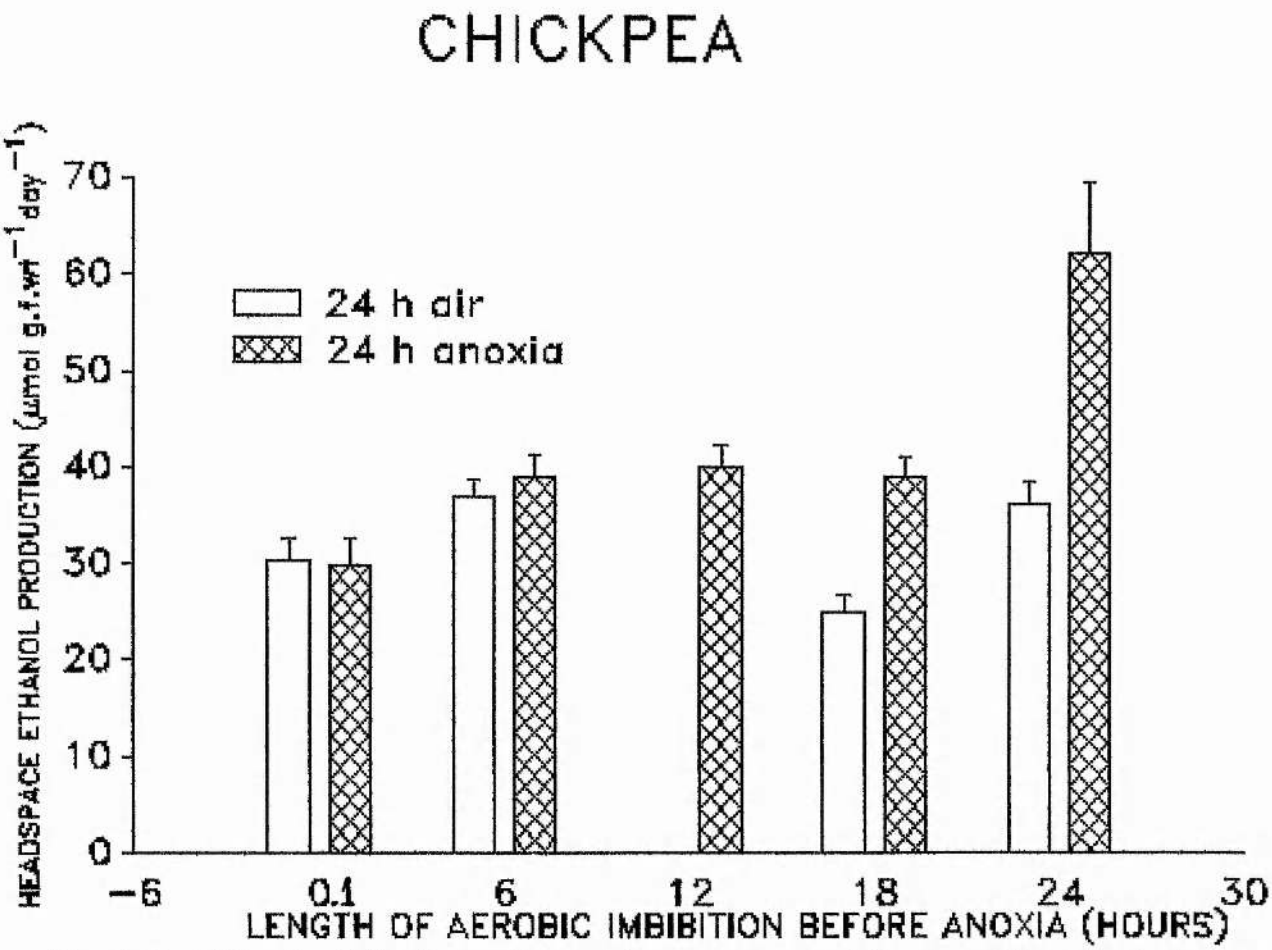

Fig.1.8. Effect of timing of anoxia, compared to aerobic controls, on the headspace ethanol production of chickpea germinating seeds. Bars show standard errors (n=5; 3 seeds per replicate). No available data for 12; 24h air. Figures are displayed in the annexe, Table 1.9a.

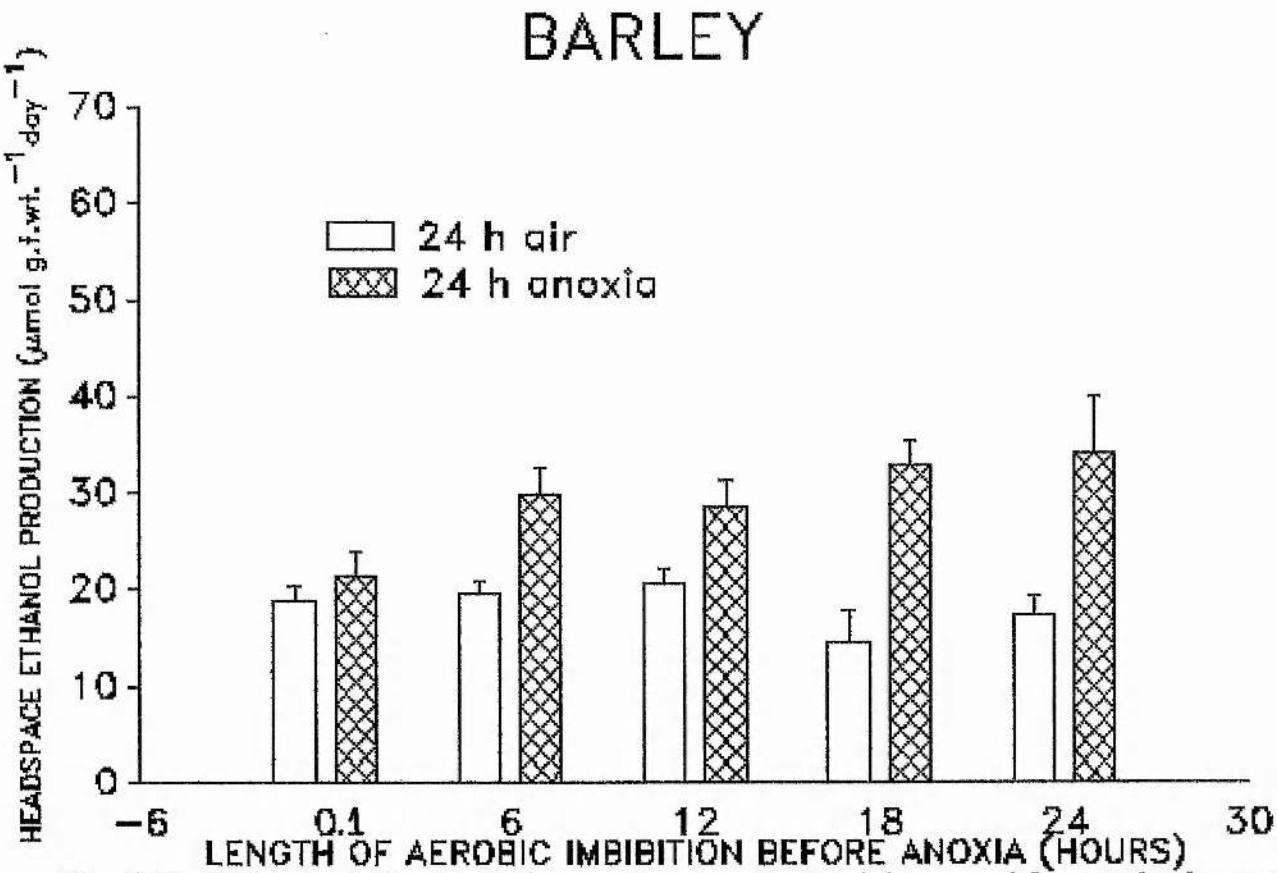

Fig.1.9. Effect of timing of anoxia, compared to aerobic controls, on the headspace ethanol production of barley germinating seeds. Bars show standard errors (n=5; 3 seeds per replicate). Figures are displayed in the annexe, Table 1.9a.

anoxia on anaerobic respiration. Unfortunately, no comparison can be made for the 12-hour stage, which has no available measurements of ethanol production after 24 hours in air. In barley (fig.1.9), the 24-hour anoxic treatment causes an increase in ethanol production that is already substantially higher when it begins within 6 hours of imbibition. Despite some variability, the effect of anoxia on carbon dioxide emission on chickpea seedlings (fig.1.6) mirrors the results on ethanol production, where the divergence between aerobically and anaerobically produced ethanol develops mostly after 18-24 hours of seed aerobic imbibition, when carbon dioxide output is mostly accelerated. In the case of barley, however, this similarity does not always occur. For instance, the lack of indication of Pasteur effect for seeds at the stages reached at 6 and 24 hours of aerobic imbibition, contrasted with the stimulation of ethanol production observed in seeds at these same stages.

3.4 *Imbibition power*

Table 1.3 records the effect of oxygen availability on seed imbibition at the early stages of germination. After 2 hours imbibing water aerobically, the onset of anoxia seems to reduce slightly the subsequent water imbibition of chickpea seeds. This effect repeats itself as the length of the anoxic treatment increases. The same occurred when 2-hour anoxia was imposed after 12-hour aerobic imbibition, and when seeds were left for 10 days under anoxia previously to imbibition. However, in none of these cases the differences between seed increase in fresh weight after aerobic imbibition and after anoxic imbibition appeared to be expressive, apparently due to a large individual variation. For barley, however, anoxia does not even show a tendency to reduce water imbibition.

Table 1.3: Initial weight of dry seeds (mg; mean±st.dev) of chickpea (***Cicer arietinum*** **L.**) and barley (***Hordeum vulgare*** **L.cv. Golden Promise**) and respective increase in seed fresh weight (mg; mean increase in fw±st.dev) after determined periods of aerobic imbibition compared with seeds which imbibition, at some stage, was carried out under anoxia (n=100).

IMBIBITION	CHICKPEA		BARLEY	
	(weight in mg±st.dev)			
	INITIAL	INCREASE	INITIAL	INCREASE
2h AIR	373.9±45.4	115.0±32.7	40.2±7.2	10.4±2.8
0h AIR + 2h ANOX	363.3±46.8	113.1±29.6	39.8±7.3	10.1±2.7
4h AIR	384.3±41.6	161.2±40.6	38.1±7.7	13.1±2.9
2h AIR + 2h ANOX	381.9±45.1	144.7±38.1	38.0±7.0	13.2±2.9
6h AIR	396.1±45.5	188.2±50.2	39.4±7.8	13.2±3.2
2h AIR + 4h ANOX	377.0±48.4	180.1±50.9	40.0±7.8	11.7±2.9
12h AIR	400.4±38.6	287.7±46.3	-	-
2h AIR + 10h ANOX	393.8±44.4	260.9±62.4	-	-
14h AIR	378.2±40.8	300.1±47.9	-	-
12h AIR + 2h ANOX	381.5±49.4	282.3±54.6	-	-
4h AIR	384.7±40.0	148.5±36.8	-	-
10 days ANOX(1) + 4 h ANOX	370.7±51.5	138.2±42.5	-	-

AIR=aerobic imbibition; ANOX=anoxic imbibition.

(1) In this treatment, during the first 10 days of anoxia the seeds were not in contact with water. Imbibition only took place during the subsequent 4-hour anoxia.

3.5 *Oxygen uptake*

An additional experiment assessing embryo oxygen uptake was found to be necessary in order to attempt to explain the significantly 36% higher survival after 4 days anoxia of chickpea seeds which have previously imbibed water aerobically for 6 minutes than seeds with no prior aerobic imbibition (see fig.1.3). This experiment assessed whether the ability of chickpea embryos to take up oxygen is affected by imposition of anoxia before or after the start of imbibition.

A comparison between treatments *i*) and *v*) in Table 1.4 shows that when the imbibition of chickpea seeds is started under anoxia, the ability of the embryos to immediately take up oxygen when returned to air 12 hours later is significantly lower (ca. 38% less) than the embryos of seeds imbibing water aerobically for 12 hours, which was expected. The comparison that more conclusively links the trend in survival described above with differences in metabolic activity due to short-term aerobic imbibition prior to anoxia, is that between treatments *i*) and *iv*); *i.e.* the oxygen uptake of embryos after 12 hours anoxic imbibition and the oxygen uptake of embryos allowed 2 hours aerobic imbibition prior to 12 hours anoxic imbibition. The embryos allowed this short period of aerobic imbibition prior to anoxia showed a significantly higher oxygen uptake (ca. 27%) at the end of the 12-hour anoxic period, than those that were allowed no previous aerobic imbibition.

A comparison between some of the other treatments, although not showing differences which are statistically significant, further indicate a tendency of survival at the early germination stages to be linked to embryo oxygen uptake for chickpeas. When after the 12 hours of anaerobic imbibition seeds are allowed a full 2 hours of aerobic imbibition, the

embryo oxygen uptake still remains reduced, even if compared with seeds aerobically imbibed only for 2 hours (no anoxic treatment), which showed an 11% higher average figure (treatment *ii vs. iii* in Table 1.4). Conversely, when seeds are subjected to 12 hours anoxia having had previously imbibed water aerobically for 2 hours (treatment *iv*), embryo oxygen uptake remains similar to the 2 hours aerobic control (treatment *iii*) and 12% higher (still a non-significant difference) than that of the embryos of seeds subjected to the 12-hour anoxia followed by 2-hour air treatment (treatment *ii*).

The experiments on embryo oxygen uptake, therefore, suggest a possible metabolic trend which matches the survival results, where post-anoxic survival in chickpea seeds is favoured when anoxia imposition occurs after aerobic imbibition has already started. The notable fact being that a significant improvement in post-anoxic survival results by having a short delay after the start of imbibition, before imposing an anoxic stress.

Table 1.4: Oxygen uptake of embryos (μl O_2.gfw^{-1} of embryo; mean per sample ± st.error) of chickpea seeds which were subjected to distinct anoxic/ imbibition treatments (n=6). The mean fresh weight (g) ± st.error of the samples used (30 embryos in each) is also given.

TREATMENT	EMBRYO F.WT.(g)	O_2 UPTAKE (μl.gfw^{-1})
i) 12 HOURS ANOXIC IMBIBITION	0.198 ± 0.001	531.42 ± 17.78
ii) 12 h ANOXIC IMBIB.+ 2 h AEROBIC IMBIB.	0.193 ± 0.002	570.59 ± 34.92
iii) 2 HOURS AEROBIC IMBIBITION	0.169 ± 0.005	640.46 ± 21.98
iv) 2 h AEROBIC IMBIB.+ 12 h ANOXIC IMBIB.	0.186 ± 0.001	644.43 ± 17.02
v) 12 HOURS AEROBIC IMBIBITION	0.204 ± 0.004	855.19 ± 26.96

t-test:			
i < *v*	(*, p<0.001)	*i* < *ii*	(ns)
i < *iv*	(*, p<0.05)	*ii* < *iii*	(ns)
i < *iii*	(*, p=0.006)	*ii* < *iv*	(ns)
ii < *v*	(*, p<0.001)	*iii* < *iv*	(ns)

4) DISCUSSION

The present study shows that tolerance of anoxia in germinating seeds, as measured through post-anoxic survival, decreases with the length of time that has elapsed since the onset of the imbibition period. The expected result that anoxia-tolerance decreases once the seedlings are in the active phase of germination with radicle emergence and tissue growth is found for both chickpea and barley seedlings, with chickpeas being more rapidly affected than barley. **DeBell & Naylor (1972)** made similar observation with submerged swamp tupelo (***Nyssa sylvatica***) seedlings.

The surprising result, however, is that in the first few hours of imbibition an oxygen deficit can have a subsequent effect on post-anoxic survival. This early sensitivity to anoxia is unexpected in view of the observation that respiration during the early stage of imbibition proceeds with respiratory quotients (carbon dioxide output : oxygen uptake) well above unity **(Morohashi, 1978)** and that oxidative metabolism is limited by the reduced respiratory capacity of the mitochondria. The relative inactivity of the mitochondria at this stage would suggest that the absence of oxygen would be unlikely to have a deleterious effect **(Simon, 1984)**. Moreover, causes of injury to seeds during the initial stages of imbibition are often attributed to physical processes such as high rate of water influx **(Powell & Matthews, 1978; 1981, McCormac & Keefe, 1990)** and not to mechanisms where the absence of oxygen would be thought to have a deleterious effect.

4.1 *Germination phases*

Returning to the germination phase classification of **Koller & Hadas (1982)**, the detailed examination of seedling survival to anoxia in the early stages of germination

(fig.1.2) showed that in chickpea it is in the transition phase, between initial imbibition and the onset of tissue growth, that the seedlings present the greatest anoxia-tolerance. Fig.1.1. shows that barley did not show any significant differences in survival between the earlier germination stages tested (from 0.1 to 24 h imbibition). However, fig.1.3 shows a significantly higher post-anoxic survival for germinating seeds submitted to anoxia at the 24-hour aerobic imbibition stage. The results of mean shoot fresh weight of surviving individuals (fig.1.4) show a small advantage to those seedlings not subjected to anoxia until the very beginning of the growth phase (18 h aerobic imbibition). These are possible indications that barley could possibly show a greater anoxia-tolerance just as the radicle protrusion commences. Submitting these seeds at different germination stages to an anoxic period longer than four days could be useful in further testing this hypothesis, and deserves future attention.

According to **Koller & Hadas (1982)**, germination can be blocked during the transition phase depending on the external conditions, and then be resumed later, which may be, at least in part, an expression of the physiological tolerance of reduced oxygen supply by chickpea at this stage. The fact that barley shows high anoxia-tolerance in the earlier phases of germination before radicle protrusion irrespective of the precise time it is subjected to anoxia, might reflect a difference in the seed coat permeability between the two species.

4.2 *Ethanol production and natural anaerobiosis*

The present results suggest that there is a pronounced period of natural anaerobiosis within chickpea seeds, which is not so readily evident in barley seeds. When chickpea seeds are placed under anoxia for 24 hours after either 0.1-

or 6-hour aerobic imbibition, they produce the same amount of ethanol as if they were kept in air suggesting that even under air the seeds are partially anaerobic. In barley, by contrast, the imposition of anoxia increases ethanol production. This difference in behaviour may be due to differences in seed coat permeability between the two species, and if this is the case, chickpea seed coat would be less permeable than barley seed coat. **Drew & Lynch (1980)** nevertheless suggest the existence of a period of anaerobiosis in barley germination due to competition with microorganisms. They claim that oxygen reaches barley's embryo through a small gap in the husk, which is also the pathway for exudated material from the seed, and which is therefore densely colonised by the spermosphere microflora. The result is oxygen deficiency around the embryo causing more exudation, more microbial growth and increased competition for oxygen by the microorganisms.

It is tempting to speculate that the period of natural anaerobiosis which a seed coat with low permeability can impose on the seed, acclimatises or conditions the embryo to be anoxia-tolerant (see discussion below). The impermeable-coated chickpea seeds have a greater tolerance of anoxia during the initial and transition stages of germination before the onset of radicle protrusion, while barley seeds, which are possibly more permeable than chickpea, do not show significant differences in terms of anoxia-tolerance between the early phases of germination, including the moment of protrusion of the radicle. This possible difference in seed coat permeability does not necessarily mean that chickpea is more anoxia-tolerant than barley, or that impermeable-coated seeds are, in general, more tolerant than permeable-coated seeds. Actually, throughout the studies conducted in this thesis barley was often more tolerant than chickpea. The indication given by the present results is that impermeable-coated seeds are likely to be more anoxia-tolerant in the

early germination stages before rupture of the testa than later.

From the respiration results, an additional argument appears in favour of the natural anaerobiosis hypothesis. The initial peak in carbon dioxide output observed in several treatments after 1-hour anoxia is possibly due to the impact on the metabolism of the germinating seeds caused by the abrupt change in the atmosphere, which goes from fully aerobic to totally anoxic as the plants enter the anaerobic chamber. Subsequently, the tolerant plants would possibly be able to regain control over their respiratory activity and reduce their carbon dioxide output. If anoxia establishes itself gradually, this first impact would possibly not occur, which could mean in many cases improved survival conditions during and after the anoxic period. Natural anaerobiosis or hypoxic conditions within the seed in the initial germination stages, which is here claimed to occur in the chickpeas studied, could be translated in a period of acclimatisation to lack of oxygen which would reduce the impact of abrupt anoxia. Indeed, there is no peak of carbon dioxide output after 1-hour anoxia for chickpeas at the 0.1 stage, and in barley the output is actually reduced at that stage. Obviously, since natural anaerobiosis is in itself a stress, it can only turn out to be beneficial in very precise circumstances, where it is not prolonged by external factors or where it does not predispose the germinating seed to be vulnerable to other sources of stress. Further discussion on stress acclimatisation can be seen in Chapter Five.

4.3 *Imbibition*

Although the seeds were uniformly selected, the large standard deviation found in the imbibition experiments here described could be due to intraspecific variation in seed

biomass. **Hendrix *et al.*(1991)** describe several differences in seed germination and seedling emergence between smaller and larger seeds of a same species.

McCormac & Keefe (1990) recently investigated the imbibition effects on cauliflower by using a modern technique of *machine vision*, which apparently reduces possible errors by measuring weight increase, particularly for small seeded plants, as those shown by the imbibition experiments here described. They concluded that imbibition damage appears to be a function of embryo quality as well as rapid water influx. It could be that what these authors call *embryo quality* is actually the physiological and metabolic importance of the early stages of germination emphasized in the present Chapter.

4.5 *Respiratory activity*

4.5.1 Whole-seed carbon dioxide output

The capacity of germinating seeds to reduce carbon dioxide output when placed under anoxia appears from these experiments to be related to the length of the aerobic imbibition period before the imposition to anoxia. Although in the survival experiments discussed above, plants were subjected to 2 and 4 days of anoxia, the 24-hour anoxia to which plants were submitted in the respiration experiments was already sufficient to indicate a direct relationship between anoxia-tolerance of particular stages (as determined by the survival experiments) and respiratory activity. This was particularly evident in chickpea. From the survival experiments it appears that chickpea is more anoxia-tolerant when submitted to the 4-day anoxic treatment after 6 hours of aerobic imbibition. Seeds at this germination stage, despite an initial 34% increase in carbon dioxide output after 1-hour anoxia, reduced this figure by 56% after 24

hours of submission to anoxia (33% less than the control). These results are in harmony with earlier views on flooding tolerance **(Crawford, 1966; 1982)**, which associated greatest tolerance of oxygen deprivation with low metabolic rates.

After 12, 18 and 24 hours of aerobic imbibition, anoxia had the reverse effect on chickpeas with carbon dioxide emission increasing approximately 46, 48 and 41% respectively, representing a substantial Pasteur effect after 24 hours of anoxia. From the survival results it appeared that in chickpea, the germination stages reached at 12 and 24 hours of aerobic imbibition seemed to be the particularly anoxia-sensitive after 4 days of anoxia, which matches the present respiration results. However, seeds at the 18-hour stage showed significantly higher survival after 4 days anoxia than seeds at the 12 and 24-hour stages, despite their similarly high Pasteur effect after 24 hours of anoxia. It is possible that, as anoxia progresses, seeds at the 18-hour stage are somehow able to reduce their respiratory rates, whereas seeds at the 12 and 24-hour stages are not.

The great deal of energy spent by the plant after 12-hour aerobic imbibition is likely to be associated with the radicle protrusion which occurs in the subsequent hours. Compared to other early germination stages, barley seeds at the 12-hour stage also showed lowest survival after 4 days anoxia, reduced shoot fresh weight after 4 days anoxia followed by 20 days of growth in air, and the highest Pasteur effect after 24 hours anoxia. Although for barley these differences were never significant, these data are a further indication of the importance of the 12-hour stage as a key moment in the germination process where subjection to anoxia can be lethal.

The little variation in respiratory response after 24-anoxia in barley reflects the reduced variation in survival shown

after 4 days of anoxia among seeds in the early germination stages.

Some controversy on seed respiration emerges from the literature. **Koller & Hadas (1982)**, although recognising a rapid increase in the respiratory rate of seeds in the initial phases of germination, affirm that respiratory activity and oxygen requirements are much higher in the growth phase. **Kramer & Kozlowski (1979)**, on the other hand, consider that seeds usually need higher oxygen concentrations for germination than that required for subsequent growth. In the present study, seed oxygen requirements were greater during the growth phase.

4.5.2 Embryo oxygen uptake

The respiration experiments provided a more conclusive link between post-anoxic survival in the early germination stages and seed metabolic activity than the studies on ethanol damage and imbibition impairment. The carbon dioxide output results offer an explanation for the variation in the survival of seeds subjected to anoxia at different moments within the first 24 hours of aerobic imbibition. However, they still do not explain the result showing that 6 minutes of aerobic imbibition prior to anoxia resulted in a 36% increase in post-anoxic survival compared with seeds anaerobically imbibed. The measurements of oxygen taken up by embryos of chickpea seeds subjected to different imbibition/anoxia treatments, however, indicated that the presence or absence of oxygen even during the initial stages of imbibition can have a significant metabolic effect. It was shown that the ability of an embryo to take up oxygen during the first 12 hours of imbibition is affected by anoxia, depending on when anoxia is imposed. An initial 2 hours of aerobic imbibition before 12-hour anaerobic imbibition guarantees, when the seeds are returned to air,

an embryo oxygen uptake significantly higher than that of the embryos of seeds returned to air after 12-hour anoxic imbibition, without a previous 2-hour aerobic imbibition.

This study shows that a short period of aerobic imbibition prior to anoxia is sufficient to account for improved survival conditions in the post-anoxic period, which finds a possible explanation in the fact that even a few hours of aerobic imbibition prior to anoxia result in an enhancement of the embryo oxygen uptake when the seed is returned to air, compared with seeds not previously allowed to imbibe aerobically. This evidence contrasts with earlier studies suggesting that in the initial stages of imbibition respiratory quotients are high **(Morohashi, 1978; Collins & Wilson, 1972)**, and mitochondrial activity is rather reduced and slow to reach its full potential **(Simon, 1984)**. The significance of oxygen during the first stages of imbibition may be connected with the need to establish and maintain adenine nucleotide ratios sufficient to maintain energy charge values at levels of 0.8 as is usually found in normal oxygen metabolising tissues **(Pradet, Narayanan & Vermeersch, 1968; Hourmant & Pradet, 1981)**. In lettuce seeds, these authors showed that the energy charge of dry seeds (0.2) rose to normal values (0.8) within 30 minutes of soaking in aerated water.

The mechanisms of soaking injury in seeds have been a cause for much speculation during the past seven decades **(Small *et al.*, 1991)**. The investigations conducted here prompt the conclusion that even the initial moments of seed imbibition are not exclusively the domain of physical processes such as water absorption and tissue hydration, but that oxygen demanding metabolic activity is already involved and essential for maximum viability. The evidence presented in this Chapter of the differential response to lack of oxygen in the initial germination stages adds to the scepticism

towards the hypothesis of rapid water uptake being the main factor leading to seed inviability (see **McCormac & Keefe, 1990; and Small *et al.*,1991**), and suggests that further research to elucidate the mechanisms of seed soaking injury should concentrate on physiological rather than physical injury to the imbibing seed.

4.5 *Experimentation*

The present results show the importance with experiments on germinating seeds and seedlings being tested for anoxia survival, of emphasizing the stage of germination of the seeds that are being used. Furthermore, according to the purposes of the experiment, particular attention should be paid to the choice of the stage of germination to work with, in order to avoid misinterpretations.

ANNEXE

Table 1.5a: Chickpea (***Cicer arietinum*** L.) and barley (***Hordeum vulgare*** L.) seedlings subjected to 2 and 4 days of anoxia at 20°C, at germination stages differing from each other by hours of aerobic imbibition prior to anoxia. After anoxia, the seedlings were planted and were allowed to grow for 20 days in air before assessing survival and shoot fresh weight.

i) Percent survival (n=60).

STAGE[1]	CHICKPEA		BARLEY	
d.u.a[2]	2	4	2	4
0.1	55.0	26.0	95.0	88.3
	ns	*	ns	ns
6	45.0	60.0	96.7	93.3
	ns	*	ns	ns
12	38.3	25.0	96.7	83.3
	*	*	ns	ns
18	68.3	45.0	100.0	88.3
	ns	*	ns	ns
24	60.0	25.0	95.0	93.3

statistical test: chisquare; * significant at $p < 0.05$.
[1] STAGE = hours of aerobic imbibition prior to anoxia;
[2] d.u.a.= days under anoxia.
The data are displayed in Figs.1.1.(barley) and 1.2. (chickpea).

ii) Mean shoot fresh weight (mg;mean±st.error) of surviving seedlings (**n** is indicated below each figure).

STAGE	CHICKPEA		BARLEY	
d.u.a	2	4	2	4
0.1	695.5±56.3	755.6±94.6	160.4±5.5	131.7±6.0
n	33	16	57	51
6	895.7±76.2	776.5±54.1	153.0±5.1	138.4±5.0
n	27	36	58	56
12	672.0±83.9	679.0±114.0	161.6±5.1	125.3±5.4
n	23	15	58	50
18	936.6±59.5	625.8±71.1	183.2±5.3	159.3±5.4
n	41	27	60	51
24	772.4±62.6	549.7±97.3	165.4±5.5	123.4±5.2
n	36	15	57	56

The data obtained for both species after 4 d anoxia are expressed as percentage of maximum mean shoot f.wt. of surviving individuals at harvest in Fig.1.4.

Table 1.6a: Chickpea (***Cicer arietinum*** L.) and barley (***Hordeum vulgare*** L.) seedlings subjected to 2 and 4 days of anoxia at 20°C, at germination stages differing from each other by days of aerobic imbibition prior to anoxia. After anoxia, the seedlings were planted and were allowed to grow for 20 days in air before assessing survival and shoot fresh weight.

i) Percent survival (n=60).

STAGE[1]	CHICKPEA		BARLEY	
d.u.a[2]	2	4	2	4
0	76.7	51.7	95.0	70.0
	ns	*	ns	ns
0.1	73.3	85.0	95.0	71.7
	ns	*	ns	*
24	83.3	20.0	93.3	91.7
	*	*	ns	*
48	50.0	5.0	96.7	63.3
	ns	ns	*	*
72	36.7	0.0	86.7	0.0
	ns	ns	*	ns
96	28.3	0.0	56.7	0.0

statistical test: chisquare - * significant at $p < 0.05$

[1] STAGE = hours of aerobic imbibition prior to anoxia.

2 d.u.a.= days under anoxia.

The data are displayed in Fig.1.3.

ii) Mean shoot fresh weight (mg;mean±st.error) of surviving seedlings (**n** is indicated below each figure).

STAGE	CHICKPEA		BARLEY	
d.u.a	2	4	2	4
0	910.5±55.9	721.1±61.5	132.7±5.0	136.0±6.8
n	46	31	57	42
0.1	863.3±47.7	806.5±50.5	152.6±8.4	154.5±8.3
n	44	51	57	43
24	441.3±49.9	451.0±79.8	138.7±4.4	182.5±5.7
n	50	12	56	55
48	220.4±55.2	81.0±58.9	99.0±6.7	98.7±12.0
n	30	03	58	38
72	237.4±42.0	-	79.8±5.6	-
n	22	-	52	-
96	213.3±38.7	-	54.2±7.5	-
n	17	-	34	-

The data obtained for both species after 4 d anoxia are expressed as percentage of maximum mean shoot f.wt. of surviving individuals at harvest in Fig.1.5.

Table 1.7a: Rate of carbon dioxide emission (mean±st.error) of chickpea (***Cicer arietinum*** L.) seeds subjected to 0, 1 and 24 hours of anoxia, at different germination stages (n=5, 3 seeds used in each repetition).

GERMIN. PHASE	STAGE[1]	RESPIRATION RATE (μmol CO_2 gfw^{-1} h^{-1}) hours of anoxia		
		0	1	24
transition	0.1	4.67±0.47	4.87±0.27	7.13±0.71
	6	6.32±0.28	9.49±0.23	4.29±0.16
	12	6.62±0.32	7.78±0.41	12.31±1.39
growth	18	5.54±0.30	7.03±0.10	10.57±0.80
	24	6.99±0.07	8.36±0.24	11.90±0.99

[1] STAGE = hours of aerobic imbibition prior to anoxia.
The data are displayed in Fig.1.6.

Table 1.8a: Rate of carbon dioxide emission (mean±st.error) of barley (***Hordeum vulgare*** L.) seeds subjected to 0, 1 and 24 hours of anoxia, at early germination stages (n=5, 3 seeds used in each repetition)[1].

GERMIN. PHASE	STAGE[2]	RESPIRATION RATE (μmol CO_2 gfw^{-1} h^{-1}) hours of anoxia		
		0	1	24
transition	0.1	34.00±1.16	30.01±0.74	9.60±1.90
	6	23.61±2.25	34.05±7.45	9.32±0.72
	12	12.79±1.04	17.80±1.07	12.45±0.77
growth	18	16.71±0.62	17.97±0.85	12.39±0.33
	24	40.08±1.55	32.72±1.86	13.31±2.21

[1] the %survival of the above treatments ranged from 86.7 to 100% after planting.
[2] STAGE = hours of aerobic imbibition prior to anoxia.
The data are displayed in Fig.1.7.

Table 1.9a: Headspace ethanol production (μmol.gfw^{-1}.day^{-1}; mean±st.error) of chickpea (***Cicer arietinum*** L.) and barley (***Hordeum vulgare*** L.) seeds subjected to 24 hours of anoxia or 24 hours of atmospheric air at different germination stages (n=5, 3 seeds used in each repetition).

	ethanol production (μmol.gfw^{-1}.day^{-1})			
Species	CHICKPEA		BARLEY	
STAGE[1]	AIR	ANOXIA	AIR	ANOXIA
0.1	30.41±2.21	29.69±2.82	18.95±1.40	21.36±2.45
6	37.03±1.59	38.87±2.31	19.54±1.42	29.89±2.59
12	-[2]	39.92±2.41	20.67±1.54	28.51±2.77
18	24.92±1.82	39.00±1.87	14.62±3.07	32.95±2.45
24	36.26±2.16	62.09±7.36	17.39±2.05	34.09±5.84

[1] STAGE = hours of aerobic imbibition prior to anoxia.
[2] data not available.
The data are displayed in Fig.1.8. (chickpea), and in Fig.1.9 (barley).

Table 1.10a: Percentage survival (n=15) and mean shoot fresh weight-MSFW (mg; mean±st.error) of surviving chickpea (***Cicer arietinum*** L.) seedlings allowed to grow for 20 days after being used in the ethanol production experiment (n for the shoot fresh weight measurements is shown beside each figure).

	24 h AEROBIC		24 h ANAEROBIC	
STAGE[1]	% surv	MSFW	% surv	MSFW
0.1	80.0	475.1±96.1 (12)	73.3	599.8±98.5 (11)
6	86.6	578.9±98.3 (13)	80.0	599.1±54.1 (12)
12	66.6	378.8±58.5 (10)	86.6	406.8±78.1 (13)
18	80.0	455.8±65.8 (12)	73.3	509.2±82.3 (11)
24	73.3	562.1±60.0 (11)	86.6	433.7±49.0 (13)

statistics: chisquare test for survival- no significant difference at $p < 0.05$.
[1] STAGE = hours of aerobic imbibition prior to anoxia.
These data are not displayed in any Fig.

Table 1.11a: Percentage survival (n=15) and mean shoot fresh weight-MSFW (mg; mean±st.error) of surviving barley (***Hordeum vulgare*** L.) seedlings allowed to grow for 20 days after being used in the ethanol production experiment (n for the shoot fresh weight measurements is shown beside each figure).

	24 h AEROBIC			24 h ANAEROBIC		
STAGE[1]	% surv	MSFW		% surv	MSFW	
0.1	100.0	205.5±12.0	(15)	100.0	176.0±9.7	(15)
6	100.0	180.9±8.1	(15)	100.0	169.1±9.3	(15)
12	100.0	241.5±11.8	(15)	80.0	169.5±10.5	(12)
18	93.3	199.4±10.0	(14)	100.0	180.0±12.9	(15)
24	93.3	169.7±13.2	(14)	100.0	167.3±8.2	(15)

[1] STAGE = hours of aerobic imbibition prior to anoxia. These data are not displayed in any Fig.

* CHAPTER TWO *

ANOXIA-SENSITIVITY OF YOUNG SEEDLINGS DETERMINING THE ABSENCE OF *Parkia pendula* FROM FLOODED AREAS IN THE NEOTROPICS [1]

1) INTRODUCTION

With the advent of snow melt in the Andes and the commencement of the rainy season in North Brazil, the water level of the rivers of the Amazon region may rise by 10 metres or more **(Furch & Otto, 1987)** flooding extensive areas of tropical rain forest for up to 8-9 months of the year **(Worbes & Junk, 1989)**. The variations in predictability and regularity, as well as the differences in nutrient concentration of the waters of the distinct rivers involved, led **Prance (1979)** to name seven different types of amazonian forests subject to inundation. These distinct types of flooding have resulted in the present existence of a mosaic of habitats in the Amazon floodplains, determining plant distribution and species diversity **(Junk, 1989)**. In this region, some species have developed different ecotypes and a number of genera have evolved different species to colonise distinct habitats. A typical example is the pantropical genus ***Parkia***, which has to date 17 species described for the neotropics **(Hopkins, 1986)**, most of which are tall forest trees **(Hopkins & Hopkins, 1983)**.

Parkia has six widespread species (although three of these are infrequent) and, according to **Hopkins (1986)**, the remaining 11 have a restricted or very restricted

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distribution. The specific habitat occupied by each of these species ranges from coastal vegetation between beach and rain forest (*restingas*) to savanna-like vegetation (*caatinga* and/or *cerrado*) and from periodically flooded areas (*igapós* and/or *várzeas*) to unflooded areas (*terra-firme* forests).

Coutinho & Struffaldi (1971) studied the effects of flooding and submersion on seed germination and seedling growth of ***Parkia discolor***, a flood-tolerant species restricted to seasonal *igapó* areas (forests periodically flooded by nutrient poor and low pH *black* water). The present Chapter investigates the effects of similar treatments on ***Parkia pendula*** which, although widespread in Central America and in the South American Amazon region, rarely occurs on flood-prone areas but, rather, is characteristic of the non-flooded forests which are called *terra-firme* in Brazil. This Chapter is an attempt to determine the ecophysiological factors which may cause the absence of ***Parkia pendula*** from flood-prone areas.

The good quality hard wood of ***Parkia pendula*** has multiple uses **(Paula *et al.*, 1980)**, which adds an economic relevance to this study.

2) METHODOLOGY

Seeds of ***Parkia pendula*** **(Willdenow) Bentham ex Walpers** collected in *terra-firme* forests (*Reserva Ducke*, Pará, Brazil), were obtained from *Cenargen-Embrapa*, Brasília, Brazil. Seed germination after submersion and subjection to anoxia and seedling survival after periods of root flooding and total submersion were tested as follows.

2.1 *Seed germination after submersion and anoxia*

Fifty seeds were exposed to submersion+anoxia and fifty were used as controls which received neither a soaking nor an anoxia treatment. The submersion+anoxia treatment was provided by placing the sterilised seeds in a dish filled with non-aerated distilled water (pH=5.2) and subsequently submitting the ensemble to fully anaerobic conditions. After 30 minutes, the dish containing the submerged seeds was placed in an anaerobe jar. The jar was placed in a 25°C incubator, in the dark.

The seeds were left seven months under these conditions, before being allowed to germinate in air, after mechanical scarification. The control seeds were stored during this period in plastic bags, at room temperature, in the dark. Germination also took place in air after mechanical scarification.

2.2 *Seedling waterlogging and submersion tolerance*

One-month old seedlings of ***Parkia pendula***, grown individually in pots (7.5 cm deep; 7.4 cm larger diameter; 5.3 cm smaller diameter in the bottom) were submitted to root flooding, total submersion or regular watering conditions. At this age, the seedlings were ca. 5.0 cm tall

and had from 3 to 4 leaves formed (mostly 4). Each treatment included ten seedlings.

For the submersion treatment, the pots were placed in plastic tanks (32 cm length x 25 cm width x 13 cm depth) fully covered with tap water. The submerged seedlings were kept in the dark, since this is probably also the case for any young seedling submerged in field conditions by the Amazon rivers, due to the depth of water (up to 10 m or more; **Furch & Otto, 1987**), and to the large amount of sediment present both in black and clear water rivers. The seedlings subject to root waterlogging were maintained with tap water up to soil level and were exposed to a low light intensity of 40 $\mu mol.m^{-2}.s^{-1}$, which is a figure commonly found in the shaded floor of tropical forests (**Kwesiga, Grace & Sandford, 1986**), as well as the controls. The control seedlings were watered at 2-day intervals and were also maintained at the light intensity of 40 $\mu mol.m^{-2}.s^{-1}$.

Leaf emergence, as an indicator of growth, was assessed at weekly intervals for the treated plants and controls over a four-week period. Loss of leaflets, as an indicator of sensitivity, was also assessed at weekly intervals, but over a five-week period. Leafless seedlings with darkening of the stem (necrosis) were considered dead, which was confirmed by returning them to aerobic conditions at this stage and observing if they would recover or not over a period of approximately two weeks.

3) RESULTS

3.1 *Seed germination after submersion and anoxia*

Seven months of seed submersion+anoxia prior to return to air and scarification did not impair germination, and treated and control seeds gave the same germination percentages (Table 2.1).

After scarification, ***Parkia pendula*** seed coats in contact with water, became gelatinous and radicle protrusion rapidly occurred after ca. 72 hours.

3.2 *Seedling waterlogging and submersion tolerance*

Compared to the non-germinated seeds, one-month old seedlings of ***Parkia pendula*** showed little tolerance to root flooding or submersion. Root flooding up to the third week did not produce any evident negative effect since leaf emergence of the root-flooded seedlings was unimpaired up to that point, and no other symptoms of stress were observed. By the fourth week, however, dropping of leaflets indicated the onset of a rapid decline, which by the fifth week was irreversible. Return to unflooded conditions at this time did not promote seedling recovery and the plants died (Table 2.2). It was not tested whether this negative effect could be reversible if flooded plant were returned to unflooded conditions earlier than by the fifth week.

Full submersion immediately halted leaf emergence of ***Parkia pendula*** seedlings. The first external symptoms of stress, however, were more apparent after two weeks, when loss of leaflets started. By the third week, the now leafless submerged seedlings were effectively dead, since return to air at this point did not permit recovery (Table 2.2).

Table 2.1: Seed germination of ***Parkia*** species after submersion and anoxia regime prior to scarification, compared to control seeds.

Species	Treatment	% germination
Parkia pendula (n=50)	submersion+anoxia (7 months)	80
	control	80
Parkia discolor[1] (n=50)	submersion (6 months)	86
	control	86

[1] from **Coutinho & Struffaldi (1971).**

Table 2.2. Leaf emergence, progress of stress symptoms and mortality of ***Parkia pendula*** one-month old[1] seedlings subjected to root flooding or full submersion, in comparison with unflooded controls over a period of 5 weeks. Results expressed in percentage of seedlings tested showing each of the examined parameters in a determined week (n=10).

	LEAF EMERGENCE[2]				LOSS OF LEAFLETS[3]					MORTALITY[4]		
weeks	1	2	3	4	1	2	3	4	5	3	4	5
CONTROLS	100	00	30	40	00	00	10	20	-	-	-	-
ROOT-FLOODED	80	00	10	30	00	00	00	30	100	-	-	100
SUBMERGED	00	00	00	00	20	70	100	-	-	100	-	-

[1] The individuals had 3-4 leaves (mostly 4) at the start of the experiment.

[2] Leaves in most cases emerged in pairs at a time. Under the growth conditions given to the control plants, emergence of new leaves took place approximately every fortnight, with some individual variation.

[3] Leaflets from the four older bottom leaves often dropped first, without previous yellowing.

[4] Leafless plants, with darkening of the stem (necrosis) were considered dead, which was confirmed by bringing them back to fully aerobic conditions and observing if they would recover or not over ca. two weeks.

4) DISCUSSION

The present results show that ***Parkia pendula*** seeds are clearly adapted to long-term submersion. Its seedlings by contrast can survive only a few weeks under root flooding or whole-plant submersion. Although at the early age of one-month old this ability to survive without an adequate oxygen supply for ca. one month can be considerably advantageous in many flood-prone habitats of the world, in the amazon floodplains, where flooding can last up to eight months or more, this would be advantageous only for marginal sites. This fact may account for the absence of ***P.pendula*** from flood-prone areas in the Amazon, contrasting with several of the other neotropical species of the genus. One-month old seedlings of ***Parkia discolor***, for instance, are able to establish themselves in the amazonian *igapós* by tolerating seven-month submersion **(Coutinho & Struffaldi, 1971)**.

The common ability of seeds of both species to endure long-term submersion is therefore not sufficient to determine habitat colonisation. The tolerance or sensitivity of young seedlings to prolonged flooding or submersion may determine the ability of ***P.discolor*** to survive in *igapós* (but not necessarily its absence from terra-firme) and the almost complete absence of ***P.pendula*** from flooded habitats. However, ***P.pendula*** has the widest distribution of the neotropical species of ***Parkia***, which may reflect a lack of habitat specialisation. By contrast, the remarkable adaptation to long-term flooding or submersion by ***P. discolor*** at any stage of its life cycle may be associated with the restricted distribution of this species to the Amazonian *igapós*.

The distribution of ***P.pendula*** extends from Honduras southwards to the Amazon, and to isolated patches of seasonal rain forest in the northeastern coast of Brazil

(Hopkins, 1986). Interestingly, the species can also be found in flooded riverine forests of white and clear water rivers in the Venezuelan Amazon (**Huber, pers.comm.**). If one-month old seedlings are killed by a few weeks of flooding while, paradoxically, trees of this same species can be found in periodically flooded forests, it must be assumed that for this species flood-tolerance increases with age, as reported for tree species in some North American flooded bottomland forests (**Crawford, 1992**). Possibly, the later ***P.pendula*** is subjected to flooding, after having germinated, the higher the chances of survival and establishment. Whether this is due to a rapid growth and increase in size or to a slow growth and accumulation of reserves in the pre-flooding period, remains to be investigated.

In this context, it is interesting to compare the period of seed production of ***P.pendula*** and ***P.discolor*** with the time of the onset of flooding. Fruiting of ***P.pendula*** takes place from August to December in most areas in the Brazilian Amazon (**Hopkins, 1986**). Seed dispersal, therefore, is likely to coincide with the flood season, which usually occurs from December to May (**Junk, 1989; Ribeiro & Adis, 1984**). The indehiscent pods of ***P.discolor*** also mature and drop during the flood season (**Hopkins & Hopkins, 1983**). Thus, in order to germinate, the seeds of both species in natural conditions, in flooded areas, would have to remain viable during ca. 6 months submersion, which they both proved to be capable of accomplishing. When the water level falls, both species would be able to germinate, having a maximum period of ca. 6 months to grow before the next flood comes. In the case of ***P.discolor***, **Coutinho & Struffaldi (1971)** have shown that one-month old seedlings and six-month old saplings are able to survive seven months submersion, however showing no growth. One-month old ***P.pendula*** seedlings, as shown in the current experiments, are not capable of surviving more than a few weeks of submersion. An interesting experiment would

be to test submersion-survival of ***P.pendula*** seedlings for a range of ages, in order to find out from which stage they are able to tolerate long-term submersion.

A study conducted by **Pearce & Jackson (1991)**, compared the strategies of rice and ***Echinochloa oryzoides*** (a rice crop mimic species) to survive submersion: while rice shows a rapid shoot extension under submersion, allowing the plant to have access to atmospheric oxygen, ***E.oryzoides*** uses a metabolically less expensive strategy of preserving its coleoptile length, and consequently conserving assimilates, under poorly aerated conditions. ***P.discolor***, as seen above, would appear to be using a strategy comparable to that of ***E.oryzoides***. For ***P.pendula***, the length of time between seed germination and seedling submersion is possibly the key factor determining whether or not the species is able to resort successfully to a strategy similar to that of ***P.discolor***. This possibility is indicated by the fact that adult trees of ***P.pendula*** can be found in some areas of periodically flooded forests, as noted previously. More detailed studies on which possible strategies under which particular set of circumstances would possibly allow ***P.pendula*** to establish itself in flooded areas in the Amazon, could be of high relevance for the understanding of the ecology and management of this species.

In short, the fact that ***P.pendula*** is capable of germination after long-term seed submersion may allow this species to perhaps colonise upper reaches of *várzea* or *igapó* forests if the flood regime in a given period does not subject young seedlings to longer than one month of waterlogging or submersion, or if seedlings are submitted to flooding at a later age. This seedling establishment could take place in these areas either naturally (depending on the seed bank available) or even by planting, provided that the saplings are introduced at a later age.

It is not only in terms of young seedling flood-tolerance that ***P.pendula*** and ***P.discolor*** differ from each other. The indehiscent pods of ***P.discolor*** are adapted to water dispersal, whereas the dehiscent pods of ***P.pendula*** contain seeds which are possibly dispersed by arboreal animals **(Hopkins & Hopkins, 1983)**. Curiously, however, despite a difference in size (***P.pendula*** has smaller and lighter seeds), the seeds of the two species are physiologically similar, in that they are both adapted to long-term submersion. Although **Hopkins & Hopkins (1983)** stated that ***P.pendula*** is one of three species in the genus that does not become gelatinous in contact with water and also, quoting Rizzini (1977), claimed that its seeds do not need scarification to germinate, this is not the case. The present study has shown that ***P.pendula*** seeds, like those of ***P.discolor***, germinate only after scarification, and in contact with water become gelatinous. **Hopkins (pers.comm.)** recently confirmed the observations currently made regarding the germination properties of ***P.pendula***.

Junk (1989) has proposed that predictability of flooding in the Amazon favoured the development of physiological adaptations in plants. He suggested that such physiological adaptations to flooding may have been acquired relatively quickly as indicated by the fact that some Amazonian species which occur in non-floodable habitats have evolved flood-tolerant ecotypes in *várzea* and *igapó*. ***P.pendula***, although mostly widespread in dry forests, may be one such species which has given rise to flood-tolerant ecotypes, in which case its seed long-term submergence-tolerance would have played a role in allowing such a process. If this is the case, it is of interest that ***P.pendula*** is one of the two species belonging to the Section ***Platyparkia***, a group which is rather distant phylogenetically from the majority of the flood-tolerant species of the genus, which belong to Section ***Sphaeroparkia*** (all the species in this group appear in

flooded areas) and Section ***Parkia*** (where 5 out of 10 species occur in flooded areas), according to **Hopkins (1986)**. However, its relative in Section ***Platyparkia***, ***P. platycephala*** is a species of *cerrado* (neotropical savanna) areas which are prone to waterlogging.

Hopkins (1986) discusses the controversy involving the origin of the genus ***Parkia***. Some argue that the genus is ancient, having evolved in the Cretaceous, while others describe it as a genus of recent origin, having achieved its pantropical distribution by transoceanic dispersal. Hopkins defends the likelihood of the transoceanic dispersal hypothesis at least for ***Parkia*** section ***Parkia***, where the species produce indehiscent pods. ***P.discolor*** is a member of this section and, as discussed before, has hydrocorous pods. The fact that ***P.pendula***, although belonging to a section not adapted to water dispersal (***Parkia*** section ***Platyparkia***), possesses seeds tolerant to long-term submersion, is certainly an additional fact to consider when analysing the evolutionary paths of the genus ***Parkia***.

Comparative studies on the ecophysiology of species within a same genus colonising different habitats, as outlined in the present Chapter, can be used as a tool to comprehend the evolution and ecology of a genus, as well as to provide information of substantial economic importance for plant breeders and plant growers.

* CONCLUSION *

The ontogenetic factors examined in this Section clearly influence plant response to flooding or anoxia. The broad classification of species as tolerant or intolerant to oxygen stress can be misleading if it disregards ontogenetical nuances. The following points are worth emphasizing:

CHAPTER ONE: SEED SENSITIVITY TO ANOXIA DURING IMBIBITION

1) After 2 and 4 days of anoxia, anoxia-tolerance of germinating seeds varied according to the length of the aerobic imbibition period previous to the anoxic shock. After 1 day of anoxia, no such variation occurred.

2) Chickpea and barley, expectedly, are more sensitive to oxygen stress while in the growth phase of germination, particularly in the stages following 48 hours of aerobic imbibition, when roots and shoots are already present. However, a marked variation on anoxia-tolerance within the early stages of germination (before radicle protrusion) was surprisingly evident in chickpeas.

3) Chickpea is more anoxia-tolerant during the transition phase of germination (as defined by **Koller & Hadas, 1982**), a moment when germination can be blocked and resumed later depending on external conditions. By contrast, barley did not show variation in survival among the several stages submitted to anoxia before radicle protrusion. This difference in response between the two species maybe due to a difference in seed coat permeability.

4) In the early stages of germination of chickpea seeds, the embryo may be exposed to a natural anaerobiosis as suggested by the similar amounts of ethanol produced aerobically and after 24 hours of anoxia by seeds in those stages (namely the stages reached after 0.1 and 6 hours of aerobic imbibition). This process does not seem to occur in barley seedlings, again suggesting a possible difference in seed coat structure between the two species.

5) Carbon dioxide output of germinating seeds after 24 hours of anoxia mirrored the survival results after 4 days anoxia. In barley, the reduced variation in survival among the early germination stages tested was matched by an almost uniform reduction of carbon dioxide output under anoxia. In chickpea, seeds at the germination stage with highest percentage survival after 4 days anoxia were the only ones to reduce carbon dioxide output under anoxia.

6) A short period of aerobic imbibition (6 minutes) prior to anoxia resulted in improved post-anoxic survival conditions, which can be explained by the fact that even a few hours (2 hours) of aerobic imbibition prior to anoxia resulted in the enhancement of the embryo oxygen uptake when seeds were returned to air, compared to seeds not allowed to imbibe aerobically prior to the anoxic treatment.

CHAPTER TWO: ANOXIA-SENSITIVITY OF YOUNG SEEDLINGS DETERMINING THE ABSENCE OF *Parkia pendula* FROM FLOODED AREAS IN THE NEOTROPICS.

1) The seeds of the Amazonian species ***Parkia pendula***, survive at least seven-month submersion without loss of germination viability, however its young seedlings do not withstand submersion or waterlogging for longer than three weeks, which may account for the species rare occurrence on

flooded areas in that region. The submersion-tolerance of ***Parkia pendula*** seedlings, however, may increase with age as indicated by the fact that some adults of this species can be found in flooded areas.

2) The fact that ***Parkia pendula*** belongs to the Section of the genus not adapted to seed dispersal by water, but its seeds are nevertheless tolerant to long periods of submersion, might support the hypothesis of transoceanic dispersal of the genus.

SECTION II

ENVIRONMENTAL OSCILLATIONS AND ANOXIA-TOLERANCE

* FOREWORD *

The extent to which environmental factors affect plant survival under complete or partial lack of oxygen is relatively well-documented. Light **(Setter *et al.*, 1987)**, temperature **(Fagerstedt & Crawford, 1987)**, salinity **(Van der Moezel *et al.*, 1988)**, flood water pH **(Prance, 1979)** and circulation of flood water **(Harms, 1973; Kozlowski, 1984)** are some of the factors that acknowledgedly influence plant response to flooding or anoxia.

This Section examines the effects on plant physiology of two, less investigated but equally important, environmental factors that often occur in combination with flooding or anoxia; namely washing and alternation of stresses.

CHAPTER THREE deals with the effects of different washing treatments on post-anoxic survival and growth of newly germinated seedlings of barley (***Hordeum vulgare*** **L.**) and chickpea (***Cicer arietinum*** **L.**) under anoxia. Anaerobic environments with circulating atmosphere often enhance survival as opposed to stagnant conditions. The technique of washing seedlings under anoxia can be related to moving anaerobic environments, and explores whether any soluble factors in the plant cell are associated with the subsequent post-anoxic survival and growth.

CHAPTER FOUR is a preliminary study of the physiological implications of an interaction between flooding, drought and anoxia. The common practice of classifying species as

tolerant or sensitive to a particular stress is often misleading under tropical conditions, where sudden climatic variations subject plants to a multiplicity of stresses sometimes within a short period of time. The responses of ***Eucalyptus camaldulensis*** **Dehnh.**, ***E.regnans*** **F. Muell.**, ***E.pellita*** **F.Muell.** and two provenances of ***E. citriodora*** **Hook** to alternations of flooding and drought are discussed from an ecophysiological point of view. This Chapter led to the investigation described in Section III, Chapter Five, which discusses the possibility of drought causing hypoxia to root tissues.

* CHAPTER THREE *

THE EFFECTS OF WASHING ON SEEDLING POST-ANOXIC SURVIVAL

1) INTRODUCTION

The enhancement of plant survival under anoxia due to circulation of the anaerobic environment, as opposed to survival impairment under more stagnant conditions, has been documented by several authors. Anoxia-sensitivity of chickpea seedlings has been associated with increased ethanol accumulation **(Crawford & Zochowski, 1984)**, however, by circulating the anaerobic atmosphere, which prevents ethanol accumulation, survival can be enhanced **(Crawford, Monk & Zochowski, 1987)**. In field conditions, adventitious roots, as in rice, in contact with free water, allow more than 98% of the anaerobically produced ethanol to diffuse into the surrounding medium **(Bertani, Brambilla & Menegus, 1980)**. ***Pinus*** **(Kozlowski, 1984)** and ***Nyssa*** **(Harms, 1973)** are injured much more by stagnant conditions than by moving water. In experiments with ***Nyssa aquatica*** and ***Nyssa sylvatica***, **Harms (1973)** showed that stagnant water has both the highest carbon dioxide and the lowest oxygen concentrations, which affected the trees adversely.

Moving anaerobic environments however, might represent negative aspects for plant survival. In field conditions, flowing water may push over, bury in mud, uproot and cause seedlings to float away **(Kozlowski, 1984)**. **Van Steveninck (1975)** showed that washing allowed key regulatory substances to escape from the plant tissues, affecting negatively the endomembrane system, ion transport, organelle structure, protein synthesis and hormone balance of the cells.

The onset of anoxic or post-anoxic injury is frequently diagnosed by membrane leakage **(Crawford & Wollenweber-**

Ratzer, 1992). Studies of maize roots have shown that injuries or cold shock drives calcium (Ca^{2+}) from stabilising sites on the plasmalemma surface into the cytoplasm, depolarising the membrane and opening ion channels causing leakage **(Zocchi & Hanson, 1982).** Washing solutions with Ca^{2+}, a membrane tightner, offers protection against destabilisation of the plasmamembrane. Acidic solutions, conversely, increase destabilisation **(Hanson, Rincon & Rogers, 1986).**

The present work examines the effects of washing barley and chickpea seedlings subjected to different periods of anoxia under distinct conditions of temperature, and washing solution pH and ionic composition. In the case of barley, responses of seedlings submitted to anoxia at different stages of their germination process were also tested, as well as different frequencies of washing. The main purpose was to assess if washing under anoxia alters seedling post-anoxic survival and to explore what soluble factors in the plant cell are associated, either positively or negatively, with the subsequent post-anoxic survival and growth. Thus, ethanol concentration in seedlings after different washing treatments was also measured.

2) METHODOLOGY

2.1 *Sterilisation, washing and anoxia*

Barley and chickpea seeds were sterilised (see the Material and Methods section), before germination in Petri dishes lined with moistened filter paper. Seedlings (see below for species, germination and stages) were taken one by one with forceps and carefully rinsed in each of three dishes filled either with distilled water (pH=5.2) or calcium solution (5 mM Ca^{2+}; 0.86 g $CaSO_4$ in 1 l of distilled water; pH=5.1) or acidic solution (0.05 N H_2SO_4 with pH adjusted to 3.0), inside an anaerobic incubator. After each washing the seedlings were transferred to clean Petri dishes with moistened filter paper. The seedlings stayed in anaerobe jars during anoxia at 20°C or 5°C incubators, in the dark.

After the anoxic period and the washing treatments, the washed seedlings and the non-washed controls were planted in a 1:1 professional compost (Levington M2)/sand mixture, and watered with tap water daily. They were allowed to grow for 20 days, when percentage survival and shoot fresh weight were scored. For the chickpeas under 4.0 and 6.0 days of anoxia, shoot dry weight and root fresh and dry weight were also measured.

2.2 *Chickpea*

Seeds from a commercial source of unknown provenance were allowed 72 hours germination, when they reached ca. 1 cm length radicle, before subjection to anoxia from 1 to 6 days, at 20°C. During anoxia, they were either washed in distilled water (pH=5.2) or non-washed (120 seedlings were washed and 120 were not; a total of 12 treatments, 2 replicates per treatment with 10 individuals each). The seedlings subjected to 1; 1.5; 2; and 2.5 days of anoxia

were washed every 12 hours. Those subjected to 4 and 6 days of anoxia were washed every 24 hours.

2.3 *Barley*

The seeds were germinated in Petri dishes which were subjected to anoxia and placed in anaerobe jars. Experiments were conducted at two different temperatures: 20°C and 5°C.

At 20°C, seedlings were submitted from 1 to 5 days of anoxia, and at 5°C from 2 to 10 days of anoxia, in both cases in the dark. The effects of washing were tested at two different frequencies: washing once a day during the anoxic period, or washing only before return to air at the end of the anoxic treatment. Thus, a seedling left under anoxia for 5 days would be washed five times in the former case, and only once in the latter situation.

The seedlings were submitted to anoxia in the growth phase of germination (according to **Koller & Hadas (1982)** classification) when the coleoptile was already 1.0 cm long. At 5°C, an additional experiment of everyday washing was carried out with seedlings at an earlier stage in the growth phase of germination, at the commencement of radicle protrusion.

At 20°C, seedlings were washed in distilled water or non-washed. At 5°C, the treatment used to test for cold shock calcium release, seedlings were washed either in distilled water, or in calcium solution, or in acidic solution or non-washed (300 seedlings for each washing solution and control: 5 treatments with 3 replicates with 20 individuals each).

The everyday washing experiments were done with ***Hordeum vulgare* L.cv. Kustaa.** This variety was chosen for this particular set of experiments for having previously proven

to be relatively anoxia-tolerant **(Fagerstedt & Crawford, 1987)**. The washing only before return to air experiments were done with ***H. vulgare*** **L.cv. Golden Promise.**

Significant differences were tested at $p<0.05$ by chisquare for all survival experiments.

2.4 *Ethanol content*

Barley (***H.vulgare*** **L.cv. Golden Promise**) seedlings at a late growth phase of germination (roots + coleoptile present) were submitted to 8 days of anoxia at 5°C, in the dark. In the last day of anoxic treatment 100 seedlings were washed in water, 100 in Ca^{2+} solution and 100 non-washed. After washing, groups of 10 seedlings were removed from the incubator, fresh weight was measured and ethanol was extracted from the tissues. Nine samples of each of the ten extracts were analysed in a gas-liquid chromatograph (see details in the Material and Methods section).

3) RESULTS

3.1 *Ambient temperature*

Both barley and chickpea seedlings, when subjected to an anaerobic environment at 20°C, failed to show any significant differences in survival between washed in distilled water and non-washed treatments (figs.3.1; 3.2). In the case of barley this occurred irrespective of the frequency of washing. However, there was a significant difference in survival among the treatments of barley seedlings at 5°C (figs.3.3; 3.4).

For barley, this indicates that washing at a lower temperature (5°C) significantly alters seedling post-anoxic survival (it will enhance or reduce survival depending on the frequency of washing, as shown in 3.2 below), whereas at a higher temperature (20°C) washing has no effect. Barley, however, was more anoxia-tolerant at lower temperatures, confirming the observation of **Fagerstedt & Crawford (1987)**. At 20°C, 11.6% of the seedlings survived 4 days of anoxia, while at 5°C the same amount of seedlings (11.6%) survived 12 days (data not shown).

3.2 *Frequency of washing*

Fig.3.4 clearly shows that washing (irrespective of type of solution) may enhance post-anoxic survival of barley seedlings if carried out only immediately before the seedlings return to air. Everyday washing during subjection to anoxia instead, is more damaging than not washing.

3.3 *Type of solution*

Barley seedlings at 5°C responded differently to non-washing, washing in distilled water, washing in calcium

solution and washing in acidic solution, both when washing was carried out everyday and when washing was conducted just before return to air. As seen before, everyday washing was damaging irrespective of the treatment if compared with non-washing. However, fig.3.3 shows that among the washing treatments, both calcium and acidic solutions resulted in significantly higher percentage survival than distilled water for barley seedlings at a late growth phase of germination (1.0 cm coleoptile). Such a difference was not significant for seedlings at an early growth phase (radicle protrusion commencing) which is discussed in the item 3.5.

Washing just before return to air was not damaging when done with distilled water, and significantly enhanced post-anoxic survival when done with calcium or acidic solution. The effect of calcium and acidic solution on seedling post-anoxic survival did not differ significantly (fig.3.4).

Curiously, of the barley seedlings that survived, growth in shoot fresh weight after planting in air did not show marked differences between treatments irrespective of seedling germination stage at the time of anoxia (annexe, Table 3.6a). Similarly to what occurred to chickpeas in Chapter One, it would appear that for those seedlings able to survive in the post-anoxic phase, irrespective of the anoxia/washing treatment imposed, shoot growth (as expressed by the shoot fresh weight measurements) is not impaired. Again, the cost of shoot growth under this conditions, could be at the cost of impaired root growth, which was to some extent apparent for chickpea (annexe, Table 3.3a), but was not assessed for barley.

3.4 *Ethanol removal by washing*

Table 3.1 shows that the washing of seedlings at a late growth phase of germination (roots + coleoptile present),

only in the last of 8 days of anoxia is not sufficient to promote a significant difference in the post-washing ethanol concentration between the washing treatments and the non-washed control.

3.5 *Seedling germination phase*

Fig.3.3 shows that seedlings at different phases of germination respond differently to washing under anoxia. There was no significant difference between washing in water and washing in calcium for seeds at an early growth phase of germination (radicle protrusion starting), whereas washing in calcium was significantly more beneficial than washing in water for seeds at a later growth phase (coleoptile and roots present). This beneficial effect is apparently not linked to ethanol removal, as seen in the item 3.4 above.

Table 3.1: Ethanol content (μmol.gfw^{-1}; mean±st.error) in barley (***Hordeum vulgare* L.cv.Golden Promise**) seeds after washing treatments applied only once in the last of 8 days of anoxia, prior to return to air (n=10).

treatments	μmol of eth.gfw^{-1}
NON-WASHED	17.50 ± 0.31
WASHED in H_2O	18.55 ± 0.31
WASHED in Ca^{2+}	19.35 ± 0.30

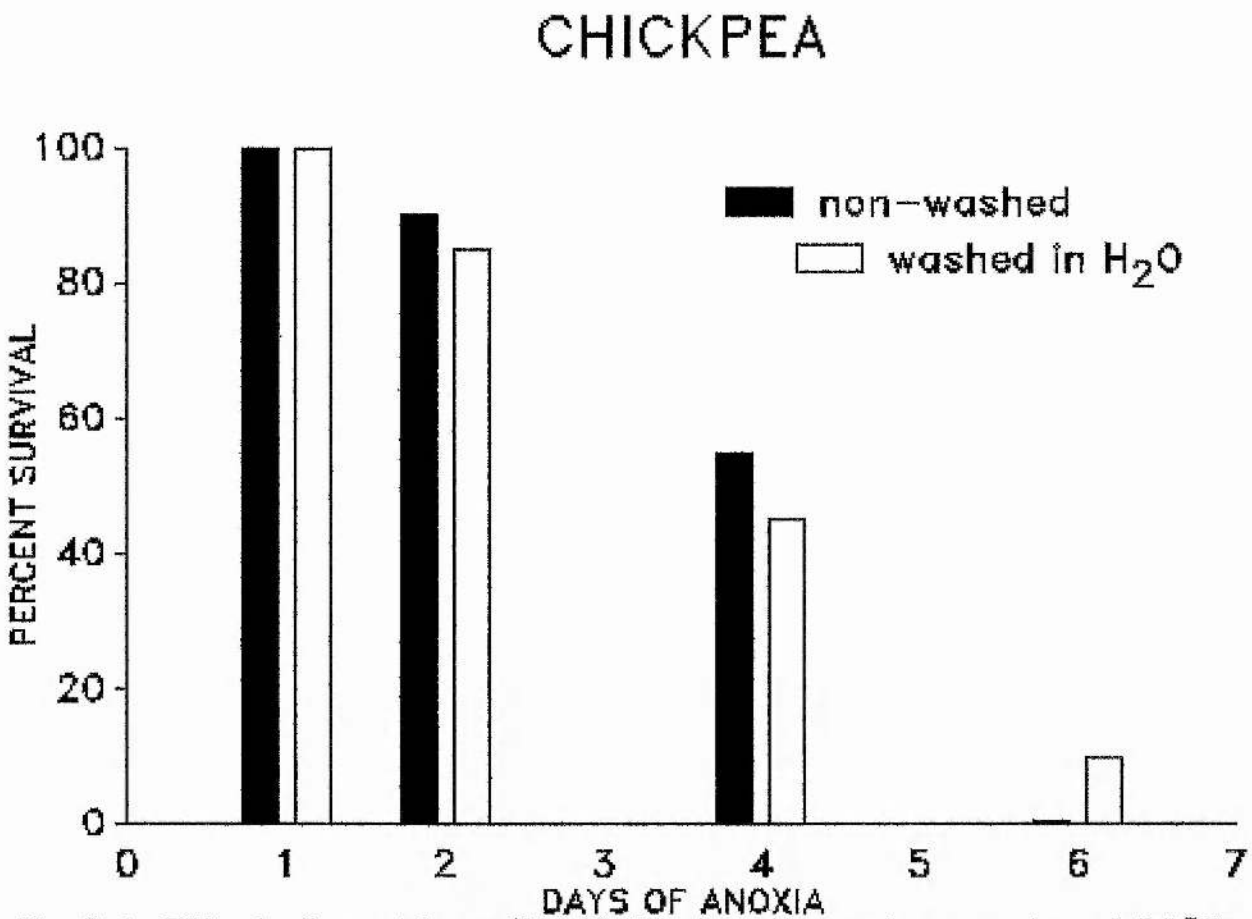

Fig.3.1. Effect of washing with distilled water under anoxia, at 20°C, on post−anoxic survival of newly germinated chickpea seeds (1 cm radicle) n=20. Figures are displayed in the annexe, Table 3.2a. No significant difference between washing and non−washing treatments (chisquare).

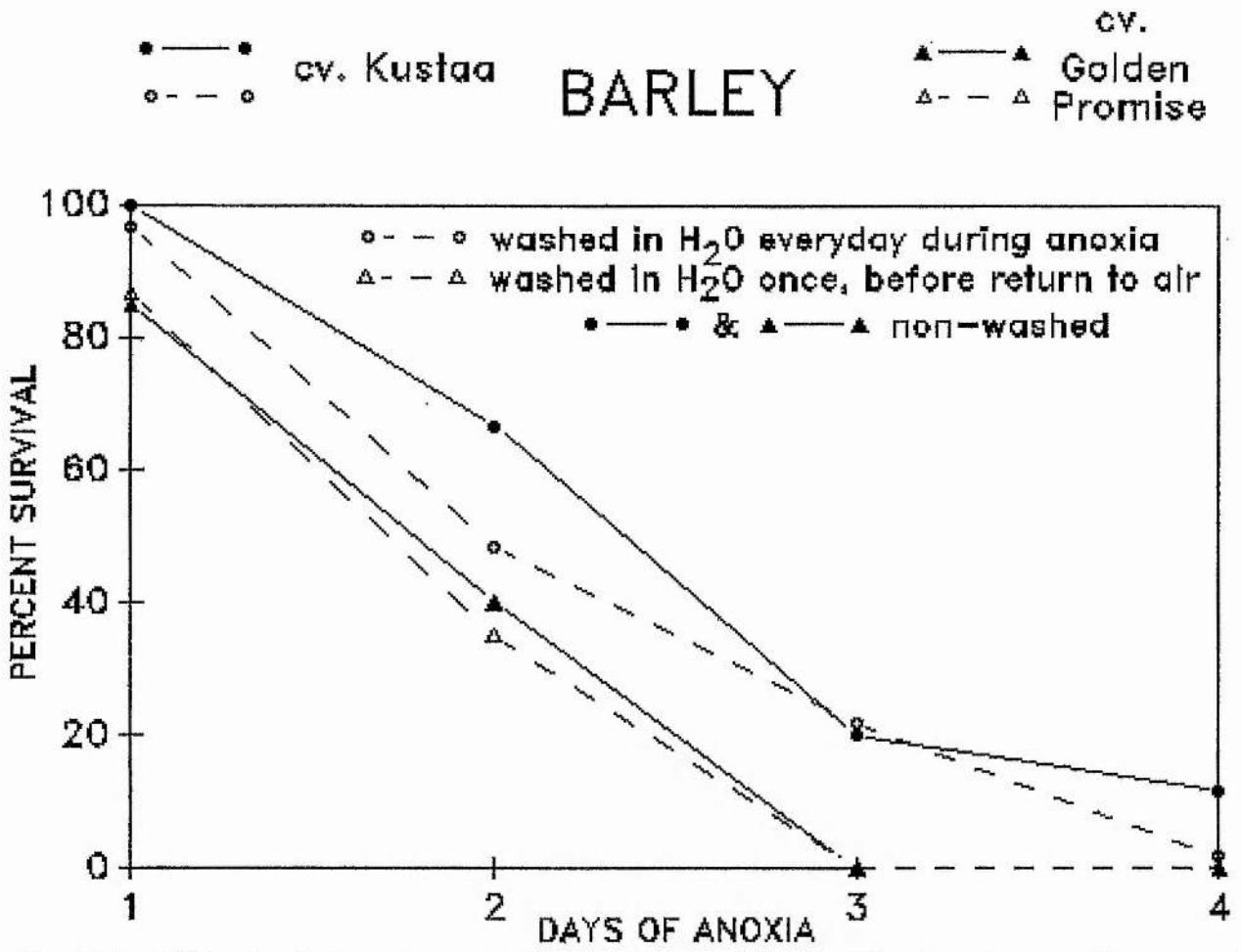

Fig.3.2. Effect of frequency of washing with distilled water under anoxia, at 20°C, on post-anoxic survival of newly germinated barley seeds (1 cm coleoptile). n=60. Figures are displayed in the annexe, Table 3.4a. No significant differences between washing and non-washing treatments. (chisquare).

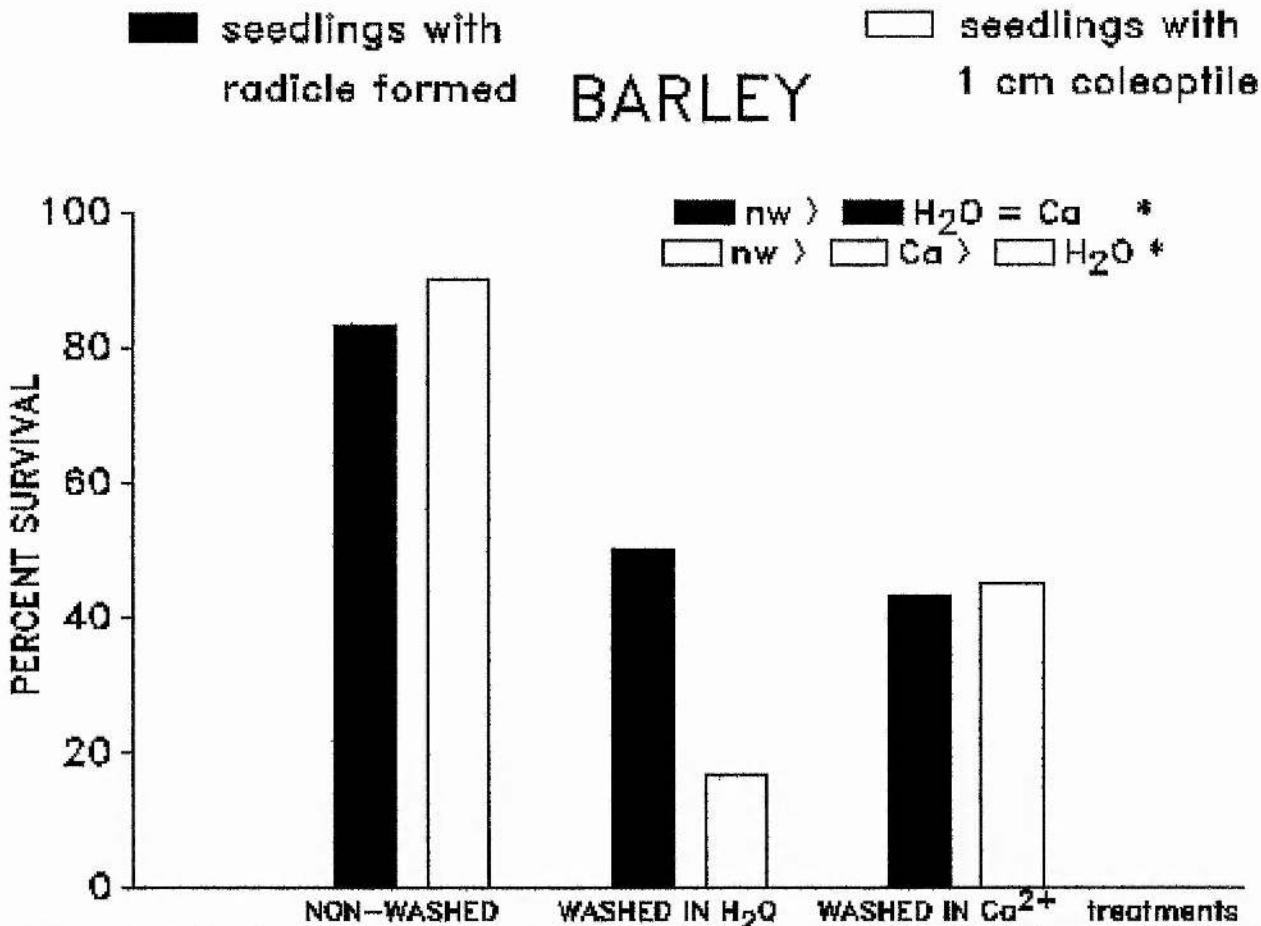

Fig.3.3. Effect of daily washing during 6 d anoxia, at 5°C, on post-anoxic survival of barley seeds at 2 different germination stages. Comparison between 2 washing solutions and non-washed controls (n=60). Figures are displayed in the annexe, Table 3.5a*i*. Significant differences (*) tested by chisquare ($p<0.05$).

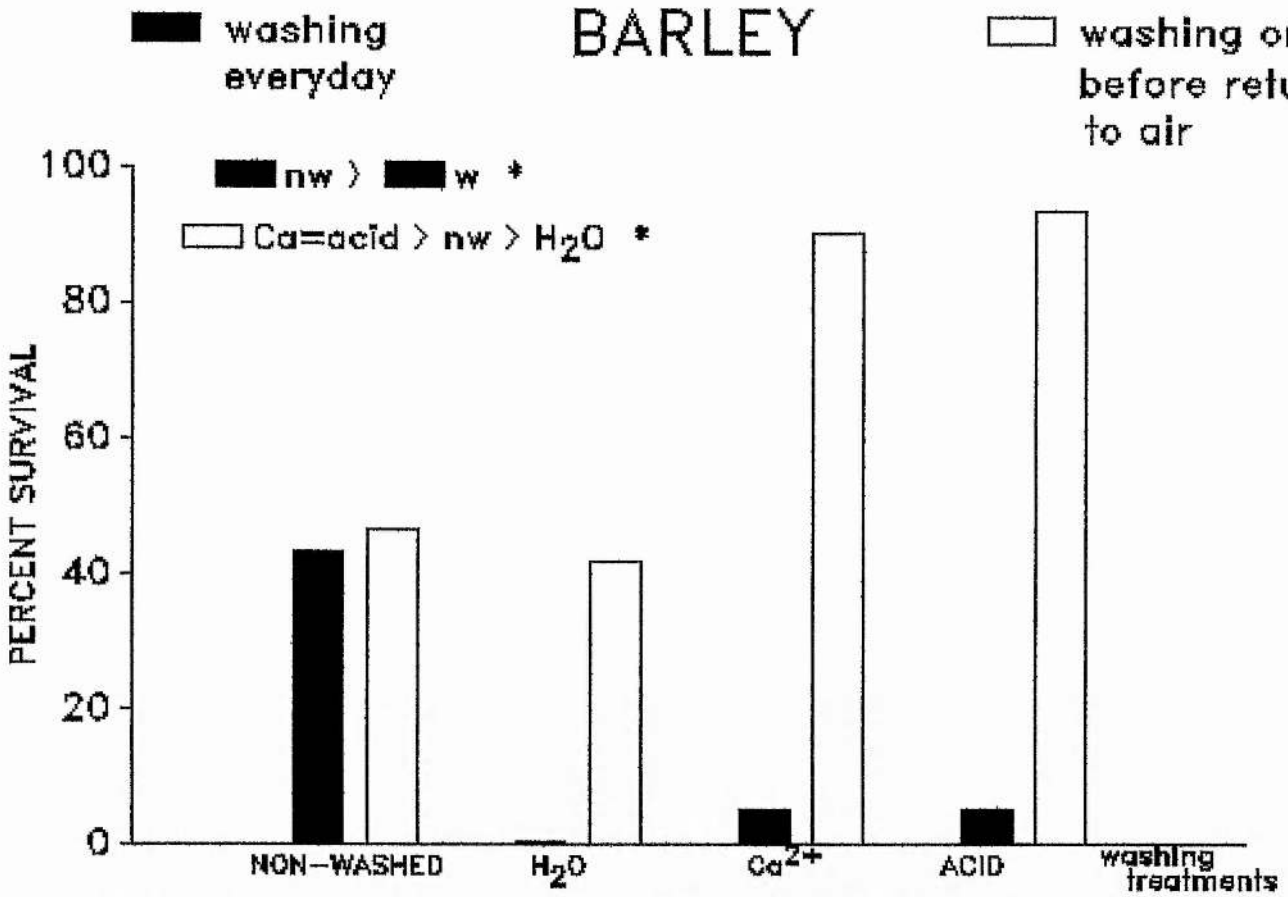

Fig.3.4. Effect of frequency of washing along 8 d anoxia, at 5°C, on post-anoxic survival of newly germinated barley seeds (1 cm coleoptile). Comparison between 3 washing solutions and non-washed controls (n=60). Figures are displayed in the annexe, Table 3.5a & a. Significant differences (*) were tested by chisquare ($p<0.05$).

4) DISCUSSION

The present results show that there are certain precise seedling stages and washing treatments where washing under laboratory conditions can enhance post-anoxic survival of barley and chickpea seedlings. The improvement in survival depends on: *i*) the frequency of washing; *ii*) ambient temperature; *iii*) the type of washing solution used (nutrients, pH); and *iv*) the seedling germination phase at the moment of washing. Each of these factors is examined below, together with an assessment of the degree of importance of ethanol removal by washing.

4.1 *Frequency of washing*

Fig.3.4 shows that washing (irrespective of type of solution) may enhance, or at least not impair, post-anoxic survival of barley seedlings if carried out only immediately before the seedlings return to air. Everyday washing during subjection to anoxia instead, is more damaging than not washing, irrespective of the washing solution. Excessive washing with deionized water may remove Ca^{2+} during rinses, upsetting the stability of the plasmalemma and consequently killing the plant **(Van Steveninck, 1975)**. In this case washing would cause damage prior to anoxia. Studying the effects of washing in plant tissues, Van Steveninck argued that the various negative effects aggravated by washing in laboratory would not occur in the same extent under the more homeostatic field conditions. Indeed, several articles report the beneficial effects of flood-water movement in the field **(Crawford, 1978; Harms, 1973; Kozlowski, 1984; Ponnamperuma, 1984)**.

4.2 *Ambient temperature*

It is interesting to examine the barley seedlings washed

everyday at cold temperature (5°C). Although more tolerant to anoxia at low temperatures, the percentage survival drops when washing is done daily. **Hanson, Rincon & Rogers (1986)** and **Zocchi & Hanson (1982)** suggest that cold shock or injury causes a Ca^{2+} efflux from the membrane and influx to the cytoplasm, destabilising the plasmamembrane. Although 5°C does not represent a metabolic cold shock for barley, it is possible that a combination of low temperature and excessive washing is lethal. Excessive washing is the possible source of injury also referred to by the authors mentioned above as a cause of plasmamembrane damage. If washing in the laboratory were to be carried out with less frequency at this temperature, as discussed in 4.1 above, it might enhance post-anoxic survival.

Similarly, a combination of high temperature and anoxia kills barley, and washing is not enough to enhance survival.

4.3 *Type of solution*

Although the present work did not attempt to measure intracellular and extracellular free Ca^{2+}, as done for instance by **Harker and Venis (1991)**, it would appear that the positive effect on post-anoxic survival of washing barley seedlings under anoxia in calcium solution could be due to the protection external calcium gives to the plasmamembrane against Ca^{2+} leakage, as suggested by **Hanson, Rincon & Rogers (1986)**. This protection, however, was not as effective when barley seedlings at a late growth phase of germination were washed everyday rather than only once before return to air. Everyday washing with calcium did not enhance post-anoxic survival but at least was less damaging than everyday washing with water (fig.3.3). A possible explanation for these facts is that everyday washing (irrespective if with calcium or not) causes physical damage

to the plamamembrane that overwhelms any protection an external calcium solution could possibly offer.

Hanson, Rincon & Rogers (1986) point out that in field conditions the presence of calcium in soil flooded with moving water is likely to enhance survival. The 5.0 mM Ca^{2+} concentration present in the calcium solution used in the present work is also the normal range found in fertile soils. **Ponnamperuma (1984)** argues that, among other factors, calcium saturation of soils favours water movement in flooded soils. Although flooded soils in which water moves are not necessarily calcium saturated, the ones that are may give better protection to the root plasmamembrane cells.

At a first glance, it appears that a washing solution with low pH has drastic effects on the post-anoxic survival of barley seedlings. **Roberts, Andrade & Anderson (1985)** show that anoxia causes acidification of the cytoplasm, which by itself is enough to reduce the viability of the plants. **Hanson, Rincon & Rogers (1986)** say that acidification of the apoplast causes an acid shock that increases Ca^{2+} influx which kills the plant. Therefore, reduction of the external pH would be likely to be even worse for survival, but the present results show the opposite. Since H_2SO_4 was used to prepare the solution, it is possible that the sulphate radicle could have a membrane tightening effect similar to that of calcium, which was however not tested. It is worth emphasizing however that, as for the calcium solution, washing everyday with acidic solution did not enhance post-anoxic survival, highlighting the extent of physical damage possibly caused by excessive shaking.

4.4 *Ethanol and post-anoxic injury*

It is not clear whether or not ethanol or other potentially toxic volatile compounds accumulated during anoxia are in

any way deleterious to the barley seedlings tested, since no differences in ethanol concentration were found between the tissues of washed and non-washed seedlings. In order to definetely prove or reject the hypothesis that potentially toxic metabolites accumulated during anoxia are removed by washing consequently enhancing survival, it would have been necessary also to measure ethanol content in the solutions after washing. This was not tested in the present work.

Considering that washing carried out only once just before return to air, in the last of 8 days of anoxia at 5°C, enhanced post-anoxic survival, it follows that the anoxic treatment in itself was not sufficient to kill barley seedlings. These seedlings while under anoxia on the eighth day, just before washing, must still have been alive, since washing would obviously not bring them back to life. Thus, it would appear that the reduced survival of non-washed seedlings (50% that of the seedlings washed in calcium or in acidic solution) in the above circumstances was due not to anoxia itself but to what occurred after anoxia, namely return to air and possibly consequent post-anoxic injuries. Therefore, whatever role in reducing plant viability played by ethanol or any other potentially toxic metabolite removed by washing under these specific conditions, would take place only with its oxidation as the plant returns to air and not during the 8 days of anoxia.

Thus, assuming that potentially toxic metabolites accumulated during anoxia may have a role to play in reducing seedling viability, even if they were eventually removed by washing (which was not proved in the present Chapter), any possible consequent enhancement of survival would not be due to the creation of a more bearable anoxic period, but to the avoidance of peroxidative damage when the plant is returned to air.

4.5 *Seedling germination phase*

The fact that seedlings at different stages of germination responded differently to washing under anoxia provides further evidence for the varying degrees of anoxia-tolerance of seedlings at different germination stages (see Chapter One). Moreover, the fact that washing with calcium solution, as compared with washing in distilled water, was effective for seedlings with coleoptile and root and yet innefective for seeds with only radicle protrusion commencing, suggests that the binding effect of calcium might work only in specific sites. In the experiments of **Hanson, Rincon & Rogers (1986)**, for instance, the beneficial effects of reducing calcium influx to the cytoplasm, and therefore preventing injury, by washing with calcium solution, was obtained for the root tissues of corn.

In short, the washing of barley seedlings in a late growth phase of germination (coleoptile 1.0 cm long), in a 5°C anaerobic environment, with a calcium solution (or surprisingly with an acidic solution), enhances post-anoxic survival provided the washing is carried out once only, before the seedlings return to air. Barley and chickpea seedlings in a 20°C anoxic environment, were indifferent to washing. Whenever washing enhanced post-anoxic survival, it seemed to be more related to protection against plasmamembrane damage (provided by addition of calcium to the washing solution) than to removal of potentially toxic volatile compounds.

ANNEXE

Table 3.2a: Newly germinated (1 cm radicle) chickpea (***Cicer arietinum*** L.) seeds subjected to different periods of anoxic dark incubation at 20°C, under two different treatments: washed in distilled water and non-washed (n=20). Measurements taken after a 20-day period of recovery and growth in air.

i) percentage survival

days of anoxia treatment	1.0	1.5	2.0	2.5	4.0	6.0
NON-WASHED	100.0	95.0	90.0	80.0	55.0	0.0
WASHED	100.0	90.0	85.0	70.0	45.0	10.0

no significant difference between washing and non-washing treatments (chisquare test).
These data are displayed in Fig.3.1., except the 1.5 and the 2.5 d anoxia data.

ii) growth - mean shoot fresh weight of live individuals (mg; mean±st.error)

treatments d.u.a	NON WASHED (n)	WASHED IN H_2O (n)
1.0	754.5 ± 12.3 (20)	865.8 ± 15.6 (20)
1.5	556.8 ± 10.9 (19)	876.1 ± 16.0 (18)
2.0	594.3 ± 11.3 (18)	473.5 ± 12.9 (17)
2.5	374.9 ± 11.7 (16)	666.7 ± 20.0 (14)
4.0	689.7 ± 20.6 (11)	461.0 ± 18.1 (09)
6.0	0.0 ± 0.0 (00)	18.3 ± 18.3 (02)

d.u.a.=days under anoxia.
These data are not displayed in any Fig.
(n) varied according to the number of live individuals in each treatment.

Table 3.3a: Mean shoot dry weight-SDW, root fresh weight-RFW and root dry weight-RDW (mg; mean ± st. error) of chickpea (***Cicer arietinum*** L.) seedlings that survived 4.0 and 6.0 days of anoxic dark incubation at 20°C, under two different treatments: washed in distilled water (W) and non-washed (NW). Measurements taken after a 20-day period of recovery and growth in air. Seeds were subjected to anoxia when they showed 1 cm radicle.

days of anoxia measurement		4.0	(n)	6.0	(n)
SDW	NW	71.3 ± 18.0	(11)	0.0 ± 0.0	(0)
	W	41.3 ± 16.0	(9)	24.0 ± 14.0	(2)
RDW	NW	22.1 ± 6.0	(11)	0.0 ± 0.0	(0)
	W	14.0 ± 5.3	(9)	0.0 ± 0.0	(0)
RFW	NW	364.5 ± 98.0	(11)	0.0 ± 0.0	(0)
	W	203.1 ± 92.0	(9)	0.0 ± 0.0	(0)

n varied according to the number of live individuals, and to the presence or absence of root (note for 6 d.u.a., the lack of root formation in the two plants which formed shoots). These data are not displayed in any Fig.

Table 3.4a: Percentage survival of newly germinated (1 cm coleoptile) barley (***Hordeum vulgare*** L.) seeds subjected to different periods of anoxic dark incubation, at 20°C, under two different treatments (washed in distilled water and non-washed) and under two different regimes of washing (n=60).

i) everyday washing (**cv.Kustaa**).

days of anoxia treatment	1.0	2.0	3.0	4.0	5.0
NON-WASHED	100.0	66.7	20.0	11.7	0.0
WASHED	96.7	48.3	21.7	1.7	0.0

No significant differences (chisquare).

ii) washing only before return to air, in the last day of anoxia (**cv. Golden Promise**).

days of anoxia treatment	1.0	2.0	3.0	4.0	5.0
NON-WASHED	85.0	40.0	0.0	0.0	0.0
WASHED	86.7	35.0	0.0	0.0	0.0

No significant differences (chisquare). The data from both tables are displayed in Fig.3.2.

Table 3.5a: Percentage survival of barley (***Hordeum vulgare*** **L.**) seedlings subjected to different periods of anoxic dark incubation at 5°C. Treatments: non-washed; washed in distilled water (pH= 5.2); washed in 5.0 mM Ca^{2+} solution (pH=5.1); and washed in 0.05 N H_2SO_4 solution (pH=3.0).

i) everyday washing (**cv.Kustaa**); plants tested at two different germination phases (n=60).

d.u.a.[1] treatment	g.p.[2]	2.0	4.0	6.0	8.0	10.0
NON-WASHED	E	93.3	95.0	83.3	75.0	53.3
	L	93.3	78.3	90.0	43.3	16.7
WASHED H2O	E	90.0	85.0	50.0	11.7	3.3
	L	90.0	61.7	16.7	0.0	0.0
CALCIUM	E	91.7	65.0	43.3	10.0	5.0
	L	100.0	95.0	45.0	5.0	0.0
ACID	E	-	-	-	-	-
	L	100.0	88.3	50.0	5.0	0.0

[1] d.u.a.= days under anoxia; [2] g.p.= germination phase; E= early growth phase (radicle protrusion); L= late growth phase (coleoptile 1.0 cm).
Plants at an early germination phase were not tested under the washing with acid treatment.
Statistics (chisquare; * significant at $p<0.05$):
2d anoxia: no significant differences (ns).
6d anoxia: NW E >W E=Ca E (*); NW L > Ca L=A L > W L (*)
8d and 10d anoxia: NW E > NW L > all other treatments (*)
Starting at 4 d anoxia: W E > W L (*)
More visible from 6 d anoxia onwards: Ca E x Ca L (ns)
The 6 d anoxia data are displayed in Fig.3.3.
The 8 d anoxia data are displayed in Fig.3.4. together with the 8 d anoxia data of *ii*) below.

ii) washing only before return to air, in the last day of anoxia (**cv. Golden Promise**); only late growth phase of germination ('L' in *i* above) tested (n=60).

treatment/d.u.a.[1]	2.0	4.0	6.0	8.0	10.0
NON-WASHED	100.0	98.3	96.7	46.7	18.3
WASHED H_2O	100.0	100.0	86.7	41.7	5.0
CALCIUM	96.7	95.0	98.3	90.0	76.7
ACID	96.7	98.3	100.0	93.3	78.3

[1] d.u.a.= days under anoxia;
Statistics: chisquare (significant difference * at $p<0.05$; ns= no significant difference). Significant differences between treatments start after 8 d anoxia: Ca=A > NW=W(*)

Table 3.6a: Mean shoot fresh weight (mg; mean ± st.error) of live individuals of barley (***Hordeum vulgare* L.**) allowed to recover and grow for 20 days after subjection of newly germinated seeds to different periods of anoxic dark incubation at 5°C. Four treatments: non-washed (**NW**); washed in distilled water (H_2O; pH=5.2); washed in 5.0 mM Ca^{2+} solution (Ca; pH=5.1); and washed in 0.05 N H_2SO_4 solution (A; pH=3.0).

i) everyday washing (**cv.Kustaa**); plants at two different germination phases tested.

t[2]	d.u.a.[1] g.p.[3]	2.0	4.0	6.0	8.0	10.0
NW	E	180.1±5.7	137.7±8.1	54.8±7.5	71.3±8.4	19.9±1.9
	n	56	57	50	45	32
	L	143.1±9.3	83.9±8.6	72.8±7.9	61.2±9.6	18.4±4.5
	n	56	47	54	26	10
H_2O	E	177.3±6.0	107.8±8.9	24.0±3.4	10.1±3.1	5.4±4.6
	n	54	51	30	07	02
	L	126.6±9.0	81.4±10.1	21.4±5.5	0.0	0.0
	n	54	37	10	0	0
Ca	E	156.8±6.4	85.3±9.7	45.2±9.0	10.0±2.2	9.7±1.5
	n	55	39	26	06	03
	L	155.4±11.5	113.7±5.9	38.7±7.2	26.0±14.5	0.0
	n	60	57	27	03	0
A	E	-	-	-	-	-
	L	186.9±11.0	111.8±8.0	67.1±8.5	13.0±2.1	0.0
	n	60	53	30	03	0

[1] d.u.a.= days under anoxia; [2] washing treatment; [3] g.p.= germination phase;
E= early growth phase (radicle protrusion); L= late growth phase (coleoptile 1.0 cm).
n: varied according to the number of live individuals of each replicate.
These data are not shown in any fig.

* CHAPTER FOUR *

ALTERNATION OF STRESSES AFFECTING *Eucalyptus* ANOXIA-TOLERANCE

1) INTRODUCTION

The numerous descriptions of species in the literature as tolerant or intolerant to a particular stress, contrast with a lack of information on interaction between stresses. However, in natural conditions, the latter is more likely to occur, particularly in the tropics.

Climatic variations in the tropics can be quite drastic, subjecting plants to a series of stresses that may be lethal, depending mainly on the duration of the stresses and the moment when they occur in relation to the plant ontogeny. While a plant is recovering from one stress, another one may appear to such an extent that the defences of the plant are not ready at that moment to tolerate this new impact.

The present Chapter studies, in a preliminary level, the responses of ***Eucalyptus camaldulensis*** **Dehnh.**, ***E.regnans*** **F.Muell.**, ***E.pellita*** **F.Muell.**, and two different provenances of ***E.citriodora*** **Hook**, to oscillations of the soil water status ranging from flood to drought. These responses are assessed in terms of survival, shoot extension, root extension and root respiratory activity. Additionally, the response of excised roots of treated plants to a subsequent short-term anoxia was also assessed in terms of respiratory activity and ethanol production, in order to observe the influence of stress alternation on plant response to anoxia.

The ca. 600 ***Eucalyptus*** species, vary among themselves in relation to responses to stresses like drought, flooding,

herbivory, salinity and frost. This fact, associated with the economic importance of the genus in many countries, for oil, charcoal, wood and paper production **(Lima, 1987)**, justifies the search for wider physiological and ecological knowledge of ***Eucalyptus*** species.

The Rio Doce valley is a traditional eucalypt stand in southeastern Brazil, used to produce charcoal for steel factories. Every year eucalypt planters suffer huge production losses due to a phenomenon known as *Seca de Ponteiros*, which means *drought of the stem apex*. The problem however, has recently been described not as drought, nor as an effect of air pollution as suggested by some hypothisers, but as the result of a short and heavy rainy season that saturates the soil, followed by a severe dry season **(Kozlowski, 1988)**, affecting species known as drought-tolerant as well as flood-tolerant ones. This situation provides a practical example of the effects that alternation of stresses may have.

Another curious example in relation to ***Eucalyptus*** is given by **Van Der Moezel *et al.*, (1988)** and **Van Der Moezel, Watson & Bell, (1989)**, who found that the combined effect of flooding and salinity is more severe on flood-tolerant species than on flood-sensitive ones. Flood-tolerant and salinity-tolerant ***E.camaldulensis*** is not as tolerant to a combination of the two factors as it is to either on its own.

This Chapter displays an extensive range of preliminary data collected with the purpose of finding patterns worth of subsequent detailed studies. The investigations described in the following Chapter Five, on the possibility of drought causing hypoxia, were stimulated by observations made in the course of the present experiments. However, despite the reduced number of replicates and consequent lack of

statistical basis to support the trends here observed, it seemed appropriate to register and call the attention of the reader to a few aspects which may stimulate future studies. In this context, the present Chapter deals less with conclusive evidence than with theoretical speculation around some of the trends observed.

2) METHODOLOGY

2.1 *Plants grown in tubes*

Six-month old saplings of ***E.camaldulensis*** **var.** ***obtusa***, ***E. regnans*** and ***E.citriodora*** were obtained from the St. Andrews Botanic Gardens. ***E.pellita*** seeds were provided by *Cia. Agrícola e Florestal Sta.Bárbara* (Brazil) and were grown for six months. Six plants of each of the first three species and eight ***E.pellita*** were then planted in transparent acrylic tubes (40 cm depth x 10 cm diameter), which were kept inside dark boxes (fig.4.0; see also fig.0.1 in the Material and Methods section).

The dimension of the tubes allowed good depth and lateral root growth. Only the shoots remained out of the dark boxes. The boxes were kept in a greenhouse, where the temperature fluctuated between 25°C (day) and 20°C (night). In addition to the natural light, mercury vapour lights were used, which allowed the plants to receive 12 hours of light per day. The light intensity under these circumstances ranged from 220 to 270 $\mu mol.m^{-2}.s^{-1}$.

For ***E.camaldulensis***, ***E.citriodora*** and ***E.regnans***, the experiments started for each of the species by keeping the soil in two tubes containing one sapling each under *normal* conditions (here and throughout the Chapter, *normal* will often be used to describe the non-stressed condition to which control plants were submitted; *i.e.* daily watering) and by flooding the soil in four of the tubes, also containing one plant each. After six weeks, one of the control plants for each of this species was submitted to 4-week drought (5 in the case of ***E.regnans***) and the other remained under normal conditions until it reached 10 weeks. For ***E.camaldulensis*** and ***E.citriodora***, from the four flooded plants, after the first six weeks, one was returned to

Fig.4.0. Example of the method used to allow visual observation of roots of ***Eucalyptus*** saplings: transparent acrylic tubes kept inside light-proof boxes, which were opened weekly, as in this photograph, for brief inspection periods. Note also the sensitivity of ***E.regnans*** to one-week flooding (tubes on the left-hand and right-hand corners), compared to a non-flooded plant (third tube from left to right). The second plant from left to right is still not showing symptoms of leaf injury after 2-day flooding.

normal conditions for four weeks, one remained flooded until it reached 10 weeks, and two were submitted to drought for four weeks. One of these two latter plants was subsequently submitted to other six weeks of flooding. For ***E.regnans***, due to the species high sensitivity to flooding, it was decided to start the stress alternation earlier. After one week of flooding, one plant was subjected to one week of drought, one remained flooded for another week, and two of the plants were allowed to return for normal conditions for five weeks. One of these two latter plants was subsequently submitted to four weeks of drought followed by five weeks of flooding. The 5-week drought-treated ***E.regnans*** plant was subsequently submitted to four weeks of flooding, followed by three more weeks of drought.

For ***E.pellita***, from the eight plants available four were submitted to flooding, two to drought and two were kept as controls. The controls were kept under normal conditions for eight weeks. From the drought-treated plants, after four weeks one plant was returned to normal conditions for four weeks and one was submitted to four weeks of flooding. From the flooded plants, after four weeks one plant was returned to normal conditions for other four weeks, one was exposed to drought for four weeks, and two remained flooded until the eighth week.

The removal of rubber stoppers sealing holes at the bottom of the tubes made it possible to control the soil saturation. It usually took one week for a soil to go from flooded to 35.5% of its field capacity (fig.4.1).

Survival, root and shoot extension and stress-symptoms were observed weekly. Shoot extension was measured individually from the stem basis to the apical bud. The measurements of root extension were made possible by the transparency of the tubes in which the plants were grown. Before the beginning

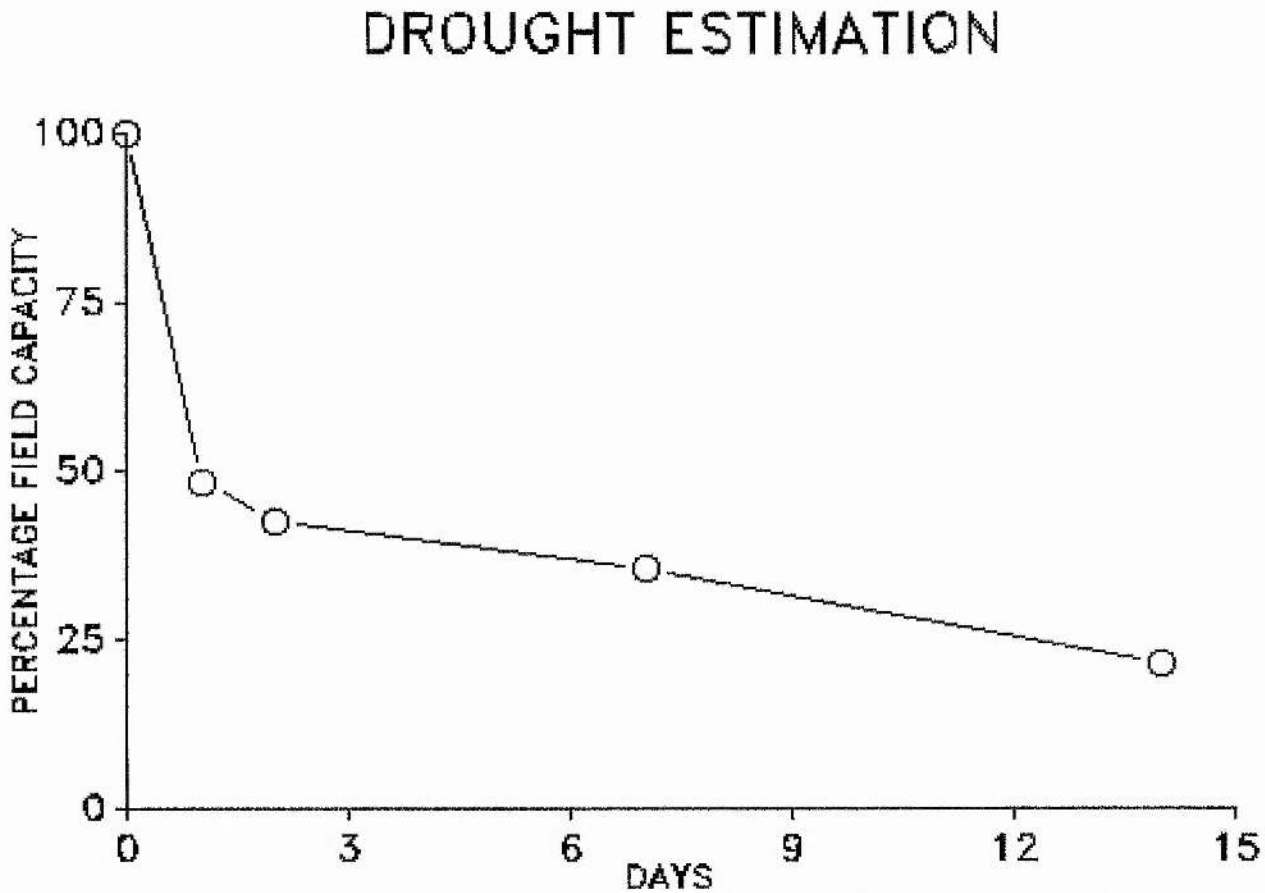

Fig.4.1. Estimation of percentage of field capacity at successive days after release of flood water from soil inside the growth tubes, following the method of Nikolskii (1964).

of the treatments, as many main roots as there were visible in the soil/tube interface (normally the number of roots measured ranged from 10 to 20 per plant) were numbered and had the position of their tip marked (see detail in fig.0.1 in the Material and Methods section). Weekly, it was observed if each marked root extended its length beyond the previous week's mark. When that was the case, the length of the extended segment of the root was measured and registered, and the new position of the tip marked again. This procedure was repeated every week until the end of the experiments. Through this method, it was possible to know for each plant, and consequently for each species, root and shoot extension per week for each treatment. It is important to mention that in view of the length of the experiments, new roots and adventitious roots were formed for some species. These roots were also marked and monitored. The data referring to root extension per week displayed in figures and tables (see "Results"), however, takes into account all the growing roots available in a determined period, irrespective if they were original main roots or newly formed roots. It is, however, mentioned to which type of root the root extension figure is mostly due.

It is important to clarify at this stage how the final calculations of root and shoot extension were made, in view of the multiplicity of stresses. For instance, using the example of ***E.camaldulensis***: a) plant 1: flooded 10 weeks; b) plant 2: flooded 6 weeks + normal 4 weeks; c) plant 3: flooded 6 weeks + drought 4 weeks; d) plant 4: flooded 6 weeks + drought 4 weeks + flooded 6 weeks. In the calculation of weekly root and shoot extension for flooded ***E.camaldulensis***, the 10 weeks of flooding of plant 1, and the first 6 weeks of flooding of plants 2, 3 and 4 were considered. Therefore, in this case n=4. For plant 2, flood-normal, only the measurements taken during the final 4 weeks of normal watering regime were considered in the

calculations of weekly root and shoot extension of ***E.camaldulensis*** under a flood-normal treatment. Therefore, in this case n=1. In the calculation of weekly root and shoot extension of ***E.camaldulensis*** under a flood-drought treatment, the measurements taken during the 4 weeks of drought for plants 3 and 4 were the data to be considered. Therefore, n=2. For ***E.camaldulensis*** treated with flood-drought-flood, only the root and shoot extension detected during the final six weeks of flooding were considered. Therefore, in this case n=1. The same procedure was adopted to all the other species.

At the end of the final stress, roots were excised for measurements of aerobic respiration and measurements of anaerobic respiration after 1-hour anoxia and anaerobically produced ethanol after 24-hour anoxia. Care was taken to ensure that only a few minutes elapsed between excision and physiological measurements. Ethanol production after 24 hours of anoxia (at 20°C) was measured by the headspace method. For both respiration and ethanol measurements, three segments of three roots were used per plant (0-5, 5-10 and 10-15 cm, from the root tip upwards). Here, distinction was made between main roots, adventitious roots, new roots and regenerating roots (see introductory paragraph of the "Results" section in this Chapter for description of each of these types).

As mentioned above, the physiological measurements were carried out at the end of the final stress treatment. Here, it was not possible to repeat the procedure adopted in Chapter One, where respiration and ethanol of barley and chickpea seedlings were measured at the beginning of the stress period. In that Chapter, in view of the facilities of working with germinating seeds as opposed to six-month old trees, it was possible to use one lot of seeds in the survival and growth experiments (assessed after 4 days of

anoxia), another lot of seeds for the respiration experiments and still another lot for the ethanol experiments (both assessed after 1-day anoxia). Here, the same reduced number of trees was being used to investigate survival, growth, respiration and ethanol production. Thus, the physiological parameters could not be measured at the beginning of the final stresses, since they involved harvesting of roots which would be most likely to have a negative effect on growth and survival. Consequently, it was considered to be more appropriate to do the physiological analysis at the end of the final stress treatments, and to reduce any possible error due to decay and death of roots by a careful selection of the roots to be excised. Additionally, considering the need to allow only a minimum amount of time to elapse between excision and measurement, no viability test was conducted for the roots, and the choice of which root material to use was, therefore, visual. Roots that were obviously darkened or injured were not used. For this reason, whenever root dieback was markedly high, the number of roots selected to perform a certain reading would be reduced to two rather than three, or even no measurement of that particular plant or root segment would be taken. For this reason, the fresh weight of the samples normally ranged from 50 to 250 mg per root segment analysed, both for respiration and for ethanol production.

Since the physiological measurements were always made at the end of the final stress the plants were submitted to, the number of plants used per treatment in this case was invariably one. In order to increase the reliability of the results, however, whenever possible more than one set of three roots was measured for the same plant. The fact that more than one root segment was excised and measured per plant also increased the reliability of the results, which nevertheless, as highlighted before, are for this reason nothing more than a possible indication of trends.

Table 4.1 shows the treatments applied to each species, as well as the measurements carried out, the observations made, and the number of plants involved in each case.

Ethanol production and respiration rate measurements are described in details in the Material and Methods section.

2.2 *Plants grown in pots*

The study was conducted with 6-month old ***E.citriodora*** grown from seeds obtained from *Cia. Agrícola e Florestal Sta.Bárbara* (Brazil). It is important to stress at this stage that these plants were morphologically different from the ***E.citriodora*** (St.Andrews) grown in tubes mentioned above. The provenance from Brazil had long, narrow, hairy leaves. The one from St.Andrews had wide, glabrous leaves. There is however no trace of doubt that the two provenances belonged to the same species, considering their reliable source, and the distinct odour produced by the ***E.citriodora*** leaves, which is unique within that genus and was observed for both provenances.

Nine plants were used and subjected to different treatments: 1 & 2) controls; 3 & 4) drought during 4 weeks; 5 & 6) drought-normal-flooding, 4, 2 and 2 weeks respectively; 7) flooding during 4 weeks; 8 & 9) flooding-normal, 4 and 1 weeks respectively. A tenth plant was flooded during the whole course of the experiment (8 weeks): an ***E.citriodora*** from St.Andrews.

The high sensitivity of the Brazilian provenance to both stresses did not allow the application of some of the treatments originally planned (see "Results").

Survival, shoot extension, leaf abscission and other shoot symptoms were observed. The treatments flood, drought and

one of the controls had root aerobic respiration, anaerobic respiration after 1-hour anoxia and headspace ethanol production after 24 hours of anoxia measured, as in 2.1 above. An additional measurement was also carried out for the drought-treated plants: apart from the ethanol production after 24 hours of anoxia, ethanol production in air after a 24-hour aerobic incubation was also measured. Also within the drought treatment, one of the two plants measured, was already severely injured at the time of these measurements, having reached the wilting point, while the other individual was only moderately affected.

Table 4.1: Treatments applied to ***E.camaldulensis***, ***E. regnans*** and ***E.citriodora*** (St.Andrews) and ***E.pellita*** (Brazil): length (in weeks) of each stress, measurements and observations done, and number of plants[1] involved in each measurement.

treatments	CAMALDULENSIS				CITRIODORA				REGNANS				PELLITA			
	L	S	R	E	L	S	R	E	L	S	R	E	L	S	R	E
NORMAL	10	2	1	1	10	2	1	1	10	2	1	1	8	2	1	1
DROUGHT	4	1	1	1	4	1	1	1	5	1	-	-	4	2	1	1
DROUGHT-NORMAL	-	-	-	-	-	-	-	-	-	-	-	-	4-4	1	1	1
DROUGHT-FLOODING	-	-	-	-	-	-	-	-	5-4	1	-	-	4-4	1	1	1
DRT-FLOOD-DRT	-	-	-	-	-	-	-	-	5-4-3	1	-	-	-	-	-	-
FLOODING	10	4	1	1	10	4	1	1	1.5[2]	4	-	-	8	4	1	1
FLOOD-NORMAL	6-4	1	1	1	6-4	1	1	1	1-5	1	-	-	4-4	1	1	1
FLOODING-DRT	6-4	2	1	-	6-4	2	-	-	1-1[2]	2	-	-	4-4	2	1	1
FLD-DRT-FLD	6-4-6	1	1	1	6-4-6	1	1	1	(*)	1	1	-	-	-	-	-

Codes: L = length in weeks of each stress; S = measurements of survival and growth and observation of symptoms; R = measurements of respiration of roots; E = measurements of root headspace ethanol production after 24 h anoxia; - = data not available.

(*): ***E.regnans*** flooding-drought-flooding treatment had an inclusion of a 5 weeks period of normal watering after the first week of flooding. The following drought and flooding stresses lasted respectively 4 & 5 weeks.

1. Adding the **n** in the 'S' column, indicates a higher number than the respective 6, 6, 6, and 8 plants used per species, since for the single stress treatments, the results obtained in the first weeks of single stress of plants that were subsequently used for multiple stress experiments, were also considered (see "Methodology"). Whenever the addition in the 'R' and 'E' columns is less than the number of plants used, that is due to the fact that a plant under a particular combination of stresses, may not have had roots in a suitable condition for physiological measurements.

2. Death at the end of the treatment.

3) RESULTS

In the following description of results, four types of roots will be often referred to: *i*) main roots: the original primary and secondary roots of the saplings; *ii*) adventitious roots: roots formed under flooding, above the root-stem interface; *iii*) regenerating roots: fine roots grown out of decaying main roots; *iv*) new roots: roots newly formed at the root-stem interface.

3.1 *Plant survival, root and shoot extension and general symptoms*

3.1.1 *Stress-tolerant species*

Table 4.1 shows that ***E.pellita***, ***E.citriodora*** (St.Andrews), and ***E.camaldulensis*** proved to be tolerant of either 10 weeks of flooding (***E.pellita*** was tested only up to 8 weeks) or 4 weeks of drought (***E.pellita*** was tested up to 5 weeks).

Flooded ***E.camaldulensis*** and ***E.pellita*** showed substantial shoot extension (fig.4.2) but the existing roots almost ceased to grow. The marked root extension of ***E.pellita*** shown in fig.4.2 is due to the formation of adventitious roots, which began by the fifth week of flooding, and their subsequent rapid extension (see also annexe, Table 4.2a). ***E. camaldulensis*** also formed adventitious roots, in this case by the sixth week of flooding, which however did not match the extension shown by ***E.pellita*** (fig.4.2 and annexe, Table 4.2a). By contrast, flooded ***E.citriodora*** showed no root or shoot extension and did not produce adventitious roots.

During drought, ***E.citriodora*** and ***E.pellita*** showed continued shoot extension, which was not matched by ***E.camaldulensis***. The roots of these three species did not seem to be severely affected by drought (fig.4.2). The existing roots showed

continued extension until the fourth week, when a reduction in growth was observed coinciding with the moment when the shoots started wilting (data not shown).

As soon as the flooded ***E.camaldulensis*** and ***E.pellita*** trees were returned to a normal soil water regime, regenerating roots were formed and, together with the adventitious roots, showed a marked extension, also seen in the shoot (fig.4.2). ***E.citriodora*** did not recover as rapidly showing small root and shoot extension (fig.4.2). When drought was followed by normal watering, ***E.pellita*** (annexe, Table 4.2a) showed continued shoot extension (80% of the control's figure).

When flooding was followed by drought, ***E.camaldulensis*** and ***E.pellita*** could still grow, but by the third week, symptoms of leaf damage, namely wilting, started to appear. ***E. citriodora*** did not show external symptoms of damage, however it did not show any considerable root or shoot extension (fig.4.2). Conversely, when drought was followed by flooding, ***E.pellita***, despite a minimal root extension showed a continued shoot extension (annexe, Tables 4.2a; 4.3a).

3.1.2 *Stress-intolerant species* (annexe, Tables 4.2a;4.3a)

E.regnans and ***E.citriodora*** (Brazil) proved to be, in an initial analysis, flood-intolerant and drought-intolerant. A week of flooding severely damaged ***E.regnans***: death of roots and wilting, desiccation and appearance of black spots on the leaves (example of leaf damage can be seen on fig.4.0.). In the second week of flooding of ***E.citriodora*** (Brazil), the leaves were drying out and wilting, contrasting with a potted ***E.citriodora*** (St.Andrews) showing no symptom of injury throughout eight weeks of flooding.

Similarly, drought also killed roots and caused leaf wilting and necrosis in ***E.regnans*** and leaf wilting and desiccation

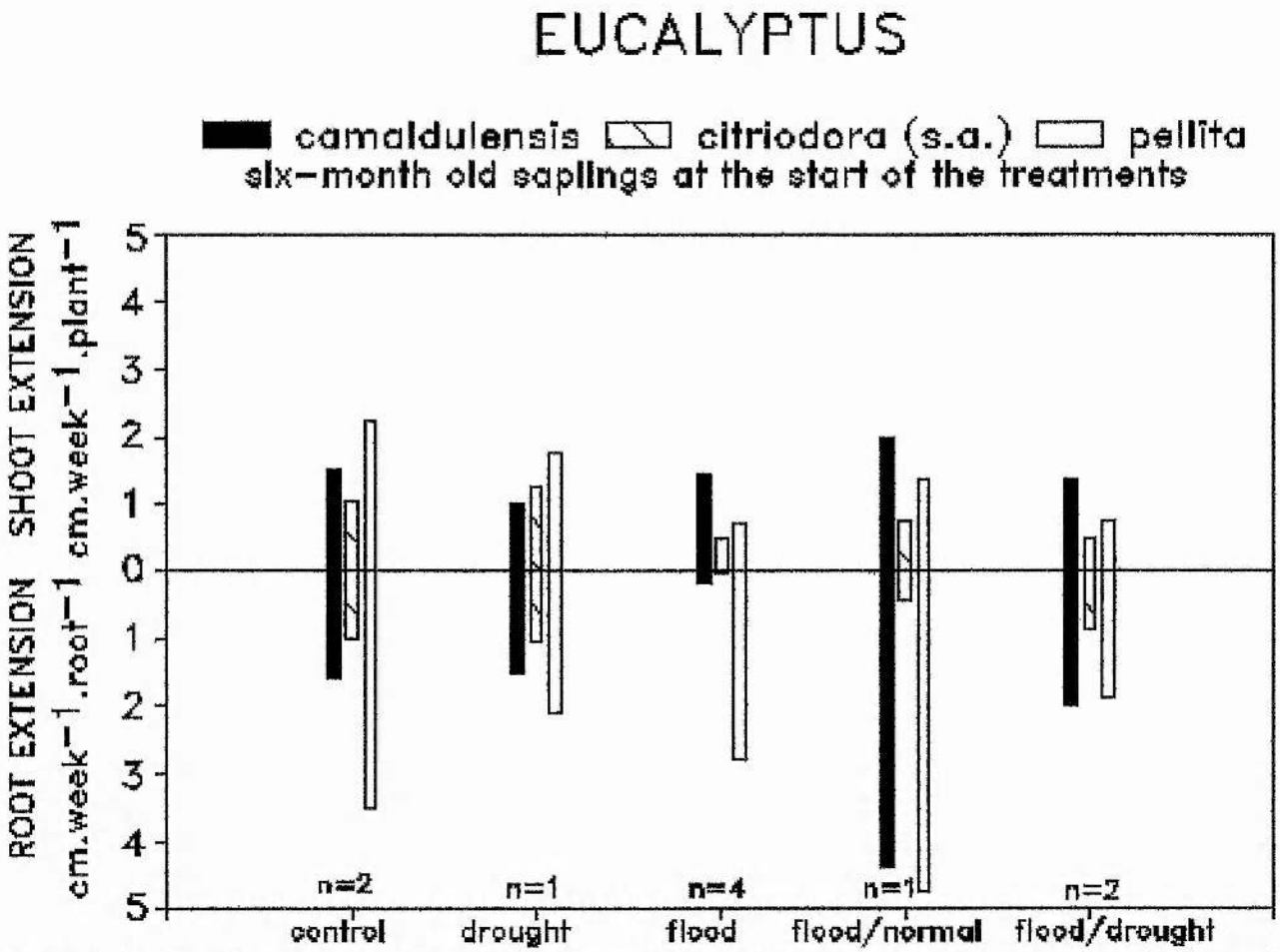

Fig.4.2. Root and shoot extension of Eucalyptus species under different soil water regimes. Multiple stress treatments measured during the final stress. Root extension data were based on the weekly monitoring of 10–20 roots per plant, irrespective of type of root. Length of stresses is on Table 4.1. The complete set of collected data, and additional comments on type of roots involved, can be seen in the annexe, Tables 4.2a and 4.3a.

in ***E.citriodora*** (Brazil). In ***E.regnans*** however, the symptoms did not progress, differently from ***E.citriodora*** (Brazil).

Removing the excess of water and returning the plant to a normal water regime after the first week resulted in the recovery of ***E.regnans***. Root regeneration and alleviation of shoot damage were noticeable, nevertheless the plant did not resume the growth achieved before flooding, only showing 26% of the control's shoot extension. ***E.citriodora*** (Brazil), however, suffered an aggravation of the injuries soon after the removal of the flood water, which resulted in death. Therefore, a flood-drought treatment could not possibly be tested for this species.

When flooding of ***E.regnans*** was immediately followed by drought, the damage became irreversible and the plants died in a few days. Curiously, however, when a 5-week period of normal watering was inserted between 1 week of flooding and 4 weeks of drought, ***E.regnans*** survived showing some shoot extension during the drought period.

Drought followed by flooding caused leaf wilting and necrosis in ***E.regnans***, but the plants, as shown in Table 4.1, were able to withstand flooding longer (4 weeks) than plants that were not subjected to drought before flooding (11 days). A reduced root and shoot extension, contrasting with the non-existent extension observed under other stress treatments, was also observed (see fig.5.4., Chapter Five).

Another interesting fact emerges from the sequence given to the treatments mentioned in the two paragraphs above. When the flooding-normal-drought treatment was followed by a further flooding, the plant once again survived up to 4-5 weeks although the first symptoms of injury appeared on the second week. When, after the drought-flooding treatment, an immediate drought was applied, the plant survived 3 weeks,

which is more than the flooding-drought treatment and less than the drought treatment (Table 4.1).

3.2 *Root respiration*

3.2.1 *Comparison of the effects of the different treatments among the different species.*

a. control

Table 4.8a, in the annexe, shows that ***E.citriodora*** (Brazil) under non-stressed conditions has the highest root aerobic respiration rates of the species studied and, therefore, highest metabolic activity. ***E.regnans*** and ***E.citriodora*** (St. Andrews) had only 20% of the rate of ***E.citriodora*** (Brazil) and ***E.camaldulensis*** and ***E.pellita*** only 15%. The species with highest respiration rates were not necessarily the ones with fastest growth: ***E.pellita***, with a lower respiration rate, showed higher root and shoot extension than the others (fig.4.2).

The roots of control plants of all the species studied showed a Pasteur effect when submitted to one hour of anoxia (fig.4.3). This, however, was particularly marked in ***E.citriodora*** (Brazil) where the main roots had an anaerobic respiratory rate 29% higher than the aerobic one. Such an increase in CO_2 output would induce a 4-fold acceleration of carbohydrate consumption. However, the Pasteur effect shown by the new roots of this species was minimal compared with that of the main roots (annexe, Table 4.7a). Conversely, in the anoxia-tolerant ***E.pellita*** a pronounced Pasteur effect was found in the new roots as opposed to the main roots of this species (annexe, Table 4.6a).

b. drought

Drought-treated roots of all species tested showed some degree of Pasteur effect after one-hour anoxia, which was more marked in ***E.pellita*** and ***E.camaldulensis*** than in the two provenances of ***E. citriodora*** (fig.4.3).

c. flooding

Table 4.8a, in the annexe, shows that the flooded roots of ***E.citriodora*** (Brazil) showed higher respiratory rates than ***E.citriodora*** (St.Andrews), both aerobically (38% higher) and anaerobically after one-hour anoxia (79%), and that ***E.camaldulensis*** had the lowest rates. Fig.4.3 shows that ***E.citriodora*** (St.Andrews) was the only species to show no Pasteur effect after one-hour anoxia and that ***E.citriodora*** (Brazil) and ***E.pellita*** (both adventitious and main roots) showed a more marked Pasteur effect than ***E.camaldulensis***.

d. flooding-normal

The Pasteur effect after one-hour anoxia for the species under this treatment was minimal, and the aerobic and anaerobic respiratory rates showed similar figures for ***E.citriodora*** (St.Andrews), ***E.camaldulensis*** (fig.4.4) and ***E.pellita*** (fig.4.5) roots (see also annexe, Table 4.8a).

e. flooding-drought

Table 4.8a, in the annexe, shows that the root aerobic respiratory rate after a flood-drought treatment was much higher in both main roots (64% higher) and regenerating roots (74% higher) of ***E.pellita*** than in ***E.camaldulensis***. However, after one hour of anoxia ***E.camaldulensis*** (fig.4.4) showed a remarkable Pasteur effect with its anaerobic respiratory rate 71% higher than the aerobic for the main

roots and 38% higher for the regenerating roots. ***E.pellita*** presented a reduced Pasteur effect which was practically non-existent in the case of the regenerating roots (fig.4.5).

f. flooding-drought-flooding

After this treatment, the root aerobic respiratory rate of ***E.camaldulensis***, measured for adventitious and regenerating roots, was over 70% higher than the other species measured (annexe, Table 4.8a). However, after 1 hour of anoxia the CO_2 emission reduced to a sixth in the adventitious roots and to a fifth in the regenerating roots (figs.4.4; 4.7; and annexe Table 4.8a). In contrast, ***E.citriodora*** (St.Andrews) showed some Pasteur effect for both main and regenerating roots (figs.4.6; 4.7).

3.2.2 *Comparison of the effects of the different treatments within the same species*

a. *E.camaldulensis*

Table 4.4a, in the annexe, shows that the aerobic respiratory rate of the root tips of this species was ca. 33% higher during drought than during normal, flooding or flooding-normal treatments, which were similar to each other, for this species. The other segments of the roots presented similar figures for all these treatments. Roots under a flood-drought regime presented reduced aerobic respiratory rates (ca. 50% lower than the controls), contrasting with the high rates of those under a flood-drought-flood regime (ca. 80% higher than the controls). However, fig.4.4 shows that after one hour of anoxia, the flood-drought-flood treated roots of ***E.camaldulensis*** showed a remarkable ability to reduce its anaerobic respiratory rates to less than a third of the aerobic rates. Conversely,

the flood-drought treated plants showed a considerably higher Pasteur effect, which was minimal or non-existent in the other treatments.

b. ***E.citriodora*** (St.Andrews)

With the exception of the flood-drought-flood treatment, most single or multiple stress treatments on this species showed similar results, *i.e.* a reduced or non-existent Pasteur effect after one hour of anoxia (fig.4.3). Main roots subjected to the flood-drought-flood treatment, however, showed a stunningly high Pasteur effect (fig.4.6). Under this treatment, regenerating roots also showed considerable Pasteur effect (fig.4.7).

c. ***E.citriodora*** (Brazil)

The high aerobic respiration rates obtained under a normal soil water regime for this species dropped dramatically (ca. 70%) when the plants were under flooding or drought. A pronounced Pasteur effect after one-hour anoxia was shown by both controls and flooded plants, whereas drought-treated plants presented little or none (fig.4.3).

d. ***E.regnans***

After a flooding-normal-drought-flooding treatment, ***E. regnans*** showed root aerobic respiration rates 90% lower than the control roots. In both cases the Pasteur effect was reduced after one-hour anoxia (annexe, Table 4.7a).

e. ***E.pellita***

Fig.4.5 shows that one-hour anoxia induced Pasteur effect in drought-treated as well as flood-treated plants of this species. However, when after flooding or drought the plants

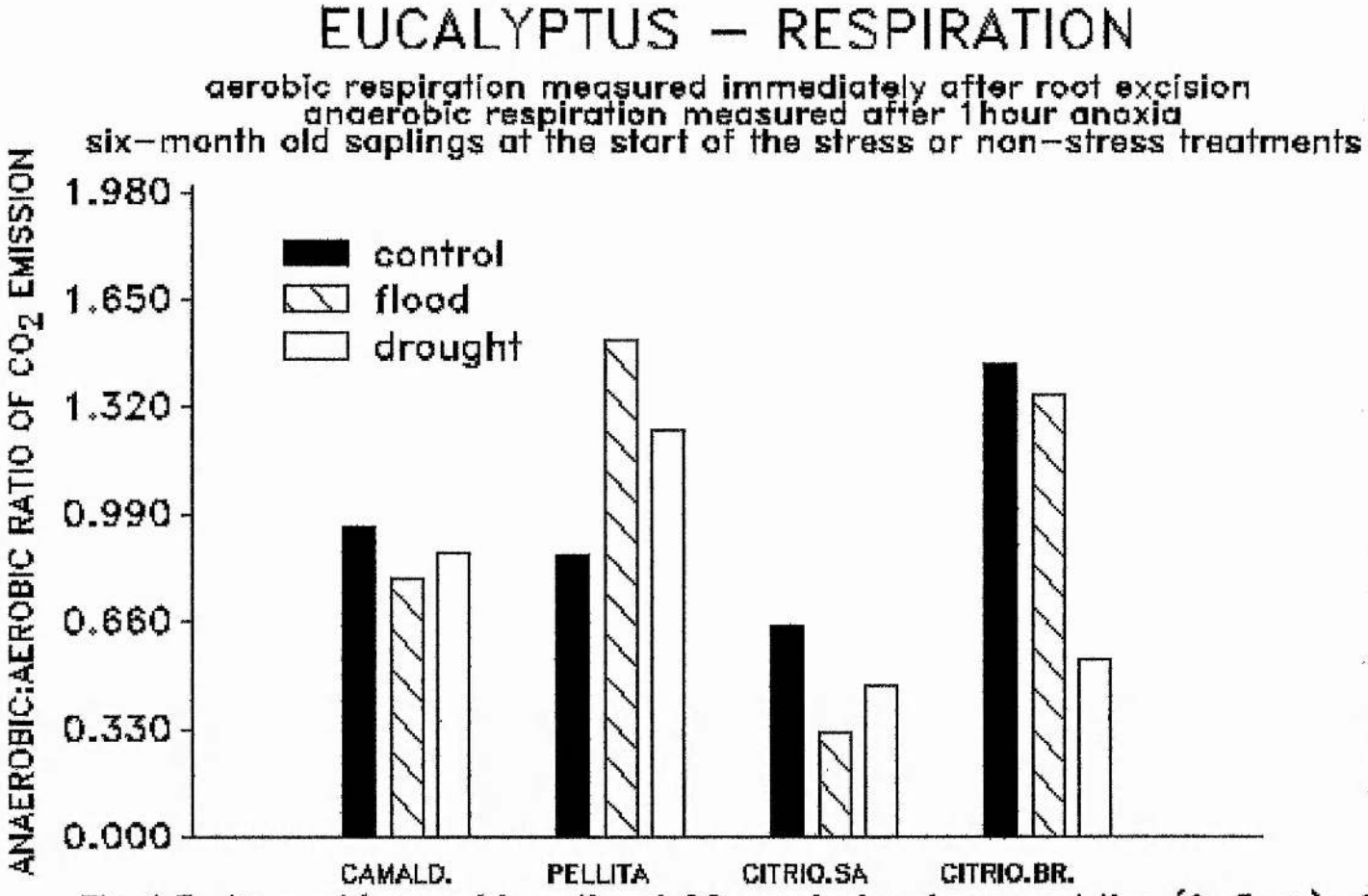

Fig.4.3. Anaerobic:aerobic ratio of CO_2 emission from root tips (0–5cm) of Eucalyptus species grown under different soil water regimes (n=1). Figures above 0,33 in y-axis indicate Pasteur effect. Length of stresses is on Table 4.1 (for E.cit.Br. see page 93). See complete set of collected data in the annexe, Tables 4.4a(camald.), 4.5a(cit.sa.), 4.6a(pel.), and 4.7a(cit.br.).

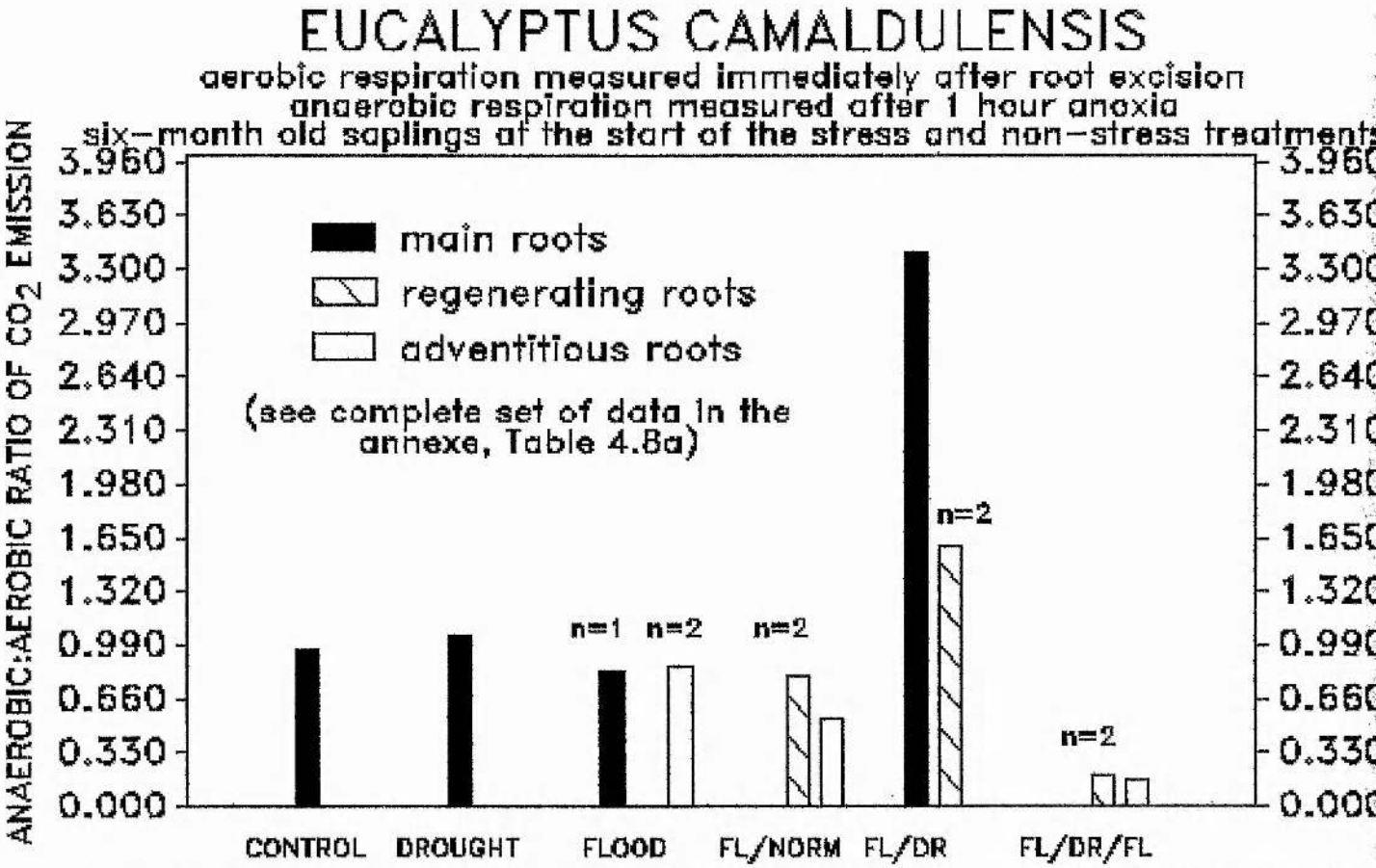

Fig.4.4. Anaerobic: aerobic ratio of CO_2 emission from 3 types of roots of E.camaldulensis under different soil water regimes. Multiple stress treatments measured at the end of the final stress. Figures above 0.33 in the y-axis indicate Pasteur effect. One plant per treatment; no. samples per plant (n)=3, unless otherwise. Length of stresses is on Table 4.1.

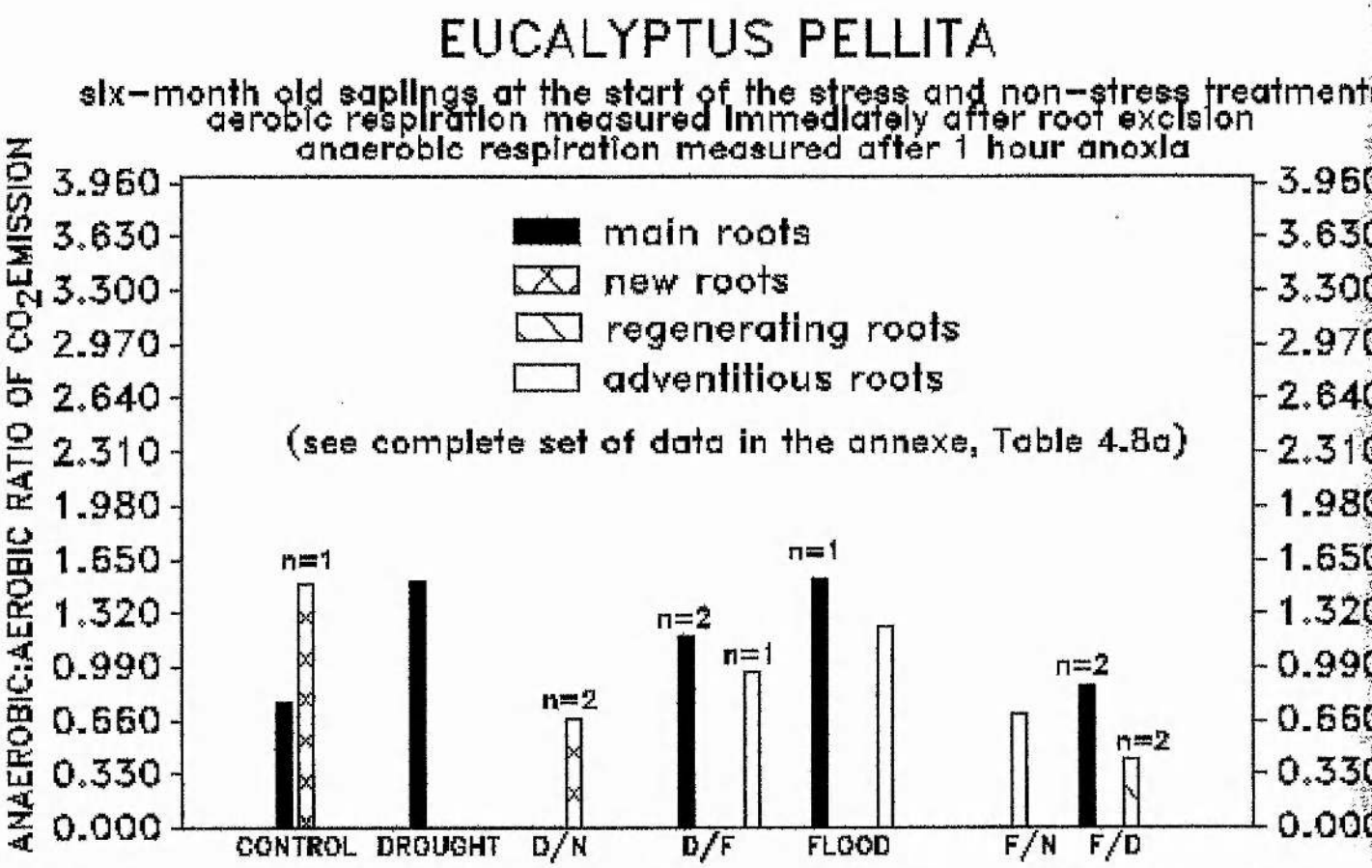

Fig.4.5. Anaerobic:aerobic ratio of CO_2 emission from 4 types of roots of E.pellita under different soil water regimes. Multiple stress treatments measured at the end of the final stress. Figures above 0.33 in the y-axis indicate Pasteur effect. One plant per treatment; no. of samples per plant (n)=3, unless otherwise. Length of stresses is on Table 4.1.

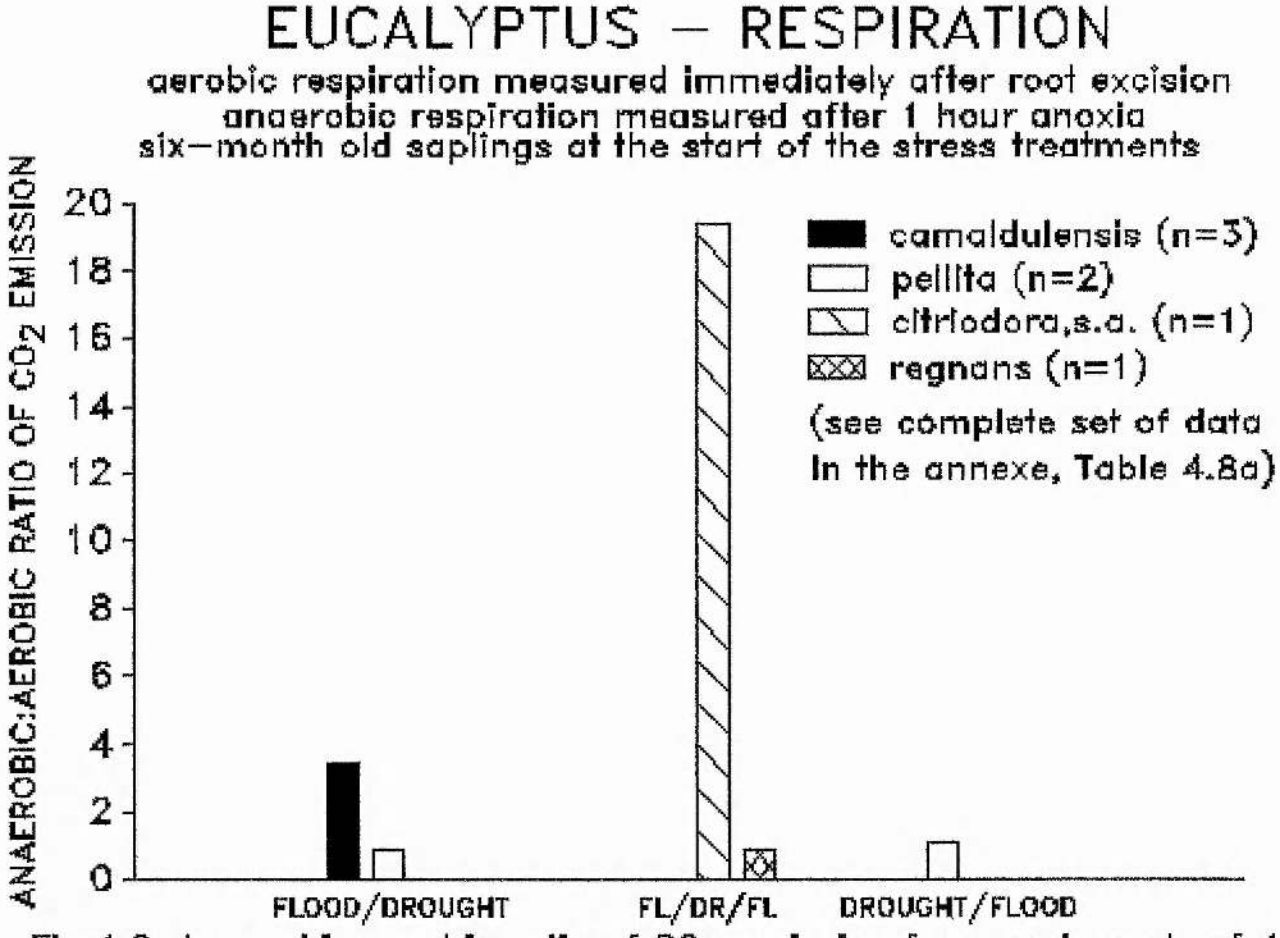

Fig.4.6. Anaerobic:aerobic ratio of CO_2 emission from main roots of 4 Eucalyptus species in alternating soil water regimes, measured at the end of the final stress. All treatments indicate some level of Pasteur Effect (all figures above 0.33 in the y–axis). One plant per treatment; no. samples per plant (n) as above. Length of stresses is on Table 4.1.

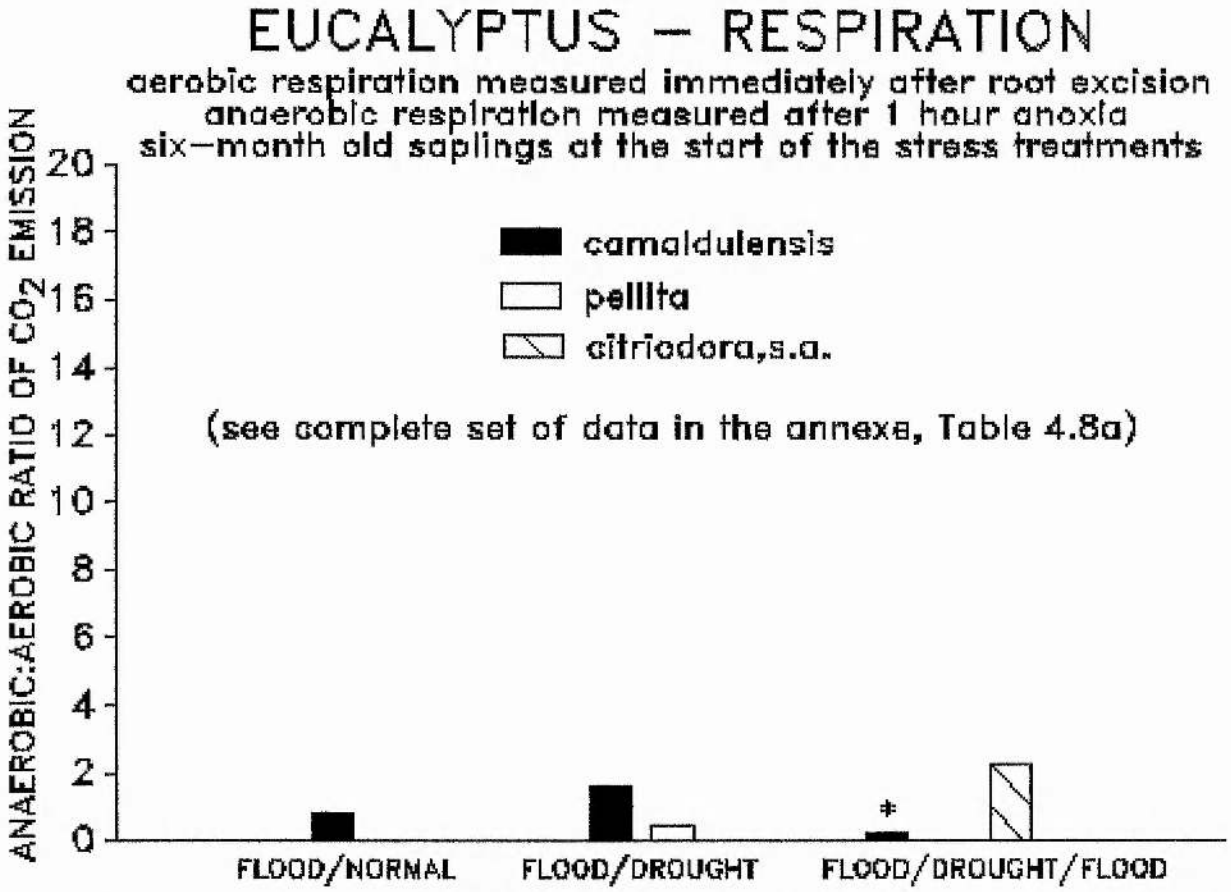

Fig.4.7. Anaerobic:aerobic ratio of CO_2 emission from regenerating roots of 3 Eucalyptus species in alternating soil water regimes, measured at the end of the final stress. All but one (*) treatment indicate some level of Pasteur effect (ratio higher than 0.33). One plant per treatment; no. of samples per plant (n)=2. Length of stresses is on Table 4.1.

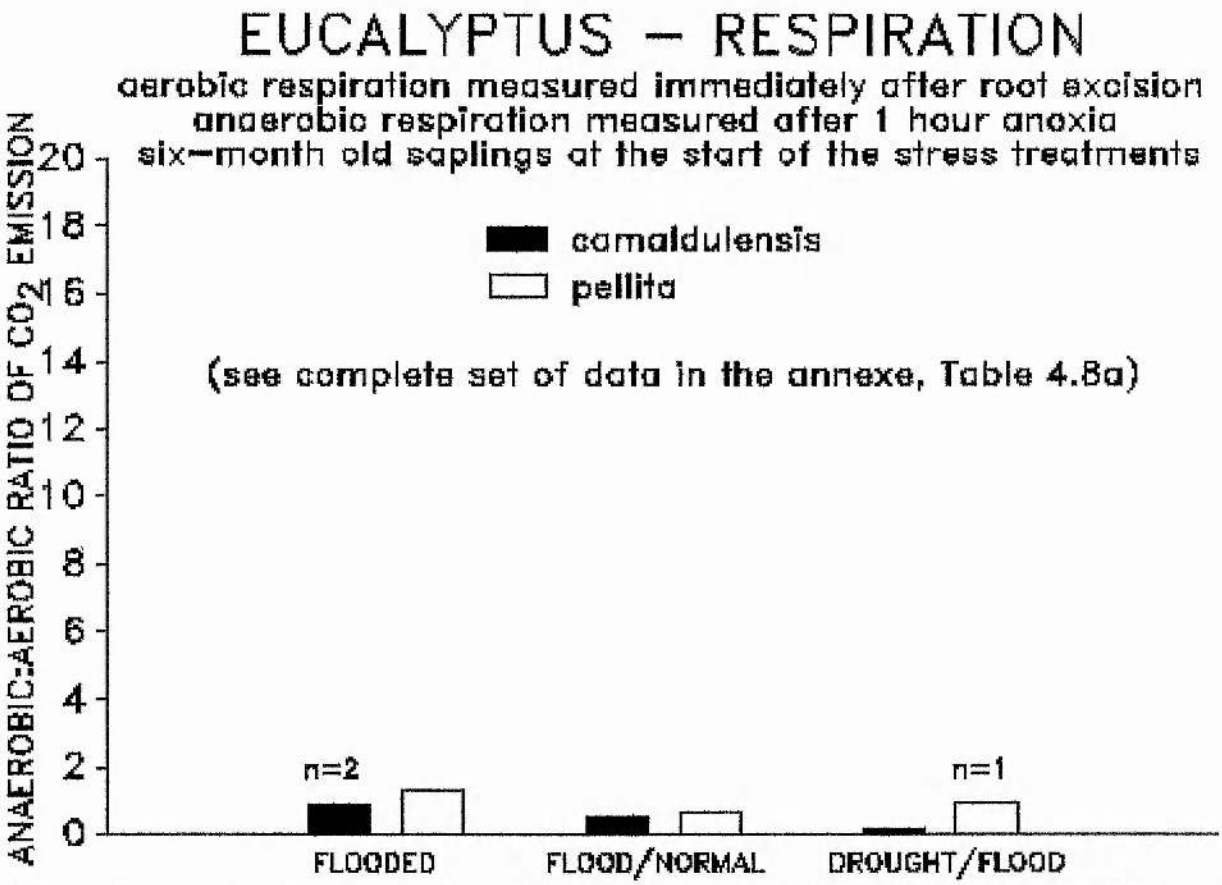

Fig.4.8. Anaerobic:aerobic ratio of CO_2 emission from adventitious roots of 2 Eucalyptus species in alternating soil water regimes, measured at the end of the final stress. All treatments show some level of Pasteur effect (ratio higher than 0.33). One plant per treatment; no. of samples per plant (n)=3, unless otherwise. Length of stresses is on Table 4.1.

were returned to a normal watering regime, this effect was reduced after one-hour anoxia. The highest aerobic respiration rates were shown by the roots under drought. However, when after drought the roots were submitted to flooding, the rates decreased to levels even lower than the control (annexe, Tables 4.6a and 4.8a). In addition, the Pasteur effect in this case was less than the more pronounced Pasteur effect showed under the drought treatment (fig.4.5). Conversely, when after flooding ***E.pellita*** saplings were submitted to drought, the aerobic respiration rates increased slightly (annexe, Tables 4.6a and 4.8a), but once again the Pasteur effect after one-hour anoxia was reduced, particularly for regenerating roots. Adventitious and regenerating roots for most treatments were particularly able to avoid or minimise Pasteur effect after one-hour anoxia (fig.4.5; 4.8).

3.3 *Ethanol production in the roots*

3.3.1 *Comparison of the effects of the different treatments among the different species.*

a. control

Fig.4.9 shows that the flood-intolerant and drought-intolerant species (***E.regnans*** and ***E.citriodora*** Brazil) produced after 24 hours of anoxia the highest amounts of ethanol among the non-stressed controls. ***E.citriodora*** (St. Andrews) showed a low ethanol production under these circumstances. The high values found for ***E.camaldulensis*** (annexe, Table 4.9a) were rather unexpected.

b. flooding and flooding-normal

Among the flood-treated roots of the species tested, the adventitious roots of ***E.camaldulensis*** and ***E.pellita*** showed

the highest ethanol production after 24-hour anoxic incubation (fig.4.10). The main roots of ***E.camaldulensis***, ***E.citriodora*** (Brazil) (fig.4.10) and ***E.citriodora*** (St.Andrews) (fig.4.13) showed comparatively low values. When 24-hour anoxia was imposed after a flood-normal treatment, root ethanol production was comparatively reduced for ***E.camaldulensis*** (fig.4.11), ***E.pellita*** (fig.4.12) and ***E.citriodora*** (St.Andrews)(fig.4.13). This reduction was not as marked for ***E.citriodora*** (St.Andrews) since it already produced a reduced amount of ethanol after 24-hour anoxia following the flooding treatment.

c. drought

Drought-treated roots of ***E.pellita*** and especially ***E.citriodora*** (Brazil), produced high amounts of ethanol after 24-hour anoxia (fig.4.10) compared with non-stressed controls (fig.4.9). ***E.camaldulensis*** also showed a comparatively high ethanol production.

d. flooding-drought-flooding

Both adventitious roots of ***E.camaldulensis*** (fig.4.11) and main roots (10-15 cm segment) of ***E.citriodora*** (St.Andrews) (fig.4.13) produced less ethanol after the 24-hour anoxia following this treatment than when previously subjected only to flooding. The anaerobic ethanol production of ***E.citriodora*** was lower than that of ***E. camaldulensis***.

3.3.2 *Comparison of the effects of the different treatments within the same species.*

a. ***E.camaldulensis***

The main roots of the control (annexe, Table 4.9a) and of the drought-treated saplings of this species produced more

ethanol after 24 hours of anoxia than the flooded ones (fig.4.10). This could mean that the roots of the flooded sapling measured had reduced viability at the time of the readings. However, flooded adventitious roots produced similar amounts of ethanol to the former treatments.

When flooded adventitious roots were returned to a normal soil water regime, the ethanol produced after the subsequent 24-hour anoxia showed a 10-fold reduction compared to flooded adventitious roots not returned to a normal water regime (fig.4.11). The roots that regenerated after flooding (during the normal watering weeks), produced ca. 4 times higher amounts of ethanol after 24-hour anoxia than the adventitious roots (annexe, Table 4.13a).

The flooding-drought-flooding treatment often induced in this species lower ethanol production after 24-hour anoxia than flooding or drought alone (Fig.4.11). In most cases, root tips produced more ethanol than the upper segments (annexe, Table 4.9a).

b. *E.citriodora* (St.Andrews)

This provenance of ***E.citriodora*** often produced less ethanol than the other species after 24 hours of anoxia following the four treatments examined: normal (fig.4.9), flooded, flood-normal and flooding-drought-flooding (annexe, Table 4.13a). Fig.4.13 shows that the amounts produced by the main roots of the control, flooding-normal and flooding-drought-flooding treatments after 24-hour anoxia were smaller than the amounts produced by the flood-treated roots. Regenerated roots under the flooding-drought-flooding treatment produced amounts of ethanol similar to the flooded main roots after 24-hour anoxia. There was little distinction between the root ethanol production of the different segments of main roots within each different treatment (annexe, Table 4.10a).

c. *E.regnans*

The roots of ***E.regnans*** under a normal watering regime, had a uniformly high ethanol production after 24 hours of anoxia in the three segments examined (over 100 μmol.gfw^{-1}.day^{-1}), compared with the other species (fig.4.9 and annexe, Table 4.10a).

d. *E.pellita*

The drought-treated tip and upper segments of the main roots of ***E.pellita***, produced considerably higher amounts of ethanol than the control after 24-hour anoxia (fig.4.9; 4.10). Only the intermediate segment showed a surprisingly low result (annexe, Table 4.11a). When after drought the plant was returned to a normal watering regime, new roots were formed and, after 24-hour anoxia, they produced amounts of ethanol similar to the average of the drought-treated plants (fig.4.12).

The return to a normal watering regime after flooding resulted in the reduction of the amount of adventitious root ethanol production after 24-hour anoxia. Examining the adventitious root tips alone (annexe, Table 4.11a), drought before flooding resulted in a high ethanol production after 24 hours of anoxia, which was, however, smaller than that of the flood treatment. Drought before flooding also resulted in a comparatively low amount of ethanol produced by the main root after 24-hour anoxia (fig.4.12).

The lowest production of ethanol after 24-hour anoxia, in this species, was shown by regenerated roots under the flooding-drought treatment (fig.4.12).

e. *E.citriodora* (Brazil)

The ethanol production results obtained for this species stimulated the study developed in Chapter Five, and the illustrations are, therefore, contained in that Chapter (see fig.5.1). Strangely, flooded roots, despite the strong Pasteur effect observed (see item 3.2.2 c), produced less ethanol than the control after 24-hour anoxia, which may have been caused by reduced viability of the excised flooded roots used. However, probably the most interesting experimental results were obtained with this stress-intolerant provenance. The drought-treated roots produced much higher amounts of ethanol after 24-hour anoxia than the control. Additionally, the droughted roots that after excision were subjected to 24 hours of air produced on average 4 times more ethanol than the ones that were exposed to 24 hours of anoxia (annexe Table 4.12a, and Chapter Five, fig.5.3). Unfortunately, for the control roots, ethanol production was not measured after 24-hour aerobic incubation and, therefore, little could be said in relation to this particular experiment. From these curious results, however, the studies on Chapter Five started to be developed, which shall be seen later.

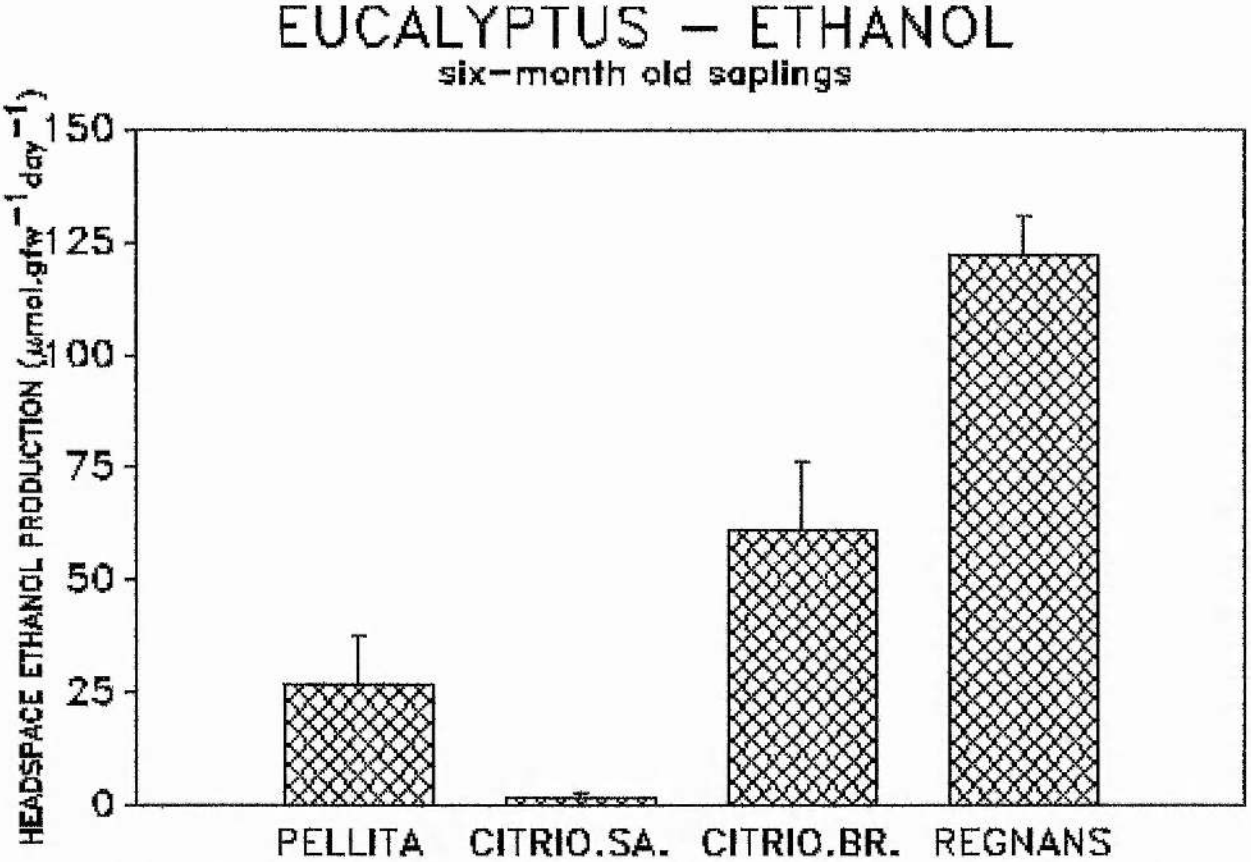

Fig.4.9. Headspace ethanol production after 24 h anoxia of main roots of 4 non-stressed Eucalyptus species (controls). One plant per species; no. of samples per plant (n)=3. Bars indicate standard error. Data are displayed in the annexe, Table 4.13a.

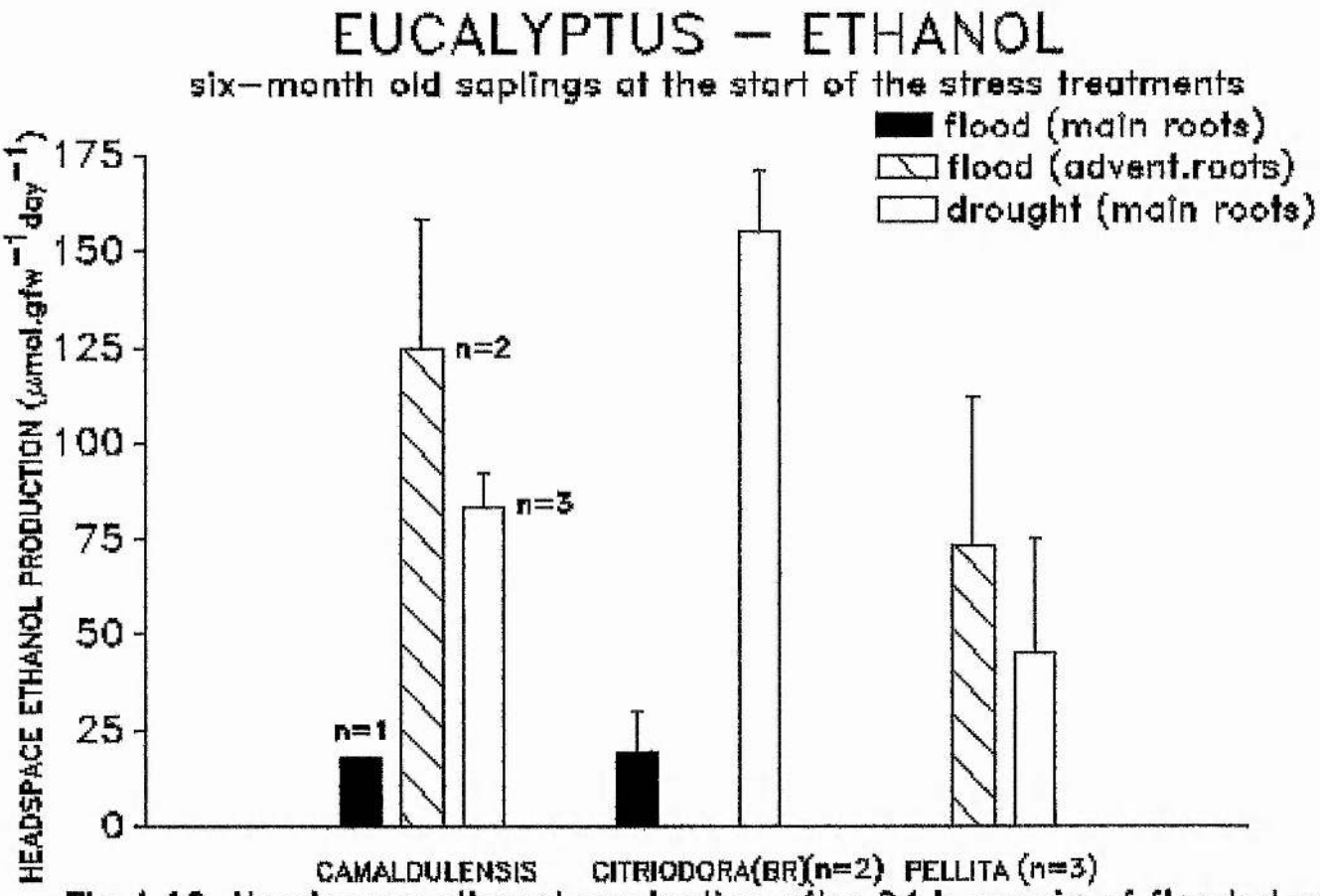

Fig.4.10. Headspace ethanol production after 24 h anoxia of flooded and drought–treated roots of 3 Eucalyptus species. One plant per treatment no. of samples per plant (n) as above. Bars indicate standard error. Length of stresses is on Table 5.1 (for E.cit.Br. see page 93). Data are displayed in the annexe, Table 4.13a.

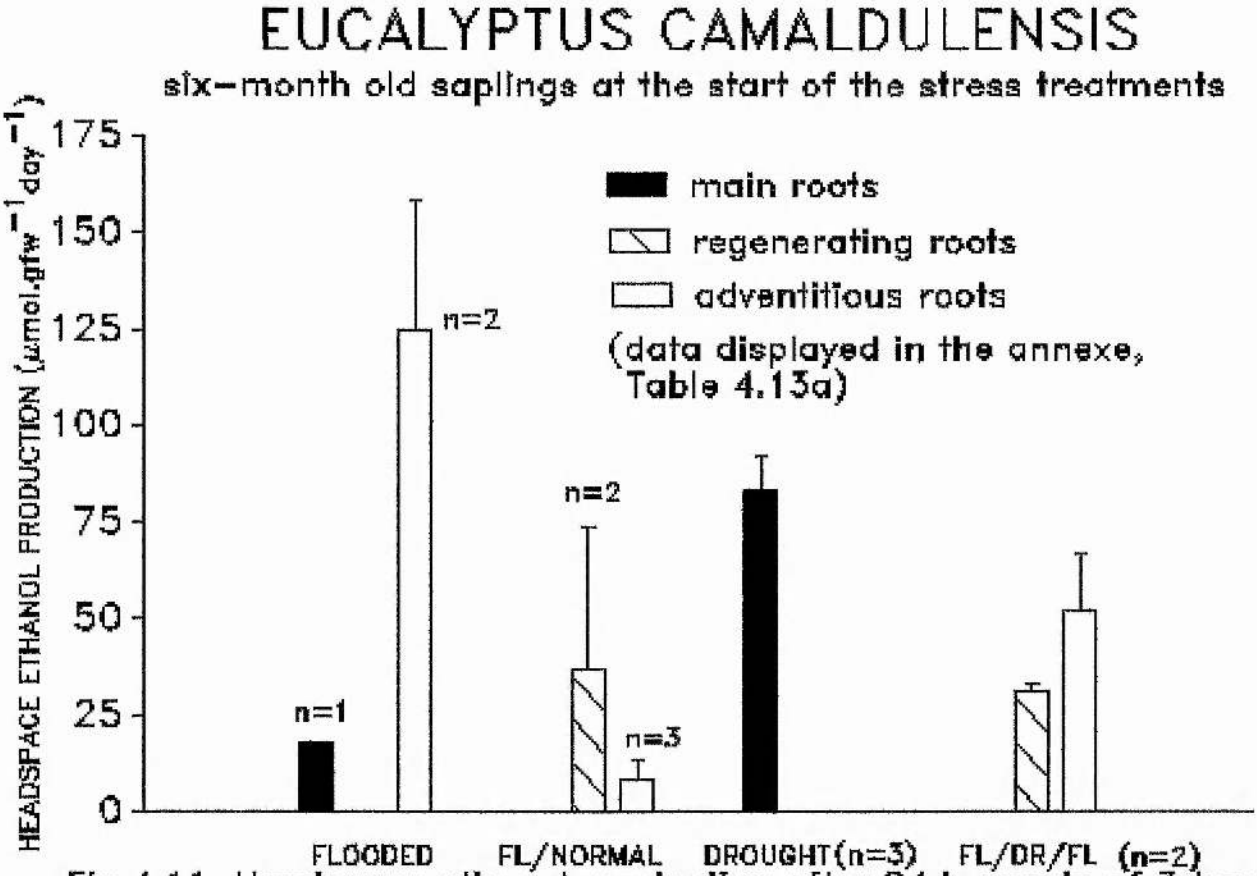

Fig.4.11. Headspace ethanol production after 24 h anoxia of 3 types of roots of E.camaldulensis previously subjected to different soil water regimes. Multiple stress treatments measured at the end of the final stress. One plant per treatment; n=no. of samples per plant. Bars show standard error. Length of stresses is on Table 4.1.

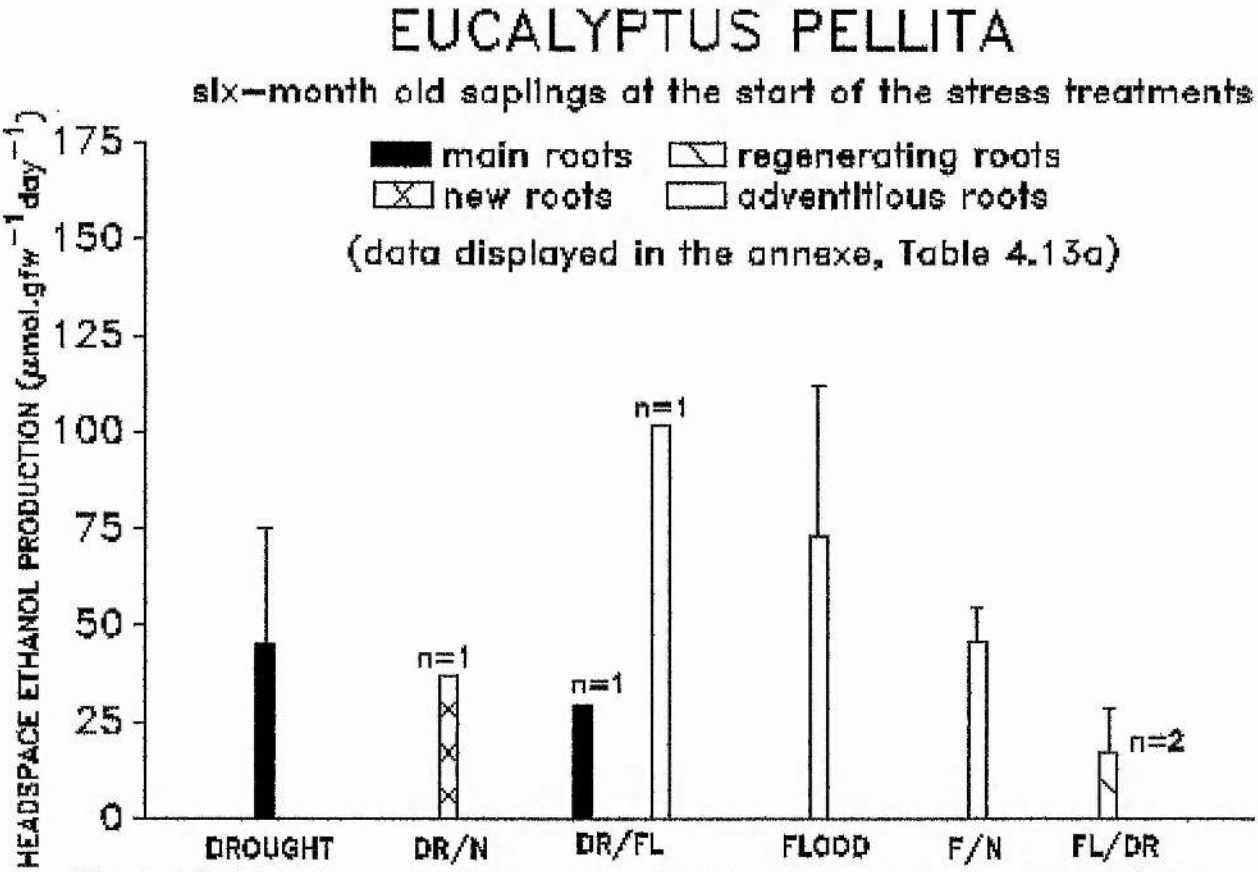

Fig.4.12. Headspace ethanol production after 24 h anoxia of 4 types of roots of E.pellita previously subjected to different soil water regimes. Multiple stress treatments measured at the end of the final stress. One plant per treatment; no. of samples per plant (n)=3, unless otherwise. Bars show standard error. Length of stresses is on Table 4.1.

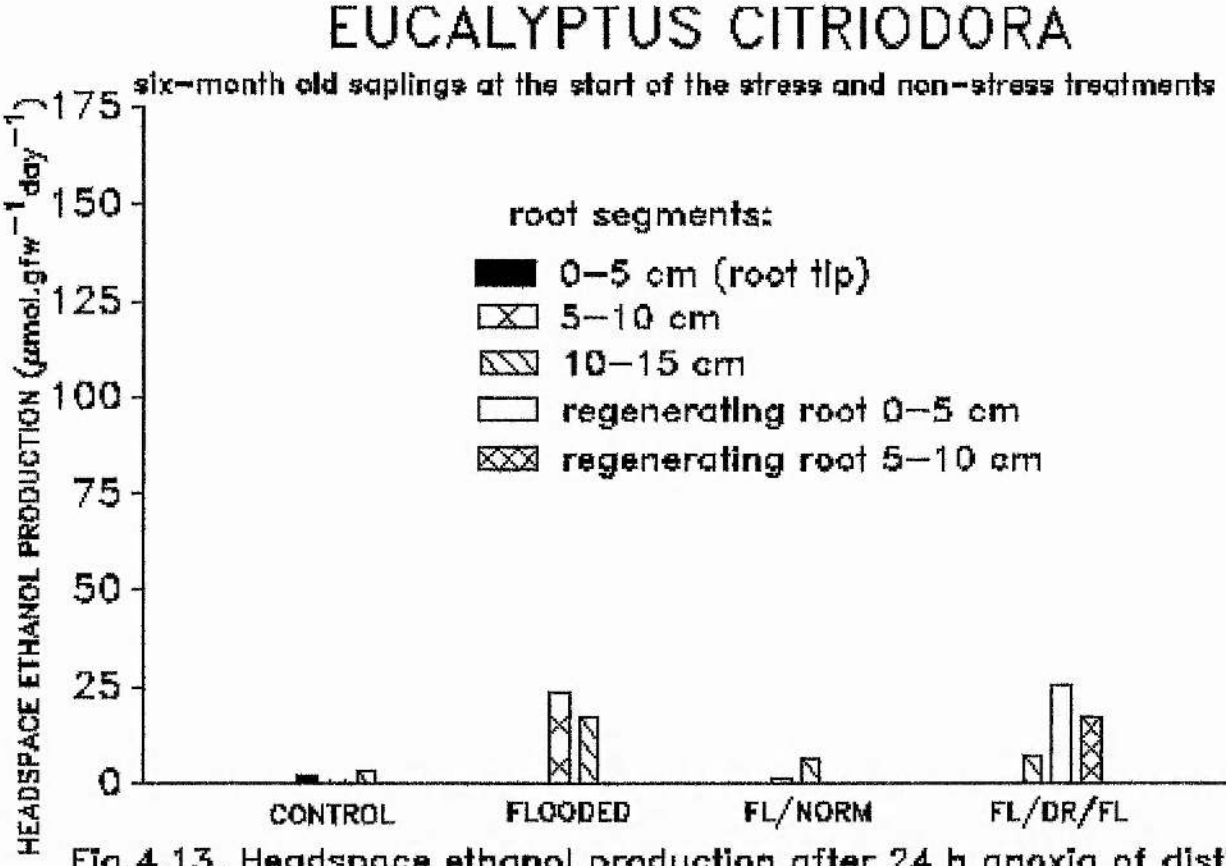

Fig.4.13. Headspace ethanol production after 24 h anoxia of distinct root segments of E.citriodora (St.Andrews) previously subjected to different soil water regimes. Multiple stress treatments measured at the end of the final stress. n=1. Length of stresses is on Table 4.1. Data displayed in the annexe, Table 4.10a.

4) DISCUSSION

The pilot study of which consists this Chapter, despite its statistical limitations, allowed the observation of some trends in relation to the effect of alternating extreme soil water regimes on ***Eucalyptus*** species. The plant responses to a determined stress were often affected by a previous stress, and these responses varied largely according to the ***Eucalyptus*** species involved. In addition, the anaerobic metabolism of roots often varied according to the stress or set of stresses the plant was submitted to prior to subjection to 24-hour anoxia. These observations are examined in more details in the following topics of discussion.

4.1 *The interaction of stresses*

E.camaldulensis, tolerant to both flooding (agreeing with **Blake & Reid, 1981; Kozlowski, 1984; Pereira & Kozlowski, 1977;** and **Van Der Moezel *et al.*, 1988)** and drought, showed symptoms of injury when after flooding the trees were subjected to drought. The same was true to ***E.pellita*** also tolerant to flooding and drought. However, ***E.regnans***, intolerant both to flooding and drought, could survive flooding at least 3 times longer (28 days) when subjected to drought previously (Table 4.1). Conversely, when the inverse situation occurred, *i.e.* flooding followed by drought, the plants rapidly died. Nevertheless, when ***E.regnans*** was given a period of recovery between flooding and drought it survived well. The two provenances of ***E.citriodora*** showed divergent behaviour. The Brazilian provenance of ***E.citriodora*** , even more intolerant than ***E.regnans***, did not withstand nor recover from any of the stresses. The St.Andrews provenance of ***E.citriodora***, conversely, was the only group of plants to which stress alternation did not cause immediate visible injuries. Its individuals survived

flooding, drought, flooding-drought and flooding-drought-flooding showing no root or shoot extension, but however without showing any morphological symptom of injury.

Drought immediately after flooding seemed to be damaging (as in ***E.pellita*** and ***E.camaldulensis***) or even lethal (as in ***E.regnans*** and ***E.citriodora***, Brazil). Conversely, drought immediately before flooding had a positive effect on flooding survival of ***E. regnans*** and was not hazardous to ***E.pellita***. The former effect was expected, since possibly when drought follows a period of flooding (which consumes plants' reserves, causes root dieback, etc.), it causes initially post-anoxic injuries (evident in ***E.regnans*** which most roots died after 24 hours of drainage), followed by starvation, since most of the reserves are used up during flooding. However, the latter effect of a pre-flooding drought treatment ameliorating flooding survival was rather unexpected, particularly in the case of the highly flood-sensitive ***E.regnans***. This observation became even more intriguing when later it was seen that drought stimulated ethanol production in roots of ***E.camaldulensis*** and ***E.pellita*** under anoxia. Considering the potential toxicity of ethanol, a pre-flooding drought treatment in this case would be likely to be even more negative to anoxic survival and, therefore, a pre-flooding drought would also be likely to have negative effects in a subsequent flooding survival.

The results obtained for ***E.citriodora*** (Brazil) provided additional curious information. After excision, the roots of drought-treated plants allowed 24 hours of air produced ca. 4 times more ethanol than roots subjected to 24 hours of anoxia. Although for non-stressed plants ethanol production of aerobic incubated roots was not tested, leaving the above experiment without a control for comparison, these data indicated the possibility that drought by itself could stimulate high ethanol production. At this stage however,

the whole basis of data available to intellectually put together these different pieces of observation, was still very weak, and will not be further discussed in this Chapter. Nevertheless, in view of the curiosity arising from these results, it was decided to investigate the subject further, as carried out in Chapter Five.

4.2 *Root metabolism following single or multiple stresses, before and after subsequent anoxia*

The metabolic responses of roots of ***Eucalyptus*** to stress alternation and to subsequent anoxia, varied largely between species.

The anaerobic root respiration rates of ***E.camaldulensis*** for most treatments compared to the controls seemed unchanged. The outstanding exceptions were the adventitious and regenerating roots of a flood-drought-flood-treated sapling, which showed at least ca. 5-fold higher readings than the average of the other treatments (annexe, Table 4.8a). Curiously, those were the very roots to show a remarkable ability in reducing their anaerobic respiration rate by a third of the aerobic rates after submission to 1-hour anoxia, differently from roots of this species in all other treatments and control which presented some degree of Pasteur effect (fig.4.4). The ethanol production of the flood-drought-flood-treated roots, after 24-hour anoxia, was also comparatively low (fig.4.11).

Another stress-tolerant species, ***E.pellita***, also showed similar aerobic rates for most treatments, with the exception of the fast-respiring newly formed control roots, and regenerating roots under the flood-drought treatment, which were at least ca. 2-fold higher than the average of the other treatments (annexe, Table 4.8a). After 1-hour anoxia, the regenerating roots previously flood-drought-

treated showed a minimal Pasteur effect (fig.4.5), and after 24-hour anoxia ethanol production was also reduced (fig.4.12).

In ***E.citriodora*** (St.Andrews), another survivor of long-term flooding, the opposite situation seen for the two species above occurred. Flood-drought-flood-treated main roots showed a minute aerobic respiration, compared to roots in other treatments and controls. After 1-hour anoxia, anaerobic respiration accelerated provoking the highest Pasteur effect of all roots tested (fig.4.6).

If low metabolic rates under stress conditions can be an indication of tolerance, as proposed by **Monk, Crawford & Braëndle (1984)** for conditions of oxygen deficiency, the reduced aerobic respiratory rates of ***E.citriodora*** (St.Andrews) could indicate a certain degree of tolerance to the flood-drought-flood treatment. However, the remarkable Pasteur effect shown by the roots of this species when subjected to 1-hour anoxia following the previous stress, are indication of anoxia-intolerance. In this case, it can possibly be said that the previous flood-drought-flood treatment rendered the roots sensitive to subsequent anoxia. In the case of ***E.camaldulensis*** and ***E.pellita***, however, the paradoxical switch from a possible initial sensitivity during respectively flood-drought-flood and flood-drought (as evidenced by the increase in the aerobic respiratory rates compared to control roots) to the subsequent signs of anoxia-tolerance following 1- (as evidenced by the ability of avoiding Pasteur effect) and 24-hour anoxia (as evidenced by the low root ethanol production shown), is not as easily explained. This paradox clearly deserves further investigation. It is possibly no coincidence that the roots involved in these unexpected responses of ***E.camaldulensis*** and ***E.pellita*** were mainly regenerating and adventitious roots, contrasting with the more expected response of main

roots of ***E.citriodora*** (St.Andrews). The next topic (4.3) discusses the relationship between root formation under stress and possible survival strategies.

4.3 *Root formation under stress and possible survival strategies*

During flooding, ***E.camaldulensis*** and ***E.pellita*** invest in the production of adventitious roots, while the main roots stop growing and show some dieback. Shoot extension still takes place under these conditions (fig.4.2 and annexe, Table 4.2a). Adventitious root formation under flooding is a characteristic commonly found in flood-adapted species **(Hook & Scholtens, 1978; Kozlowski, 1984)**. These structures, due to the presence of aerenchyma tissues and to their high porosity (these roots characteristically show a high ratio surface area : tissue volume) are more likely to easily liberate their own anaerobically-produced potentially toxic metabolites in the surrounding medium, than less porous types of roots **(Armstrong, 1979)**. Indeed, adventitious roots in rice allow 98% of the anaerobically produced ethanol to diffuse into the surrounding water **(Bertani, Brambilla & Menegus, 1980)**.

For adventitious roots of ***E.camaldulensis*** and ***E.pellita*** it was found that anaerobic ethanol production was often high compared to other types of roots (figs.4.11; 4.12; 4.13), however an account of the balance between ethanol production and diffusive loss for these species cannot be given, since only production was measured and not accumulation of ethanol.

Reduction of metabolic activity is a characteristic commonly associated with plants well-adapted to flooding **(Crawford, 1989; Pradet & Bomsel, 1978)**, which was seen for ***E.citriodora*** (St.Andrews) that does not produce adventitious

roots under flooding, but not seen for the adventitious root forming ***E.camaldulensis*** and ***E.pellita***. It is tempting to speculate that these two tolerant species can afford to keep the same levels of metabolic activity under flooding, and actually promote shoot extension, due to their ability to develop adventitious roots. Although the main roots would eventually decay, stop extension and possibly die, the adventitious roots could survive and grow (see annexe, Table 4.2a) possibly for their capacity to liberate potentially toxic compounds formed by anaerobic respiration. **Monk, Crawford & Braëndle (1984)** suggest that a low metabolic rate under anoxia will not be of importance to anoxia-tolerant plants that readily eliminate ethanol from the tissues into the medium, provided that carbohydrate supplies are sufficient for prolonged fermentation. In this case, an active alcoholic fermentation can be of advantage in maintaining a viable energy charge in the tissue **(Pradet & Bomsel, 1978)**. At this stage, however, the present discussion cannot go beyond speculation. Future studies, focusing the relationship between the rate productivity:loss of ethanol in the different types of ***Eucalyptus*** roots and their survival and physical responses to flooding, could provide interesting insight into these species survival strategies.

Root regeneration also merits examination. When drainage follows flooding, ***E.camaldulensis*** regenerates new roots (adventitious roots may cease to function) with the onset of water stress (in this case one week later). The capacity of ***E.regnans*** to regenerate and form new roots when returned to a normal watering regime after a short flooding period, may have aided posterior drought survival. In this case, differently from ***E. camaldulensis***, ***E.regnans*** was allowed a longer period of recovery after flooding (five weeks) before subjection to drought. When the onset of drought took place, these new roots probably had accumulated enough reserves to

withstand the water stress. This is also likely to be true of ***E. camaldulensis***, if allowed a longer recovery period after flooding.

4.4 *The natural habitat of the species studied*

The natural habitat of the species studied may allow additional understanding of the different physical and metabolic responses to stress alternation shown. ***E.camaldulensis*** grows along river margins being periodically flooded **(Lima, 1987)**, and is also known as a plant of great tolerance to drought, high temperatures, herbivory and salinity. It is the most widespread of all eucalypt species ranging over some 23° of latitude **(Penfold & Willis, 1961)**. ***E.pellita***, according to the latter authors, is found on light, sandy coastal soils, from sea level to ca.750 m altitude, in well-drained soils. The fact that these authors do not mention the occurrence of this species in flooded areas is rather surprising in view of the capacity of these species to form adventitious roots, so characteristic of plants well-adapted to flooding. ***E.citriodora*** is found on poor gravely soils, but grows in almost any soil **(Penfold & Willis, 1961)**. ***E.regnans***, known for its frost resistance, is almost restricted to cool mountains **(Ashton, 1958)**, although some individuals appear naturally on swamps where they grow poorly, seldomly reaching the mature stage **(Ashton, 1975)**.

As seen for the ***Parkia*** species in Chapter Two, each of these ***Eucalyptus*** species may have evolved distinct strategies to withstand the different stresses their habitats frequently subject them to. This does not imply that they are not able to tolerate other kinds of stresses, as the case of ***E.camaldulensis*** shows, but probably means that if the strategies to tolerate a particular stress are specific, the plants may have difficulties in withstanding any sudden variation in climate and/or soil water status. Drastic

climatic changes are characteristic of tropical countries, where a short and heavy rain may occur in the middle of the dry season, and a warm and dry period may appear in the middle of the rainy season. Thus, in areas subject to periodic stress or stress alternation, tree-planters should take extra care in the choice of the tree species to be planted. Another factor often taken into account when choosing tree species for planting, is how fast a species can grow. ***E.regnans***, the world's tallest latifoliate **(Lima, 1987)**, and ***E.citriodora*** (Brazil), the highest metabolic activity of the species studied, were highly sensitive to both flooding and drought at the age of six months.

4.9 The Brazilian *Seca de Ponteiros do Vale do Rio Doce*

Dianese, Haridasan & Moraes (1984) and **Kozlowski (1988)**, studying the Brazilian *Seca* (see "Introduction") suggested from field experiments and observation that ***E.camaldulensis*** is tolerant, while ***E.citriodora*** is very susceptible to the *Seca*, but shows rapid recovery. If the *Seca* is really due to a heavy rainy season being followed by a severe drought, as proposed by **Kozlowski (1988)**, the present preliminary results do not entirely match these field observations. ***E.camaldulensis***, although tolerant to flooding and drought, showed symptoms of injury and increased ethanol production and respiration rate during the treatment flooding-drought. However, it recovered quickly. ***E.citriodora*** showed that between provenances the difference can be drastic. The St.Andrews provenance survived single stresses or combination of stresses, often with an impairment of growth, whereas the Brazilian provenance was affected by all the stresses without recovery. However, the works above cited refer to adult trees, while the present work used six-month old saplings.

ANNEXE

Table 4.2a: Average root extension[1](cm.week^{-1}.root^{-1}) of ***E.camaldulensis***, ***E.citriodora*** (St.Andrews), ***E. regnans*** and ***E.pellita*** submitted to different soil water regimes. Multiple stress treatments, were measured during the final stress[2].

SOIL SATURATION	CAMALD.	CITR.SA	REGNANS	PELLITA
CONTROL	1.57	1.00	2.13	3.49
DROUGHT	1.51	1.04	0.00	2.08
DROUGHT-NORMAL	-	-	-	2.77
DROUGHT-FLOOD	-	-	0.57	0.31
DROUGHT-FLOOD-DROUGHT	-	-	0.00	-
FLOOD	0.18[3]	0.01	0.00	2.78[3]
FLOOD-NORMAL	4.39[4]	0.43	0.96	4.75[4]
FLOOD-DROUGHT	1.99[5]	0.87	0.00	1.86[5]
FLOOD-DROUGHT-FLOOD	0.30	0.00	0.00[6]	-
FLOOD-NORMAL-DROUGHT	-	-	2.15	-

[1] Root extension data was based on the weekly monitoring of 10-20 roots per plant, irrespective of type of root.
[2] for the duration of each stress, and n see Table 4.1.
[3] any extension registered was mainly due to adventitious roots formed after the fifth week of flooding.
[4] any extension registered was mainly due to regenerating roots formed when plants were returned to normal conditions, and to previously existent adventitious roots.
[5] the extension registered took place mostly during the first week of drainage; as drought conditions progressed extension was halted.
[6] there was a 5 weeks period of normal water regime between the first flood and drought.

Some of the above data referring to ***E.camaldulensis***, ***E.citriodora*** and ***E.pellita*** are displayed in fig.4.2. The data referring to ***E.regnans*** are in displayed in Chapter Five, fig.5.4.

Table 4.3a: Average shoot extension ($cm.week^{-1}.plant^{-1}$) of ***E.camaldulensis***, ***E.citriodora*** (St.Andrews), ***E.citriodora*** (Brazil), ***E.regnans*** and ***E.pellita*** submitted to different soil water regimes. Multiple stress treatments measured during the final stress[1].

SOIL SATURATION	CAMALD.	CITR.SA	CITR.BR	REGNANS	PELLITA
CONTROL	1.52	1.05	2.13	2.25	2.22
DROUGHT	1.00	1.25	0.00	0.00	1.78
DROUGHT-NORMAL	-	-	0.00	-	1.25
DROUGHT-FLOOD	-	-	0.00	0.50	1.25
DROUGHT-FLOOD-DROUGHT	-	-	-	0.00	-
FLOOD	1.42	0.48	0.00	0.00	0.72
FLOOD-NORMAL	2.00	0.75	0.00	0.60	1.37
FLOOD-DROUGHT	1.38	0.50	0.00	0.00	0.75
FLOOD-DROUGHT-FLOOD	1.42	1.17	-	0.00[2]	-
FLOOD-NORMAL-DROUGHT	-	-	-	0.75	-

[1] for the duration of each stress see Table 4.1, except for ***E.citriodora*** (Brazil) which is shown in page 93.
[2] there was a 5 weeks period of normal water regime between the first flood and drought.

Some of the above data referring to ***E.camaldulensis***, ***E.citriodora*** (St.Andrews) and ***E.pellita*** are displayed in fig.4.2. The data referring to ***E.regnans*** are displayed in fig.5.4, Chapter Five.

Table 4.4a: Carbon dioxide emission (μmol CO_2 h^{-1} gfw^{-1}) of distinct ***E.camaldulensis*** root segments under different soil water regimes. Aerobic respiration measured immediately after root excision at the end of a stress or, for multiple stress treatments, at the end of the final stress[1]. Anaerobic respiration measured after 1-hour anoxia following stress(es) treatment (n=1).

SOIL SATURATION	root segm(cm)	aerobic resp.	anaerobic resp.
CONTROL	0- 5	17.52	16.75
	5-10	13.51	12.67
	10-15	14.75	14.75
FLOODED	main root	13.32	10.52
	adv. 0- 5	11.11	9.96
	adv. 5-10	12.39	10.11
DROUGHT	0- 5	47.09	41.10
	5-10	11.27	26.90
	10-15	20.65	14.57
FLOOD-NORMAL	adv. 0- 5	17.07	12.39
	adv. 5-10	20.93	9.14
	adv.10-15	18.73	9.42
	reg. 0- 5	15.26	5.63
	reg. 5-10	20.20	22.46
FLOOD-DROUGHT	0- 5	8.10	11.23
	5-10	9.03	34.44
	10-15	8.99	43.22
	reg. 0- 5	15.47	18.94
	reg. 5-10	10.11	22.12
FLOOD-DROUGHT-FLOOD	adv. 0- 5	85.02	15.18
	adv. 5-10	106.19	15.26
	reg. 5-10	58.07	10.22
	reg.10-15	89.20	17.34

[1]. for the length of each stress see Table 4.1
Some of these data can be seen in fig.4.3.
Table 4.8a shows the averages per treatment and per type of root.

Table 4.5a: Carbon dioxide emission (μmol CO_2 h^{-1} gfw^{-1}) of distinct root segments of ***E.citriodora*** (St. Andrews) under different soil water regimes. Aerobic respiration measured immediately after root excision at the end of a stress, or for multiple stress treatments, at the end of the final stress[1]. Anaerobic respiration measured after 1-hour anoxia following stress(es) treatment (n=1).

SOIL SATURATION	root segm.(cm)	aerobic resp.	anaerobic resp.
CONTROL	0- 5	23.60	15.33
	5-10	25.89	16.24
	10-15	25.93	25.93
FLOODED	5-10	28.91	9.29
	10-15	29.49	14.15
DROUGHT	0- 5	18.96	8.85
	5-10	9.82	9.64
	10-15	16.71	13.70
FLOODING-NORMAL	0- 5	24.22	15.93
	5-10	22.81	11.06
	10-15	27.19	13.60
FLOOD-DROUGHT-FLOOD	main	1.03	19.36
	reg. 0- 5	19.21	29.29
	reg. 5-10	10.90	39.96

1. for the length of each stress see Table 4.1.
Some of these data can be seen in fig.4.3.
Table 4.8a shows the averages per treatment and per type of root.

Table 4.6a: Carbon dioxide emission (μmol CO_2 h^{-1} gfw^{-1}) of distinct root segments of ***E.pellita*** under different soil water regimes. Aerobic respiration measured immediately after root excision at the end of a stress or, for multiple stress treatments, at the end of the final stress[1]. Anaerobic respiration measured after 1-hour anoxia following stress(es) treatment (n=1).

SOIL SATURATION	root segm(cm)	aerobic resp.	anaerobic resp.
CONTROL	0- 5	18.63	16.11
	5-10	19.98	15.58
	10-15	18.05	12.21
	new	45.17	67.74
FLOODED	main	16.59	25.39
	adv. 0- 5	20.38	13.19
	adv. 5-10	16.47	16.80
	adv.10-15	24.40	49.74
DROUGHT	0- 5	27.87	34.84
	5-10	28.39	47.85
	10-15	33.43	53.09
FLOOD-NORMAL	adv. 0- 5	18.58	16.52
	adv. 5-10	20.25	16.08
	adv.10-15	30.20	12.48
FLOOD-DROUGHT	0- 5	20.99	19.65
	5-10	29.95	24.45
	reg. 0- 5	31.73	14.35
	reg. 5-10	74.29	29.20
DROUGHT-NORMAL	new 0- 5	26.41	24.45
	new 5-10	21.58	8.99
DROUGHT-FLOOD	0- 5	11.39	9.35
	5-10	6.26	9.60
	adv.	14.45	13.94

[1]. for the length of each stress see Table 4.1.
Some of these data can be seen in fig.4.3.
Table 4.8a shows the averages per treatment and per type of root.

Table 4.7a: Carbon dioxide emission (μmol CO2 h^{-1} gfw^{-1}) of distinct root segments of the stress-intolerant species ***E.regnans*** and ***E.citriodora*** (Brazil) under different soil water regimes. Aerobic respiration measured immediately after root excision at the end of a stress or, for multiple stress treatments at the end of the final stress[1]. Anaerobic respiration measured after 1-hour anoxia following stress(es) treatment (n=1).

SOIL SATURATION	root segm(cm)	aerobic resp.	anaerobic resp.
E.regnans	0- 5	21.04	19.17
NORMAL	5-10	27.75	13.88
	10-15	22.30	18.58
FLD-NORM-DRT-FLD	main	2.84	2.41
E.citriodora (Br.)	0-10	111.50	162.60
NORMAL	10-20	118.93	161.05
	new	56.63	49.55
FLOODED	0-10	30.51	41.60
	10-20	63.42	64.89
DROUGHT (severely injured)	0-10	20.20	17.17
	10-20	26.90	20.53
DROUGHT (moderatly injured)	0-10	35.40	19.27
	10-20	23.36	14.57

1. the length of each stress for ***E.regnans*** is on Table 4.1; for ***E.citriodora*** (Brazil) each treatment lasted 4 weeks.

Some of these data can be seen in fig.4.3.

Table 4.8a shows the averages per treatment and per type of root.

Table 4.8a: Mean root carbon dioxide emission (μmol CO_2 h^{-1} gfw^{-1}) of different root segments of main (m), adventitious (a),regenerating (r) and newly formed (n) roots of five ***Eucalyptus*** species under distinct soil water regimes. Aerobic respiration measured immediately after root excision at the end of a stress or, for multiple stress, treatments, at the end of the final stress[1]. Anaerobic respiration measured after 1-hour anoxia following stress(es) treatment (n ranged from 1 to 3).

SPECIES	CAMALD.		CITRIOD.SA		CITRIOD.BR		REGNANS		PELLITA	
SOIL H_2O	air	anox.	air	anox.	air	anox.	air	anox.	air	anox.
CONTROLm	15.3	14.7	25.1	19.2	115.2	161.8	23.7	17.2	18.9	14.6
n	-	-	-	-	56.6	49.6	-	-	45.2	67.7
DROUGHTm	26.3	27.5	15.2	10.7	26.5	18.9	-	-	29.9	45.3
FLOODEDm	12.3	10.2	29.2	11.7	47.0	53.3	-	-	16.6	25.4
a	11.7	10.0	-	-	-	-	-	-	20.4	26.6
FLD-NORm	-	-	24.7	13.5	-	-	-	-	-	-
a	18.9	10.3	-	-	-	-	-	-	23.0	15.0
r	17.7	14.1	-	-	-	-	-	-	-	-
FLD-DRTm	8.7	29.6	-	-	-	-	-	-	25.4	22.1
r	12.8	20.5	-	-	-	-	-	-	53.0	21.8
FL-D-FLm	-	-	1.0	19.4	-	-	2.8	2.4	-	-
a	95.6	15.2	-	-	-	-	-	-	-	-
r	73.6	13.8	15.1	34.6	-	-	-	-	-	-
DRT-NORn	-	-	-	-	-	-	-	-	24.0	16.7
DRT-FLDm	-	-	-	-	-	-	-	-	8.8	9.5
a	-	-	-	-	-	-	-	-	14.5	13.9

[1]. for the length of each stress see Table 4.1, except for ***E.citriodora*** (Brazil) which is on page 93.
These data are separately shown in figs.4.4; 4.5; 4.6; 4.7 and 4.8.

Table 4.9a: Headspace ethanol production after 24 hours of anoxia (μmol.gfw^{-1}.day^{-1}) of distinct root segments of ***E.camaldulensis*** under different soil water regimes. In multiple stress treatments the production was measured at the end of the final stress[1].

SOIL SATURATION	root segment(cm)	headspace ethanol
CONTROL	0- 5	-
	5-10	82.75
FLOODED	main root	17.64
	adv. 0- 5	158.30
	adv. 5-10	91.20
DROUGHT	0- 5	100.39
	5-10	71.08
	10-15	78.01
FLOODING-NORMAL	adv. 0- 5	6.74
	adv. 5-10	0.00
	adv.10-15	17.88
	reg. 0- 5	73.98
	reg. 5-10	0.00
FLOOD-DROUGHT-FL.	adv. 0- 5	66.81
	adv. 5-10	38.06
	reg. 5-10	29.50
	reg.10-15	32.86

[1]. for the length of each stress see Table 4.1.
Table 4.13a shows the averages per treatment and per type of root, which were used in the drawing of figures.

Table 4.10a: Headspace ethanol production after 24 hours of anoxia (μmol.gfw^{-1}.day^{-1}) of distinct root segments of ***E.citriodora*** (St.Andrews) and ***E.regnans*** under different soil water regimes. In multiple stress treatments the production was measured at the end of the final stress[1].

SOIL SATURATION	root segment(cm)	headspace ethanol
E.regnans	0- 5	126.77
CONTROL	5-10	134.44
	10-15	104.96
E.citriodora St.Andrews	0- 5	1.71
CONTROL	5-10	0.00
	10-15	3.26
FLOODED	5-10	23.78
	10-15	17.24
FLOODING-NORMAL	0- 5	0.00
	5-10	1.39
	10-15	6.58
FLOOD-DROUGHT-FLOOD	reg. 0- 5	25.15
	reg. 5-10	17.09
	main	7.27

[1]. for the length of each stress see Table 4.1.
The data for ***E.citriodora*** are displayed in fig.4.13.
Table 4.13a shows the averages per treatment and per type of root.

Table 4.11a: Headspace ethanol production after 24 hours of anoxia (μ mol.gfw^{-1}.day^{-1}) of distinct root segments of ***E.pellita*** under different soil water regimes. In multiple stress treatments the production was measured at the end of the final stress[1].

SOIL SATURATION	root segment(cm)	headspace ethanol
CONTROL	0- 5	6.44
	5-10	32.67
	10-15	41.86
	reg.	74.35
FLOODED	adv. 0- 5	150.17
	adv. 5-10	45.21
	adv.10-15	25.12
DROUGHT	0- 5	30.94
	5-10	2.61
	10-15	102.68
FLOODING-NORMAL	adv. 0- 5	64.07
	adv. 5-10	37.05
	adv.10-15	36.80
FLOODING-DROUGHT	reg. 0- 5	28.62
	reg. 5-10	6.08
DROUGHT-NORMAL	new 5-10	36.84
DROUGHT-FLOODED	0-10	29.34
	adv. 0-10	101.91

[1]. for the length of each stress see Table 4.1.
Table 4.13a shows the averages per treatment and per type of root, which were used in the drawing of figures.

Table 4.12a: Headspace ethanol production after 24 hour-anoxia and 24 hour-air (μmol.gfw^{-1}.day^{-1}) of distinct root segments of ***E.citriodora*** (Brazil) under different soil water regimes.

SOIL SATURATION	root segment(cm)	headspace ethanol air	anoxia
CONTROL	0-10	-	75.45
	10-20	-	31.05
	new	-	76.67
FLOODED	0-10	-	30.02
	10-20	-	8.86
DROUGHT (severely injured)[2]	0-10	617.45	92.04
	10-20	442.50	89.26
DROUGHT (moderately injured)	0-10	319.93	171.12
	10-20	441.23	139.46

1. the length of each treatment was 4 weeks.
2. severely injured plants had reached the wilting point by the time of the measurements, differently from the moderately injured which were only showing signs of leaf desiccation.

Table 4.13 shows the averages per treatment and per type of root.

These results are displayed in Chapter Five, figs.5.1 and 5.3.

Table 4.13a: Mean headspace ethanol production (μmol.gfw^{-1} day^{-1}) of different root segments of main (m), adventitious (a), regenerating (r) and newly formed (n) roots of five ***Eucalyptus*** species, at distinct soil water regimes. Production measured after subjecting the excised roots of the treated plants to 24 hours of anoxia (and also after 24 hours of air in the case of ***E.citriodora***, Brazil). In multiple stress treatments, the measurements were done at the end of the final stress[1] (n ranged from 1 to 3).

SPECIES / SOIL H_2O		CAMALD. anox	CITRIOD.SA anox	CITRIOD.BR. air	CITRIOD.BR. anox	REGNANS anox	PELLITA anox
CONTROL	m	82.75	2.5	-	53.3	122.1	27.0
	n	-	-	-	76.7	-	-
	r	-	-	-	-	-	74.4
DROUGHT	m	83.2	-	455.3[2]	123.0[2]	-	45.4
FLOODED	m	17.6	20.5	-	19.4	-	-
	a	124.8	-	-	-	-	73.5
FLD-NOR	m	-	2.6	-	-	-	-
	a	8.2	-	-	-	-	46.0
	r	37.0	-	-	-	-	-
FLD-DRT	r	-	-	-	-	-	17.4
FL-D-FL	m	-	7.3	-	-	-	-
	a	52.4	-	-	-	-	-
	r	31.2	21.1	-	-	-	-
DRT-NOR	n	-	-	-	-	-	36.8
DRT-FLD	m	-	-	-	-	-	29.3
	a	-	-	-	-	-	101.9

1. for the length of each stress see Table 4.1, except for ***E.citriodora*** (Brazil) which is shown in page 93.
2. average of moderately and severely injured plants.

These data are separately displayed in figs.4.9; 4.10; 4.11 and 4.12.

* CONCLUSION *

The environmental factors examined in this Chapter clearly influence plant response to flooding or anoxia. The broad classification of species as tolerant or intolerant to oxygen stress can be misleading if it disregards environmental nuances. The following points are worth emphasizing:

CHAPTER THREE: THE EFFECTS OF WASHING ON SEEDLING POST-ANOXIC SURVIVAL.

1) Washing treatments only enhanced post-anoxic survival of barley seedlings when done with a very precise combination of additional factors, namely: low ambient temperature (5°C); low frequency of washing (only once, before return of the seedlings to air); and the use of a calcium or, surprisingly, an acidic solution, rather than distilled water only. However, this combination of factors enhanced survival of seedlings that had coleoptile already formed at the moment they were exposed to anoxia, and was, conversely, deleterious to seedlings in the earlier germination stage of radicle protrusion.

2) Whenever washing enhanced survival, it did not appear to be through the removal of potentially toxic volatiles. The positive responses in this cases could be related to the protection offered to the cells' plasmamembrane integrity, probably through the addition of external calcium. However, more investigation is necessary to establish the degree of importance of ethanol removal and that of restauration of membrane integrity by washing in enhancing plant post-anoxic survival. From the present results it can be said, however, that if ethanol has a role to play in reducing barley post-

anoxic viability, this occurs when the plant is returned to air; and that the possible role of calcium in protecting the plasmamembrane against leakage is impaired when washing is conducted excessively, probably due to irreversible physical damage.

CHAPTER FOUR: ALTERNATION OF STRESSES AFFECTING *Eucalyptus* ANOXIA-TOLERANCE.

The pilot experiments conducted in this Chapter, despite the reduced number of replications, allowed the preliminary observation of some trends regarding the ***Eucalyptus*** species used, which deserve future attention:

1) A previous stress tended to affect the responses of a determined species to a subsequent stress. The nature of these responses varied greatly according to the species involved. For instance the flood-tolerant and drought-tolerant species ***E.camaldulensis*** and ***E.pellita***, showed symptoms of injury when submitted to a combination of flooding followed by drought. ***E.regnans***, sensitive to both flooding and drought, showed a surprising increase in the length of flooding survival (at least ca. 3-fold) when previously subjected to drought. The possible reasons for this latter situation are examined in the next Chapter.

2) No single pattern was observed in terms of metabolism following a single or multiple stress, varying again according to the species involved. For ***E.camaldulensis*** and ***E.pellita***, stress survival often seemed to be related to their capacity to form adventitious and regenerating roots. For ***E.citriodora*** (St.Andrews), it may possibly be linked to reduced metabolism. Again, no single pattern was observed in terms of anaerobic metabolism following a single or multiple stress. Expectedly, in some cases, certain combination of

stresses appeared to affect negatively the metabolic responses to a subsequent short-term anoxia (Pasteur effect and high ethanol production). However, more surprisingly, in particular situations a previous stress alternation allowed roots to avoid Pasteur effect and increased ethanol production under anoxia, as found for ***E.camaldulensis*** (flood-drought-flood) and ***E.pellita*** (flood-drought).

SECTION III

CASE STUDIES

* FOREWORD *

The present Section consists of two case studies which provide a basis for exploring a new theoretical approach to the study of plant stress ecophysiology.

CHAPTER FIVE presents and discusses the curious evidence that roots of several plant species subjected to drought produce high amounts of ethanol, often coinciding with a contraction in the root diameter. The results led to the formulation of a hypothesis of drought-induced hypoxia, which is debated in comparison with some of the ecophysiological work produced in recent years.

CHAPTER SIX investigates physical and metabolic responses of ***Enterolobium contortisiliquum*** to flooding and drought. The species is among the few woody species to appear in both dry and periodically waterlogged soils in Brazilian neotropical savannas (*cerrados*).

* CHAPTER FIVE *

A HYPOTHESIS OF DROUGHT-INDUCED HYPOXIA IN ROOT TISSUES AND ITS IMPLICATIONS

1) INTRODUCTION

Drought is undoubtedly a synonym for water deficiency, and research on the physiology of drought has always described plant symptoms and responses to drought as a function of lack or insufficiency of water **(Bradford & Hsiao, 1982; Crawford, 1989; Kozlowski, 1976; Schulze, 1986)**. Water stress is known to affect many physiological and developmental processes, including cell division, cell expansion and primordium development **(Quarrie & Jones, 1977)**. However, the present work claims that there is more to drought than water stress only. This Chapter raises the hypothesis that drought causes hypoxia in root tissues, by discussing results obtained with several plant species and relating recent findings in literature with this phenomenon.

Irrespective of the discussion of whether toxicity to plant tissues is caused by ethanol *per se* or its transformation to acetaldehyde **(Crawford, 1989; Jackson, Herman & Goodenough, 1982; Perata & Alpi, 1991)**, ethanol accumulation provides a useful indication of the level of oxygen stress in plant tissues **(Crawford, 1989; Kimmerer & Stringer, 1988)**. Root ethanol production was used as a parameter to assess root hypoxia under drought for several plant species including trees and agricultural crops.

Faiz & Weatherley (1982) working with root contraction in transpiring plants, showed that considerable shrinkage of maize roots occurs when water stress is increased by raising the transpiration rate of the leaves. It would therefore appear that soil drought, by inducing plant water stress,

could cause root shrinkage. It is here proposed that this tissue contraction could occur to such an extent that the root permeability to gas exchange and absorption of oxygen would be severely reduced, thus causing hypoxia. This Chapter, by combining investigation on root ethanol content, root shrinkage and root lignification under drought, proposes the hypothesis that roots during drought are physically impeded to absorb oxygen , which consequently leads to hypoxia in the tissues. This hypothesis is presented and related to other findings in literature. The possible consequences of this phenomenon are examined, particularly an indication that drought may acclimatise plants to subsequent flooding. Additionally, some of the missing links necessary to conclusively confirm or reject this proposed hypothesis, are debated, for instance, the controversies around ethanol production as a stress signal, and the possible role of drought-induced abscisic acid.

2) THE PRELIMINARY INVESTIGATIONS

Enterolobium contortisiliquum **(Vell.) Morong** is a species present in the *cerrados* (neotropical savanna) of Central Brazil and appears both in dry soils and in the periodically flooded soils of the gallery forests **(Joly, 1990;** see detailed discussion about distribution of this species in Chapter Six**).** ***Eucalyptus citriodora*** **Hook** is a species that is not particular in soil requirements, growing well in any soil **(Penfold & Willis, 1961).** The Brazilian provenance used, as seen in Chapter Four, seemed particularly sensitive to both flooding and drought.

In a pilot experiment, ***Enterolobium contortisiliquum*** and ***Eucalyptus citriodora*** six-month old saplings, grown in transparent acrylic tubes kept in light-proof boxes (figs. 0.1 and 4.0) and pots respectively, were submitted either to

flooding or drought. ***Enterolobium contortisiliquum*** was treated for 8 weeks in each case and ***Eucalyptus citriodora*** (Brazil) for 4 weeks. Submission to flooding and drought took place as described in the Material and Methods section. Drought was monitored by measuring percentage of field capacity according to the method described by **Nikolskii (1964)**. Usually, it took a week for a soil to dry down to 35% of its field capacity (see previous Chapter, fig.4.1). Control plants were regularly watered. The experiments were carried out in a greenhouse at 20°C and 12 hours of light per day (220-270 $\mu mol.m^{-2}.s^{-1}$).

Headspace ethanol production of different root segments was measured. In the case of ***Enterolobium contortisiliquum***, three plants were used per treatment, whereas one ***Eucalyptus citriodora*** (Brazil) was used per treatment (see Chapter Four). After the treatments, roots were excised and submitted to 24-hour anoxia (and to 24-hour air in the case of ***E.citriodora***), at 20°C, in 30 ml glass phials (75 mm depth x 24 mm diameter) closed with serum cap stoppers (*Suba Seal*) with moist filter paper, which was followed by the headspace ethanol measurements (see Material and Methods section). For ***Enterolobium contortisiliquum***, three different 5 cm segments of the main roots from the tip upwards, and regenerating roots were analysed. Each phial used to examine a particular root segment, contained segment samples from each of three distinct roots per plant. Headspace ethanol production was a function of the fresh weight of the sample used. For each segment sampled of the main roots of this species, the fresh weight of the sample often ranged from 100 to 200 mg. For the regenerating root samples the fresh weight ranged from 300 to 500 mg. In the case of ***Eucalyptus citriodora***, two 10 cm segments from the tip upwards were analysed, and each phial also contained samples from three distinct roots per plant. For this species, each sample per root segment showed a fresh weight ranging from 50 to 150

mg. The variation in the amount of root segment sampled per plant responded to availability of the material under a determined treatment.

Figs.5.1 and 5.2 show that the roots of both species produced high amounts of ethanol per gram fresh weight of root under anoxia following drought. The root tips of ***Enterolobium contortisiliquum*** (0-5 and 5-10 cm segments) produced more than twice the amount of anaerobically produced ethanol by both control and flooding treatment. The upper root segment (10-15 cm) produced slightly inferior amounts than both control (ca. 1.2 times) and flooding treatment (ca. 1.5 times). ***Eucalyptus citriodora*** produced ca. 3 times more ethanol after 24-hour anoxia under moderate drought than the control, and at least 5 times more than the flooded treatment. After a more severe drought (plant reaching the wilting point) the result was a less pronounced production of ethanol, although still higher than the control and flooded plants. Conversely, when after drought the roots were not subjected to anoxia, the ethanol produced during 24-hour aerobic incubation was higher for the roots under severe drought (fig.5.3). Experiments with two other eucalypt species, ***E.camaldulensis*** and ***E.pellita***, also showed production of ethanol after 24-hour anoxia following drought matching the production following flooding for a same type of root (see fig.4.10, Chapter Four).

From these preliminary results, two trends were observed. First, drought seemed to stimulate ethanol production under anoxia. Secondly, the experiments with ***Eucalyptus citriodora***, although lacking controls for comparison, showed an indication that drought by itself could induce high ethanol production (fig.5.3). Although this latter observation was still rather fragile at this stage, it was decided to pursue this line of investigation, by examining

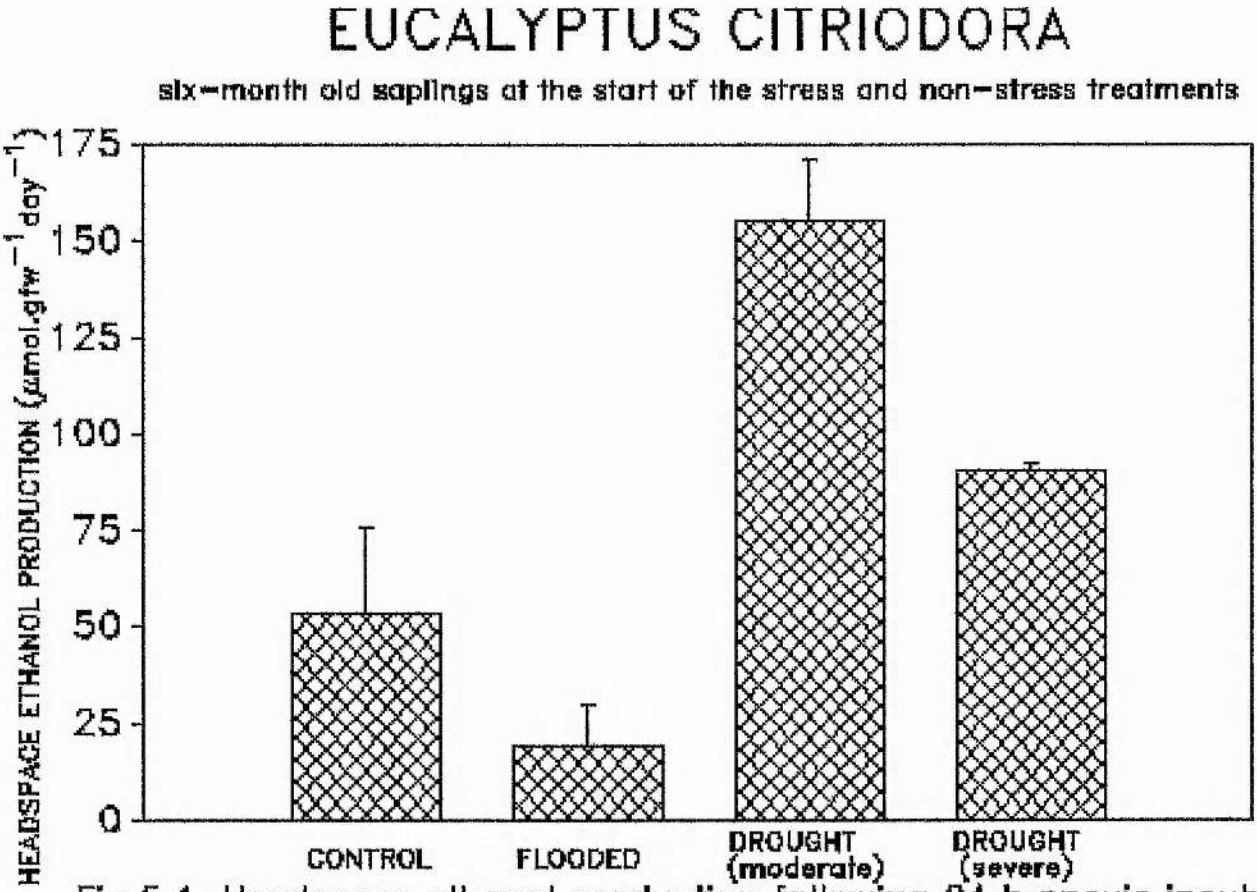

Fig.5.1. Headspace ethanol production following 24 h anoxic incubation of roots of E.citriodora (Brazil) previously subject to 4 weeks of flooding, drought or no stress. One plant per treatment; no. of samples per plant (n)=2. Bars show standard error. Data are displayed in the annexe of Chapter Four, Table 4.12a.

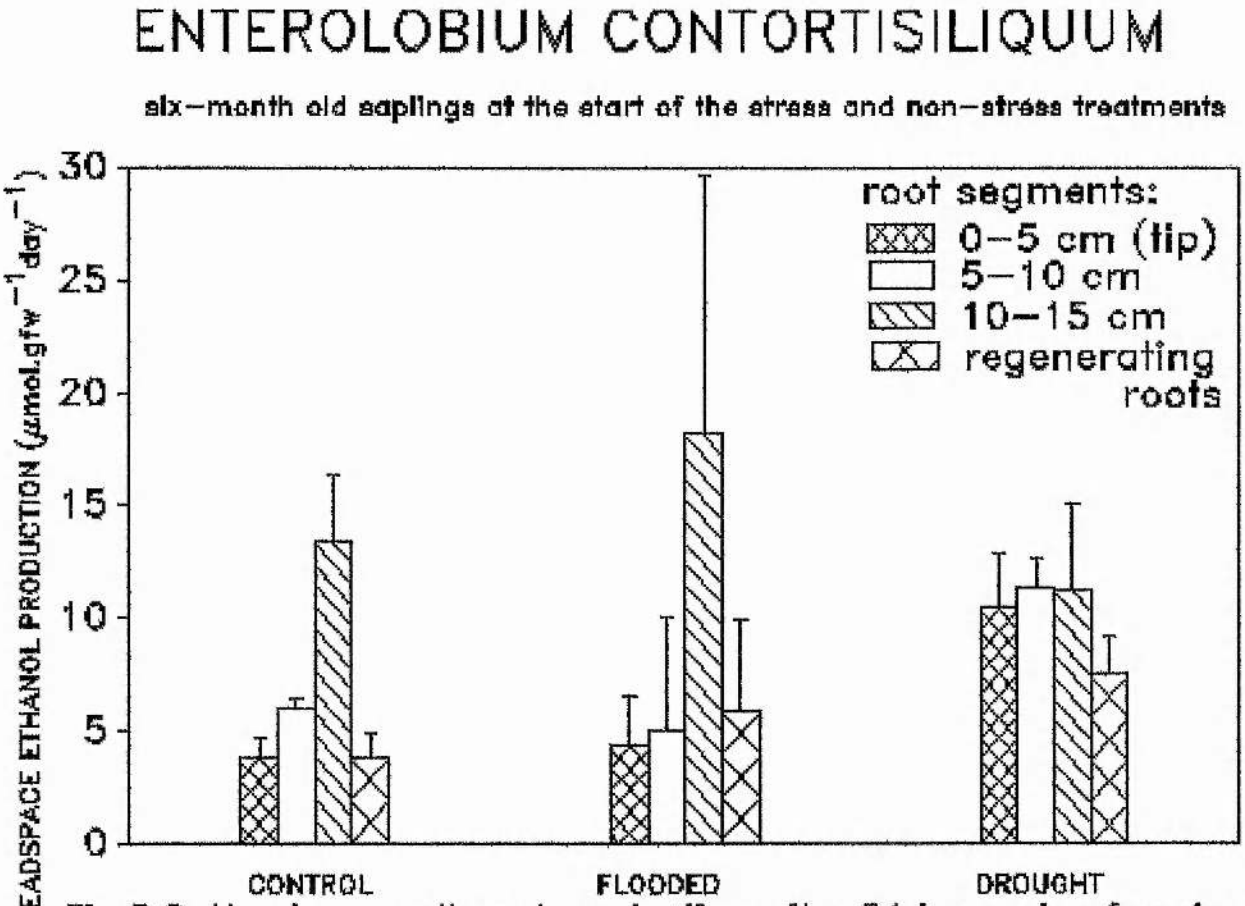

Fig.5.2. Headspace ethanol production after 24 h anoxia of roots of E. contortisiliquum previously subjected to 8 weeks of flooding, drought, or no stress. Three plants per treatment. Bars show standard errors.

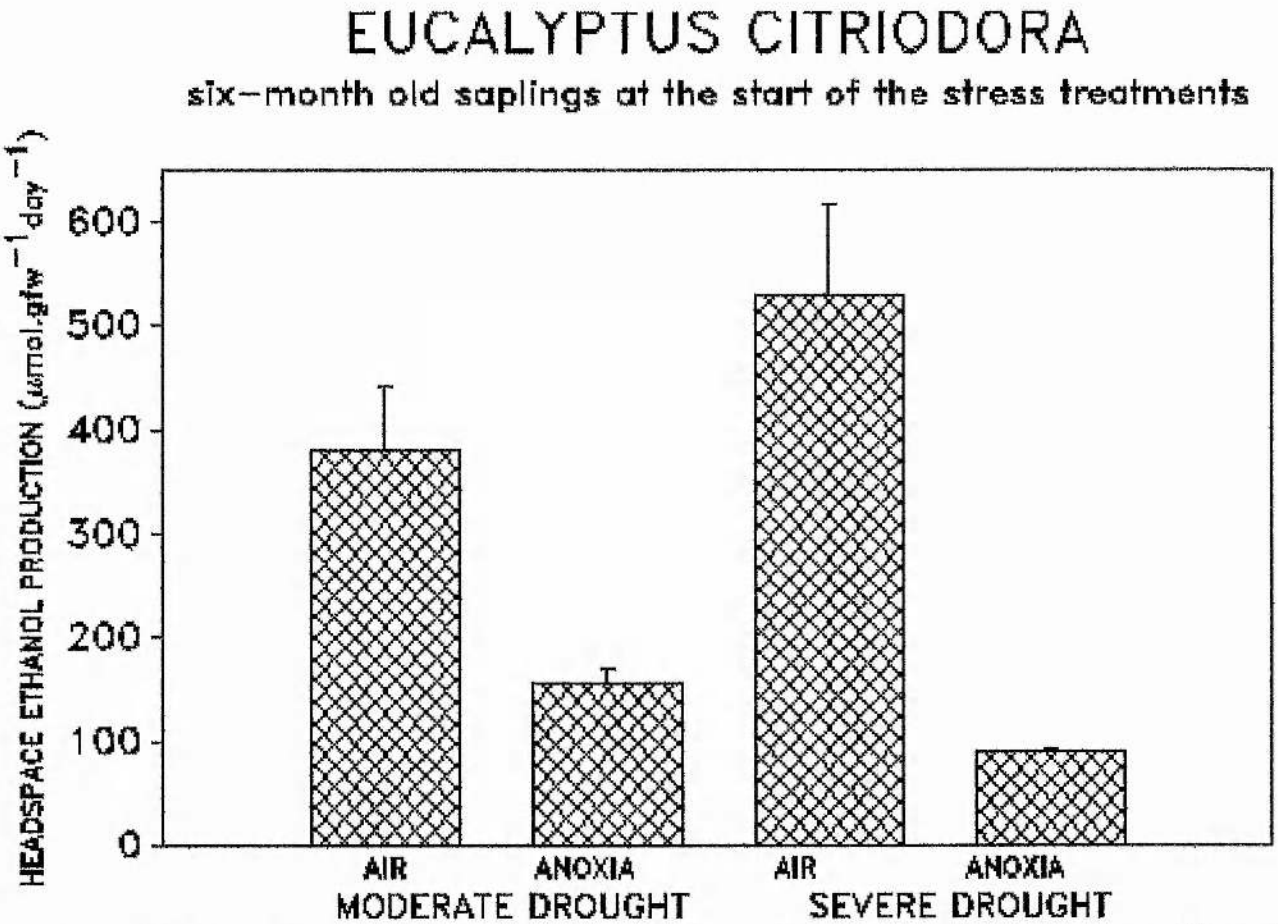

Fig.5.3. Headspace ethanol production of roots of E.citriodora (Brazil) incubated for 24 h under anoxia or 24 h in air, following 4 weeks of drought. One plant per treatment; no. of samples per plant (n)=2. Bars show standard error. Data are displayed in the annexe of Chapter Four, Table 4.12a.

ethanol content in roots of several agricultural crop species under drought.

3) THE DROUGHT-INDUCED HYPOXIA HYPOTHESIS

3.1 *Drought-induced increase in root ethanol concentration*

In order to simultaneously reach a more significant number of replicates and a wider range of plant species, ethanol content was measured for roots of agricultural crop species rather than tree species, for the obvious logistic facilities derived from the use of smaller plants. Five-week-old seedlings of chickpea (***Cicer arietinum*** **L.**), carrot (***Daucus carota*** **L.**), french bean (***Phaseolus vulgaris*** **L.**), barley (***Hordeum vulgare*** **L.**) and maize (***Zea mays*** **L.**) were grown from seeds in pots (15 cm depth x 15 cm larger diameter on the top x 11 cm smaller diameter on the bottom part of the pot) filled with the same mixture as the trees above, and under the same temperature and light conditions (see also the Material and Methods section). For one species, usually four plants were grown per pot. Within a same pot, seedlings would be subjected to drought or to non-stressed conditions. The drought treatment was applied by not watering the seedlings, and it took ca. two weeks for wilting to start, which was the point where roots were excised for the ethanol analysis. Control seedlings were regularly watered.

The ethanol content of 1 cm root tips of the agricultural crop species was measured by extracting the ethanol from the tissues and proceeding as in the Material and Methods section. Each control or treated plant was sampled by collecting a known amount in weight of 1 cm root tips (see legend in Table 5.1). The root material, after excision was gently cleaned to remove soil particles.

The root tips of chickpea, carrot and french bean produced significantly higher amounts of ethanol under drought than under the controlled non-stressed conditions (Table 5.1 and 5.2). In barley however, there was no significant difference between root tip ethanol production of control and drought-treated plants, and in maize the ethanol concentration was significantly higher in the roots of the control plants than in the drought-treated plants (Table 5.1).

Table 5.1. Concentration of ethanol in root tips of control and drought-treated seedlings (wilting point) of five agricultural crop species (μmol.gfw^{-1}).

SPECIES	CONTROL (n)	DROUGHT (n)	
Cicer arietinum	0.74 ± 0.02 (10)	1.41 ± 0.10 (10)	*
Daucus carota	1.55 ± 0.27 (10)	3.09 ± 0.40 (09)	*
Phaseolus vulgaris	0.75 ± 0.18 (08)	3.76 ± 1.19 (07)	*
Hordeum vulgare	0.71 ± 0.06 (05)	3.37 ± 1.50 (05)	ns
Zea mays	4.48 ± 0.51 (09)	1.86 ± 0.31 (09)	*

Statistics: * = significant difference between controls and drought treatment. T-test: $p<0.001$ (C.a.; Z.m.); $p<0.01$ (D.c.); $p<0.05$ (P.v.).

n = no. of plants sampled. Sampling consisted of harvesting several 1 cm root tips. The fresh weight (mg; mean±stdev) of the root samples per treatment were:

	control	drought
Cicer arietinum	305.0 ± 33.0	150.0 ± 26.0
Daucus carota	150.0 ± 56.0	70.0 ± 15.0
Phaseolus vulgaris	592.0 ± 33.0	337.0 ± 29.0
Hordeum vulgare	452.0 ± 98.0	117.0 ± 42.0
Zea mays	134.0 ± 65.0	164.0 ± 43.0

3.2 Drought-induced root shrinkage

Roots from four of the five agricultural crop species above (***Zea mays*** excluded) were harvested and gently cleaned, before being quickly immersed in liquid parafin to avoid water loss and to remove any soil particle still adhering to the roots. The apical region of the roots (1 cm) was excised when the shoots of the drought-treated plants were beginning to wilt. The diameters of individual root segments were measured by using a microscope equipped with a micrometer. The root segments were then submerged in distilled water for 4 hours before determining the turgid diameters, which allowed the estimation of root shrinkage in terms of percentage of the root diameter when root was fully turgid. It is important to mention that the plants used for the shrinkage experiments were not the same as the ones used for the ethanol experiments. These plants were also five-week old seedlings. The number of root tips tested for shrinkage per plant per species ranged from 2 to 4 and the number of plants tested ranged from 5 to 8 (Table 5.2).

The results in Table 5.2 a and b show that root shrinkage under drought was more marked for chickpea and barley. Barley, however, did not show significant increase in ethanol content under drought. The results obtained for ***Daucus carota*** and ***Phaseolus vulgaris*** were less clear. Although the control seedlings of carrot showed a two-fold higher root tip diameter than the drought-treated seedlings, a subsequent submergence of the roots in water for four hours revealed that the difference between the percentage of the fully turgid diameter for control roots was similar to the drought-treated ones. For french beans the inverse situation occurred: the root diameter of control and drought-treated root tips was similar, however the percentage of the fully turgid diameter was ca. 10% higher for the control plants.

Table 5.2. Estimation of contraction of root tip (the apical 1 cm) diameter of control and drought-treated (wilting point) seedlings of agricultural crop species.

a) root diameter (mm, mean ± stdev) measured immediately after excision.

SPECIES	CONTROL (N; n)	DROUGHT (N; n)
Cicer arietinum	0.61 ± 0.09 (16;8)	0.33 ± 0.05 (16;8)
Daucus carota	0.48 ± 0.12 (14;7)	0.21 ± 0.06 (14;7)
Phaseolus vulgaris	0.31 ± 0.04 (32;8)	0.29 ± 0.04 (28;7)
Hordeum vulgare	0.32 ± 0.04 (20;5)	0.19 ± 0.04 (20;5)

N = no. of roots tested.
n = no. of plants used to harvest the above roots.

b) percentage of the diameter of fully turgid roots, after submersion in water for 4 hours, of control and drought-treated root diameter prior to submersion.

SPECIES	CONTROL	N	n	DROUGHT	N	n
Cicer arietinum	91.7%	16	8	67.8%	16	8
Daucus carota	92.9%	14	7	88.6%	14	7
Phaseolus vulgaris	79.7%	32	8	69.9%	28	7
Hordeum vulgare	82.5%	20	5	65.8%	20	5

N = no. of roots tested.
n = no. of plants used to harvest the above roots.

3.3 Drought-induced root lignification

For root tips of french bean seedlings, in addition to the root shrinkage measurements, the degree of tissue lignification was estimated by using a staining method for five control plants and five drought-treated plants (a total of ten 1 cm root tips per treatment). In this experiment, rather than waiting until the wilting symptoms started to appear, as done for the ethanol and shrinkage experiments, roots were excised after 72 hours of drought. The staining method described by **McLean and Cook (1941)** was used. Phloroglucin was dissolved in 95% alcohol and concentrated hydrochloric acid was added gradually until the solution began to precipitate. Five root sections were placed in a Petri dish containing the above solution and those with lignified tissues stained red. The test allowed the visual observation that, invariably, the root tip cells of drought-treated french bean were highly lignified, including the epidermal layers. The control plants, by contrast, showed only a minor lignification in the vascular bundle region.

3.4 *Drought-induced hypoxia*

Based on the results displayed above, a hypothesis is proposed that drought may induce hypoxia in root tissues of some plant species. The increase in ethanol content of root tissues in 3 out of 5 species submitted to drought, in comparison with control plants, may be linked with a possible insufficiency of oxygen availability to the these same root tissues. One of the processes that could gradually allow the oxygen stress to take place, may be that of root shrinkage. The contraction of the root tissues caused by the drought-induced water deficiency, could possibly reduce their permeability to external oxygen, creating a hypoxic condition for the root tissues, and in particular the root tips. Tissue lignification under drought is possibly an

additional factor reducing root tip gas exchange. Lignification, however, was only tested for one of the species used and shrinkage was not directly confirmed for all species used. These possible exceptions are discussed later in the item 3.5.3.

In one of the few works relating drought to oxygen stress, **Nir, Poljakoff-Mayber and Klein (1970)** found that reduction in the water content of maize roots was accompanied by a reduction in oxygen uptake. They related the inability of roots to consume oxygen to mitochondrial damage. Thus, it is likely that roots during drought may be either physically impeded to absorb oxygen (by root shrinkage and lignification) or this impediment could occur at the cellular level through mitochondrial damage. It is also plausible to say that, in some cases, the two phenomena could occur simultaneously.

Although mitochondrial damage was not currently tested, it seems reasonable to propose that drought may indirectly induce hypoxia by possibly causing root shrinkage, root lignification and/or mitochondrial damage, which are factors likely to reduce the ability of the tissues of some plant species to capture free-oxygen.

3.5 The questions facing the drought-induced hypoxia hypothesis

It is proposed in 3.4 that drought may indirectly induce hypoxia in root tissues of some plant species by creating a physical or metabolic barrier preventing a satisfactory tissue oxygen uptake. Three main points concerning this hypothesis are largely debatable, and it will now be attempted to examine them.

3.5.1 *The controversy around ethanol as a stress signal*

The present hypothesis is largely based on the concept, mentioned in the "Introduction" that ethanol production provides a useful indication of the level of oxygen deficiency in plant tissues. Although this concept is probably true in face of the numerous evidences confirming it **(Crawford, 1978; Kimmerer & Stringer, 1988)**, it is questionable whether oxygen shortage is the **only** inducer of ethanol production. **Kimmerer & Kozlowski (1982)** demonstrate that water deficit, freezing, ozone exposure and SO_2 induce production of ethanol, irrespective of whether the environment is hypoxic or not. They admit, however, that a substantial alteration of respiratory metabolism occurs in the stressed plants. As argued before, it may be claimed that these authors overlooked the possibility, in the case of drought, of water deficit causing oxygen deficiency in the root tissue itself and not in the environment around the roots. In relation to freezing, it has been shown that ice encasement induces anaerobic stress, and that a low temperature flooding pretreatment (hypoxia) increases tolerance to the subsequent more severe hypoxic or anoxic stress of ice encasement **(Andrews & Pomeroy, 1983; 1989)**. Due to lack of experimental data available, no relationship can be promptly claimed to exist between ozone exposure and hypoxia, or SO_2 and hypoxia. However, it appears that perhaps the most challenging question emerging from this debate is not that of whether oxygen deficiency is the only inducer of ethanol production, but rather that of whether any sort of stress that stimulates ethanol production, has an indirect component of impairing plant tissue oxygen uptake, as we propose to exist for drought. Answering both these questions would most certainly contribute, not only for a more comprehensive understanding of the issues discussed in this Chapter, but also for a step forward in

the understanding of the mechanisms involved in stress physiology.

3.5.2 *Similarities and differences between drought and flood-induced oxygen shortage*

If drought does induce hypoxia, why are responses such as adventitious root formation and stem hypertrophy not observed? ***Enterolobium contortisiliquum***, for instance, a tree species relatively tolerant to both stresses, shows lenticel hypertrophy under flooding but not under drought (see Chapter Six). ***Eucalyptus camaldulensis*** forms adventitious roots under flooding but not under drought (Chapter Four). This difference in response undoubtedly reflects a difference between the physiology of the two stresses, which is not denied by the drought-induced hypoxia hypothesis presented here. In the case of flooding, where the above responses are often observed, oxygen stress is possibly a primary effect: the environment lacks oxygen and the roots, therefore, cannot uptake the amounts necessary to perform aerobic respiration. The plant tissues under these circumstances are more likely to remain turgid, and adventitious roots and stem hypertrophy are more characteristic of turgid rather than flacid tissues **(Pereira & Kozlowski, 1977)**. In the case of drought, where these structures are not formed, oxygen stress, if the present hypothesis is correct, is an indirect effect of water deficiency: the environment lacks water and the plant suffers water loss through transpiration, which is possibly related to root shrinkage **(Faiz & Weatherley, 1982)**, lignification and mitochondrial damage, which altogether may restrict root oxygen absorption, although the soil is not necessarily hypoxic. Under these circumstances, however, the tissues are likely to be flacid, which possibly does not favour the formation of adventitious roots and stem hypertrophy. It would be interesting to know, however,

assuming that drought does cause hypoxia as proposed, whether tissue morphology and anatomy are the only factors involved in the different responses mentioned, or whether hormones and metabolism would also be a component factor.

In short, the answer proposed to the question posed at the beginning of this topic of discussion of, if drought is supposed to cause hypoxia, why does it not induce formation of adventitious roots and stem hypertrophy in plants as flooding does, is that hypoxia, when drought-induced, is a consequence of water deficiency, whereas in the case of flooding it is a direct consequence of shortage of free-oxygen in the environment. This may represent major differences in terms of morphological and anatomical responses (turgidity *vs.* flacidity of tissues) and possibly also in terms of hormonal balance and metabolism, which may account for the different responses to flooding and drought, although both could affect plant oxygen uptake. Injury of plants due to drought can only partially be attributed to oxygen stress. Water stress definitely gives a major contribution to drought injury.

It is likely, however, that the fact that roots under drought may produce large amounts of ethanol (irrespective of whether this is linked to hypoxia or not), will subject them to similar risks as those of flooded plants. One of these dangers is that of peroxidative damage on return to fully aerated conditions. The increase of plant sensitivity to peroxidative damage on return to air is due to the fact that, during anoxia, the enzymes that protect tissues against oxygen toxicity may suffer a reduction of activity. In this case, the oxidation of anaerobically accumulated metabolites such as ethanol, may generate potentially harmful products such as acetaldehyde, by the activity of catalase in the presence of hydrogen peroxide **(Crawford, 1989)**. If drought causes hypoxia, the same situation is

likely to occur, when the soil is re-hydrated and the root recovers its ability to assimilate oxygen.

Le Prince *et al.* (1990) showed that desiccation treatment resulted in peroxidative damage to lipids in germinating maize seeds. Desiccation during germination, they conclude, suppresses the activity of enzymes critical to the processing and protection from activated oxygen. Moreover, these authors say that protection from drought-induced damage in wheat and eleven other species, has been linked to the accumulation of the antioxidants tocopherol and glutathione. Therefore, it seems that desiccation, drought and anoxia share the common effect of causing peroxidative damage in plant tissues. The suggestion that drought may cause hypoxia at least in root tissues, might be the missing link between desiccation and peroxidative damage not found by Le Prince and colleagues. Desiccation or drought, by possibly inducing hypoxia and consequent impairment of antioxidants, could lead to peroxidative damage as germination progresses and conditions become more aerated.

3.5.3 *Universality of the hypothesis*

A third controversial aspect that emerged from the results obtained is that not all species showed a stimulation of ethanol production under drought. In the case of barley the root ethanol content under drought was not significantly higher than the controls. In the case of maize, even more unexpectedly, the controls showed significantly higher ethanol content than the drought-treated roots. Although it can be argued that in the case of barley the result could be possibly attributed to a smaller number of replicates, and in the case of maize the drought-treated roots may have lost their viability before the actual readings, the present evidences are not sufficient to claim that the proposed drought-induced hypoxia phenomenon is true for all plant

species. If interspecific difference exists in that sense, it would be interesting to know whether the factors involved in promoting them, would be linked to root morphology and/or metabolism. Additionally, if the present hypothesis is correct and some plant species are subjected to drought-induced hypoxia, it would be important to know the primary causes of this phenomenon. From the present data, there seems to be interspecific differences in terms of root shrinkage which, at this early stage in the investigations, already suggests that there could be more than one cause of drought-induced hypoxia.

4) AN INDICATION OF A POSSIBLE PROCESS OF DROUGHT ACCLIMATISATION TO FLOODING

4.1 The case of *Eucalyptus regnans*

Six-month-old saplings of ***Eucalyptus regnans*** **F.Muell.** were grown in tubes, as the other ***Eucalyptus*** species described in Chapter Four, and were subjected to different stresses and combination of stresses. Survival and shoot and root extension were monitored over a nine-week period, as described in Chapter Four. The removal of the stopper in the bottom end of the tubes allowed a quick drainage of the soil water when necessary. Monitoring of the drought conditions of the soils was done as described before in this Chapter.

This was again a pilot experiment, which details are described in Chapter Four. The results, although still very preliminary in their nature, allowed a new insight into the drought-hypoxia subject, which will be here discussed since it may stimulate future research in the subject. One week of flooding was enough to cause severe damages in the flood-sensitive ***E.regnans***, namely death of roots and wilting, drying and appearance of black spots on leaves, which

resulted in death shortly afterwards. Similarly, drought also killed roots and caused leaf wilting and necrosis, but in this case the plant survived, regenerating new roots and stopping leaf damage. Subjection to drought after one week of flooding was lethal to ***E.regnans***. The most astonishing results, however, came out of the treatment of drought followed by flooding. ***E.regnans*** subjected to flooding after 5 weeks of drought, survived at least ca. 3 times longer than when not pre-treated with drought (Table 4.1, Chapter Four). Moreover, a small amount of root and shoot growth, not observed in the other stress treatments, was also observed (fig.5.4) in flooded plant pre-treated with drought.

It has been shown that determined periods of hypoxic pre-treatment acclimatise roots of maize to subsequent anoxia **(Saglio, Drew & Pradet, 1988; Johnson, Cobb & Drew, 1989).** This fact could explain the unexpected result of a drought pre-treatment prolonging flooding survival of ***E.regnans***. Since pre-hypoxia prolongs anoxic survival, in the event of drought inducing hypoxia, it is likely that a pre-flooding drought treatment would prolong flooding survival. The drought pre-treatment in ***E.regnans*** could be progressively reducing the root ability to capture oxygen, establishing a gradual onset of hypoxia. When the plants are subsequently subjected to flood-induced anoxia, the roots are possibly acclimatised to low oxygen levels and, thus, able to survive longer. Which mechanisms allow this possible process of acclimatisation, however, is matter of some controversy, which will be examined in 4.3, after some other possible cases of acclimatisation are discussed in 4.2.

E.regnans is commercially planted in many tropical and subtropical countries. The results here discussed may stimulate research that could contribute to development of new forestry practices and planning concerning this species.

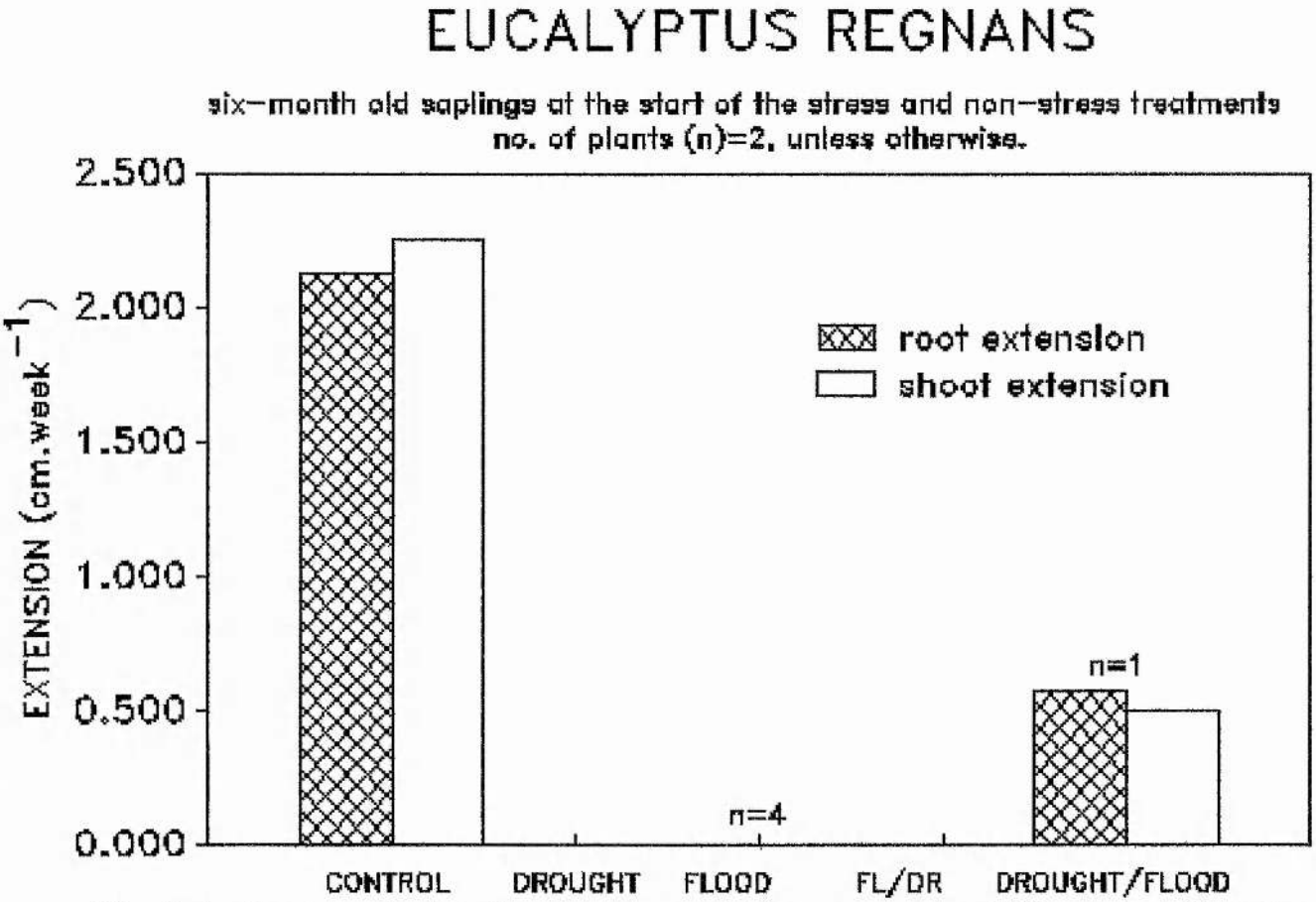

Fig.5.4. Root and shoot extension of E.regnans in different soil water regimes. Multiple stress treatments measured during the final stress. Root extension data were based on the weekly monitoring of 10-20 roots per plant. Length of stresses can be seen in Chapter Four, Table 4.1. The numerical data are in the annexe of that Chapter, Tables 4.2a and 4.3a.

4.2 *Other possible cases of hypoxic pre-treatment prolonging anoxic survival*

The hypothesis of hypoxia pre-treatment prolonging anoxia survival, and in the context of this Chapter, of drought prolonging flooding survival, finds some parallels in the literature. **Nobel (1990)** studying soil oxygen and carbon dioxide effects on the viability of roots of three desert succulents (***Agave deserti***, ***Ferocactus acanthodes*** and ***Opuntia ficus-indica***), found that survival was hardly affected by one month of anoxia (0% oxygen). Instead, cellular inhibitory effects were more rapidly detected at elevated carbon dioxide levels. He concludes that the general restriction of desert succulents to well-aerated soils is a restriction to low carbon dioxide levels in the soil gas phase. Nobel did not attempt to explain why these desert plants survived for so long in the absence of oxygen, a condition more common in completely distinct environments such as mangroves, marshes, *etc*. Since desert conditions can be immediately related to drought, the present hypothesis that drought causes hypoxia that, as a pre-treatment, prolongs anoxic survival, seems to be a possible explanation for the survival of desert roots in anaerobic conditions. Although the portion of the soil where these roots occur is well-aerated, root contraction caused by drought could impair oxygen absorption by the tissues.

Crawford (1989) explains the concept of natural anaerobiosis within impermeable seed coats. The early stages of germination, before rupture of the testa, have a demand for oxygen exceeding the rate of replacement, therefore submitting the embryo to a state of natural hypoxia. The results of Chapter One show that if anoxia is artificially prolonged during the period when the seeds are naturally anaerobic, chickpea survives longer than if anoxia is imposed later during an aerobic growth phase of germination.

Again, the pattern hypoxia prolonging anoxic survival seems plausible. **Reisman-Berman, Kigel & Rubin (1989)** however, suggest that hypoxia within the seed may cause the synthesis of inhibitors or may limit the oxidation of inhibitors already present in the seed, resulting in inhibition of germination. This is distinct from embryo acclimatisation to anoxia, although the final effect of favouring post-anoxic survival is the same.

4.3 *The questions facing the hypothesis of drought-acclimatisation to flooding*

The only evidence in favour of the argument that drought may acclimatise plants to subsequent flooding comes from data collected for only one plant of one ***Eucalyptus*** species, ***E.regnans***. This plant showed a minimum of ca. 3-fold increase in the length of flooding survival when previously subjected to 5-week drought. This lack of replicates, and also the fact that only one species was tested, are not the only aspects demanding further experimental investigation. Even if a given length of drought pretreatment improves subsequent flooding survival for a determined species or group of species, the process allowing such a phenomenon to take place would still be rather obscure. The question to be immediately posed would be how can this process of acclimatisation take place. Three possibilities seem to be plausible. Two of them, however, are directly related to the formation of anaerobic polypeptides, but they seem to differ about how these proteins are formed:

4.3.1 *Hypoxia-induced formation of anaerobic polypeptides*

Saglio, Drew & Pradet (1988) and **Johnson, Cobb & Drew (1989)** propose that the gradual decline in oxygen availability imposed by hypoxia, which is more likely to occur under natural conditions than the abrupt decline in energy

metabolism imposed by anoxia, allows for some species the induction of a set of stress proteins, the anaerobic polypeptides, which at times can aid survival. Such a process characterises acclimatisation and, assuming that the drought-induced hypoxia is correct, it would provide an explanation for the fact that drought may acclimatise plants to subsequent flooding: drought, by gradually inducing hypoxia, would allow the formation of anaerobic polypeptides which would possibly increase subsequent flood-tolerance.

4.3.2 *Hypoxia-induced effect on glycolytic activity*

As the onset of drought seems to be progressive, so is that of hypoxia under these conditions, always assuming that the hypothesis of drought inducing hypoxia is correct. This could provoke a gradual reduction in the roots' metabolic rate and a consequent reduction in the production of potentially toxic metabolites, which could possibly remain reduced when of the onset of anoxia due to flooding. The evidence to support this hypothesis is, however, rather controversial. Fig.5.3 shows that plants subjected to severe drought produced more ethanol than those subjected to moderate drought. However, when the roots were submitted to 1-hour anoxia after the drought treatments, the result was the opposite: those roots previously subjected to more severe drought produced lower ethanol. As the onset of hypoxia under drought seems to be progressive, plants under more severe drought would be in a more advanced stage of hypoxia and possibly more able to acclimatise rapidly to anoxia. This, however, cannot be promptly confirmed by the present results in view of the reduced replication and lack of controls for this particular experiment.

The opposite case, of drought-induced hypoxia stimulating glycolytic activity, appears to be more likely in view of the data presently collected. As seen for the tropical

trees, in most cases a pre-anoxia drought treatment did induce high ethanol production under the subsequent anoxia. The generation of metabolic energy in the absence of oxygen by glycolysis is likely to have a positive effect on survival. However, if ethanol is at all phytotoxic, irrespective of whether its toxicity is expressed during anoxia or in the post-anoxic phase, drought should have an even more negative effect on subsequent anoxic or post-anoxic survival, and the same should possibly be true in the case of a subsequent flooding. It is possible that, as claimed by **Andrews & Pomeroy (1989)** for plants under ice encasement, a fine balance could be achieved under the drought conditions described between these two opposing factors, *i.e.* generation of metabolic energy and production of the potentially toxic ethanol. This paradox could be solved with more detailed investigation and higher number of replicates, which could show, for precise conditions of length of stresses and species involved, whether a pre-flooding drought treatment may affect glycolytic activity during flooding and how this process would take place, and also whether or not the drought-induced ethanol production is relevant in terms of plant survival.

4.3.3. *Drought-induced abscisic acid formation and a possible consequent formation of anerobic polypeptides*

Hwang & Van Toai (1991) have shown that abscisic acid induces anaerobiosis tolerance in corn seedlings and that the induced tolerance was probably mediated by a significant increase in alcohol dehydrogenase enzyme (ADH) activity before anoxia. Additionally, they have shown that increase in anoxic survival due to treatment with abscisic acid was significantly higher than the increase in anoxic survival due to a hypoxic pretreatment (seedling soaked in water in an open tray). Since drought induces abscisic acid

production **(Hiron & Wright, 1973)**, it is possible that in doing so it also gradually increases ADH activity, which may prolong flood-survival.

This possibility, if true, does not rule out the present drought-induced hypoxia hypothesis. The main difference from the argument discussed in 4.3.1 is in terms of what is it that allows a pre-flooding drought treatment to acclimatise plants to the subsequent flooding. Here, it is argued that it does not need to be necessarily hypoxia which causes acclimatisation. Even if hypoxia takes place under drought, it could be the ABA formation stimulated by drought, irrespective of hypoxia, which induces the formation of the anaerobic proteins necessary for survival under the subsequent flooding.

These three explanations offered for the possible process of drought-acclimatisation to flooding do not rule out one another. However, even if these three aspects occur at the same time under drought; namely *i*) gradual onset of hypoxia allowing formation of anaerobic polypeptides prior to flood-induced anoxia, *ii*) gradual onset of hypoxia somehow affecting metabolic activity prior to flood-induced anoxia and *iii*) drought inducing ABA formation which mediates formation of anaerobic polypeptides prior to flood-induced anoxia; a hierarchy of importance could not possibly be established at the present stage.

5) POSSIBLE PRACTICAL APPLICATIONS

The hypothesis developed in this Chapter and its possible consequences are summarised in fig.5.5. If what is being proposed is correct, drought may induce hypoxia which is an additional burden to the plant under these circumstances, which however can turn out to be favourable if drought,

after a given amount of time, is followed by flooding. The deleterious effects of anoxia would in this case be minimised, since plant roots would possibly be acclimatised by the previous drought and/or hypoxia.

Succession of stresses, as mentioned previously, is a common feature of tropical areas where, within short periods in the middle of a dry season, a plant may experience both drought and flooding due to heavy tropical showers. Particularly for these areas, if the hypothesis above is confirmed as a fact, it could have important practical applications.

Plants cultivated in dry regions are commonly submitted to the practice of hardening, which basically consists of growing seedlings under moderate water stress. Supposedly, this practice enhances plant ability to withstand drought when planted in the field. Therefore, if the assumptions made here in terms of the possibility of drought acclimatising plants to subsequent flooding are true, it seems reasonable to expect that submission of seedlings in the nursery to controlled levels of drought could have a beneficial effect in growing these seedlings in areas subjected to periodical waterlogging, which would be of great importance for both forestry and agriculture.

Some of the issues raised here are currently being tested, particularly those related to acclimatisation and to the possibility of drought causing mitochondrial damage. Further research on this subject should possibly provide useful information to aid in the solution of some of the theoretical and practical problems concerning the effects of drought on plants. However, it seems that before the hypothesis here discussed is either confirmed or rejected, several theoretical aspects, which raised much controversy in the past two decades, need further understanding. For instance, *i*) does accelerated ethanol production necessarily

signalise oxygen stress? (see **Kimmerer & Kozlowski, 1982**, and discussion in this Chapter, 3.5.1); *ii*) is ethanol phytotoxic, in which circumstances, and when is this toxicity expressed? (see **Crawford 1967, 1989; Jackson, Herman & Goodenough, 1982; Perata & Alpi, 1991**; and discussion in Chapter Three); *iii*) do anaerobic proteins, like ADH, have a role to play in anoxia-tolerance? (see **Crawford, 1967; Hwang & Van Toai, 1991**).

The fact that in the present Chapter possibly more questions have been posed than answers were given, highlights both a practical and a theoretical necessity of further research involving the subjects discussed above.

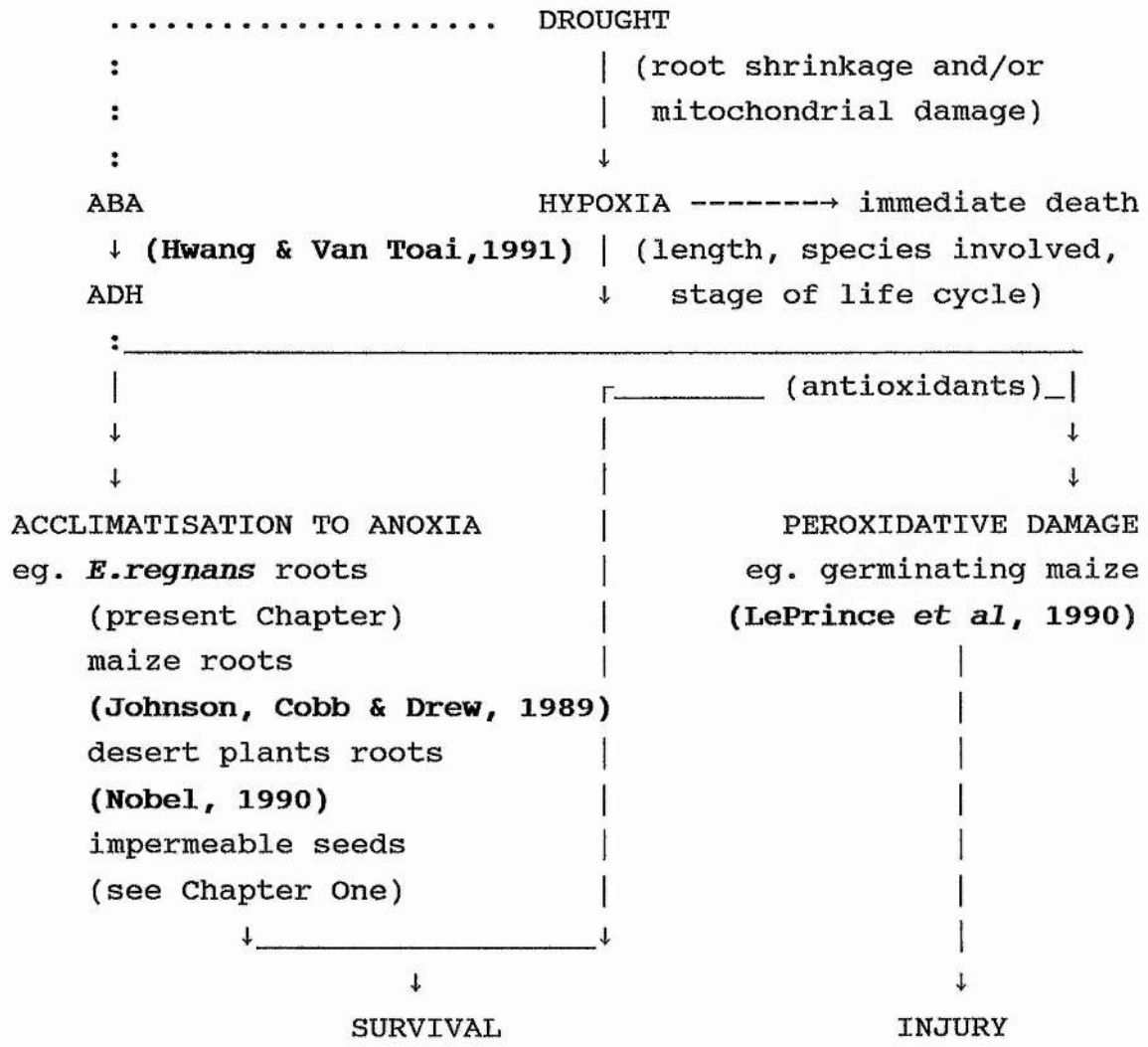

Fig.5.5. Drought-induced hypoxia hypothesis

* CHAPTER SIX *

PHYSICAL AND METABOLIC RESPONSES OF A *CERRADO* SPECIES OF *Enterolobium* TO FLOODING AND DROUGHT.

1) INTRODUCTION

The present Chapter examines the morphological and metabolic responses to flooding and drought of a *cerrado* (neotropical savanna) species of ***Enterolobium***, discussing whether or not these responses constitute adaptations to the stresses.

Cerrado is a semideciduous xeromorphic vegetation dominant in Central Brazil, covering ca. 23% of the whole country or 40% of the non-Amazonian part of Brazil. It is the second most important vegetation of that South American country. *Cerrado* has approximately 800 species of trees and large shrubs, but it is not so floristically diverse at the generic level **(Eiten, 1972; Furley & Ratter, 1988; Silberbauer-Gottsberger & Eiten, 1987).**

The unique appearance of *cerrado* is due to the thick bark, leaning and twisted trunks and open crowns that the trees often present. It shows a large variety of physiognomic vegetation types, ranging from closed forest-like forms to pure grassland **(Silberbauer-Gottsberger & Eiten, 1987).** *Cerrado* species normally live on poor and well-drained soils **(Eiten, 1972).** The tree species with higher tolerance to waterlogging form a distinct community on the margins of *cerrado* with wet *campo* (grassland) and on raised islands of ground in wet *campos* **(Furley & Ratter, 1988).** The distribution of the latter, according to those authors, forms a landscape known as *campo de murunduns*, consisting of an expanse of wet *campo* dotted with a regular pattern of raised earthmounds bearing *cerrado* trees, shrubs and often termitaria. Gallery forests (vegetation similar in

physiognomy and composition to the dense and tall tropical rain forests) follow the streams that cross the *cerrado* valleys and some of the species there present live on swampy soils. *Cerrado* either contacts the gallery forest directly or is separated from it by a strip of seasonally saturated grassy *campo* **(Silberbauer-Gottsberger & Eiten, 1987).**

It is probably due to the fact that the flood-prone areas in *cerrado sensu lato* are comparatively less representative of the vegetation as a whole than the vast areas periodically subjected to drought and/or fire, that few researchers have examined flood-tolerance in *cerrado* species, as carried out for instance by **Joly & Crawford (1982).** However, the few tree species that live on both typical dry *cerrado* and wet *campo de murunduns* and/or flood-prone gallery forests, are of enormous ecological interest for their ability to cope with distinct stresses and distinct types of competition. ***Enterolobium contortisiliquum*** is one of these cases and will be here examined.

2) TAXONOMIC CONTROVERSY

The seeds that gave origin to the nine trees used in the present work were collected in *cerrado* in the State of São Paulo, Brazil. The material was classified by the collectors as ***Enterolobium contortisiliquum.***

However, only three references were found in the literature mentioning the existence of this species in *cerrado* areas: **Joly (1990)** working with plant ecophysiology; **Pereira (1982)** investigating occurrence of potential ornamental plants in the rio São Bartolomeu basin in Central Brazil; and **Filgueiras & Pereira (1990)** who listed the species as present in Brasilia, although not mentioning in which type of vegetation. **Prance & Schaller (1982)** found ***Enterolobium***

contortisiliquum in dry arid areas of a *Chaco*-like vegetation type of the Brazilian *Pantanal*. Several floristic surveys of different physiognomic types of *cerrado* and in different regions in Brazil did not find this species **(Furley, Ratter & Gifford, 1988; Ratter *et al.*, 1988a, Ratter *et al.*, 1988b)**. Even in gallery forests the species was not found **(Gibbs, Leitao Fº & Abbott, 1980; Paula *et al.*, 1990)**. The species is found by most of these surveys in deciduous forests or in calcareous ground. Instead, **Ratter *et al.* (1988a)** found ***Enterolobium ellipticum*** in *cerrado* areas.

Burkart (1979) affirms that ***Enterolobium contortisiliquum*** can be found in Brazil (from the northeast, in the State of Ceará, to the southern-most State of Rio Grande do Sul), Uruguay, Argentina, Paraguay and Bolivia. Most of the work done with this species comes from the moist forests of southern Brazil and Argentina, and stresses its economic importance: light wood with multiple uses ranging from making furniture to boats; saponin-rich fruit and bark used as soap; forageous dried fruit and leaves; frequent use as ornamental tree; fast-growing heliophile potentially useful for reafforestation; and potentially useful on recovering degraded habitats for the quick decomposition of litter and root nitrogen-fixation **(Burkart, 1952; Finger & Minussi, 1980; Klein, 1972; Meyer, 1963; Reitz, Klein & Reis, 1983)**.

If all the works mentioned are talking about the same species - ***Enterolobium contortisiliquum*** - it can be said that it occurs, in higher or smaller frequency, almost everywhere in Brazil. However, it seems unlikely that all these works are talking about the same species. **Rizzini (1971)** studying vicariance said that ***Enterolobium ellipticum*** from *cerrado* originated from ***Enterolobium contortisiliquum*** from the Amazon and Atlantic Rain Forest. If that is the

case, it would be unlikely to find the latter in *cerrado* areas.

Lewis (1987) says that ***E.timbouva*** is a synonym of ***E.contortisiliquum*** and that ***E.tamboril*** is a synonym for ***E. timbouva* var.*canescens***. He suggests that possibly a new combination is required at a variety level, or else, ***E.tamboril* Mart.** can regain its original status as species, for its recognisable distinction from typical ***E.contortisiliquum***. The ***E.tamboril*** herbarium material examined by Lewis was collected by Martius in 1866 in waterlogged forest by the São Francisco river, Bahia, Brazil.

However, according to **Lewis (1987)**, the genus, that has from six to eight species in the West Indies, Central and South America, was at that time being revised by A.L.Mesquita (UTAM, Manaus, Brazil) and apparently still is. As there is considerable amount of controversy around the taxonomy of the genus at the moment, this thesis will call the material studied ***Enterolobium contortisiliquum* (Vell.) Morong** as it was identified in the occasion of seed harvest in the field (*cerrado* from São Paulo). The reader should however bear in mind the discussion made in the present topic for future reference.

3) FLOOD AND DROUGHT SURVIVAL: PHYSICAL ADAPTATIONS

3.1 *Growth and symptoms*

Enterolobium contortisiliquum, as suggested by **Joly (1990)**, occurs throughout the amplitude of the moist gradient determined by the fluctuations of the water-table in *cerrado* areas. This means that this species, unlike most of its *cerrado* counterparts, survives and reproduces in the dry

poor soils of *cerrado sensu strictu* as well as in the moister and/or flood-prone sites presented by some of the other physiognomic vegetation types of *cerrado sensu lato*.

The present investigation consisted of placing 9 six month-old *Enterolobium contortisiliquum* trees in transparent acrylic tubes (see fig.0.1 and 4.0), and submitting three of them to flooding, three to drought and three to normal watering regime during eight weeks. These treatments were carried out as described in the Material and Methods section. Root extension, shoot extension and stress-symptoms were monitored as for the ***Eucalyptus*** species (Chapter Four, pages 89 and 90).

Both flooding and drought impaired root and shoot extension. The controls showed an average shoot extension of 2.33 cm per plant per week and the root extension was of 4.14 cm per week per root system measured. Flooded roots did not show any extension and drought-treated roots grew only at an average of 0.04 cm per root per week (no growth was registered after one week). The shoots of the drought-treated plants showed no extension whereas the shoot extension of the flooded plants was reduced (0.33 cm per plant per week; no growth was registered after one week).

Although the treated plants showed hardly any extension during the eight weeks of treatment, survival was not affected. During the second week the response of the individuals to the stresses started to become apparent. Some of the flooded roots assumed a dark colour. The leaves of the drought-treated plants became chlorotic, with the symptoms progressing upwards along the shoot. By the fifth week, the drought-treated plants were entirely leafless as opposed to the flooded plants that showed no abnormal loss of leaflets compared to the control plants. The flooded plants showed a striking lenticel hypertrophy at the stem

basis, which became more apparent after the first four weeks of flooding.

3.2 *Flooding and lenticels hypertrophy*

Impairment of growth and lenticel hypertrophy caused by flooding were previously observed for ***Enterolobium contortisiliquum*** by **Joly (1990)**, who suggested a possible role for lenticels in diffusing oxygen from shoot to root. There is much debate, however, on what is the role played by lenticels in flooded trees. **Armstrong (1968)** working with flooded cuttings of the woody species ***Salix atrocinerea*** and ***Salix fragilis***, has shown that a progressive obstruction of lenticels above the water line, by lanolin or by immersion of the given stem segment in water, resulted in a progressive reduction of oxygen diffusion from the roots. **Hook & Scholtens (1978)**, however, stressed that up to that year the quantitative data available to evaluate the adequacy of the role of lenticels in gas exchange was rather conspicuous. **Chirkova (1978)** and **Pradet & Bomsel (1978)** claimed that lenticels play a role in detoxification by liberating ethanol and other potentially toxic volatile compounds. **Kozlowski (1984)** argues that lenticel function varies according to the species involved. In ***Salix alba***, flood-induced lenticels assist both aeration and liberation of toxic compounds, whereas in ***Populus petrowskiana*** they do not provide any assistance. **Crawford & Finegan (1989)** working with ***Pinus contorta*** showed that lenticels did not contribute significantly to root detoxification in trees, although analysis of ethanol exiting from them provided a sensitive indicator of the existence of an oxygen deficit within roots. No clear indication is available so far to claim that the hypertrophied lenticels of ***Enterolobium contortisiliquum*** aid oxygen diffusion, detoxification or both.

3.3 *Drought and leaf abscission*

Loss of leaves during the dry season is a common feature of many Brazilian *cerrado* species and, in most cases, it takes place gradually never leaving the trees entirely leafless **(Rizzini, 1979)**. In the present experiments, leaf loss in ***Enterolobium contortisiliquum*** was gradual up to the fifth week of drought, when the individuals were totally leafless, as already mentioned. **Jordan, Morgan & Davenport (1972)** believe that moisture stress contributes both to a physical inhibition of auxin transport and stimulation of ethylene production, which further reduces auxin supply and induces synthesis of hydrolytic enzymes in the abscission zone provoking the separation of the petiole. **Suttle & Hultstrand (1991)** suggest that apart from being the result of ethylene sensitivity, leaf abscission involves differential response to indolacetic acid as well. Leaf abscission is clearly a characteristic of drought-avoiders, which restricts transpirational water loss.

3.4 *Ethylene as a possible aid for both flood and drought survival*

Both flooding **(Bradford & Dilley, 1978; Jackson & Campbell, 1976; Jackson, 1990; Wadman-Van Schravendijk & Van Andel, 1986)** and drought **(Jordan, Morgan & Davenport, 1972)** can induce increased ethylene production in plants. **Jordan, Morgan & Davenport (1972)** and **Suttle & Hultstrand (1991)** suggest that ethylene production is partly responsible for leaf abscission in plants during drought. **Kozlowski (1984)** says that lenticel hypertrophy seems to be induced by ethylene produced during flooding. If both hypotheses are correct, it would seem that the stimulation of the production of this hormone under flooding and drought is a possible link between ***Enterolobium contortisiliquum*** survival to subjection to both stresses. This work, however, did not

conduct any investigation on ethylene production, which is currently being done for the same species by Dr. Maria Isabel Seródio and group, at the University of Lisboa, Portugal.

4) FLOOD AND DROUGHT SURVIVAL: METABOLIC ADAPTATIONS

4.1 *Root respiration*

Carbon dioxide output of roots was measured immediately after root excision under aerobic conditions. Subsequently, these roots were submitted to 1-hour anoxia for measurement of anaerobic carbon dioxide output (see details in the Material and Methods Section). The sampling and the root segments used were the same as for the ethanol measurements described in Chapter Five. The results (fig.5.2) and comments on ethanol production were already presented in the previous Chapter.

Although ***Enterolobium contortisiliquum*** is characteristically a fast-growing species, this was not translated in terms of high respiratory rates as opposed to other fast-growing species studied in this thesis. The low aerobic and anaerobic root respiratory rates presented by non-stressed trees of ***Enterolobium contortisiliquum*** remains practically unchanged during flooding or drought (Table 6.1). The ability of root cells to maintain their energy status anaerobically is decisive for long-term plant survival under flooding **(Crawford, 1992)**. Such an ability is probably equally important for plants under drought stress.

If carbon dioxide consumption remained constant and all anaerobic respiration was by glycolysis to ethanol, then anaerobic emission rates should fall under nitrogen to a third of their aerobic level, which did not occur in any of

the treatments. Although control and treated roots after 1-hour anoxia showed Pasteur effect to some extent, in the flooded roots this was less pronounced. The anaerobic emission of CO_2 after 1-hour anoxia was ca. 1.1 times lower than the aerobic emission. Examining the different root segments individually, provides further indication of a tendency in ***Enterolobium contortisiliquum*** to adapt more readily to flooding than to drought through reduction of respiratory rate, even although the anaerobic emission of CO_2 of none of the segments was reduced to a third of their aerobic emission, signalising some Pasteur effect. The metabolically active root tips, reduced their CO_2 emission under anaerobiosis by a factor of 1.4 when previously subjected to flooding, which was not seen under drought or normal water saturation. Only the secondary roots of flooded plants seemed to have some difficulty in keeping low rates of Pasteur effect after 1-hour anoxia, similarly to the controls and drought-treated plants.

4.2 *Carbohydrate reserves and the possible role of the xylopodium* (Table 7.2).

Underground organs are abundant in *cerrado* plants and, as well as serving vegetative reproduction, play an important role in the storage of food, particularly carbohydrates **(Figueiredo-Ribeiro *et al.*, 1986)**. The xylopodium, a rigid woody tuber present in some 100 genera of the Brazilian *cerrado* **(Rizzini, 1979)**, is one of these organs. It is seen as a traumatic growth form induced by fire injury **(Sarmiento, Goldstein & Meinzer, 1985)** often associated with drought-tolerance and as a means of ensuring plant survival and re-growth after the periodic fires which are characteristic of the vegetation **(Eiten, 1990)**. For the plants studied, however, xylopodia formation took place under the growth conditions described in the Material and Methods Section, without needing to submit the plants to any

kind of shock to induce their formation. The xylopodia of plants studied were formed two months after the seeds germinated.

The object of this experiment was to analyse the total carbohydrate concentration in three different parts of the underground system: xylopodium, primary root and secondary root. The three parts were sampled as follows: *i*) xylopodium: one 5 cm sample from each plant collected close to the stem; *ii*) primary root: six 15 cm samples from each plant (root tip); *iii*) secondary root: six 5 cm samples from each plant. The samples were reduced to a fine powder for analysis. The weight of primary and secondary root samples ranged from 25 to 50 mg of powder, and for the xylopodium samples it ranged from 100 to 300 mg (see details in the Material and Methods section).

The wide variation among the three plants within each treatment and the unlikely higher carbohydrate concentration figures in the stressed plants than in the controls (Table 6.2), suggest a methodological deficiency. Although the plants used were similar in height and general morphological attributes, it is possible that some differences occurred in genetic source or development during the growth period. The sampling method used and the number of repetitions was, therefore, not entirely satisfactory.

Despite these methodological problems, a pattern of carbohydrate allocation within treatments can still be traced. The controls and the drought-treated plants showed a carbohydrate concentration in the xylopodium higher than the primary roots by a factor of only 1.4. The flooded plants, however, presented a carbohydrate concentration of ca. 4 times higher in the xylopodium than in the primary roots, due to an apparent increase in the xylopodium carbohydrate concentration. The high carbohydrate concentration present

Table 6.1: Carbon dioxide emission (μmol CO_2.gfw^{-1}.h^{-1}; mean ± st.error) of distinct root segments of ***Enterolobium contortisiliquum*** under drought, flooding and non-stressed conditions (n=3). Aerobic respiration measured immediately after root excision at the end of the 8-week treatments. Anaerobic respiratory rate measured after one-hour anoxia following treatments.

TREATMENTS	root segment(cm)	RESPIRATORY RATE AEROBIC	ANAEROBIC
CONTROL	0- 5	4.30 ± 1.01	5.55 ± 0.91
	5-10	6.02 ± 1.25	6.10 ± 0.87
	10-15	7.88 ± 1.29	8.90 ± 1.51
	secondary	3.03 ± 0.28	3.40 ± 0.19
	x	**5.31 ± 1.05**	**5.99 ± 1.13**
FLOODED	0- 5	5.91 ± 0.99	4.19 ± 0.58
	5-10	5.42 ± 0.30	4.98 ± 0.27
	10-15	5.69 ± 0.75	5.43 ± 0.69
	secondary	3.58 ± 0.92	4.50 ± 1.12
	x	**5.15 ± 0.53**	**4.77 ± 0.27**
DROUGHT	0- 5	5.06 ± 0.21	6.22 ± 0.88
	5-10	5.84 ± 0.51	6.34 ± 1.41
	10-15	7.76 ± 1.48	8.71 ± 3.10
	secondary	4.96 ± 1.99	5.52 ± 1.57
	x	**5.91 ± 0.65**	**6.70 ± 0.70**

Table 6.2: Total carbohydrate concentration (mg.gdw^{-1}; mean ± st.error) in xylopodia, primary roots and secondary roots of ***Enterolobium contortisiliquum*** under flooding, drought and non-stressed conditions (n=3).

	XYLOPODIA	PRIMARY ROOTS	SECONDARY ROOTS
CONTROL	154.4 ± 37.6	112.7 ± 6.0	30.4 ± 4.6
FLOODED	513.8 ± 64.4	105.6 ± 41.5	45.3 ± 12.5
DROUGHT	339.8 ±141.0	201.0 ± 74.6	69.9 ± 16.2

in the samples extracted from xylopodia in this experiment suggest a possible role for this underground organ in the flood-tolerance of ***Enterolobium contortisiliquum***. At present however, this hypothesis does not go beyond speculation. Probably a larger number of repetitions and measurements of the carbohydrate concentration of the underground parts as a whole, instead of sections, would minimise the errors and provide valuable information for the species, as well as providing a further insight into the functions of the xylopodium.

5) ADAPTATION OR SYMPTOM?

Hook (1984) stresses that it is confusing whether or not a list of plant responses to flooding consists of positive survival adaptation to high water tables. The same can be said in relation to plant responses to drought.

Under flooding, ***Enterolobium contortisiliquum*** displays a series of possible adaptations, both metabolic (reduced root metabolic rates and a possible strategy of carbohydrate storage and consumption) and physical (hypertrophy of lenticels, possibly facilitating gas exchange). **Jackson, Herman & Goodenough (1982)** suggest that the development of certain morphological attributes may play a more important role in tolerance than metabolism. The present investigations, however, cannot establish a hierarchy of importance for positive adaptations. **Hook (1984)** believes that tolerance is not conveyed by a single adaptation, but by a combination of adaptations, which seems likely be the case for the species currently studied.

On the other hand, the degree of adaptation of the species to drought is not as evident. It is hard to tell if the loss of leaves, that takes place as soon as the soil water status

drops, consists of an adaptation or merely a symptom of injury. The presence of xylopodium (known as a feature of drought-adapted plants) is likely to be favourable to survival, however the results available for carbohydrate concentration, although not entirely satisfactory at the moment, do not suggest any strategy of distribution or consumption of resources, which could be the case for the xylopodia of flooded plants.

Enterolobium contortisiliquum seedlings are, therefore, clearly adapted to survive determined periods of flooding, and are also able to survive drought, even if not entirely adapted. It would seem however, that this ability to withstand drought for certain periods was enough to allow seeds of this species coming from moist forests to establish themselves in *cerrado* areas, even if in small numbers, and possibly, to allow selection for truly drought-tolerant forms such as ***Enterolobium ellipticum***.

* CONCLUSION *

CHAPTER FIVE: A HYPOTHESIS OF DROUGHT-INDUCED HYPOXIA IN ROOT TISSUES AND ITS IMPLICATIONS.

1) Three out of five agricultural crop species studied presented high ethanol concentration in the root tips of drought-treated seedlings, as compared with non-stressed controls. High ethanol concentration was sometimes accompanied by a considerable degree of root shrinkage and, as tested for ***Phaseolus vulgaris***, root lignification. Based on these results it is hypothesized that root contraction caused by water stress, might create an impediment to oxygen diffusion to the tissues, thus causing hypoxia. The main question facing this hypothesis is that of whether ethanol production is only induced by oxygen shortage or whether it can be induced by stress in general, irrespective of causing hypoxia.

2) A drought pretreatment prolonged considerably the survival of flood-intolerant ***Eucalyptus regnans*** under waterlogging. This experiment needs to be repeated and also tested for other plant species. However, if a given period of drought may acclimatise plants to a subsequent flooding, it is important to know how this process of acclimatisation takes place, whether it is due to drought causing hypoxia which, for its gradual establishment, may stimulate the formation of anaerobic polypeptides or a gradual alteration of metabolism before flood-induced anoxia, or whether it is not related to the onset of hypoxia but, rather, to the formation of abscisic acid induced by drought, which mediates an increase in ADH activity before flood-induced anoxia.

CHAPTER SIX: PHYSICAL AND METABOLIC RESPONSES OF A *CERRADO* SPECIES OF *Enterolobium* TO FLOODING AND DROUGHT.

1) The flood-tolerance of ***Enterolobium contortisiliquum*** seedlings seems to be a result of a combination of physical (hypertrophy of lenticels) and metabolic adaptations (low metabolic activity and a possible strategy of controlled allocation and use of carbohydrate reserves in the underground organs).

2) The species does not seem to be as well adapted to drought as it is to flooding, but some of its characteristics (dropping of leaves on the onset of drought and xylopodium formation) may have allowed the establishment of the species in the dry *cerrado* areas, possibly giving origin to more drought-tolerant species as ***Enterolobium ellipticum***.

FINAL SECTION

DISCUSSION

* FOREWORD *

The next Section aims to discuss the results displayed in the previous Sections under three different perspectives: *i*) the insufficiency of oxygen shortage by itself to kill plants and the primary causes of anoxic injury (**CHAPTER SEVEN**); *ii*) the problems of oversimplified labelling of plants as stress-tolerant or -intolerant (**CHAPTER EIGHT**); and *iii*) the contribution of applied research to the development of ecophysiological theory (**CHAPTER NINE**).

* CHAPTER SEVEN *
PLANT DEATH IN OXYGEN-DEPRIVED ENVIRONMENTS

1) INTRODUCTION

The need for an adequate supply of oxygen for the accomplishment of major vital functions characterises aerobic organisms. Anaerobiosis impairs several vital processes in these organisms, including cell division. Although total lack of oxygen (anoxia) consistently kills tissues of aerobic organisms, this condition is less likely to occur in nature than a partial lack of oxygen (hypoxia), according to **Johnson, Cobb & Drew (1989)**. Plants and animals are, however, able to survive determined periods with little or no oxygen. This ability varies according to the species involved, and may involve a combination of physical and metabolic adaptations.

2) DOES OXYGEN SHORTAGE BY ITSELF KILL PLANTS?

Oxygen shortage might seem the key factor reducing plant viability in a periodically or permanently hypoxic habitat, however, this is not always the case. **Crawford (1992)** points out that although oxygen shortage in flooded soils can be shown to be a predominant causal effect in creating distinctive communities, it does not mean that all species living in flooded areas are tolerant of oxygen deficiency. In a previous work, **Crawford (1982)** divided plants into two groups: *i*) those absent from flood-prone sites which can either be a) directly injured by inundation of their roots or b) physiologically viable but inefficient competitors; *ii*) those present in flood-prone sites which either a) grow actively in flooded soils or b) remain quiescent or dormant. From this classification, it is clear that those species in

groups *ii* (a and b) and *i* b (killed by competition) are not necessarily damaged directly by oxygen shortage. Group *i* a, containing plants absent from flood-prone sites which can be directly injured by inundation of their roots, is probably more relevant for study in order to try to answer the question whether oxygen shortage by itself is sufficient to kill plants.

Among the plants studied in this thesis, ***Eucalyptus regnans*** is probably the one that most obviously fits in the group of plants mentioned above. As seen in Chapters Four and Five, this characteristically flood-intolerant tree species was able to survive flooding a minimum of ca. three times longer when previously subjected to drought. Drought, irrespective if by inducing hypoxia or by stimulating abscisic acid production (see discussion in Chapter Five), apparently acclimatised the roots for the subsequent flooding. In this case, oxygen shortage created by flooding was not sufficient to kill ***E.regnans*** seedlings by itself. Although lack of oxygen in non-adapted plants triggers acceleration of glycolysis, eventually leading to the accumulation of possibly lethal toxic volatile compounds, this malfunction will be conditioned to a set of environmental and ontogenetic circumstances before and during the hypoxic period. If non-adapted plants as ***E.regnans*** are not subjected to the damaging effects of hypoxia due to other external factors, the opposite situation of typically hypoxia-adapted plants to be injured during hypoxia due to other external factors, is also likely to occur. Moreover, adapted plants can be killed by external factors other than oxygen shortage even before the onset of the latter, as seen for instance in Chapter Three, where barley plants under anoxia were killed by excessive washing and a possible destabilisation of the plasmamembrane rather than by anoxia directly.

It would appear, therefore, that for a given species and a given length of hypoxic treatment, oxygen shortage would only be lethal depending on environmental conditions, flooding phenology and plant ontogeny. The set of environmental factors pertaining during hypoxia (temperature, movement of anaerobic environment, shade, *etc.*) have long been recognised as affecting the former. However, the present results add to this the perspective of the plant's *past* (previous stresses, development, growth) also determining its present response to hypoxia. For this reason, studies on hypoxia and flooding should be aware of the possibility that the plant studied may have been previously subjected to other sources of stress. Therefore, studies on combination of stresses are likely to uncover mechanisms closer to those existent in nature, than studies on isolated stresses.

3) PRIMARY CAUSES OF ANAEROBIC DEATH

Once established that anaerobiosis is the factor leading to death, the next step would be to detect which is the primary cause of death to plant tissues as a result of oxygen deprivation. It is possible that a universal cause or common target of anoxic injury does not exist and that species or tissues differ in the nature of their anoxia-sensitivity. Speed of death from oxygen deprivation in comparable tissues varies greatly between species, from hours to months, and it is likely that this in itself reflects different causes of death **(Crawford, 1992)**. When death is rapid, cellular malfunction under anoxia may be due to the immediate decline in energy charge from which some anoxia-sensitive species appear to be unable to recover **(Hourmant & Pradet, 1981)**. In tissues where anaerobic death is not so rapid, there can be a stabilisation of energy charge values and, in these cases carbohydrate starvation, has been suggested as the principal

limitation to anaerobic survival **(Braëndle & Crawford, 1987; Steinmann & Braëndle, 1984)**. In other cases, toxic products of anaerobiosis have been considered as potentially hazardous to plants that are unable to prevent their accumulation either by metabolic control or dispersal **(Studer & Braëndle, 1987, 1988; Monk, Crawford & Braëndle, 1984; Crawford, 1992)**. Additionally, oxygen shortage can render the plant sensitive to peroxidative damage **(Crawford, 1989)**.

In this thesis, various plant species were studied under three physiological parameters (respiratory rate, ethanol production, carbohydrate consumption), which cover the possible causes of death listed above. As suggested by **Crawford (1992)**, no common cause of anoxic injury was found. The ability to keep root respiratory rates at low levels under anoxia, that appeared to play a role in survival of ***Enterolobium contortisiliquum*** under flooding, was however not seen in the tolerant species ***Eucalyptus camaldulensis*** and ***Eucalyptus pellita***. Increased ethanol production may have played a role in reducing viability of the intolerant species ***Eucalyptus regnans*** and ***Eucalyptus citriodora*** (Brazilian provenance), which was, however, not proven for barley. As for carbohydrate reserves, this parameter was only measured for ***Enterolobium contortisiliquum*** underground organs, where a possible role of maintaining adequate carbohydrate reserves to survive anoxia is still to be confirmed.

If there are different causes of anaerobic injury, plants are likely to have different strategies to adapt to oxygen deprivation. Depending on the nature and effectivity of the survival strategy, plants are classified as tolerant, avoider or sensitive. The adequacy of this classification is examined in the next Chapter.

* CHAPTER EIGHT *
TYPIFYING ADAPTATION MECHANISMS

1) INTRODUCTION

Confronted with a puzzlingly wide range of morphological and functional variation in nature, in the cosmos and within himself, Man, since his origin, feels a necessity to name and divide things into groups in order to attempt to subsequently understand them.

This process of classification is probably true of all tools Man created to interprete reality, but it was and still is particularly used and developed by Science. Although the advantages of classification can be seen in many organisational aspects of modern life, some disadvantages can appear in any field where classification becomes oversimplification.

The common practice among plant physiologists to divide plants as either tolerant or sensitive to a determined stress, is an example on how classification may lead to oversimplification. The present Chapter discusses the practical risks of labelling plants as stress-tolerant or stress-sensitive.

2) PROBLEMS WITH THE TOLERANCE AND AVOIDANCE CONCEPTS

2.1 *Definitions*

As defined by **Crawford & Baines (1977)**, anoxia-tolerance in perennial plants is characterised by the ability of roots to endure a period of total or partial anoxia during which growth does not necessarily take place, yet the tissues must

survive in a condition fit enough to resume growth when aeration is restored.

Anoxia-avoiders are species that do not present the characteristics above but, nevertheless, survive the adverse period, either by exploiting regions of the soil restricted to surface layers where the consequences of flooding are minimal (both in terms of soil toxin and oxygen deficits) or by restricting root growth to those seasons when water tables are at their lowest **(Crawford, 1989)**.

Hook (1984) names those plants able to adapt metabolically to stress as *truly tolerant*, those which use morphological and physiological adaptations to avoid a stress as *apparently tolerant*, and those unable to adapt as *intolerant*. **Jackson, Herman & Goodenough (1982)**, however, believe that the development of certain morphological attributes rather than metabolic ones characterises tolerance. The controversies around these concepts are examined below.

2.2 *Tolerance, avoidance or chance?*

Some of the species studied in the present thesis do not entirely fit into the above groups. ***Eucalyptus regnans***, as seen in Chapters Four and Five, is a tree species commonly described as anoxia-intolerant. This species, however, survived flooding ca. three times longer than normal when previously submitted to drought. Drought may have acclimatised the plant to subsequent anoxia. The situation of suddenly passing from drought to flooding and *vice-versa*, can be seen in many tropical regions, where environmental oscillations are an ecological fact, and where the fast-growing ***Eucalyptus regnans*** is planted. This species, in the case described, does not fit in the tolerance/avoidance classification; if it is fitter to survive flooding longer

when previously subjected to drought, it is not for any particular strategy of avoidance or tolerance. Instead, it can be attributed to coincidence or chance: if by chance ***E.regnans*** is subjected to a short period of drought prior to waterlogging, the species is likely to survive this latter stress in better conditions than it would if waterlogging took place without a previous short dry period.

Enterolobium contortisiliquum, studied in Chapter Six, does not entirely belong to the tolerant/avoider groups either. This species seems to present both the morphological and metabolic attributes that would characterise respectively avoiders and tolerators, falling therefore in an intersection between the two groups. **Joly (1991)** recently pointed out that, in the majority of the cases, success in surviving flooding is achieved by a combination of morphological, anatomical and metabolic adaptations and consequently the classification of species in isolated categories is often inappropriate.

2.3 *Economy and Evolution*

It is not within the scope of this work to revise the concepts of anoxia-avoidance and anoxia-tolerance, but rather to question the range of their application both at the practical level of selection of species for planting in agricultural and forestry programmes, and at the theoretical level of evolutionary modelling.

The choice of species for planting for economic purposes in periodically stressed areas, is largely based on the classification of species as tolerant or intolerant. This classification very often overlooks important factors such as environmental oscillation and plant ontogeny. These factors, as seen in the previous Sections, might at short but significant periods in a plant's life reveal a latent

capacity (or inability) of this plant to endure a particular stress, which does not exist in other moments of the plant's existence. **Coutts (1981)** stresses that what sometimes is called interspecific difference in tolerance to waterlogging, could well be only a response to variations in the condition of the root system at the time of flooding. Agriculturists and foresters are often caught by surprise when the response of the chosen species to a determined stress is not the one expected (for illustration see Chapter Four's discussion on the *Seca de Ponteiros* of Brazilian eucalypts). Particularly in the tropics, any classification of tolerance or sensitivity that overlooks ontogeny and environmental oscillations, will be subject to error. This error in many cases may lead to economic setbacks and productivity losses. In order to avoid such hazards, that are likely to be all the more frequent as the rate of environmental change in the globe reaches unprecedented levels (see Chapter Nine in this Section), more knowledge is needed on the relationship ontogeny/stress and on multiple stresses, instead of proceeding with the standard classification of plants as stress-tolerant or intolerant.

The study of stress physiology is starting to contribute a great deal to the understanding of processes such as competition, population dynamics and evolution **(Brocklebank & Hendry 1989; Buckley, Corlett & Grubb 1980; Crawford 1992; Crawford, Studer & Studer, 1989)**. Classification of species in terms of tolerance can again be misleading in the comprehension of these processes. For instance, the fact that ***Parkia pendula*** in the Amazon appears almost strictly in well-drained soils differently from other species of the genus which are periodically flooded, would certainly lead many to assume the species flood-intolerance as indisputable (see Chapter Two). Nevertheless, the present work has shown that the supposed flood-intolerance of ***Parkia pendula*** is possibly true only for a short period in the plant's life

cycle, namely during the first few months after germination takes place, which is, however, enough to keep the species away from flooded areas. This kind of information seems to be more useful to the understanding of the evolutionary pathways of a species or a group of species than simply classifying them as tolerant or not.

* CHAPTER NINE *
PRACTICAL APPLICATION AIDS DEVELOPMENT OF ECOPHYSIOLOGICAL THEORY

1) INTRODUCTION

Probably in all forms of knowledge, and certainly in Science, there is a clear distinction between what is conventionally called basic research and practical or applied research. Basically, the former's main concern is to enhance knowledge (which might or might not become useful for the society), whereas the latter's is to solve practical problems. In principle, basic research is stimulated by curiosity, while applied research is stimulated by necessity.

These two distinct approaches to the same tool (Science), in theory should complement each other: one provides the basic knowledge that subsequently is tranformed by the other in practical application. However, things are never as clearcut as this. Practical problems many times appear previously to the existence of any background of knowledge and immediate solutions are demanded. This factor was probably the catalyst of a reaction which culminated with the present day situation where to a large extent basic and applied research are disconnected. The division between the two forms of research can be seen in terms of ideology (curiosity *vs.* necessity), of finances (basic research is financed by governments while applied research is financed by the private sector), of location (universities *vs.* companies), of objectives (enhancement of knowledge *vs.* solution of problems), and even of language and vehicles of communication (scientific journals *vs.* company reports and sometimes mass media). Obviously, several exceptions will be found within this generalisation which, however, is true to

such an extent that led to the formation of stereotypes of Science and scientists: basic research is *intellectual* and *thorough*; applied research is *commercial* and *rough*; basic researcher specialisation is seen as *social alienation*; applied researcher is seen as a *businessman*.

This division can be seen also in other forms of knowledge, such as arts (intellectual art historian or critic *vs.* temperamental artist) or religion (theories and rules created by theologicians *vs.* day-by-day social problems confronted by priests). Theory *vs.* practice seems, therefore, to be another of the countless dualities characteristic of the human nature, and as such also permeates Science.

Nevertheless, as the turn of the century approaches, some symptoms of a change in attitude begin to appear, and basic and applied research in many fields seem to be at last experimenting with some fusion.

Probably, the most striking example of the fusion between basic and applied research comes from the research on greenhouse effect and global warming. Another example (not as popular as the greenhouse effect) comes, we believe, from Plant Ecophysiology, particularly from the investigations on plant stress ecophysiology. Curiously, both subjects are closely related as drastic climatic changes will most certainly lead to changes in intensity and types of stress plants are submitted to. For this reason, it seems appropriate to discuss in this Chapter the *basic-applied* approach of both the greenhouse effect (based on literature) and plant stress ecophysiology (based on the results herewith presented) research, as well as the relationships existent between the former and the latter.

2) RESEARCH ON THE GREENHOUSE EFFECT

The increase in atmospheric concentration of CO_2, CH_4, N_2O_2, O_3 and chlorofluoromethanes in the atmosphere, leading to an additional heating of the earth surface, is known as greenhouse effect **(Henderson-Sellers, 1990)**. Man's contribution to the rapid increase in the atmospheric concentration of these gases is a fact since the Industrial Revolution around 1860 **(Jarvis, 1989)**. Nowadays, the phenomenon of *greenhouse warming* and some of its causes and consequences are already of public knowledge **(Davis, 1990)**.

Henderson-Sellers (1990) provides an interesting account on the history of research on global warming and greenhouse effect, which dates back to 1827 (curiously even before the Industrial Revolution). The emphasis during the 19th century and early 20th century was on the understanding of cyclical glacial theory. It was not until the 1950's that greenhouse theory moved towards increase in temperature and then, during the 1960's, the phenomenon became popularised.

When the greenhouse effect was finally seen as a phenomenon that could cause considerable hazard to life on earth, some knowledge already existed in the field, particularly in physics and meteorology. However, the awareness of the practical consequences of global warming dragged several other disciplines into the realm of the greenhouse effect. The result is that nowadays much research is done attempting either to indicate solutions or to predict the consequences of this practical problem, which is leading to unprecedented theoretical knowledge on many fields from physics to meteorology and from biology to agriculture. Therefore, the research on global warming stands as a prime example on how practical research can lead to theoretical knowledge.

3) RESEARCH ON STRESS ECOPHYSIOLOGY

Some of the experimental work developed in the present thesis was directly stimulated by practical problems, for instance, the research developed with ***Eucalyptus***. Although these investigations were merely attempting to contribute to an understanding and an eventual solution of a serious problem afflicting forestry in Brazil, posed by the *Seca de ponteiros* in the Rio Doce Valley (see Chapter Four), which causes major economic losses to the foresters involved, the results led to one entirely new theoretical hypothesis (that drought induces hypoxia and that, by doing so, it can acclimatise roots to subsequent flooding or anoxia).

The investigations on the ecophysiology of ***Parkia pendula*** and ***Enterolobium contortisiliquum*** also respond to a practical necessity to understand the limitations to survival of these species, since their habitats (respectively the Brazilian Amazon and *cerrado*) are being rapidly transformed by man. Nevertheless, the results also provided a contribution to the understanding of the evolution of the genus ***Parkia*** (Chapter Two), an insight on the function of the xylopodium (Chapter Six) and further evidence leading to the drought-hypoxia hypothesis (Chapter Five).

The studies on chickpea and barley were less orientated towards practical applications, however some of the results highlight the importance of choosing the right time to plant these crops (Chapter One). One of the main theoretical novelties found in these studies is that in the early stages, germination is not a purely mechanical process, as it is often described, but rather a process where seed metabolism is directly affected by the onset of oxygen stress (Chapter One). Another relevant finding is that factors other than anoxia, particularly membrane

destabilisation (Chapter Three), may reduce plant viability in moving anaerobic environment sometimes even prior to the onset of anoxia itself.

The present thesis therefore, as some of the plant ecophysiological work done in the recent years, highlights the capacity of applied research to lead to theoretical breakthroughs.

4) GLOBAL WARMING, STRESS AND EVOLUTION

Davis (1990) says that many authors consider that predictions of the effects of climatic change on biological systems are difficult at the moment, requiring considerable additional information on environmental physiology and its genetic control. **Barnett (1990)** in an article entitled "Beware Greenhouse Confusion", points out the lack of statistical confidence even to support the idea of greenhouse-gas-induced global warming.

Nevertheless, assuming the global warming is a fact, it seems likely that many plant communities will be submitted to one or more stresses that they were not commonly subjected to, and/or to different intensities of stress. **Parsons (1990)** points out that the rapid increase in world temperature will, in itself, constitute an environmental stress. **Overpeck, Rind & Goldberg (1990)** foresee climate-induced changes in disturbance leading to substantial alteration of the world forests by the first part of the next century. **Holt (1990)** suggests that plants will respond to climate change in one out of three ways: *i*) by shifting in abundance and distribution; *ii*) by going extinct; or *iii*) by evolving. To predict which of the three would occur to a determined species, the author stresses, it would be necessary to know enough relevant specific ecology,

physiology and genetics, which is not the case for most species at the moment.

Parsons (1990), however, suggests that the evolution of resistance to environmental stress, one of the key factors to guarantee survival in a warm globe, may involve genetic change associated with reduced energy expenditure. Reduced energy expenditure is a common characteristic for some of the species herewith studied and it was particularly striking on ***Enterolobium contortisiliquum*** (species found in a wide range of habitats in South America), even under stressed conditions. Although some ecological and physiological knowledge is already available for this species, little is known in terms of its genetics, which would be necessary to aid predicting the degree of adaptability of the species to climatic change. However, the species economic importance already highlighted elsewhere (Chapter Six), and its reduced energy expenditure, make of ***Enterolobium contortisiliquum*** an important species to study and to include in any genetic conservation program.

Parkia pendula appears in the environment where **Parsons (1990)** believes species extinction is going to be particularly high with future climatic changes: the species-rich rain forests. **Lean & Warrilow (1989)** simulated the regional impact of Amazon deforestation if large segments of the rain forest are substituted by pasture. According to their model, the rainfall over the region would decrease 20%, the local climate would be warmer and evapotranspiration would be reduced. Although the flooding regime of the region is mostly determined by the melting of snow in the Andes, local variations of this type would be likely to affect periodicity, extent and intensity of flooding in the region. Once again, it is too early to make any predictions, but for species like ***Parkia pendula***, where survival and distribution are so clearly affected by the

phenology of flooding, alterations would be likely to occur. The main weapon of this species to survive environmental change of any type appears to be its impermeable seed coat which would possibly allow the plant to germinate when and where conditions are more suitable. One should bear in mind that the nature of the disturbance, if natural or anthropogenic, would certainly play a role in the species speed of recovery **(Uhl *et al.*, 1990)**.

The decline in productivity due to increased moisture stress during the growing season, predicted by **Parry, Porter & Carter (1990)** to occur in the productive areas of many Third World countries, is another reason for concern. Therefore, ecophysiological research on stress-tolerance for agriculture and forestry crops, as attempted in this thesis with tropical crops like chickpea and ***Eucalyptus***, is particularly relevant.

5) RESEARCH POLICIES

The argument defended in the present Chapter that applied research leads to development of ecophysiological theory is of considerable importance for the establishment of research policies, particularly in the so-called Third World countries. In these countries, the investment on scientific research is understandably reduced in comparison with their wealthier counterparts. Where to concentrate these reduced investments so that the return is maximised is, therefore, a matter of paramount importance, and obviously of great controversy (eg. **Anderson, 1989**).

In countries characterised by poor infra-structure, unstable economies and disproportional distribution of wealth, it seems unthinkable to invest on basic scientific research. On the other hand, these countries cannot rely solely on the

knowledge produced by the so-called First World. For instance, worldwide concern has been expressed in terms of the extent to which the present rate of deforestation of the Amazon forest will contribute to global climatic changes, however little has been said (eg. **Dickinson, 1989**) or done (eg. **Lean & Warrilow, 1989**) about the local ecological and social effects. This fact shows the need for economically dependent countries to aim for a scientific independence so that they can find solutions for their own local problems.

Thus, it seems that the fusion of application and theory as discussed in this Chapter, and so well exemplified by the research on greenhouse effect, is a possible alternative for those countries striving to achieve a scientific development that will eventually lead them to improved social conditions.

The perspective that global warming might bring a decrease in productivity and an additional selective pressure on natural habitats (**Parry, Porter & Carter, 1990**), poses a substantial threat to the already deficient economy of most tropical and subtropical countries that so much depend on agriculture and their natural resources. This fact highlights the relatively urgent need of research showing possible solutions for this problem. Plant ecophysiology, as both a practical and basic field, has certainly an important role to play in achieving this objective.

* CONCLUSION *

CHAPTER SEVEN: PLANT DEATH IN OXYGEN-DEPRIVED ENVIRONMENTS.

1) Lack of oxygen by itself is often insufficient to determine plant death. Plant responses to hypoxia seem to depend on the set of environmental and ontogenetic factors before, during and after oxygen stress.

2) A common cause of anaerobic injury does not seem to exist for the plants studied.

CHAPTER EIGHT: TYPIFYING ADAPTATION MECHANISMS.

1) The common practice of classifying plants as tolerant or sensitive to stress often overlooks ontogenetic and environmental factors, and is, therefore, a simplification that may mislead plant growers.

2) In the species studied, anoxic survival seemed for some species to be related to metabolic adaptations (eg. ***Eucalyptus citriodora***, St.Andrews), to other species to morphological adaptations (eg. ***Eucalyptus camaldulensis***), or to a combination of morphological and metabolic adaptations (eg. ***Enterolobium contortisiliquum***).

CHAPTER NINE: PRACTICAL APPLICATION AIDS DEVELOPMENT OF ECOPHYSIOLOGICAL THEORY.

1) As exemplified by research on the greenhouse effect and on plant stress ecophysiology, applied research can lead to enlargement of basic knowledge, as well as basic research can lead to information useful in the solution of practical

problems. Stimulation and investment on research aiming to provide both basic and applied knowledge may be an adequate strategy for countries economically and culturally dependent.

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