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ENVIRONMENTAL STRESS AND CALCIUM NUTRITION DURING THE SEED-FILLING STAGE OF SOYBEAN

by

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Stress Physiology of Soybean Crop

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

وَاللَّهُ أَنزَلَ مِنَ السَّمَاءِ مَاءً فَأَحْيَا بِهِ الْأَرْضَ بَعْدَ مَوْتِهَا ۚ إِنَّ فِي ذَٰلِكَ لَآيَةً لِّقَوْمٍ يَسْمَعُونَ ﴿٦٥﴾ سورة النحل

In the name of Allah, the Compassionate, the Merciful. And Allah sends down rain from the skies, and gives therewith life to the earth after its death: verily in this is a Sign for those who listen.

Translations of the Qur'an, Chapter 16, verses: 65

Reference: http://www.usc.edu/cgi-bin/msasearch

This thesis is dedicated to whom I love:

To the prophet of Islam, Mohammed (ص), and his household (ع),

To my family especially my wife and my sons.

ABSTRACT

An infusion technique was used with an aqueous radiocalcium ($^{45}CaCl_2$) solution during the seed-filling stage of well-watered and moisture-stressed soybean in a greenhouse. The kinetics of infusion volume showed a quadratic reduction in absorption which approached zero on the sixth day for non-irrigated plants. The concentration of ^{45}Ca increased quadratically from the point of injection towards the apex independent of both water status and plant parts. The difference in concentration of ^{45}Ca between irrigated and non-irrigated plants was significant ($P < 0.05$) and concentrations attained the maximum values at the sixth node from the plant base. Seeds contained considerably less ^{45}Ca than either stem or leaves.

The effects of a long (LD, 16 h) and a short (SD, 12h) photoperiod with two water stress levels (SL) of stress (ST) and no stress (NS) on the distribution of ^{45}Ca in plant organs (PO) of leaves, petioles, and stem at different node number (NN) of soybean were studied during the seed-filling stage. The univariate and Manova analyses showed the main effects of photoperiod (PP), SL, and PO to be highly significant ($P < 0.001$) on Ca distribution. The long PP increased Ca concentration in top leaves compared with the short PP regardless of SL. Water stress significantly ($P < 0.001$) modified the Ca distribution and reduced its concentration in PO within NN irrespective of the photoperiod (a measure of light stress). A possible mechanism for the regulation of Ca distribution is discussed in terms of nitrate reduction.

Ca uptake was also studied by immersing the central tip of a trifoliate leaf in various concentrations of $^{45}CaCl_2$ solutions and drought conditions during the seed-filling

period of soybean. The beta-ray gauging and the diurnal leaf temperature variation showed similar characteristics for leaf water status. The activities of ^{45}Ca were significantly higher ($P < 0.0001$) at 5, 10, 20, and 30 mM concentrations for water-stressed and non-stressed leaves compared with the control. ^{45}Ca activities at 5, 10, and 20 mM Ca concentrations between stressed and non-stressed leaves were not significant, but the difference in their mean values at 30 mM Ca concentration was significant ($P = 0.0159$). The relationship between ^{45}Ca uptake and Ca concentration was parabolic for both stressed ($R^2 = 0.77$) and non-stressed ($R^2 = 0.81$) leaves. Autoradiograms indicated Ca movement through the mid-rib and veins of the tip-immersed trifoliate leaf but showed no activity in other plant parts. An activity gradient developed between seeds when a pod-tip was immersed in the radioactive solution. Solutions of ruthenium red (RR, 0.01 mM), Ethylene Glycol-bis-(β-aminoethyl ether)-*N,N,Ń,Ń*-Tetraacetic Acid (EGTA, 0.1 mM), calcium (Ca, 1 mM), and double distilled water (control) were fed through a bottom branch of soybean with (ST) and without (NS) water stress. The volume absorptions and transpiration rates were significantly higher for NS than ST plants and decreased almost linearly with time for all treatments. The transpiration rates of Ca-feeding ST plants and the control overlapped while the NS plants approached the same rate of transpiration by the third week. Ca was implicated in stomatal closure for the reduction in the transpiration rates. The relative amounts of chlorophyll decreased with time but chlorophyll was least affected for Ca-absorbing plants for both ST and NS plants. The use of RR (Ca transport blocker), and EGTA (Ca chelator) indicated the role of intracellular Ca concentrations on stomatal closure and foliar senescence at the end of the season.

RÉSUMÉ

Une solution contenant du Ca radioactif (Ca-45) a été introduite par infusion dans des plantes de soja arrosées et des plantes sous stress irriguées durant la période du développement des grains. Dans le cas des plantes non-arrosées, le volume de la solution absorbée a diminué avec le temps d'une manière quadratique et l'absorption a cessé complètement au bout de six jours. La concentration du Ca-45 a augmenté d'une façon quadratique entre le point d'injection et le bout de la plante indépendamment du niveau de stress et des différentes parties des plantes. La différence entre la concentration du Ca-45 dans les plantes arrosées et non arrosées (sous stress) a été significative ($P < 0.05$) et la concentration a atteint le maximum au niveau du sixième noeud (à compter de la base des plantes). La concentration du Ca-45 a été considérablement plus faible dans les graines que dans les feuilles et les tiges.

Par ailleurs, nous avons étudié les effets de photopériodes (PP) longues et courtes sur le soja stressé (ST) et non stressé (NS) au niveau de la distribution du Ca-45 dans les feuilles, les pétioles et les tiges, pendant la période du développement des graines. Les analyses de variance univariée et multivariée ont démontré que la PP et le niveau du stress (SL) aussi bien que les différentes parties des plantes (PO) ont influencé significativement la distribution du Ca-45 dans les plantes. Les longues photopériodes ont augmenté la concentration du Ca dans les feuilles supérieures plus que les courtes photopériodes, indépendamment du niveau du stress. Le stress a changé la distribution du Ca et a réduit sa concentration dans les PO (différentes parties des plantes) à chaque noeud considéré, indépendamment de la durée de la photopériode. Les effets de la réduction du nitrate sur la

régulation de la distribution du Ca dans les plantes sont discutés.

L'absorption du Ca dans les conditions de sécheresse a été étudiée en immergeant le bout d'une foliole centrale (feuille composée de trois folioles) dans des solutions de différentes concentrations de $^{45}CaCl_2$ (5, 10, 20 et 30 mM) pendant l'étape du développement des graines. La mesure des rayons bêta et la variation de la température nocturne de la feuille ont démontré des caractéristiques similaires au niveau du statut de l'eau dans la feuille. L'activité du Ca-45 a été plus élevée ($P < 0.0001$) dans les plantes sous stress et non stressées traitées avec les différentes concentrations que dans les plantes de contrôle. Une différence significative ($P < 0.0159$) entre l'activité du Ca-45 dans les feuilles des plantes sous-stress ou non stressées a été observée seulement dans les plantes traitées avec la solution de 30 mM. La relation entre la concentration et l'absorption du Ca par les feuilles des plantes stressées et non stressées a été parabolique dans les deux cas ($R^2 = 0.77$ et 0.81 respectivement). Les autoradiogrammes ont indiqué que le Ca reste dans les veines des folioles immergées et ne se déplace pas aux autres parties de la plante. Quand le bout des gousses a été immergé dans la solution radioactive, les graines les plus proches du bout immergé contenaient plus de Ca que les plus éloignées. Des solutions de rouge de ruthenium (RR, 0.01 mM), d'Éthylène Glycol-bis-(B aminoéthyl-éther)-N,N,N,N,- tétraacétique acide (EGTA, 0.1mM), de calcium (Ca, 1 mM), et d'eau distillée (contrôle) ont été introduites dans les branches inférieures des plantes stressées (ST) et non stressées (NS). Le volume de la solution absorbée et le taux de transpiration ont été plus élevés dans les plantes non stressées et ont diminué d'une manière presque linéaire avec le temps (pour tous les traitements). Les taux de transpiration dans les plantes stressées traitées au Ca ou à l'eau distillée (contrôle) ont été semblables (identiques), tandis que dans les plantes non stressées,

ces taux sont devenus comparables seulement après la troisième semaine. Dans les plantes non stressées, le Ca a été impliqué dans la fermeture des stomates, ce qui a abouti à une réduction dans le taux de transpiration. La quantité de chlorophylle a diminué avec le temps, mais moins rapidement dans les plantes (stressées et non stressées) traitées avec la solution de Ca. L'utilisation des solutions RR et EGTA a indiqué que la concentration intracellulaire du Ca intervient dans la fermeture des stomates et dans la sénescence des feuilles vers la fin de la saison.

STATEMENT FROM THESIS OFFICE

In accordance with the regulation of the Faculty of Graduate Studies and Research of McGill University, the following statement excerpted from the Guidelines for Thesis Preparation (McGill University 1995) is included:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, ***connecting texts that provide logical bridges between the different papers are mandatory****. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.*

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". ***The thesis must include:*** *A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.*

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, ***the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extend.*** *Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.*

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1) A stem infusion technique in combination with an aqueous solution of radiocalcium was used for the first time to study the pattern of Ca distribution and concentration in soybean plants during the seed-filling stage when drought stress prevailed. This was considered original and important because plant nutrition studies in the future may emulate the technique described in this thesis.

2) The relationship between the duration of light (an environmental stress) and the Ca distribution and concentration pattern during the seed-filling stage of soybean was explored for the first time. The long photoperiod increased Ca concentration in leaves.

3) Leaf-tip and pod-tip immersions in radioactive Ca for a period of two weeks were employed, as never before, to examine retranslocation of Ca during the seed-filling stage. It was shown that Ca was not retranslocated but it penetrated through the pod tissue ever so slowly. Thus, foliar application of Ca may not supply sufficient Ca to the seeds.

4) An unique but a simple method of branch feeding of Ca was developed which eliminated the effect of the external force used in the infusion technique. The method was described as unique because, to the best of the knowledge of the author, no researcher had used it before with the Ca modifying agents as done in the present experiments.

5) The role of Ca as a signalling agent was explored on the whole plant level in influencing plant senescence and stomatal dynamics in soybean plants undergoing environmental stress. Ca played a delaying role in foliar senescence in stressed and non-stressed soybean. Foliar concentration of Ca had no effect on stomatal movement in drought stress, while under non-stress conditions Ca influenced closure.

ACKNOWLEDGMENTS

I would like to start this thesis in the name of Allah (God), the Compassionate, the Beneficent, and the Merciful. I appreciated Allah for everything and especially for my life with love to the Prophet Mohammed (ص) and household of Mohammed (ع). Also, I should thank God for giving me an opportunity to continue my education in Iran and Canada.

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Last but not least, I would like to express sincere thanks to my wife, since she had a very important role in my life especially during my studies. I fully appreciated the stress she had undergone in trying to cope with my situation, and thank her for the patience and understanding she has exercised. I also thank my sons Hassan and Hossien for being co-operative. I cannot forget my father who had wished to see me finish Ph.D. I hope a good life for him in the next world. I thank my mother, sister and brothers.

ON AUTHORSHIP OF THE MANUSCRIPTS

Ali Sorooshzadeh was the senior author on all the four manuscripts arising out of this thesis. He performed the experiments, collected, organized, and statistically analyzed the data. Dr. N.N. Barthakur supervised the experimental work, and provided safety, technical, and administrative assistances in handling and purchasing the radioisotope. Dr. N.P. Arnold was a co-author on the first published paper by virtue of his considerable contributions as an advisor in the Ph.D. committee, and in writing and editing the manuscript.

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PREFACE

The thesis consists of eight chapters. Chapter 1 introduces the subject-matter, and states the hypotheses and the objectives of the research project. Chapter 2 contains the relevant and pertinent literature on the subject. Chapter 3 deals with the effect of environmental stress of water on calcium (Ca) concentration and distribution in soybean plants when Ca was injected into the stem. Chapter 4 describes the effect of photoperiod and moisture stress on Ca distribution when Ca was fed through the root system. Chapter 5 includes the effect of water stress on Ca translocation when Ca was introduced through leaf and pod immersions. Chapter 6 discusses the effect of increased Ca on stress physiology when it was made available through a branch. Chapter 7 surveys the integrated view of the thesis with recommendations for future research projects. Chapter 8 concludes with a summary and conclusions.

Chapter 3 to 6 contain the bulk of the experimental work of the project which provided materials for the four manuscripts published or submitted to peer-reviewed journals. However, the manuscripts have been modified to a certain extent to suit the purpose of the thesis. Additional comments and discussions were included as deemed necessary to elucidate difficult concepts or points.

CHAPTER 1

INTRODUCTION

1.1 Environmental Stress and Crop Production:

An imbalance between agricultural production and the rapid growth in human population in various parts of the world is the root cause for severe socio-economic problems and instability on the planet. Therefore, it is very important for the food production to increase in direct proportion to the increase in the population of the world to avoid regional food shortages and famines. Efforts to produce enough food to feed the world will extend farming to marginal land or on land less favourable for growing crops than the prime agricultural land now in cultivation. However, the land resource of the world for growing crops is limited. It is estimated that from more than 14 billion hectares (ha) of available land in the world only about 1.4 billion ha are classified as non-stressed or good crop land. An additional 2.9 billion ha are limited in crop productivity by mineral stress, 3.7 by drought, 3.2 by shallowness, 2 by permafrost, and 1.6 by excess water or flooding (Dudal, 1976). Thus, agricultural crops must continually cope with stress of one kind or another, and any increase in productivity will be dependent upon reducing the effect of plant stress. Stress is broadly defined as any adverse effect, e.g., reduced growth or yield of plants from any external influence or force.

Understanding response processes of plants to stress are essential in any attempt

to reduce their effects. Therefore, the study of plant response to environmental stress has become a central feature of research for plant environmental physiologists and physiological ecologists. Among the environmental stresses, droughts perhaps limit plant growth and crop production more than any other single environmental factor (Dudal, 1976; Boyer, 1982; McWilliam, 1986). It was reported that 28% of the agriculturally utilizable soils in the world, crop production was affected regularly by drought and an additional 24% of the world's soils was too shallow for drought conditions to make any difference (Dudal, 1976). Droughts frequently occur in the arid, semi-arid, and tropical regions of the world from the limited seasonal rainfall or its uneven distribution. Such reduction in rainfall leads to water stress in plants, which limit plant growth and development (Quizenberry, 1982). Although soil salinity, heat, cold, and nutrient deficiency, and other environmental stresses reduce plant growth and crop production, moisture stress will constitute the main concern of this thesis.

1.2 Plant Response to Water Stress and Role of Calcium:

Water stress adversely affects many physiological growth processes of plant directly or indirectly. One of these processes is the mineral nutrient uptake and translocation (Power, 1990). Mineral deficiency causes some nutritional disorders in plants. For example, blossom-end rot of tomatoes, bitter-pit of apples, necrosis of potato-stolons, low seed-setting of subterranean clover, and "pops" of groundnut are all

attributed to an insufficient supply of calcium (Ca) under soil moisture stress conditions. It is well-known that these disorders are due to the very slow mobility of Ca in the phloem, which limits its redistribution from old to new tissues, when the transpiration rates were reduced (Marschner, 1995). Ca transport and translocation are dependent on the transpiration stream. The effect of extracellular Ca on various physiological processes such as abscission and leaf senescence has been known for a long time (Poovaiah and Leopold, 1973; Poovaiah, 1979; Bangerth, 1979). However, recent investigations have shown that the intracellular level of Ca has a key role in plant growth and development through the regulation of different metabolic processes in plant cells (Bush, 1995). Research efforts in finding relationships between intracellular levels of Ca and the effects of different environmental stimuli (light, chilling, wind, aluminium and salt toxicities, touch) or different plant hormones (such as abscisic acid, gibbrellic acid, auxins) were both intensive and extensive during the last 15 years or so (Hepler and Wayne, 1985; Poovaiah and Reddy, 1987; Evans *et al.*, 1991; Knight *et al.*, 1991, 1992; Rengel, 1992; Rengel and Elliott, 1992). It has been established that intracellular level of Ca plays a central role in transducing the environmental and hormonal signals in plant cells (Coté and Crain, 1993). One of the most important research areas concerns relationships between stomatal closure and Ca concentration in guard cells. There is strong evidence that stomatal closure (which is one of the manifest effects of water stress) is induced by augmenting the synthesis of abscisic acid (ABA) with increased level of

Ca in cytoplasm of guard cells of stomata (McAinsh *et al.*, 1990). Thus, water stress influences both intracellular and extracellular concentrations of Ca.

1.3 Soybean Crop and Its Importance:

From a taxonomical point of view soybean belongs to the legume family (*Leguminosae*), subfamily Papilionoideae, and genus *Glycine*. This genus contains two subgenera: *Glycine* and *soja*. *Glycine* includes eight species, while *Soja* covers two species - *Glycine Soja* and *Glycine max*. Except *Glycine max* (L.) Merr. which is the cultivated soybean and all other species are wild. Soybean is native to eastern Asia, Australia, and several of the Pacific Islands. However, the soybean culture began about 2800 B.C. in China, and probably it was used as food, feed and medicinal plant at that time (Smith, 1995). The cultivation of this crop spread down to the south and southeast Asian tropics, and reached Europe in the early eighteenth century. Then in the later part of that century, it was transported from France to the USA (Norman *et al.*, 1995). In the United States, until World War II, soybean was grown as a forage crop. Prior to 1930, less than 25% of soybean planted was harvested only for seeds in that country. However, after WWII, the need for fats, oils and oilseeds in the United States increased resulting in a corresponding increase by 72% of total land devoted for soybean harvesting for seeds (Smith, 1995). The total world soybean production, within the last 25 years has been tripled (from 32 million metric tons in 1965 to 126 million metric tons in 1989) and

its area of production is doubled (from around 28 to more than 56 million ha for the same period) (Food and Agriculture Organization of United Nations: FAO, 1975, 1996). In 1990/91 the United States produced 50% of the world soybean production. Farm income from soybean in that year and country was more than any other single crop (Prevedell, 1993). In 1995, the soybean was grown commercially in nearly 90 countries and was listed as the seventh most important crop in the world (FAO, 1996). The leading soybean producers in that year were the USA, Brazil, the Peoples's Republic of China, and Argentina. In addition, India, Indonesia, Paraguay, and Canada grew considerable amounts of soybean (Table 1.1) (FAO, 1996). In Canada, soybean production doubled in a-10 year period (1986-1996) mostly by increasing the land area for production in Ontario and especially in Quebec (Table 1.2). The soybean has become an important commercial source for edible oil and protein.

The economic importance of this crop can be appreciated from the quality of the soybean seeds which contain approximately 20% oil and 40% protein on a dry weight basis. Moreover, about 40% of the world's vegetable oil comes from this crop for human consumption (Wilcox, 1987). Soybean provides a substantial amount of protein in the diets of people, particularly, in the regions where animal protein is expensive and not easily available. Specialty foods like tofu made from soybean have become a common food item world-wide. A type of ink produced from soybean could be viewed as environmentally friendly (Peprer, 1994). Thus, research on improving yield of soybean

Table 1.1 Area, yield, and total production of soybean in the world and major soybean-producing countries in 1995

Country	Area (10^3 ha)	Yield (Mt ha^{-1})	Total Production (10^3 Mt)
USA	24,952	2.35	58,569
Brazil	11,651	2.20	25,581
China	8,132	1.66	13,518
Argentina	5,914	2.04	12,088
India	5,000	0.92	4,600
Indonesia	1,503	1.12	1,689
Paraguay	830	2.77	2,300
Canada	819	2.78	2,280
World	62,285	2.02	125,930

Source: FAO (1996)

Table 1.2 Area, yield, and total production of soybean in Canada in 1986 and 1996

Province	Area (10^3 ha)		Yield (Mt ha^{-1})		Production (10^3 Mt)	
	1986	1996	1986	1996	1986	1996
Ontario	380.40	777.00	2.50	2.51	949.80	1905.1
Québec	4.40	93.00	2.27	2.57	10.00	255.0
P.E.I.	----	5.3	----	1.94	----	10.30
Total	384.80	875.30	2.49	2.51	959.80	2170.4

Source: Canada Grains Industry Statistical Handbook 1996

is very important, particularly, in the developing countries where there is a chronic shortage of edible oils and proteins for a fast growing population.

Soybean is a relatively high water-demanding crop and requires approximately 4.6 x 10^6 L/ha for transpiration. In contrast, wheat requires approximately half that of soybean (2.4 x 10^6 L/ha) (Pimentel *et al.*, 1997). Drought stress reduces by 25-40% the potential yield of soybean (Boyer, 1982). For example, in the south of the United States incidence of droughts was one of the primary factors that caused reduction in soybean production from 1979 until 1992 (Prevedell, 1993). Thus, efforts should be directed towards improving yield of this crop when drought stress prevails.

It has been known that soybean susceptibility to water stress is dependent on its stage of growth. Investigations in the past have indicated that the yield of soybean seed in response to water stress is most severe during the reproductive stage than during any other phenological stages of growth. Evidence of this phenomenon comes from both laboratory and field experiments (Korte *et al.*, 1983a). Even within different periods of the reproductive stage, response of soybean plants to drought varies considerably. For example, drought during the seed-filling stage of soybean growth, hastens senescence (Sionit and Kramer, 1977; Cure *et al.*, 1983; Cortes and Sinclair, 1986), but from the flowering to mid pod-fill stage no acceleration of senescence has been observed (Cortes and Sinclair, 1986). Physiological mechanism(s) of this response is still not known (Pell and Dann, 1991). The delaying effect of extracellular Ca on leaf senescence under normal

growth conditions has been observed (Poovaiah and Leopold, 1973; Poovaiah, 1979), while other investigations have shown that the intracellular level of Ca might enhance senescence (Leshem *et al.* 1982, 1984, 1986). Although a reduction in Ca concentration in soybean plant from drought during the seed-filling stage has been reported (Batchelor *et al.*, 1984; Smiciklas *et al.*, 1989), the effect of Ca on plant senescence during a period of water stress has never been investigated.

The objectives of this thesis were:

1.4 Hypotheses:

1. That water stress during the seed-filling stage reduces Ca concentration and changes the distribution pattern of Ca within soybean plants.

2. That during the seed-filling stage of soybean, a long photoperiod or long day (LD) increases Ca concentration in leaves.

3. That soybean plants subjected to water stress during the seed-filling stage promotes foliar senescence when Ca concentrations in leaves are reduced.

4. That leaf senescence that occurs from water stress during the seed-filling stage is delayed by increasing Ca concentrations in leaves.

Based on these hypotheses, the objectives of this thesis were.

1.5 Objectives:

The objectives of this thesis were:

1. To study the effects of water stress during the seed-filling stage on Ca concentration and distribution in soybean plants.

2. To determine the influence of photoperiod during the seed-filling stage on Ca concentration and distribution under water stress conditions.

3. To examine the translocation of Ca from leaf to seeds and vice versa under water stress condition during the seed-filling stage of soybean.

4. To investigate the role of calcium (Ca) on leaf senescence of soybean as related to water stress during the seed-filling stage.

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CHAPTER 2

LITERATURE REVIEW

2.1 Effects of Water Stress on Soybean:

The effects of water stress during different phenological stages of plant growth showed the reproductive stage to be more sensitive than the prior stages of growth (Shaw and Laing, 1966; Sionit and Kramer, 1977). Within a particular stage of growth, sensitivity to water stress may vary considerably. Thus, researchers have concentrated their investigations on the effects of moisture stress on plants at different periods of the reproductive stage. Fehr *et al.* (1971) introduced the symbol R for the reproductive stage of soybean and different periods are represented by flowering (R1), full bloom (R2), beginning of pod (R3), full pod (R4), beginning of seed filling (R5), full seed (R6), beginning of maturity (R7), full maturity (R8).

2.2 Morphological Responses:

2.2.1 Root Response

Root growth in soybean continues until the plants attain physiological maturity at which time the root growth ceases and root loss begins due to decomposition (Brown, 1984). Since the root system is the first plant organ to encounter soil moisture stress, it is understandable that investigators of field and growth chamber experiments concentrated

their efforts on root response to droughts. Moisture stress in non-irrigated treatments reduced root growth at the top layer of soil, and growth continues in deeper layers; while irrigation had just the reverse effects on root growth (Mayaki *et al.*, 1976; Sionit and Kramer, 1977; Burch *et al.*, 1978; Garay and Wilhelm, 1983; Brown *et al.*, 1985; Huck *et al.*, 1986; Hoogenboom *et al.*, 1987a). However, several of these investigators observed the above effects only when stress occurred before the pod or seed development stage (Mayaki *et al.*, 1976; Brown *et al.*, 1985; Huck *et al.*, 1986; Hoogenboom *et al.*, 1987a). The difference in results could perhaps be attributed to a decrease in root growth beneath rows and an increase in root growth between rows during pod set (Brown, 1984). Varietal difference in root growth during the reproductive stage might be another reason for the difference in the observations.

The newly developed soybean cultivars are less susceptible to water stress and have higher yields compared with the old varieties because the former are bred so that they would produce a greater root density than the latter cultivars. Carter (1989) suggested that the most effective mechanism to improve soybean tolerance to drought is to increase its ability to extract soil water via enhancing the root system. Carter and Rufty (1993) screened 300 Plant Introduction Soybean (PIS) cultivars for drought stress in two years of field experiments. They found PI 416937 cultivar from Japan to be highly drought tolerant because it had developed a relatively deep root system by the time the pod-filling stage was attained. Hudak and Patterson (1995) reported a slower growth

rate reduction of PI 416937 than the non-tolerant Forrest cultivar. They measured the root mass, the root volume, and the relative surface area of PI 416937 and showed these to be significantly higher than those for the non-tolerant cultivar.

2.2.2 Stem and Shoot Response

A soybean stem has two main growth habits, known as determinate and indeterminate. In the determinate plants, vegetative growth is nearly complete when the plant starts flowering; while in the indeterminate plants the vegetative growth does not terminate when plants enter the reproductive stage. In addition, the indeterminate plants are usually taller and have more nodes per plant than the determinate counterparts (Fageria *et al.*, 1991). The effect of water stress on plant growth is dependent on the growth habit of the stem and the phenological stage of plant development.

2.2.2.1 Indeterminate Cultivars

In case of the indeterminate soybean cultivars, irrigation at flowering until the end of the seed development stage resulted in a significant increase in plant height, nodes/plant, and lodging (Mayaki *et al.*, 1976; Momen *et al.*, 1979; Korte *et al.*, 1983a; Egli *et al.*, 1984; Hoogenboom *et al.*, 1987b). However, irrigation at the reproductive stage or a later period (seed development and later) had little effect on plant height, and nodes/plant (Korte *et al.*, 1983a; Kadhem *et al.*, 1985a). Obviously, water stress reduces

plant height of indeterminate cultivars.

2.2.2.2 Determinate Cultivars

Sionit and Kramer (1977) showed that water stress during flowering, pod formation, and seed-filling stages did not affect plant height and shoot dry weight of `Ransom' (a determinate cultivar of soybean); but decreased the shoot dry weight of `Bragg' (another determinate cultivar of soybean). Constable and Hearn (1978) supported Sionit and Kramer's (1977) findings, and suggested that soybean cultivars have large differences in their response to water stress during the pod-filling stage. These differences may arise from a plant's ability to move carbohydrates from storage in the stem to the seeds. Consistent with this report, Rawson *et al.* (1978) observed the loss in stem weight in drought-stressed `Bragg' was largely due to the re-translocation of carbohydrates. Other investigators have also shown that water stress during any period of the reproductive stage of determinate soybean cultivars had no significant effect on plant height and lodging (Korte *et al.*, 1983a; Kadhem *et al.*, 1985a; Hoogenboom *et al.*, 1987a). However, during the vegetative stage water stress may affect stem growth. Bouslama and Schapaugh (1984) observed a reduction in stem height from stress during the vegetative growth stage in determinate and indeterminate cultivars of soybean. These authors recommended that inter-node elongation and plant height can be used in evaluating soybean genotypes for drought resistance.

2.2.3 Leaf Response

2.2.3.1 Leaf Growth

The area of leaf surface per unit area of land surface is defined as Leaf Area Index (LAI). In the determinate cultivars the maximum LAI may occur near the beginning of flowering, while in the indeterminate cultivars LAI maximum reach near the end of flowering (Fageria *et al.*, 1991). Boyer (1970) showed the negative response of leaf elongation rate to water stress in soybean. On the other hand, a linear relationship between leaf elongation rate and turgor pressure during the vegetative growth of soybean have been reported by Bunce (1977), and Sivakumar and Shaw (1978).

Mayaki *et al.* (1976) reported that the maximum LAI in non-irrigated plants occurred seven days before irrigated plants, and was less than the maximum LAI of irrigated plants. Sionit and Kramer (1977) found the leaf area of two determinate soybean cultivars significantly reduced, by imposing water stress at any period of the reproductive stage but a correspondingly higher reduction occurred during the pod-filling stage. The results of Sionit and Kramer (1977) in growth chamber experiments on leaf area was supported by Constable and Hearn (1978) in field experiments. Although during two dry years of a three-year field experiment performed by Scott and Batchelor (1979), there was no significant difference in leaf area before the reproductive stage between irrigated and non-irrigated plants. However, during the reproductive stage, water stress limited leaf expansion, and the difference in leaf area between irrigated and non-irrigated was

significant. Karlen *et al.* (1982a) observed that irrigation during the reproductive stage improved leaf dry matter only at the early podding period. Although water stress influenced leaf area, other environmental factors such as duration of light period (photoperiod) may also contribute to leaf area variations as reported by Cure *et al.* (1983) for determinate soybeans cultivars. They found that under long photo-period when water stress occurred during the mid seed-filling stage, the late maturing cultivar 'Ransom' maintained 45%; the early maturing cultivar 'D72-8126' maintained 25% of their maximum leaf areas compared with 16 and 0%, respectively, for those exposed to short photoperiod. This was because the leaf drop was greatly reduced under long photo-period in both genotypes. Eck *et al.* (1987) reported that drought stress initiated from seed-filling stage (R5) to full seed stage (R6) caused immediate reduction in LAI of indeterminate soybean cultivars. Hoogenboom *et al.* (1987b) reported the sensitivity to water stress increased as the total leaf area increased. The leaf area expansion rate significantly decreased in non-irrigated crops during drought stress, and these plants produced smaller leaf than those for irrigated plants.

2.2.3.2 Leaf Movements

Leaf movements have been observed in response to environmental stimuli in some plants including leguminous soybean (Ehleringer and Forseth, 1980; Meyer and Walker, 1981). It was proposed that leaf movement could promote drought avoidance by reducing

light interception, leaf temperature, and transpiration rate, which caused increasing water-use efficiency (yield per unit of water used) (Ehleringer and Forseth, 1980). Leaf movement is due to differences in turgor within the pulvinus (or pulvinule) (a motor organ located at the base of the leaf and leaflets) (Satter, 1979).

Meyer and Walker (1981) observed movement of terminal leaflet of soybean over a range of leaf water potentials. Oosterhuis *et al.* (1985) confirmed the result of Meyer and Walker (1981), and proposed using upper canopy leaflet angles as a plant water stress indicator. However, subsequent investigations showed that paraheliotropic (light avoiding or away from the sun) leaf movement may be altered by photosynthetic photon flux density (Berg and Heachelin, 1990) or nitrogen availability (Kao and Forseth, 1991) or air temperature (Kao and Forseth, 1992). The above-mentioned works have been done during the early vegetative growth of soybean.

2.2.3.3 Stomatal Frequency

Stomatal movement has been shown to be influenced not only by genetical factors but also by environmental conditions (Quizenberry, 1982). The effect of water stress on stomatal movement is important in plant adaption processes since it has been reported that low stomatal frequency (the number of stomata per unit leaf area) was correlated with water loss (Miskin *et al.*, 1972). A significant reduction in the number of stomates during the flowering stage of soybean from water stress has been reported (Ciha and

Brun, 1975). However, stomatal number was not of great concern with subsequent investigations on soybean except in one study reported by Buttery *et al.* (1993). They observed water stress to reduce transpiration, yield, and photosynthetic activity in soybean cultivars with high stomatal density more than those cultivars with low stomatal density. These authors concluded that cultivars with a low stomatal density are more tolerant to water stress than the cultivars with a high stomatal density. However, the tolerant cultivars produced less yield than the non-tolerant cultivars when water stress was not present.

2.2.4 Effects on Reproductive Organs

Shaw and Laing (1966) reported that moisture stress at the flowering stage reduced the number of pods/plant while stress that occurred during the seed-filling stage reduced the number of pods/plant and the number of seeds/pod as well as seed size (weight per seed). Subsequent studies mostly supported these results. Sionit and Kramer (1977) observed in growth chamber experiments that soybean plants water-stressed during the flowering stage produced fewer flowers, pods, and seeds than non-stressed ones. The explanation for this phenomenon was that the flowering period was shortened and flowers were aborted. They found that the greatest reduction in pod and seed number per plant occurred when drought stress was imposed in the early pod formation period. At this period the seed-size was not affected by water stress, while stress during the pod-filling

stage produced the smallest seeds but did not reduce the number of pods or the total number of seeds. They suggested that this reduction in seed size was due to the lowering in translocation and accumulation of dry matter in the seed. Ashley and Ethridge (1978) showed that seed-size was greater for plants which received initial irrigation at the pod-filling stage than from either of the irrigation treatments during the full season and the bloom period in the field. Constable and Hearn (1978) also reported significant reduction in seed yield of water-stressed soybean plants during the pod-filling stage, mainly because seeds were smaller. Momen *et al.* (1979) imposed water stress during different periods of the reproductive stage of soybean, and observed a greater reduction in seed number and seed weight from water stress during the seed-filling stage, but a relatively smaller reduction in pod number. Although the pod number was most affected by stress during the pod-filling stage, yet stress during the flowering and early podding periods also reduced pod number.

The effect of water stress on reproductive organs might be modified by other environmental factors such as photoperiod as have been reported by Cure *et al.* (1983). They showed that under short photoperiod and when stress occurred near the middle of the seed-filling stage, the stage was shortened by nine days. Drought during the seed-filling stage induced a reduction in seed yield mainly due to the reduction in seed size when the photoperiod was short. The reduction in seed number was less than the reduction in seed size (weight per seed) compared with the control. Under water stress

when the days were long, the duration of seed-filling was not affected but there was a greater reduction in pod and seed per plant and less reduction in seed size than those for short photoperiod plants. They suggested that photoperiod may directly alter photosynthetic activity of leaves as well as sink activities of seeds.

Korte *et al.* (1983a), reported that a single irrigation at the flowering stage (F) had little effect on seed yield, whereas single irrigations at pod elongation (P) or at seed enlargement (S) phases significantly enhanced seed yield relative to the non-irrigated check. Since seed yield and maturity as influenced by irrigation were highly correlated, they suggested that a large seed yield increase induced by the P and S irrigations were related to the concomitant delay in the onset of senescence. In a later publication (Korte et al., 1983b) they reported that the seed yield increase induced by the P and S irrigations were mostly due to enhanced seed size. An F irrigation caused a large reduction in 100-seed weight, but it increased the seed number. Thus, there was not much change in yield. In contrast, a S irrigation greatly enhanced 100-seed weight but did not decrease seed number which resulted in increased seed yield. The P irrigation was neutral in its effect on 100-seed weight, but an increased seed number resulted in a higher increase of seed yield than in the case for the corresponding F irrigation. The correlation of seed yield with seed number per plant ($r = +0.46$) was lower than the correlation of seed yield with 100-seed weight ($r = +0.82$). The seed yield and 100-seed weight responses to a S irrigation were related to the extension of the seed-filling period

arising from delayed maturity. The F irrigation increased seed number by enhancing pod set, because of the effects on pollination and fertilization, whereas an S irrigation increased seed number, by reducing ovule abortion within developing pods. Both factors appeared to be involved in the seed number response to a P irrigation. They reported that irrigation treatment influenced the number of total pods/plant as well as the frequency of one-, two-, three-, and four-seed pods/plant. Irrigation during the flowering stage increased one-, two-, and four-seed pods, whereas pod elongation increased only two- and three-seed pods. Irrigation during seed enlargement had no significant effect on any pod class. Therefore, irrigation-induced increases in the number of one- and four-seed pods became smaller as irrigation was delayed. Ramseur *et al.* (1984) reported that although irrigation increased yield, there was no significant difference in soybean seed yield when plants were irrigated during all of the growing season or at the beginning of the bloom period. Irrigation also increased seed growth and decreased effective filling period. Meckel *et al.* (1984), from the results of field experiments, reported that a severe water stress treatment at the seed-filling growth stage (R5) caused a reduction in the number of seed per unit area. However, the rate of individual seed growth (mg $seed^{-1}$ day^{-1}) was not affected. They observed that water stress did not reduce the supply of assimilate to the seed when the yield was reduced by 30% from severe moisture stress. They suggested that the growth rate of soybean seed was less sensitive to water stress than other plant processes. They observed that shortening in the duration of seed-filling

was one of the mechanisms responsible for reductions in yield in drought-stressed plants. Kadhem *et al.* (1985a) studied the effects of nine single irrigation treatments during the reproductive stage of 16 soybean cultivars in a three-year field experiments. They reported that a single irrigation during the middle of pod elongation (R4.7) generally resulted in the highest yield for indeterminate cultivars, whereas multiple irrigations during the flowering (R1) until the middle of seed development stage (R6.4) resulted in the highest yield for determinate cultivars. In another article, they (Kadhem *et al.* 1985b) reported that multiple irrigations (T8) applied throughout the flowering until the middle of seed development stage (R1.1 to R6.4) maximized seed number per plant relative to a non-irrigated check (T0). A sequentially timed series of seven single irrigation treatments (T1 through T7) applied at 10-day intervals (from R1.1 to R6.4) resulted in a decreased level of seed number enhancement as the single irrigation was delayed from stage R1.1 to R6.4. The multiple irrigation T8 and single irrigation treatments T1 to T3 resulted in a smaller seed size than T0. In contrast, T4, T5, T6, and T7 (coinciding with the middle of pod and seed development stages, respectively; R4.7 to R6.4) resulted in significantly larger seed size than T0. They suggested that the period from mid-pod elongation to just before the seed enlargement was a critical phase for obtaining soybean yield response to irrigation. Seed size decreased if the single irrigation occurred before stage R4, but increased if the single irrigation was applied after stage R4. The determinate cultivars were more resistant to change than other cultivars in seed number

and seed size that were induced by irrigation treatments. Villalobos-Rodriguez and Shibles (1985) in field and greenhouse experiments, studied the effect of water stress on two determinate and two indeterminate soybean cultivars. They reported that field seed yield of the determinate cultivars was more severely affected by water stress, especially when water stress was imposed at the pre-flowering stage (beginning seven days before R1), and the beginning of the full bloom stage, than was the case for the indeterminate cultivars. However, the early indeterminate cultivars showed no advantage over either determinate or semi-determinate cultivars in recovering from stress imposed at the beginning of full pod. Both field and greenhouse experiments showed that the yield component most responsive to water stress was the number of pods per node. Early maturity cultivars of both types were more affected by water stress than were the late maturing ones. The weight of 100 seeds became important in response to water stress during the rapid seed-filling (R5-6), as reported in previous investigations. Eck *et al.* (1987), in a three-year experiment, studied the effect of water stress at different growth stages of indeterminate soybeans. They reported that drought stress during the early reproductive stages (R1, R2 ,R3) did not drastically reduce soybean yield, but the crop was more sensitive to stress during the seed development and growth (R5-R7). They suggested that water stress imposed during seed-filling was more detrimental to yield than when imposed earlier. When stress was applied later, there was no compensation for stress-induced pod abortion and for reduced seed weight. Dornbos *et al.* (1989) reported

that seed yield, and seed filling were reduced significantly and linearly by drought imposed during the seed-filling stage. Seed number, and yield were reduced at a faster rate than seed weight by severe drought. Smiciklas *et al.* (1989) reported that in a determinate soybean cultivar reduction in yield due to water stress during the seed-filling stage (R5) was more than that during the flowering, full pod (R4) or full seed stage. Dornbos and Mullen (1991) reported that as stress progressed, the weight and number of seeds produced by each plant, and individual seed weight declined linearly. At optimum air temperatures, water stress reduced seed number more than individual seed weight, but at relatively high air temperatures, water stress reduced individual seed weight more than seed number. Water or air temperature stresses caused few larger seeds and more small seeds to be produced. Vieira *et al.* (1992) studied the effect of drought and defoliation stresses during the seed development in the field. They reported that there were significant reductions in yield and seed numbers and seed size following water stress. Smiciklas *et al.* (1992) studied the effect of drought and pod position on determinate soybean cultivars in field experiments. They reported that seed number, duration of seed filling period, and yield remained similar for the R2+R5 and R2+R6 multiple stress treatments when compared with corresponding single R5 or R6 drought stress treatments. Both single and multiple stresses for stage R5, and R6 reduced yield, seed number, seed weight, and duration of the seed-filling period. Interactions between the pod position and the duration of drought stress were not significant.

Figure 2.1 summarizes the morphological responses of soybean plants to water stress during the reproductive stage.

2.3 Physiological Responses:

2.3.1 Stomatal Dynamics and Leaf Water Potential

Plant water status is a function of the amount of water absorbed relative to the amount lost by transpiration via stomata. For this reason, one of the most important studies in physiological response of plant to water stress has been stomatal movement. In soybean, the effect of water stress on stomatal dynamics has been studied by several investigators, and many of them have found the effect not uniform throughout the stages of growth. Sionit and Kramer (1977) found that when water stress occurred at a later stage of growth, wilting occurred at higher water potentials (less stress) than those stressed before or at the flowering time. During the flowering stage, plants that were under water stress (leaf water potential about -23 bar), recovered their water potential after rewatering more rapidly than the plants stressed during the pod-filling stage. Jung and Scott (1980) observed the leaf water potential to reach its maximum in early morning and the minimum value at midday in the field. As the soil moisture stress increased, the leaf water potential of the non-irrigated plants decreased, earlier in the day, and recovered more slowly at night than the irrigated counterparts. The maximum and minimum seasonal leaf water potentials found under these conditions were 3.4 and -17.1 bars (-1.71 MPa), respectively. As the stress intensified, daily differences in leaf

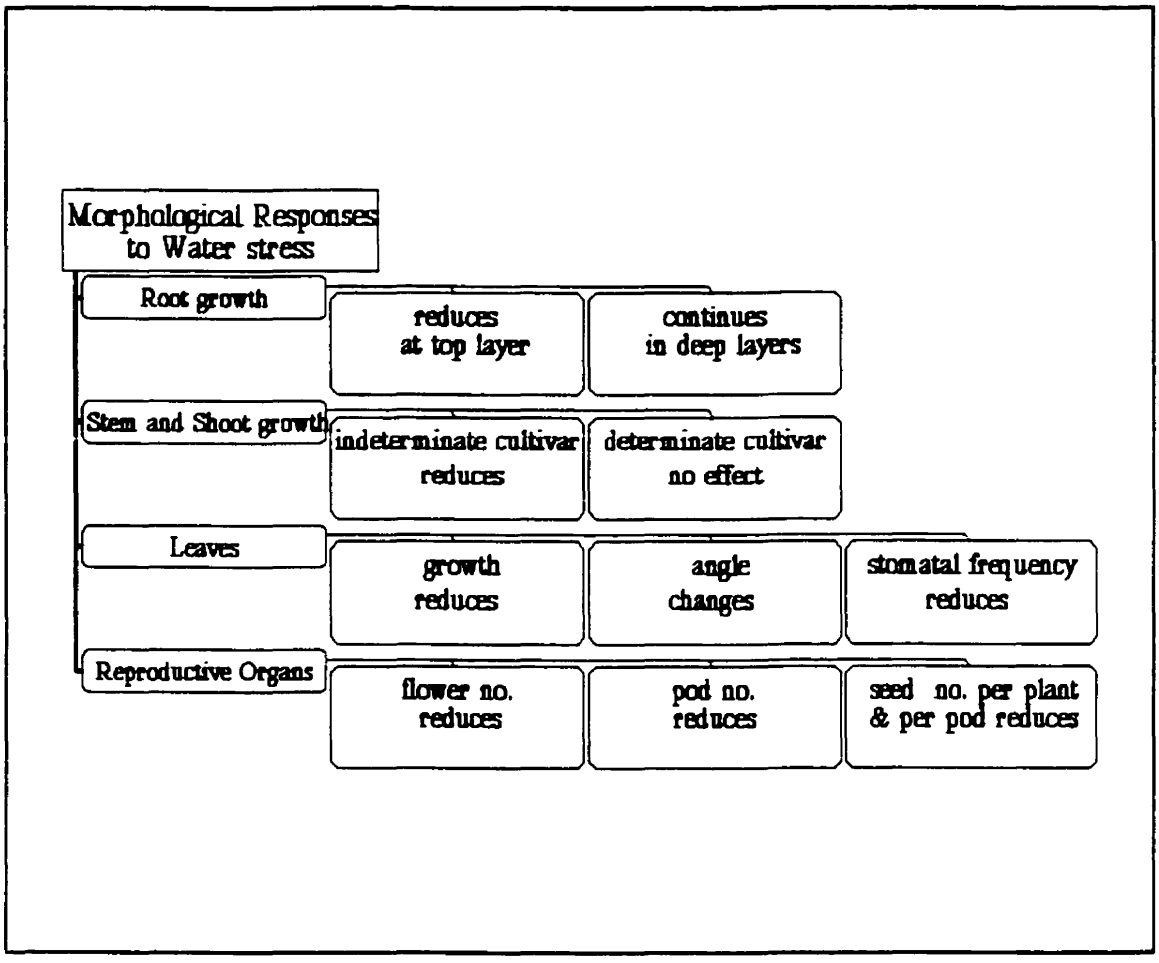

Fig 2.1 Summary of the morphological responses of soybean plants to water stress during the reproductive stage.

water potential between the irrigated and non-irrigated plants increased over the season. The maximum difference of approximately -4 bars between mid-day and mid-afternoon was found towards the end of full-size seed (R6) until the beginning of seed maturity period (R7). Stomata of non-irrigated plants in comparison with the irrigated plants were partially closed during the daylight hours and closed earlier in the afternoon. Villalobos-Rodriguez and Shibles (1985) in field and greenhouse experiments, studied the effect of water stress on two determinate and two indeterminate soybean cultivars. They reported the early morning and mid-day leaf water potentials of plants stressed at pre-flowering (beginning seven days before R1), did not decrease quite as much as did leaf water potential of plants water-stressed at R2 (full bloom) and R4 (full pod). Therefore, it seemed that water stress at pre-flowering was less severe than stressed imposed at the beginning of R2 (full bloom), and stress imposed at the beginning of R4 (full pod). Rewatering did not bring mid-day leaf water potential of stressed plants to the level of irrigated plants within one day after its application. There still were differences of 0.1 to 0.2 MPa between the two groups in the field.

2.3.2 Leaf Temperature

Transpiration of water from leaves keeps leaf temperatures relatively cooler than the ambient temperature, while stomatal closures cause reduction in transpiration which increases leaf temperature compared with the air. Therefore, the difference between air

and leaf temperature has been used as an indicator of drought stress in plants (Keener and Kircher, 1983).

A relationship between leaf temperature and leaf relative water content in soybean has been reported by Carlson *et al.* (1972). Rawson *et al.* (1978) reported that when water stress was imposed at pod-filling, leaf water potential decreased below -1.5 MPa, stomata were almost fully closed, and leaf temperature (T_L) rose to about 5 °C above air temperature. For every 0.1 MPa there was about 9% decrease in the integrated daily assimilation by leaves. Jung and Scott (1980) reported that the leaf temperature increased from a minimum in the morning to a maximum at mid-day. As the drought continued, leaf temperatures of the non-irrigated plants increased earlier in the day and decreased later in the afternoon as compared with those of the irrigated plants. The difference in leaf temperatures between non-irrigated and irrigated plants increased during the growing season. The maximum temperature difference (5.5 °C) was observed at mid-day and mid-afternoon towards the end of full-size seed (R6) stage until the beginning of the seed maturity (R7) period. Mid-day leaf temperatures of irrigated soybeans were approximately 90% of the maximum air temperatures whereas those for the non-irrigated plants were approximately the same as the maximum air temperature. Harris *et al.* (1984) reported a genotypic variation in canopy temperature of soybean in response to water stress after the flowering period. They observed that earlier maturing cultivars were cooler than the late maturing cultivars. A significant difference in leaf temperature minus

air temperature (ΔT) between water stressed and non-stressed soybean plants has been reported by Mengistu *et al.* (1987). They reported the yield of soybean to be negatively correlated with (ΔT) in two soybean varieties. They also found that for every one degree increase in the (ΔT), there was 15% reduction in soybean yield. A Stress Degree Days index (SDD) was defined by Idso *et al.* (1977) as: $SDD = A - B(\Sigma SDD_i)$ where A and B are constants and SDD_i is a daily difference between the leaf (T_L) and air temperature (T_A) at mid-afternoon. The index was used by Dornbos *et al.* (1989) as a measure of stress intensity. They observed that as SDD values during seed-filling increased, leaf resistance linearly increased, while transpiration and photosynthesis decreased linearly.

Leaf temperature might also be influenced by other environmental factors such as Mn toxicity. However, the effect on leaf temperature from water stress and Mn toxicity cannot be distinguished (Suresh *et al.*, 1989).

2.3.3 Leaf Senescence and Plant Maturity

Mayaki *et al.* (1976) reported non-irrigated soybean matured 3-4 days earlier than irrigated plants. Sionit and Kramer (1977) reported that drought stress at pod-filling shortened the length of the maturation period in determinate soybean cultivars and the leaf senescence started about one week earlier than those for the control (irrigated plants). They concluded that the leaves were more susceptible to drought at a later stage

of growth probably because the leaves were already approaching senescence. It seems that the effect of water stress on leaf senescence is dependent on the stage of plant growth during which stress occurs. For example, Cure *et al.* (1983) found that when stress occurred near the middle of seed-filling, this stage was shortened by nine days; while when stress occurred near the end of seed-filling, the stage was shortened by only two days. Korte *et al.* (1983a) observed that the maturity was delayed (2 to 6 days) by all irrigation treatments, but the delay was greater when irrigations were applied progressively later in the reproductive development stage (at pod elongation or at seed enlargement stages). Kadhem *et al.* (1985a) showed that all single irrigations postponed maturity relative to the non-irrigation treatments, but multiple irrigations resulted in the largest delay. Delays in maturity caused by irrigation supported the view that water stress at pod- and seed-fillings shortened the length of the maturation period as reported by above-mentioned researchers. Meckel *et al.* (1984) found the duration of seed-filling shortened (5 days) in some cases by water stress and apparently was more sensitive to moisture stress than the rate of dry matter accumulation. However, water stress before mid pod-filling period could not influence leaf senescence as reported by Cortes and Sinclair (1986).

Thus, the selection of early maturity soybean cultivars and early sowing dates has been proposed as one strategy for avoiding the effect of drought on soybean production in New South Wales, Australia (Rose, 1987a,b; Rose *et al.*, 1992). A similar strategy

was adopted in USA to circumvent yield reduction from drought stress during the reproductive stage (Kane and Grabau, 1992; Savoy *et al.*, 1992; Bowers, 1995; Pfeiffer *et al.*, 1995).

2.3.4 Plant Mineral Nutrient Content

Since droughts affect nutrient concentrations in soil solution, and mineralization rate, the nutrient content in plants is reduced (Nilsen and Orcutt, 1996). The effect of soil moisture on mineral nutrient content in soybean has been reviewed (de Mooy *et al.*, 1973). They concluded that environmental condition influenced soybean growth, yield, and nutrient accumulation. They stated that nutrient accumulation was dependent on the effective period of drought during the growing season and the location of the root system in the water depleted soil. Based on this review, water stress during the vegetative stage might reduce potassium (K), nitrogen (N), calcium (Ca), and phosphorus (P) concentrations in soybean. However, there has been controversy among researchers as to the validity of these results. For example, Bennie et al. (1982) observed that irrigation significantly increased N, P, K, and Cu concentrations, but decreased concentrations of Ca, Mg, and Fe in leaf, while the concentrations of these elements in seeds were not significantly affected by irrigation. Karlen *et al.* (1982a) observed that avoiding irrigation during pod-filling caused a reduction in K concentration but did not effect Ca and Mg concentrations in any plant parts. However, in another report (Karlen *et al.*, 1982b)

nonirrigation at the late vegetative or early reproductive stage of determinate soybean cultivars was found to cause a significant reduction in dry matter accumulation associated with the reductions of P, Fe, and Mg concentrations in plants. Zinc concentration was not affected by water stress. An increase in N concentration in soybean plants from irrigation has been reported by Hunt *et al.* (1983), and Hobbsets and Muendel (1983).

A three-year field experiment was designed by Batchelor *et al.* (1984) to study the effect of irrigation on leaf mineral nutrient concentrations in determinate soybean cultivars. They reported that during a wet year, irrigation did not influence concentrations of K, N, Ca, and Mg in leaves. In contrast, in the dry years, irrigation increased concentration of these elements in leaves. Smiciklas *et al.* (1989) studied the effect of withholding water during flowering (R2), full pod (R4), seed formation (R5), and full seed (R6) on mineral nutrient contents of seeds in a determinate soybean cultivar. The results indicated that water stress during the seed formation (R5), reduced yield more than any other treatments and was the only treatment that reduced the concentration of Ca deposited in the seeds compared with the non-stressed plants.

In experiments by Vasilas (1989), concentrations of macro- and micro- nutrients in different plant parts were affected by sowing dates of soybean cultivars and even the weather condition during the pod and seed-filling period. Kaspar *et al.* (1989) reported that during the seed-filling stage drying soil surface reduced K concentrations in shoot and pod more than when it occurred during flowering. Reduction in N concentrations and

total N accumulation from droughts also have been reported by De Vries *et al.* (1989).

The disagreement among investigators on the effect of water stress during reproductive stage on mineral nutrient concentration in soybean plant could be due to variation in root growth during this stage in different soybean cultivars as observed by Kaspar *et al.* (1978). A genotypical difference in uptake, translocation, accumulation and use of mineral nutrient (Clark, 1983) also could be another reason.

2.3.5 Osmotic Adjustment

Generally, through osmotic adjustments plants reduce their osmotic potential by accumulating an active solute. This mechanism enables plants to maintain cell turgor and other essential processes such as photosynthesis when soil water potential is relatively low (Hsiao *et al.*, 1976; Morgan, 1984). Therefore, it was proposed that the osmotic adjustment was a crucial mechanism in plant adaptation to water stress (Hsiao *et al.*, 1976).

Osmotic adjustment in soybean has been studied extensively. However, the subject still remains controversial as a few researchers reported evidence of osmotic adjustments in soybean plants while others could not find any such mechanism. Meyer and Boyer (1972) observed this mechanism in hypocotyls in soybean seedlings under low water potential. In contrast, Sionit and Kramer (1977) did not observe this mechanism during the pod development stage while they reported that during the vegetative growth osmotic

adjustment did occur. Turner *et al.* (1978) reported that under field conditions, water stress during grain-filling had no effect on the physiological processes in soybean, until the leaf water potential decreased to below -1.5 MPa. This reduction caused stomatal closure, decreased photosynthesis, reduced starch accumulation in leaves of two soybean cultivars. So they suggested that no osmotic adjustment occurred. Significant changes in osmotic potential of soybean under water stress condition at the mid pod-filling period was reported by Cortes and Sinclair (1987). A similar observation during the vegetative stage has been reported by Minguez and Sau (1989). They also found that osmotic potential was lower in plants which received nitrate fertilizer.

The conflicting results of the above investigations could probably be attributed to the genetic variation which was reported in other plant species (Morgan, 1980; Shackel *et al.*, 1982; Quisenberry et al., 1984). In soybean, Sloane *et al.* (1990) reported that during the pod-filling period relative drought tolerant genotypes of Plant Introduction PI 416937 maintained lower levels of solute potential and higher level of pressure and water content than non-tolerant soybean cultivars.

2.3.6 Effect on Plant Hormones

Samet *et al.* (1984) detected abscisic acid (ABA) in soybean plants from the vegetative stage to the early pod-filling stage and showed the accumulation of this hormone correlated directly to decreases in leaf water potential. They observed a drought

resistant soybean cultivar accumulated more ABA than a non-resistant cultivar. Davies *et al.* (1986) summarized the effect of water stress on the main class of plant hormones. They reported that droughts caused increasing ABA content, IAA oxidase activity, and ethylene but reduced cytokinin transport, auxin transport, and gibberellic acid (GA) activity. However, Morgan *et al.* (1990) showed that ethylene production in leaves of cotton and beans did not increase even by severe water stress.

2.3.7 Effect at the Level of Cell Membrane

It has been shown that the ultrastructures of cell membrane could be changed by water stress (Fellows and Boyer, 1978). Therefore, it has been suggested that membrane integrity has a role in the resistance of plants to water stress (Bewley, 1979). It was reported that water stress in non-tolerant soybean, the membrane integrity and cell membrane stability were reduced compared with tolerant cultivars (Senaratna and McKersie, 1983; Krishnamani *et al.*, 1984). Premachandra *et al.* (1990) reported that the percentage of injury in the polyethylene glycol (PEG) test (a test of cell membrane stability for drought tolerance by using a solution of PEG) had negative correlation with Ca concentration in leaf tissue and cell sap of soybean.

2.3.8 Effect on Photosynthesis

A reduction in the rate of photosynthesis of soybean leaves had been observed

when leaf water potential was reduced to -1.0 to -1.2 MPa (Boyer 1970, 1980; Cure *et al.*, 1983). The maximum photosynthetic rate was dependent on CO_2 concentration, water status, temperature, leaf age, interception of photosynthetically active radiation (PAR) by the total leaf area, and N level in leaves (Raper and Kramer, 1987). Water stress can influence the maximum rate of photosynthesis indirectly by affecting all or some of the above factors or by directly inhibiting biochemical processes of photosynthesis (Lawlor, 1995). For example, CO_2 per unit leaf area depended on stomatal movement, and leaf area and both of these might be influenced by water stress as discussed earlier. In addition, water stress increases leaf temperature and causes leaf senescence during the reproductive stage. On the other hand, PAR is influenced by leaf angles which, in turn, might be changed by leaf movements from water stress as discussed earlier. The N concentration in soybean leaves may also by reduced from droughts as has been reported by De Vries *et al.* (1989).

2.3.9 Effect on N Metabolism

Nitrate (NO_3) metabolism and symbiotic N fixation provide two different input systems for soybean. Both of these systems may be affected by water stress. Droughts can reduce nitrate metabolism through NO_3 uptake (Harper, 1974, 1981) or by decreasing the synthesis of nitrate reductase enzyme (NR) (Manam *et al.*, 1977; Hanson and Hitz, 1982) or by inactivating the enzyme (Kaiser and Förster, 1989).

N fixation might be reduced under water stress due to its direct effect on nitrogenous enzymes (Finn and Brun, 1980; Bennett and Albrecht, 1984) or through an indirect effect by inhibiting the energy required for N fixation which is provided by photosynthesis (Huang *et al.*, 1975). However, it has been shown that nitrogenase activity is much more sensitive to reduction in nodule water potential than to the reduction in photosynthesis (Albrecht *et al.*, 1984).

2.3.10 Effects on Seed Quality

Sionit and Kramer (1977) observed that water stress at any period of the reproductive stage did not affect the oil or protein content of the seeds. Karlen *et. al* (1982a) reported that in the dry year of a two-year field experiment, irrigation during the early podding period influenced pod growth, but did not affect on dry matter, K, Ca, and Mg concentrations of soybean pod. Egli *et al.* (1984) from field experiments, reported that water stress during seed-filling (R5), although reduced plant weight, yield, and seed size, it had no significant effect on the concentrations of N in the mature seeds, and suggested that the N metabolism in the developing seed was resistant to water stress. From a two-year field experiment, Batchelor *et. al* (1984) reported that element concentrations in stems and leaves that were significantly affected by irrigation during different periods in the reproductive stage, also were reflected in the pods and seeds.

Water stress during seed-filling can reduce seed germination and vigor of the seeds from soybean plants (Yaklich, 1984; Dornbos *et al.*, 1989; Smiciklas *et al.*, 1989). Smiciklas *et al.* (1989) indicated that water stress during the seed formation (R5), was the only treatment that reduced the concentration of Ca in the seed as compared with the non-stressed plants and that was correlated with reduction in seed germination. Westgate and Grant (1989) from greenhouse experiments reported that when water was withheld during seed-filling (R5.5), water potential, osmotic potential, and turgor of seed tissues were not significantly different from those of the controls. They concluded that the water status of developing seeds of soybean was not altered by short-term water deficits. It was important for seed growth rate exhibited by soybean under dry conditions to be conserved. Since similar observations have been made in maize, wheat, barley, and rice, they suggested that the seed water status was independent of the mother tissues and was a common phenomenon of all seeds. Westgate *et al.* (1989) showed that during the soybean seed development stage, when canopy photosynthesis completely inhibited by water stress, reserve assimilates were mobilized from leaf, stem, and pericarp tissues to support the seed growth rate. They observed cotyledon sucrose content and sucrose concentration in cotyledon apoplast of seeds from water-deficient plants decreased by approximately 50%. Embryos from seeds of stressed plants accumulated sucrose nearly twice as fast as the controls. They suggested that the decrease in apoplast sucrose concentration within the first three days after water was withheld had little effect on seed

growth rate because the embryos continued to accumulate sucrose rapidly even at the lower apoplast concentration. Thus, the water deficit had a dramatic effect on the steady-state level of sucrose supplied to the outer layer of cotyledonary cells as well as the total pool of sucrose within the cotyledons. These results indicated that both the maternal and zygotic tissues in soybean were affected directly by plant water deficits during seed-filling. Therefore, both the mobilization of reserves from vegetative tissues and rapid uptake of assimilates by the reproductive tissues at low water potential acted in concert to maintain seed growth. Egli (1990), who studied the relationships between net water uptake by soybean seed and dry matter accumulation in an in vitro system, suggested that dry matter accumulation by soybean seeds can continue only as long as there was a net uptake of water to drive cell expansion. In the absence of a net water uptake, continued dry matter accumulation caused desiccation which triggered maturation.

Figure 2.2 shows the summary of the physiological responses of soybean to water stress.

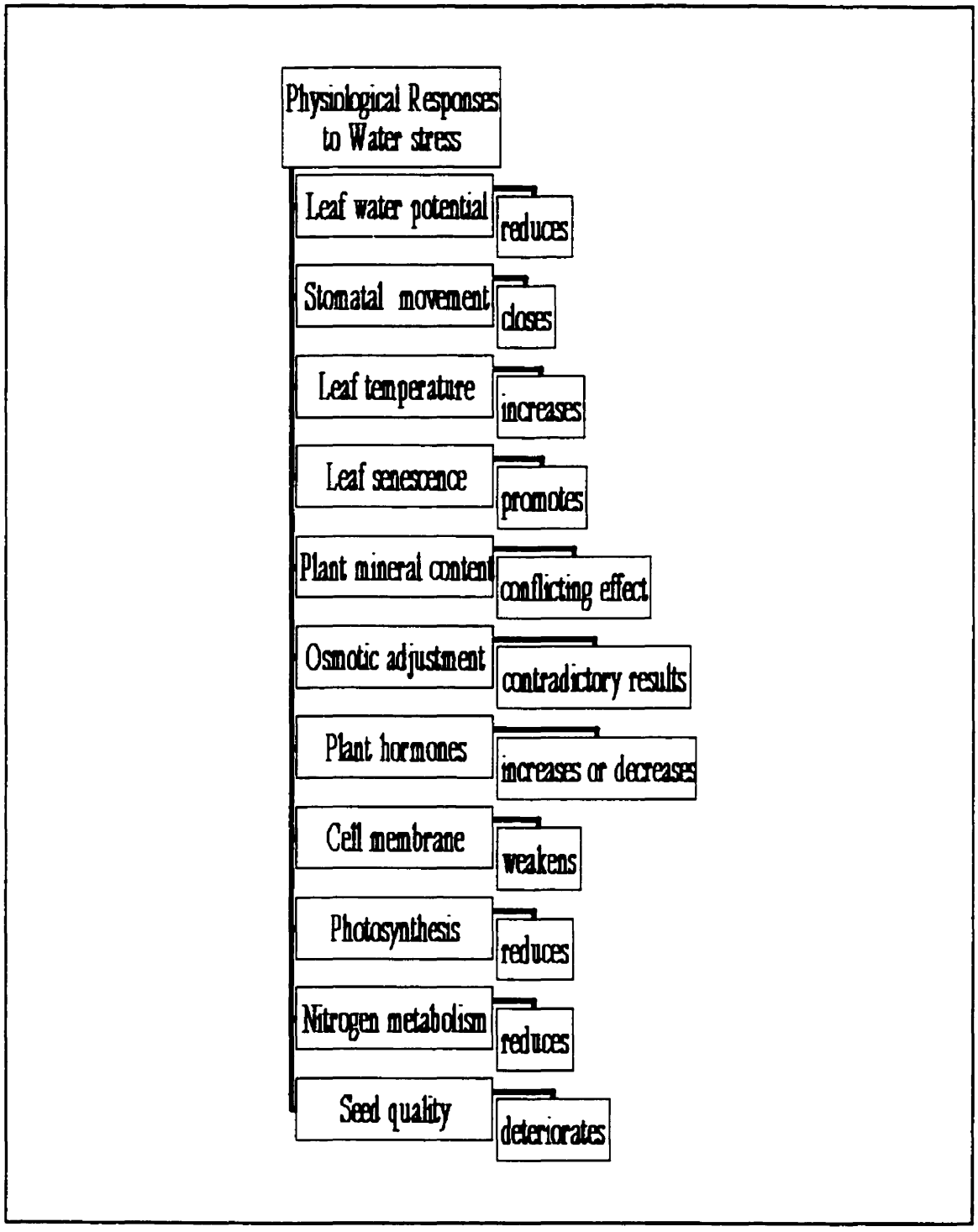

Fig. 2.2 Summary of the physiological responses of soybean plants to water stress during the reproductive stage.

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PRELUDE TO CHAPTER 3

Calcium is known to play an important role in the bodily function such as bone formation, nerve impulse transmission, the action potential, blood clot formation, membrane integrity in animal and human cells and tissues. Therefore, research on Ca as a determinant of human health has been both intensive and extensive. As a logical extension on the basis of the uniformity of life, it has been realized that Ca is also a critical element in determining structure and function of plant cells and tissues. Of particular importance now to Ca translocation in plants is the influence of environmental changes, especially, soil moisture status. Although there have been studies on Ca translocation and uptake on several species of plants, this thesis will consider soybean because uptake and translocation may not only be species-specific but also genotype- and cultivar-specific.

This chapter describes the first experiment of this thesis designed to study the effect of water stress during the seed-filling stage on Ca concentration and distribution in soybean plants. The rationale for this was that an occurrence of a drought stress during the reproductive stage of soybean could adversely affect seed quantity and quality, and seed germination. This thesis will focus on only the seed-filling stage of soybean growth with reference to Ca uptake and translocation. The results of the first experiment were published in 1995 (Sorooshzadeh, A., Arnold, N.P. and Barthakur, N.N. 1995. Calcium

distribution in soybean during seed-filling in relation to moisture stress. Journal of Plant Nutrition 18:515-522) which clearly showed that water stress during this stage of soybean growth can reduce Ca concentration in leaf and other plant parts. A unique combination of the radiotracer methodology and an infusion technique was used to investigate Ca translocation as a function of water stress during the seed-filling stage of soybean growth.

CHAPTER 3

CALCIUM DISTRIBUTION IN SOYBEAN DURING SEED-FILLING IN RELATION TO MOISTURE STRESS

ABSTRACT

The distribution of calcium (Ca) within stem, leaves, and seeds of soybean was studied with an infused radioactive $^{45}CaCl_2$ solution during seed-filling under well-watered and water stressed regimes in a greenhouse. The kinetics of infusion volume showed a quadratic reduction in absorption which was reduced to zero on the sixth day for non-irrigated plants. The concentration of ^{45}Ca increased quadratically from the point of injection towards the apex for both water status and plant parts. The difference in concentration of ^{45}Ca between irrigated and non-irrigated plants was significant ($P < 0.05$) and concentrations attained maximum values at the sixth node from the plant base. Seeds contained considerably less ^{45}Ca than either stem or leaves.

3.1 INTRODUCTION

3.1.1 Role of Calcium (Ca) in Plant Nutrition:

Calcium (Ca) is the sixth largest nutrient and its concentration in plants vary between 0.1 and 5.0 % on a dry weight basis, depending on the growing condition, plant species, and plant organ. Ca is recognized as an essential nutrient for plant growth and classified as a macro-nutrient (Streeter and Barta, 1984; Marschner, 1995). It is known that Ca influences diverse plant processes of growth, differentiation, hormone regulation, enzyme activation, ion balance, senescence, abscission, membrane permeability, cell wall and membrane stabilization, cell extension, root gravitropic response, and mitosis. A new function of Ca as a second messenger in the signaling process involved in responses to any changes in the environmental condition have been recognized during last 17 years and attracted attentions of many plant physiologists and biologists (Marschner, 1995). Thus, Ca-deficiency influences different metabolic activities in plants which can reduce growth and crop yield. In the young leaves, Ca-deficiency causes deformation and chlorosis (Mengel and Kirkby, 1978) and in fruits and roots, 35 Ca-related disorders have been listed (Shear, 1975). Examples include bitter pit in apples, and blossom-end rot in pepper and tomato. In soybean, Ca-deficiency is manifested as leaf curl, vein and root browning (Chapman, 1966), and reduces seed germinability (Keiser and Mullen, 1993). Ca concentration in most soil and growing media (20-100 mg/Kg) are sufficient

to meet Ca demand of plants (10-30 mg/Kg) (Jacques *et al.*, 1990). Therefore, these disorders are mostly due to insufficient uptake, translocation, and distribution of Ca rather than its amount present in the soil (Hanger, 1979; Kirkby and Pilbeam, 1984). Studies of translocation and distribution of Ca are very important in understanding the Ca-deficient disorders in terms of plant physiology in order to take preventative measures to avoid these disorders.

3.1.2 Uptake and Translocation of Ca in Plant:

In general, it seems that Ca absorption from the soil solution occurs passively, via the apical root zones of plants and then it moves with the water through the apoplasmic pathway and reach various plant organs with the transpiration stream through the xylem vessels (Roux and Slocum, 1982). Since Ca movement in phloem is very limited, the xylem pathway is the major source of Ca for plant growth, and a continual Ca-uptake by root apical zones and its translocation are essential to meet the demand for Ca by different plant parts especially young tissues. Therefore, not only any reduction in Ca-uptake, but also any reduction in the transpiration rate can cause a Ca-deficiency in the growing tissues. A reduction in Ca translocation within the plant xylem may occur during soil water stress and other physio-chemical changes in the soil. Relationships between the transpiration stream versus Ca-uptake, translocation and distribution have been studied for some plant species such as tomato, apple, potato, clover, and groundnut

in attempting to avoid Ca-deficiency disorders (Marschner, 1995). However, Ca demand, uptake, translocation and use are dependent on species and cultivars (Jacques *et al.*, 1990; Clark, 1983), therefore, the relationship between plant water status and Ca-uptake and movement need be investigated for the specific cultivar of interest.

3.1.3 Ca and Water Stress Relationship in Soybean:

Studies on Ca nutrition and metabolism in soybean have become increasingly important since the cultivation has now been extended to semi-arid regions of the world, where water stress is the first environmental factor that limits crop production. Water stress during the seed-filling stage of soybean not only reduce soybean seed yield, but also it may reduce germinability of soybean seeds due to a reduction in seed Ca concentration under drought stress conditions (Smiciklas *et al.*, 1989). Thus, a knowledge on the distribution pattern of Ca in different tissues of soybean during the seed-filling stage under water stress would contribute towards understanding Ca-deficiency problems in soybean. These studies are also necessary for the cultivation of soybean, particularly, in semi-arid climates. The literature contains conflicting results on the effect of water stress during the seed-filling stage on Ca concentration and distribution in the soybean plant. Karlen *et al.* (1982) reported that Ca concentration in any soybean plant parts was not influenced by water stress during the pod-filling stage. In spite of this report, Bathchelor *et al.* (1984) observed that Ca concentrations in leaves, stems, pods, and

seeds were reduced in soybean plants which were subjected to water stress during the reproductive stage. This could be due to genetic variation in Ca demand, uptake, translocation and use (Jacques *et al.*, 1990; Clark, 1983) or due to the genetic variation in the root growth during the reproductive stage (Kaspar *et. al.*, 1978). Since Ca is absorbed by young roots, its uptake and translocation are affected by the extent of root growth and soil conditions. A stem infusion technique developed by Grabau *et al.* (1986) could be used to study Ca translocation and distribution without the direct participation of the root system. The stem infusion technique has been extended to several plant species as a tool for this type of investigations (Boyle *et al.*,1991; Ma and Smith 1992). In the present experiment, a radioactive calcium was used in conjunction with the infusion technique for the first time to monitor Ca distribution in soybean plants exposed to water stress.

3.1.4 Objectives:

The main objective of this experiment was to study the effect of water stress during the seed-filling stage on Ca concentration and distribution in soybean plants. Another objective of this paper was to determine the applicability of a combined technique of radiolabelled Ca and its infusion directly into the tissue of soybean plants.

3.2 MATERIALS AND METHODS

3.2.1 Cultivar and Culture:

Soybean (*Glycine max* L. Merr.) seeds of determinate cultivar Chuusei kuro daizu (PI 416835) were obtained from USDA Soybean Germplasm Collection, Urbana, Illinois. Seeds were sown in 20-cm diameter plastic pots and experiments were conducted in a greenhouse. The substrate and plant growth conditions used were similar to those recommended elsewhere (Schussler *et al.*, 1984). Twenty g of commercial lime was added per pot. The pots were given 250 ml of 20-20-20 (N-P-K) fertilizer twice weekly. Treatments were arranged in a complete random design and replicated three times. All plants were treated similarly until the seed-filling period, phenologically classified as R-5 (Fehr *et al.*, 1971), was attained.

3.2.2 Imposition of Stress and Infusion of Radiocalcium:

At the beginning of R-5, one group of plants was irrigated daily, while the other received no water for one week. One week later leaf temperatures were measured with a portable leaf chamber analyzer (Licor, model 6100, Lincoln, NE). The difference between the leaf and the air temperatures was used as an indicator of water stress.

All plants were simultaneously injected with a radioactive Ca solution by using a modified stem infusion technique (Ma and Smith, 1992). Plastic syringes (5 ml) were

filled with 4 ml of deionized water and were suspended 1.5 m above the plant canopy. One ml of radioactive solution containing $^{45}CaCl_2$ in water (specific activity = 324.12 MBq mg^{-1} Ca) was added at the top of the perfusion system. The perfusion system was made leak proof and the solution was allowed to flow through a plastic tubing to a 26-gauge hypodermic needle. The needle was then inserted at an upward angle of 50° between the third and the fourth nodes from the bottom. Figure 3.1 shows a sketch of the experimental setup. The radioactive material with 99% radionuclide purity was purchased from ICN Biomedicals, Inc., Irvine, California. Silicone was applied lightly at the injection point to prevent leakage.

3.2.3 Sample Preparation and Radioactivity Counting:

The amount of radioactivity in stem, seeds, and leaves at nodes below and above the injection point was determined by using a liquid scintillation spectrometer (LKB, 1215 Rackbeta, Finland). Tissue samples were digested in a 0.75 ml solution of a 2:1 mixture by volume of 60% perchloric acid and 30% hydrogen peroxide in an oven at 65 °C for two hours. Interference from chemiluminescence (CL) was prevented by adjusting the spectrometer discriminators and also by storing the samples for 24 h at 30°C in the dark before counting. ^{45}Ca emits beta-rays, and the maximum energy of emission is 0.257 MeV (1 eV = 1.6 x 10^{-19}J). The mean energy of the beta-particles from ^{45}Ca is approximately 0.086 MeV, and the half-life of 163 days. Because of the relatively low

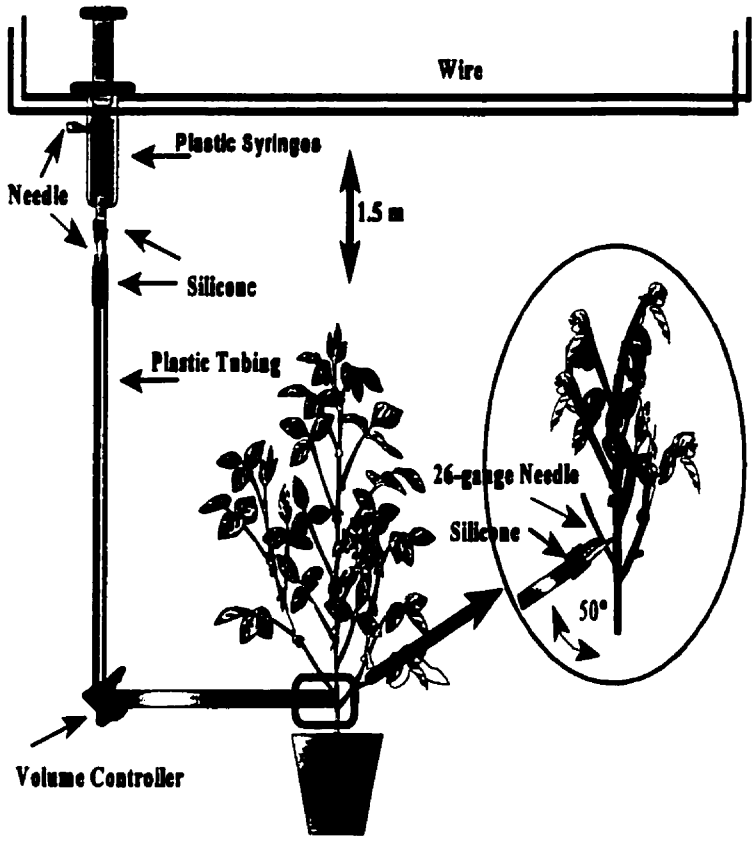

Fig. 3.1 The experimental arrangement.

energy, ^{45}Ca can be classified as a soft beta-emitter, and the pulses produced from it are similar in amplitude to the CL pulses. For this reason, it was important to prevent CL pulses from counting so that only true counts were recorded. CL arises mainly from the presence of hydrogen peroxide - a highly oxidizing agent, when it reacts with other compounds present in the scintillation cocktail including the sample. Horrocks (1974) listed several methods for eliminating CL, one of which is to store samples to allow for decay of CL (usually more rapid decay at elevated temperatures) before counting. We adopted this method for reducing CL in the samples.

The counts per minute (CPM) readings were converted to the disintegration per minute (DPM) by using a standard quench curve, and the results were expressed in the specific activity unit of DPM mg^{-1} fresh weight of the samples.

3.3 RESULTS AND DISCUSSION

3.3.1 Stress Indicator:

The average difference between the leaf and the air temperatures of the irrigated plants was - 1.2 °C, while for the non-irrigated plants the average difference was + 2.1 °C at the end of the experimental period. Similar temperature differences to produce stress in soybean were also reported (Harris *et al.*, 1984). The difference between air and leaf temperature has been used as an indicator of drought stress in plants. Plants maintain

its leaf temperatures relatively cooler than the ambient temperature through transpiration. While reduction in the transpiration rate due to stomatal closures under water stress increases leaf temperature compared with the air (Keener and Kircher, 1983). Thus, higher leaf temperatures of non-irrigated soybean plants indicate that avoiding irrigation for one week was sufficient to reduce transpiration and induce water stress in soybean plant.

3.3.2 Infusion Volume:

The infusion volume input of $^{45}CaCl_2$ solution to the plant decreased with time for both water treatment regimes (Fig. 3.2). Although there was no significant difference in absorption between stressed and control (irrigated) plants on the first day, the difference was significant ($P < 0.05$) for the remaining days of the experiments. A polynomial regression analysis showed the daily absorption volume to decrease quadratically with time for both non-irrigated and irrigated plants ($R^2 \geq 0.98$). The reduction in the infusion volumes during the experiments also have been reported in the other investigation (Boyle *et al.*, 1991, Ma *et al.*, 1994; Zhou and Smith, 1996).

The kinetics of the reduction in infusion volume of irrigated plants cannot be explained with certainty. However, the reduction in absorption could be attributed to the resistance offered by the plugging of vessels following the local vascular disruption. The resistance also may be due to damage and death of stem tissue at the infusion site. A

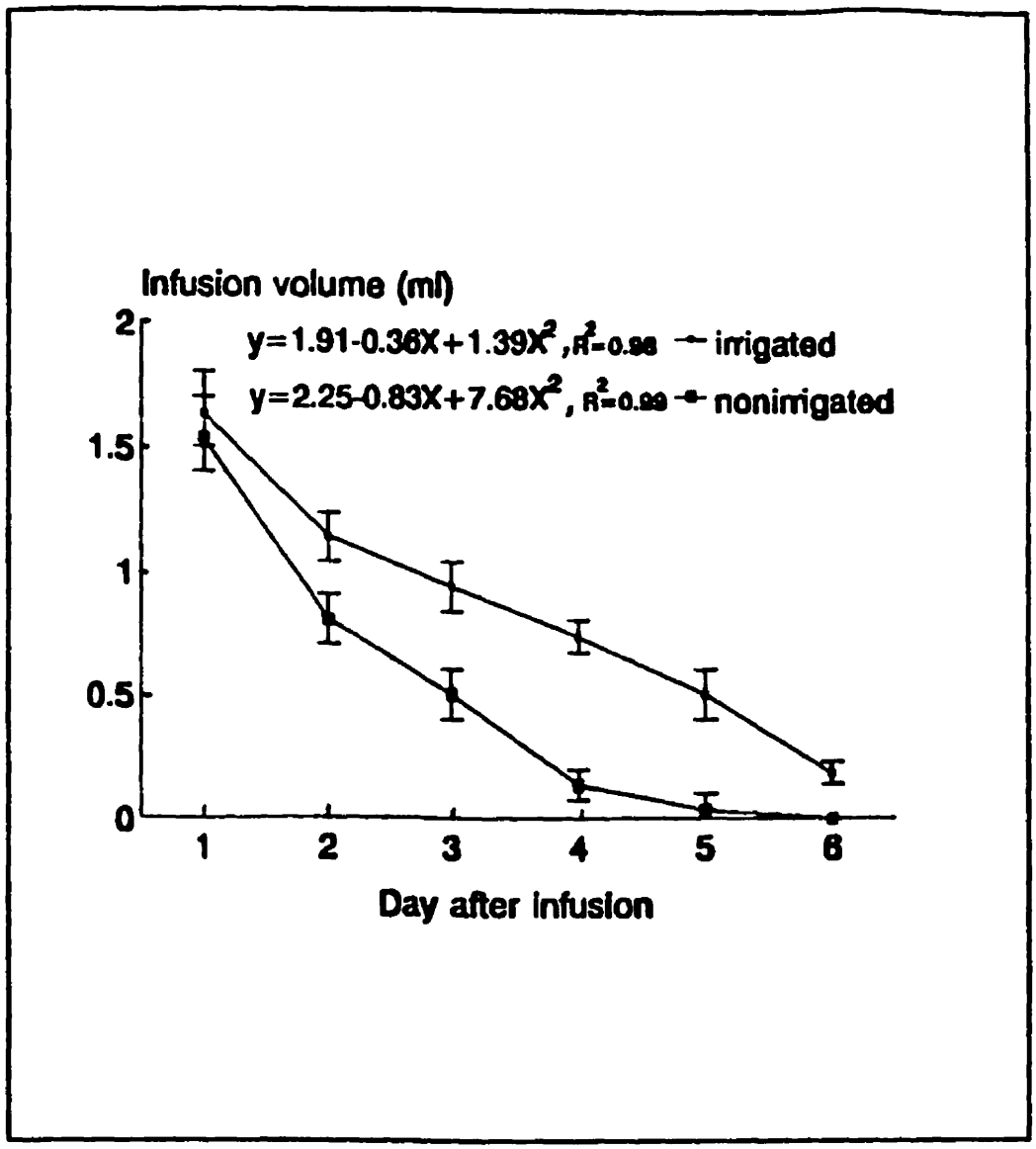

Figure 3.2 Infusion volume of irrigated versus non-irrigated plants. Error bars indicate standard deviation.

greater reduction in the infusion volume in non-irrigated plants compared with the control is probably due to additional resistance to absorption. This may have resulted from a reduction in water potential, which caused cavitation (Boyle *et al.*, 1991) due to gas bubble formation in the stem and leaf xylem (Davies, 1986). Since continuous non-irrigation reduces water potential in the xylem, xylem pressure becomes progressively negative, and the cohesion in the water columns break down and air enters into the xylem producing small gas bubbles. The flow of water can block when these gas bubbles coalesce into a larger bubble (Nilson and Orcutt, 1996). The maximum difference in absorption volume between irrigated and non-irrigated plants (0.6 ml) was observed on the fourth day of infusion. The infusion volume for the non-irrigated plants was reduced to zero on the sixth day of application. These results showed that although the infusion volume was dependent on the resistance in the vessels, however it might be influenced by the transpiration stream which agreed with past findings (Boyle *et al.*, 1991).

3.3.3 Ca Distribution:

The distribution of ^{45}Ca within stems of stressed and control plants (Fig. 3.3 and Fig. 3.4) showed a quadratic nodal increment ($R^2 \geq 0.97$) from the point of injection towards the apex. A small amount of ^{45}Ca was also detected below the injection point for irrigated plants which could be attributed to passive diffusion of the cation. No significant amount of ^{45}Ca was detected below the injection point of stressed plants.

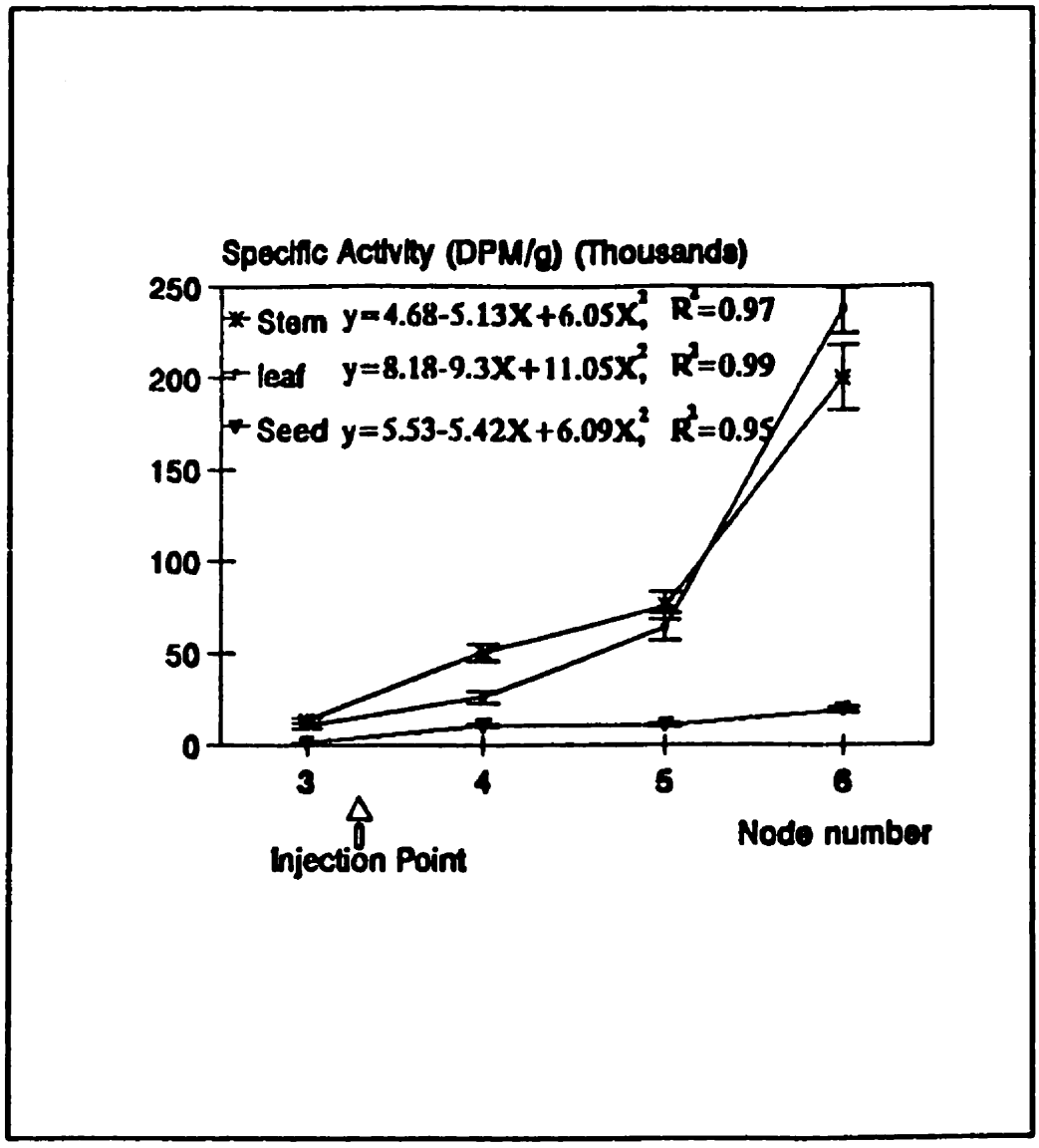

Figure 3.3 Distribution of ^{45}Ca in irrigated soybean during seed-filling. Error bars indicate standard deviation.

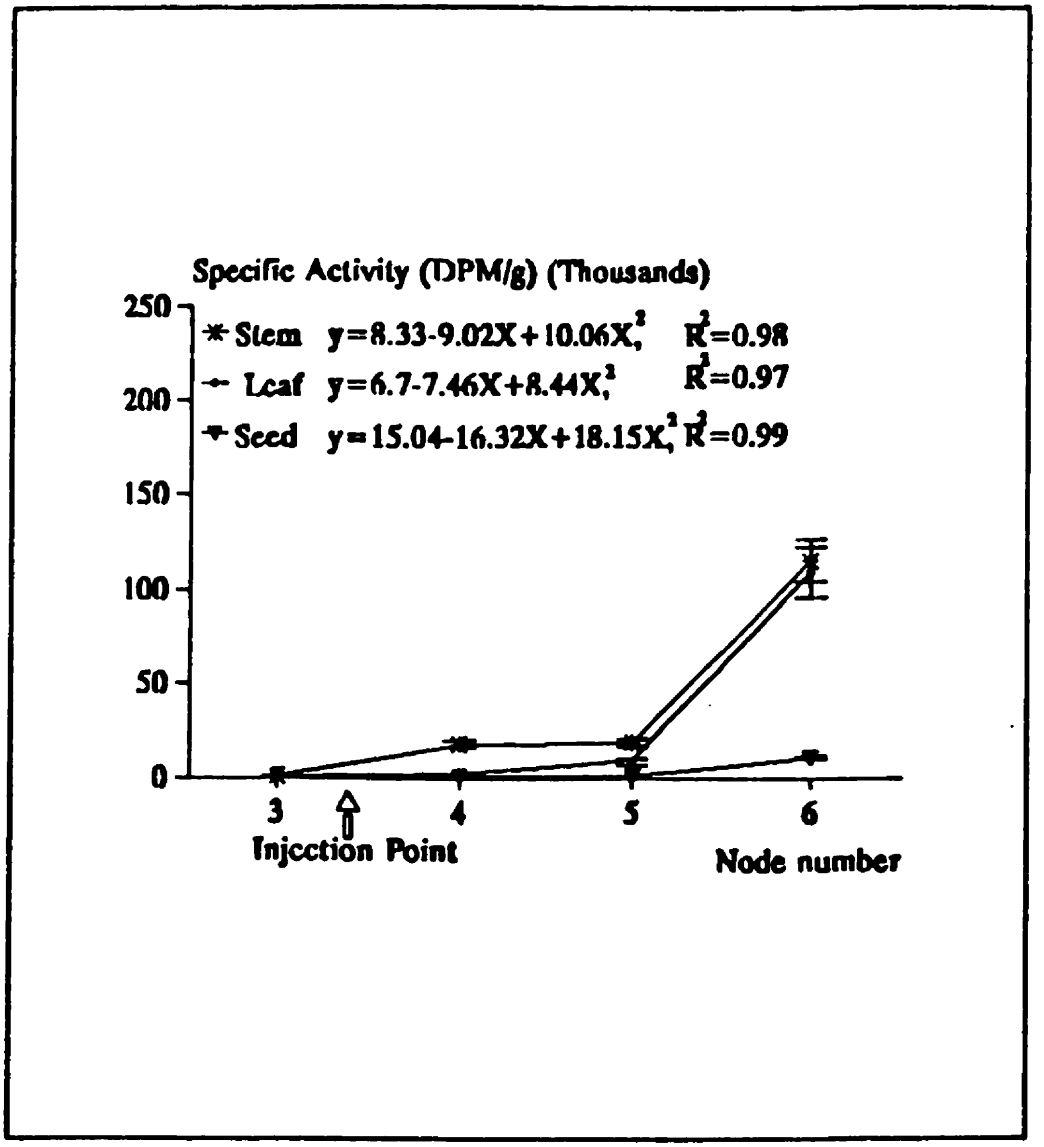

Figure 3.4 Distribution of ^{45}Ca in non-irrigated soybean during seed-filling.

Error bars indicate standard deviation.

Water stress significantly reduced the ^{45}Ca activity (DPM g^{-1} of fresh weight) but may not have changed the Ca pathway within the plant. By using the infusion technique, ^{45}Ca directly enters into the transpiration stream, thus the ^{45}Ca activities in irrigated and non-irrigated plants were not directly influenced by the root system. The reduction in ^{45}Ca activity in non-irrigated plants in comparison with irrigated plants was due to less absorption of the infusion volume which was resulting from water stress as was discussed earlier. Thus, the results of the present experiment were in agreement with those of Batchelor *et al.*, (1984) and Smiciklas *et al.* (1989), who reported that water stress during the seed-filling stage reduce Ca content in soybean plants.

Maximum accumulation of ^{45}Ca in both water treatments occurred at node number six. Although Ca movement in the xylem is mainly controlled by the transpiration stream, Ca demand in different plant tissues also might be influenced Ca translocation and distribution within the plant. This demand is dependent on cation exchange capacity (CEC) (free carboxyl group of polygalacturonic acid) at the end of xylem vessels and apoplasm of different plant tissues (Kirkby and Pilbeam, 1984). The Ca demand could arise due to (1) the formation of new binding sites from cell division in young tissues, and/or by (2) the removal of Ca from exchange sites within the xylem of the individual parts of the plants via the metabolic activity (Marschner, 1995). Thus, the various tissues can accumulate Ca in proportion to their metabolic utilization (Bell *et al.*, 1963). This might be an important factor in the accumulation of Ca in the upper part of the stems in

both stressed and non-stressed plants. The curvilinear response of ^{45}Ca distribution with plant height could be due to the high Ca demand in the actively growing tissues of upper nodes. Cell division has been considered as a driving force for Ca movement by increasing demand for Ca which must be transported to the growing sites (Marschner, 1983). Thus, Ca moved upward because the young foliage at the top were characterized by a higher rate of cell division than the matured leaves near the plant base.

The distribution pattern of ^{45}Ca within leaves at different nodes (Fig. 3.3 and Fig. 3.4) also supported this interpretation. Leaf specific activity of ^{45}Ca was significantly ($P < 0.05$) higher at the sixth node than the stem for irrigated plants. The possible explanation might be that the leaves at this node were relatively small and required more Ca for growth. For the non-irrigated plants, the specific activity difference of ^{45}Ca between leaf and stem was not significant at the sixth node probably because of the adverse effect on growth from stress. The leaf ^{45}Ca distribution pattern was similar to that of the stem.

The seed ^{45}Ca distribution at different nodes for both water regimes was similar to that of stem and leaves. The higher concentration of ^{45}Ca in the seeds from the upper part of the plant rather than the lower can also be explained by the greater need for the nutrient at actively growing sites which are located near the top.

A comparison between the distribution patterns of ^{45}Ca in stem, leaves, and seeds (Fig. 3.3 and Fig. 3.4) showed a relatively high concentration of the nutrient in the stem

and leaves for irrigated relative to stressed plants. Seeds contained considerably less amounts of Ca compared with stem and leaves.

3.4 CONCLUSIONS:

The Ca distribution pattern in soybean during the seed-filling stage demonstrated significantly reduced concentration of the nutrient in stem, leaves, and seeds for moderately water stressed compared with irrigated plants. Thus, Ca deficiency may be anticipated in soybean cultivation in semi-arid regions. The radiotracer technique combined with stem infusion provided a suitable methodology to monitor Ca movement in plants at any growth stage.

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A STATEMENT CONNECTING CHAPTER 3 AND CHAPTER 4

The common themes throughout this thesis are: the plant species of the research project i.e., soybean, and focus efforts on how environmental changes during the seed-filling stage affect soybean physiology. Chapter 3 considered Ca translocation and distribution in soybean plant when water stress occurred during the seed-filling stage, largely bypassing the complex action of the root system. Chapter 4 deals with the relationship between photoperiod and water stress on the concentration and distribution of Ca within the soybean plant without bypassing the root system during the seed-filling stage of soybean. The work described in Chapter 4 was undertaken as a result of the literature review (Chapter 2) which showed that water stress during the seed-filling stage not only reduced Ca concentration in soybean plants, but might have promoted soybean leaf senescence. This Chapter provided the materials for the manuscript entitled, "Calcium distribution in response to photoperiod and water stress" by A. Sorooshzadeh and N.N. Barthakur submitted for publication.

CHAPTER 4

CALCIUM DISTRIBUTION IN RESPONSE TO PHOTOPERIOD AND WATER STRESS DURING THE SEED-FILLING STAGE IN SOYBEAN

SUBJECT CATEGORY: ENVIRONMENTAL STRESS PHYSIOLOGY

ADDITIONAL INDEX WORDS. *Glycine max*, radiocalcium

ABSTRACT

The effects of a long day (LD, 16 h) and a short day (SD, 12h) photoperiod with two levels of water stress (SL) (stress (ST) and no stress (NS)) on the distribution of radiocalcium (^{45}Ca) in plant organs (PO) of leaves, petioles, and stem at different node number (NN) of soybean were studied during the seed-filling stage. The univariate and Manova analyses showed the main effects of photoperiod (PP), SL, and PO to be highly significant ($P < 0.001$) on Ca distribution. The long PP increased Ca concentration in top leaves compared with the short PP regardless of SL. Water stress significantly ($P < 0.001$) modified the Ca distribution and reduced its concentration in PO within NN irrespective of the photoperiod. A possible mechanism for the regulation of Ca distribution is discussed in terms of nitrate reduction.

4.1 INTRODUCTION

4.1.1 Role of Ca in Plant Growth and Development:

The role of calcium (Ca) in plant growth and development begins at the initial growth stage, because Ca is a constituent part of every plant cell wall where it occurs as calcium pectate. In addition, Ca is necessary for cell elongation, protein synthesis, and cell division (Marschner, 1995). A reduction in seed Ca concentration during the seed development can reduce germinability of seeds as was observed in soybean plants (Keiser and Mullen, 1993). Ca also can influence plant development by its involvement in different plant physiological processes such as root elongation and gravitation, leaf development (Marschner, 1995), regulation of cabohydrate translocation, (Greger and Bertell, 1992). Therefore, Ca deficiency in plants can cause disorders in plants. For example, more than 30 disorders in vegetables and fruits such as bitter pit in apples (*Malus pumila*) and blossom-end rot in peppers (*Capsicum annuum*) and tomatoes (*Lycopersicon esculentum*) have been reported (Simon, 1978). Ca has received tremendous attention of scientists because of its crucial role in plant cell responses to the environmental stimuli (Hepler and Wayne, 1986).

4.1.2 Ca Concentration and Distribution in Plants in Response to the Environment:

Ca is one of the most immobile macro-nutrients in plants, and its translocation between different tissues is not facilitated. Environmental factors influence Ca content in plants, and a steady uptake and transport of Ca are necessary to avoid disorders related to Ca-deficiency.

Water stress during the seed-filling period of soybean reduces Ca content in different plant parts (Batchelor *et al.*, 1984; Smiciklas *et al.*, 1989; Sorooshzadeh *et al.*, 1995), and promotes leaf senescence (Sionit and Kramer, 1977; Cure *et al.*, 1983; Cortes and Sinclair, 1986). Water stress-induced leaf senescence might be delayed by increasing day length (Cure *et al.*, 1983), and Ca has a central role in influencing senescence (Poovaiah, 1988). Radiation increases Ca accumulation in young tomato leaves, when the air humidity is high (Lim, 1989). Itai *et al.* (1992) suggested that Ca concentration may increase in plants with increased length of photoperiod. Thus, a long photoperiod during the seed-filling period of soybean along with increased foliar concentration of Ca might be effective in delaying leaf senescence. Ca distribution pattern, photoperiod, and water stress relationships during the seed-filling stage of soybean growth remain unknown.

4.1.3 Objective:

The objective of the present study was to explore the distribution of Ca during the seed-filling stage of soybean when day length and moisture stress varied.

4.2 MATERIALS AND METHODS

4.2.1 Plant Culture and Growth Condition:

Soybean (*Glycine Max* L. Merr.) seeds of determinate cultivar Chuusei Kuro daizu (PI 416835) were obtained from USDA Soybean Germplasm Collection, Urbana, Illinois. Seeds were germinated in petri dishes with deionized water, and germinated seeds with identical radicle length were selected and planted in a 1:1 (v/v) perlite:vermiculite medium containing commercial lime (10 g per pot). The seedlings were grown in a greenhouse with controlled environment of 26/22°C (day/night) temperatures, 400 μmol L^{-1} atmospheric CO_2, and 75% relative humidity. Light was supplemented with sodium vapor lamps with an average photon flux density of 400 μE m^{-2} s^{-1} at the canopy height. The pots were drip-irrigated twice daily with a complete nutrient solution as recommended by Keiser and Mullen (1993) until the seed-filling stage was reached as defined by Fehr *et al.* (1971).

4.2.2 Experimental Design and Arrangement:

Plants were then selected on the basis of their uniformity in growth, removed from the media and transferred to vessels containing deionized water and taken to the radiotracer laboratory. After a 24-h adaptation period to the new environment, the plants were transferred to plastic containers with 700 mL deionized and aerated water. An

activity of 0.7 MBq ^{45}Ca as $CaCl_2$ in aqueous solution was added to each container. The radioactive ^{45}Ca with 99% radionuclide purity was purchased from ICN Biomedical Inc., Irvine, California.

The split-plot design was used and each treatment was replicated three times. A long photoperiod (LD) of 16 h, and a short one (SD) of 12 h in combination with a non-limited (NS) and a limited (ST) water availability were used as factors. The day length treatments were conducted by using cool white fluorescent lamps which produced photosynthetically active radiation of 400 ($\pm$10 SD) $\mu E\ m^{-2}\ s^{-1}$ at plant height. Water stress was imposed by adding 0.5 M mannitol to the aqueous medium as an osmotic agent (Krizek, 1985), that produced an osmotic potential of -1.1 MPa as measured by a thermocouple psychrometer (Wescor Inc., model HR-33t). The solution medium and the environmental conditions were kept constant for 10 days.

4.2.3 Measurements of Plant Parameters:

At the end of the experimental period, the radioactivity in different plant organs (stems, petioles, and base leaves) at node numbers (NN) 4 (bottom), 6 (intermediate height), and 8 (top) were determined with a liquid scintillation spectrometer as described by Sorooshzadeh *et al.* (1995). Net photosynthesis, transpiration, leaf area of the top leaf, seed dry weight, and seed number were determined. A portable steady state photosynthesis system (LI-6200 COR. Inc., Lincoln NE, USA) was used to measure the photosynthetic activity and transpiration.

4.2.3 Statistical Analysis:

The data were analyzed using repeated measurement analysis of the Statistical Analysis System (SAS institute, Cary, NC) program. The statistically significant differences were separated using least square means. The repeated measurement analyses require the repeated measure data from an individual plant across time or space (NN in our case) meet the H-F (Huynh and Feldt, 1970) condition. However, the main effects of photoperiod (PP), stress level (SL), and plant organ (PO) do not require the H-F condition and so the univariate analysis could be used. The fulfilment of the H-F condition of the data can be examined by using the sphericity test (Fernandez, 1991).

4.3 RESULTS

4.3.1 Effects of Photoperiod and Water Stress on Plant Parameters:

Water stress reduced the rate of transpiration, the net photosynthetic rate, and the leaf area of the top leaf as expected, but the day length had no influence on these parameters (Table 4.1). Day length and water stress had little effect on seed dry weight and seed number.

Table 4.1 The effects of long (LD) and short (SD) day, water stress (ST) and non-stress (NS) on transpiration, net photosynthesis, leaf area of the top leaf, seed dry weight, and seed number. Standard errors are indicated.

	SD		LD	
Parameters	NS	ST	NS	ST
Photosynthesis[1]	0.81±0.03	0.12±0.01	0.79±0.03	0.13±0.01
Transpiration[2]	112.43±4	30.51±3	115.37±5	31.00±2
Leaf area[3]	49.27±2.2	43.55±1.2	50.15±2.5	44.18±1.2
Seed dry weight[4]	51.20±1.1	49.50±2.2	48.30±2.4	49.80±1.8
Seed number	49.00±4	52.00±5	50.00±4	49.00±3

1 = mg CO_2 m^{-2} s^{-1}, 2 = mg H_2O m^{-2} s^{-1}, 3 = cm^2, 4 = mg

4.3.2 Effects of Photoperiod and Water Stress on Ca Content and Distribution:

The univariate analysis showed the main effects of PP, SL, PO, and PP x SL interaction on Ca distribution were significant (Table 4.2), but PP x PO, SL x PO, and PP x SL x PO interactions were not. The sphericity test (with χ^2 approximation 2.2 with 2 df $P > \chi^2 = 0.34$) was not significant, which indicated that the H-F condition was satisfied, and the results of univariate analysis (Table 4.3) were valid. This analysis showed that leaf position (NN) and its interaction with other factors had highly significant effects on the Ca concentration in the plant. The univariate and Manova analyses (Tables 4.3 and 4.4), agreed on the significance of the effects of the factors on Ca concentration.

In LD-NS treatment (Fig. 4.1), Ca was uniformly distributed between leaves, petioles, and stem at NN4. There was no significant difference in Ca between leaves and petioles at NN8, and both significantly accumulated more Ca than the stem ($P < 0.001$). Leaves significantly absorbed more Ca ($P < 0.001$) than the stem and petioles, and there was no significant difference between petioles and stem at NN6. Ca concentration increased curvilinearly from the bottom to the top of the plant.

In LD-ST treatment (Fig. 4.2), Ca distribution was uniform between leaves, petioles, and stem at both NN4 and NN8. Leaves and petioles accumulated approximately the same Ca concentration and both significantly ($P < 0.001$) absorbed more Ca than the stem at NN6. Water stress reduced the Ca concentration in leaves and petioles at NN8 by about 50%, and significantly increased its concentration ($P < 0.05$) in petioles at NN6

Table 4.2 Univariate test analysis on the effects of photoperiod (PP), stress level (SL), and plant organ (PO) in Ca concentration.

Source	DF	SS Type III	F Value	Pr > F
PP	1	83111	40	0.001
SL	1	260604	124	0.001
PO	2	55092	13	0.001
PP x SL	1	20358	10	0.0047
PP x PO	2	1610	0.4	0.685
SL x PO	2	11683	3	0.081
PP x SL x PO	2	159	0.04	0.963

Table 4.3 Univariate test analysis on the effect of node number (NN) and its interactions with photoperiod (PP), stress level (SL) and plant organ (PO) in Ca concentration.

Source	DF	SS Type III	F Value	Pr > F*
NN	2	237769	424	0.001
NN x PP	2	53778	96	0.001
NN x SL	2	146732	261	0.001
NN x PO	4	20459	18	0.001
NN x PP x SL	2	7259	13	0.001
NN x PP x PO	4	17852	16	0.001
NN x SL x PO	4	11487	10	0.001
NN x PP x SL X PO	4	34709	31	0.001

*Sphericity test: χ^2 approximation 2.2 with 2 DF $P > 0.34$. H-F = The adjusted probability value by the Huynh-Feldt epsilon = 1.44.

Table 4.4 Manova test analysis on the effect of node number (NN) and its interactions with photoperiod (PP), stress level (SL), and plant organ (PO) in Ca concentration.

Source	λ*	F Value	Num DF**	Den DF***	Pr>F
NN	0.04	318	2	23	0.001
NN x PP	0.13	75	2	23	0.001
NN x SL	0.05	216	2	23	0.001
NN x PO	0.19	15	4	46	0.001
NN x PP x SL	0.40	17	2	23	0.001
NN x PP x PO	0.18	16	4	46	0.001
NN x SL x PO	0.39	7	4	46	0.002
NN x PP x SL x PO	0.10	24	4	46	0.001

*Wilk's lamda; **Numerator degree of freedom; ***Denominator degree of freedom

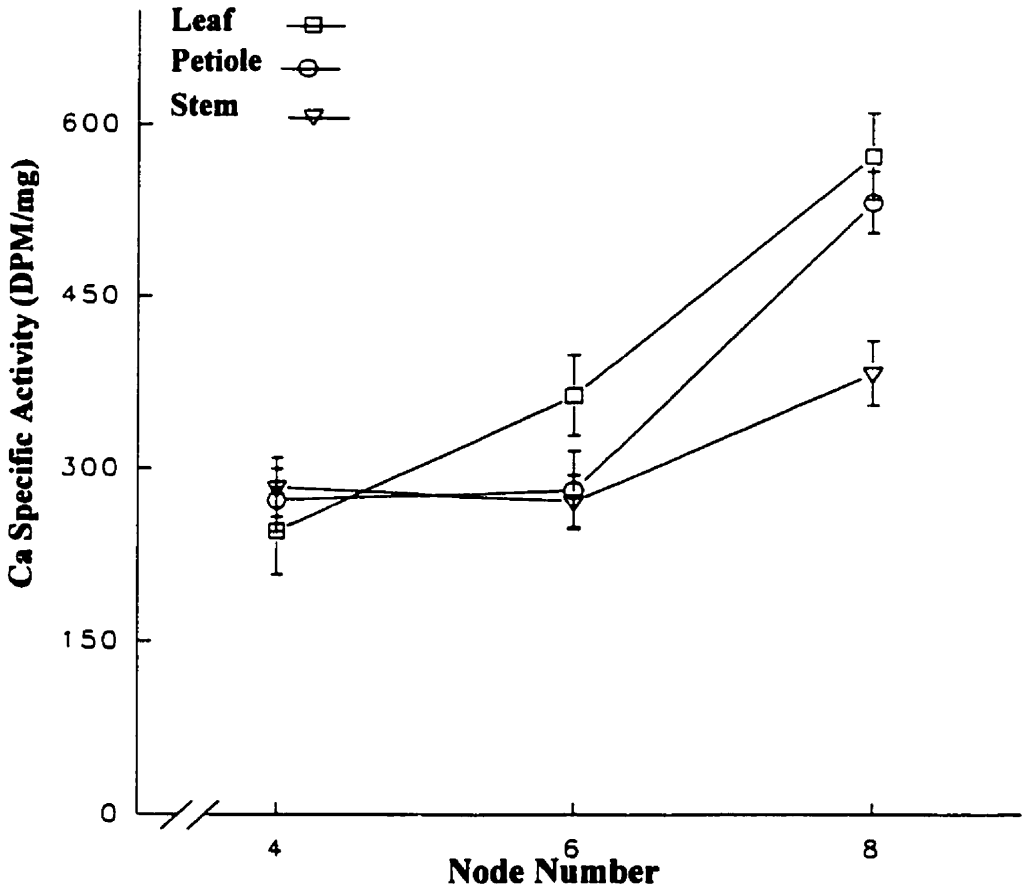

Figure 4.1 Distribution of Ca in soybean plants during the seed-filling period under 16 h photoperiod with no water stress.

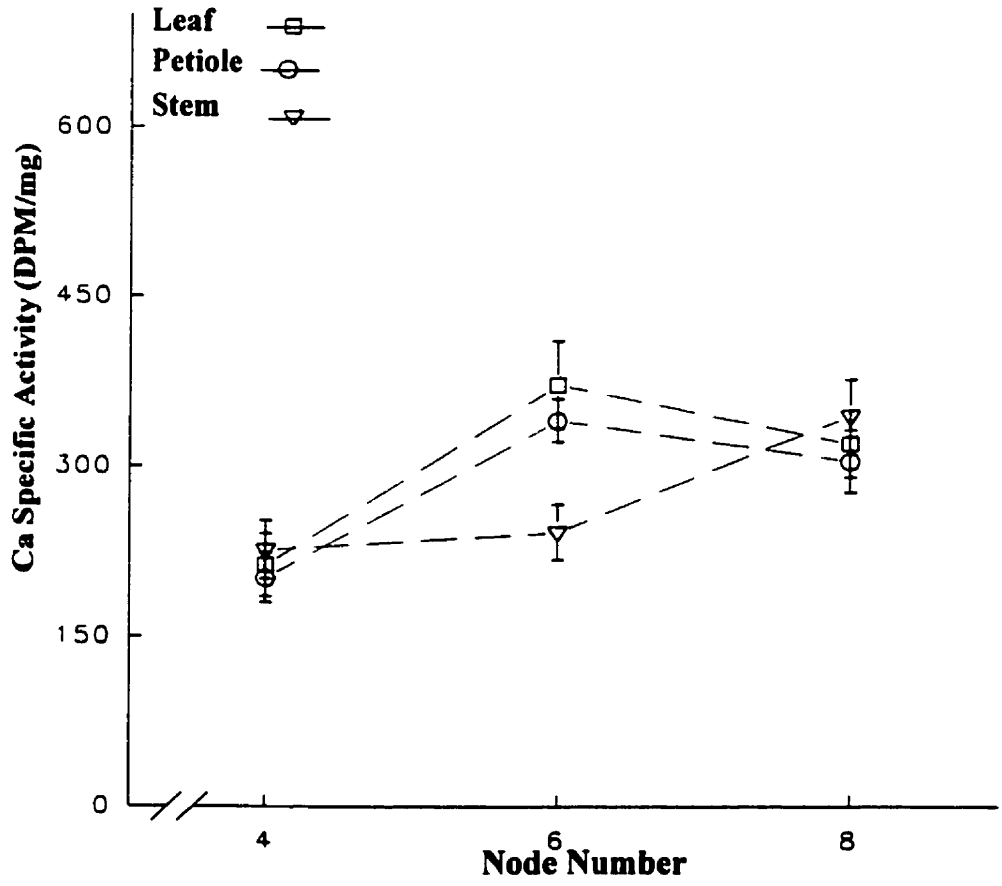

Figure 4.2 Distribution of Ca in soybean plants during the seed-filling period under 16 h photoperiod with water stress.

compared with the non-stress and long photoperiod (LD-NS) plants. Water stress changed the distribution pattern of Ca within the plant compared with the LD-NS treatment.

In SD-NS treatment, Ca distribution between different organs was similar at NN8 (Fig. 4.3). There was no significant difference in Ca concentration between leaves and petioles, and both accumulated significantly ($P < 0.001$) more Ca than the stem at NN6. The Ca concentration in the stem and petioles was almost the same and leaves had significantly ($P < 0.001$) higher Ca than either of the organs at NN4. Ca concentrations in stem at all nodes, and in leaves and petioles at NN6 and in petioles at NN4 were independent of photoperiod. Ca concentrations in leaves and petioles at NN8 were reduced while in leaves at NN4 increased significantly ($P < 0.001$) in SD-NS compared with LD-NS treatments.

The SD-ST treatment produced uniform distribution of Ca at NN4 and NN6. Leaves at NN8 absorbed more Ca than the stem and petioles (Fig.4.4). Water stress did not affect Ca concentrations in stem at NN4 and NN6 when the photoperiod was short. However, the Ca concentrations were significantly ($P < 0.001$) reduced in all plant parts at NN8, and in petioles at NN6.

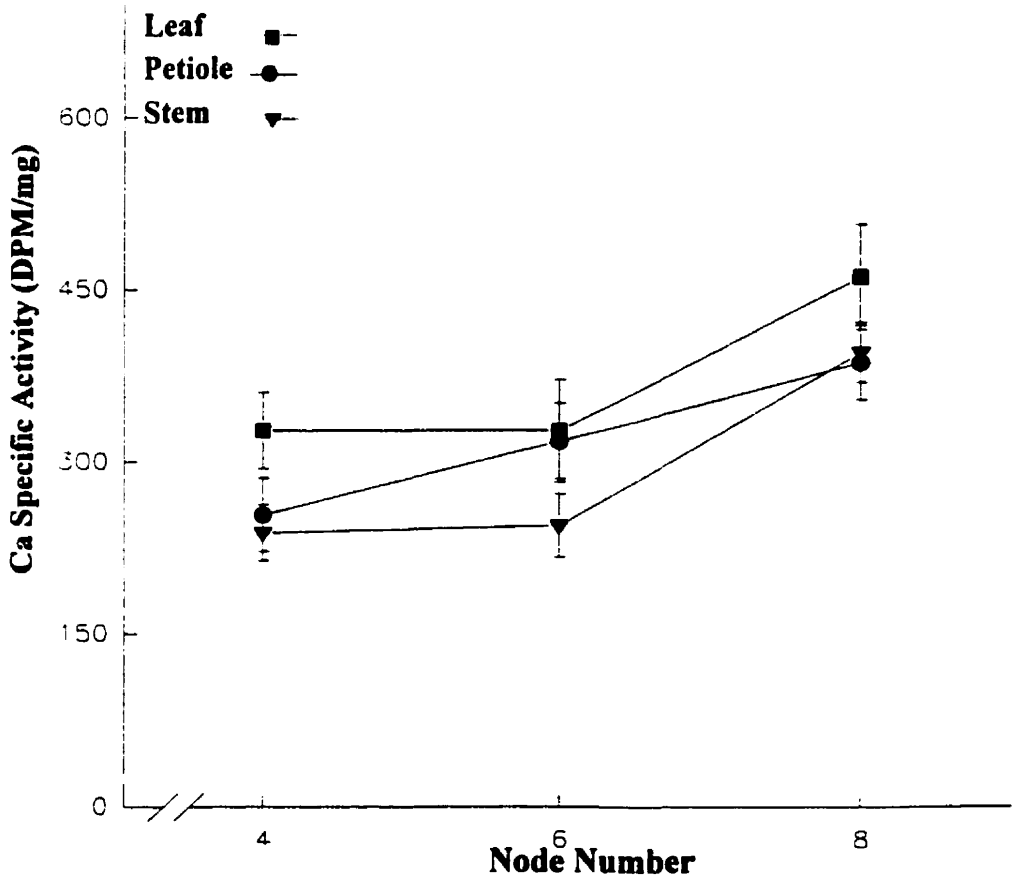

Figure 4.3 Distribution of Ca in soybean plants during the seed-filling period under 12 h photoperiod with no water stress.

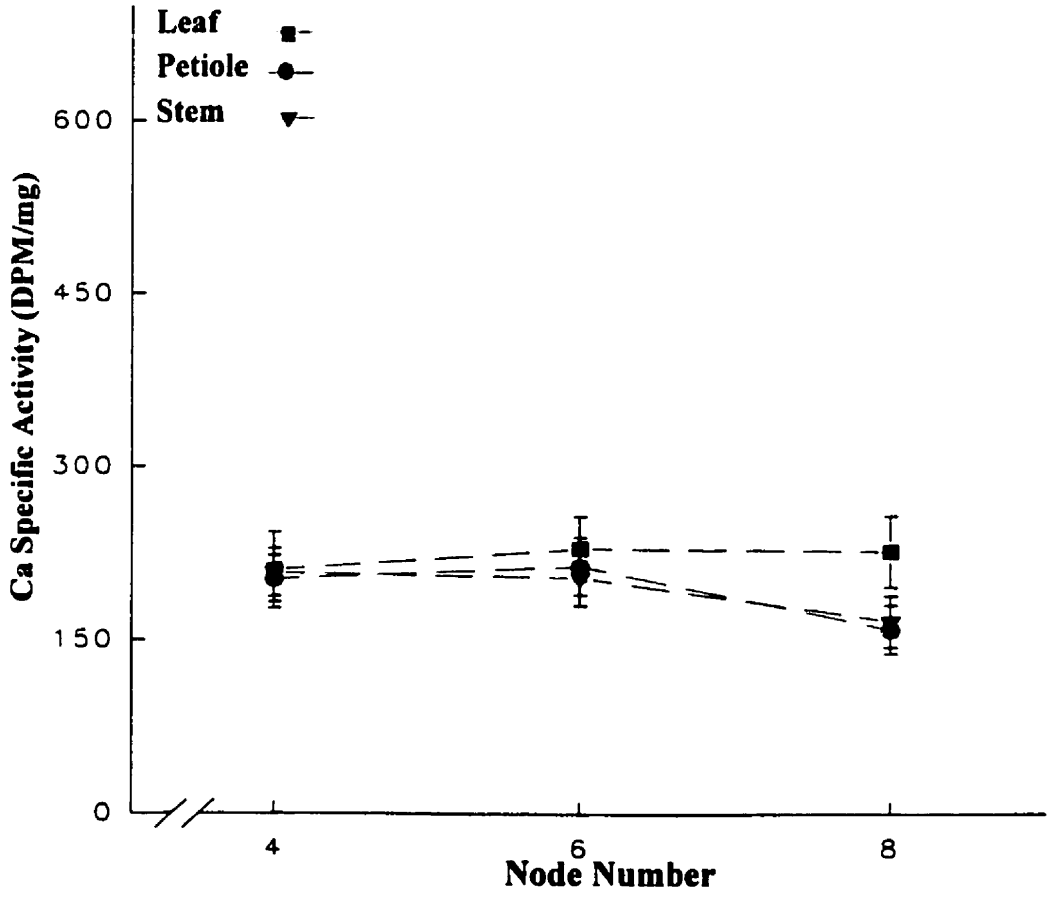

Figure 4.4 Distribution of Ca in soybean plants during the seed-filling period under 12 h photoperiod with water stress.

4.4 Discussion

Seed number and seed dry weight were unaffected by water stress in our experiments but conflicting results were reported. Momen *et al.* (1979), Cure *et al.* (1983), and Meckel *et al.* (1984) observed variations in seed number and seed dry weight from water stress. Sionit and Kramer (1977), Constable and Hearn (1978), Cure *et al.* (1983), and Korte *et al.* (1983) found no change in seed number but observed changes in seed dry weights from water stress. Seed weight depended on seed growth rate and the seed-filling duration (Egli, 1990), and these quantities were genetically and environmentally controlled (Egli *et al.*, 1987). Any decrease in soybean seed weight from water stress during seed-filling may result from its shortened duration from leaf senescence (Meckel *et al.*, 1984). The discrepancy between our results and those of past authors on the effect of water stress on seed weight and number could be due to a constant seed-filling time we set between stressed and non-stressed plants. The normal seed-filling time of our soybean cultivar was 30 days, but in the peresent experiment we shortened this period to 10 days. Because of the constant seed-filling time, seed growth rate was little affected by water stress, which offered an explanation for the non-significant difference reported here in seed weight and seed number.

Cell division and expansion were most sensitive to water stress (Hsiao, 1973), which supported the decreased area of the top leaf reported here and elsewhere (Sionit and Kramer, 1977; Constable and Hearn, 1978; Cure *et al.*, 1983). Cure *et al.* (1983)

reported that following 5-10 days of water stress during seed-filling, plants grown under long days had more leaf area and seed dry weight than those exposed to short days. The difference in the results between the present and these authors was probably due to the difference in the post-water stress growth period which was 50-55 days in their experiments compared with 10 days in ours.

Transpiration and photosynthetic rates decreased from water stress (Rawson *et al.*, 1978; Cure *et al.*, 1983). Stomatal closure, biochemical reactions of photosynthesis and the structural integrity of the chloroplast contributed to this reduction (Chaves, 1991). Cure *et al.* (1983), Huber *et al.* (1984), and Morandi (1986) observed no effect of the length of photoperiod on photosynthesis when there was no water stress. However, Cure *et al.* (1983) showed equality in the photosynthetic activity of water stressed plants under short and long days, but post-water stress recovery of photosynthetic activity was higher for the latter than the former.

Our results showed water stress and photoperiod can significantly affect the distribution and accumulation of Ca in plant tissues of soybean during the seed-filling stage. Ca is known to be transported passively with the transpiration stream, and its movement in xylem follows the upward direction of water flow (Hanger, 1979). Therefore, a reduction in transpiration from water stress can cause a corresponding reduction in Ca uptake and its accumulation in plant as reported (Batchelor *et al.*, 1984; Smiciklas *et al.*, 1989; Sorooshzadeh *et al.*, 1995). However, the movement and

accumulation of Ca may not be entirely controlled by transpiration. Root pressure and cation exchange capacity (CEC) (free carboxyl group of polygalacturonic acid of xylem and apoplasm) may each play a role in Ca transport (Kirkby and Pilbeam, 1984). Root pressure is a positive pressure in the xylem resulting from accumulation of salt in the root xylem, and occurs when transpiration rate is very low. CEC produces a sink for Ca in different plant parts and can influence Ca movement to those parts. Ca sinks could be formed by cation exchange due to (1) the formation of new binding sites from cell division in young tissues, and (2) the removal of Ca from exchange sites at the end of xylem vessels in mature tissues (Marschner, 1995). Ca can be removed from exchange sites by chelats such as EDTA or by ions such as divalent ammonium cation.

The duration of light might influence Ca movement and distribution via transpiration rate, root pressure or CEC. Plants grown under long day and without water stress have increased transpiration duration and Ca uptake. The root resistance to water flow increased for short day plants (Bunce, 1978). The effect of light on Ca accumulation and distribution through CEC might be explained in terms of nitrate reduction. Ca uptake was reduced by ammonium while it increased when nitrate was present in the growth medium (Hanger, 1979). Irradiation increased cytosolic Ca (Treyn *et al.*, 1991), and Sharma *et al.* (1994) observed increased Ca uptake by red light which stimulated the nitrate reduction activity in etiolated maize leaves. Nitrate assimilation in a plant cell involves reductive reaction which may lead to an increase in the cytoplasmic pH from

the production of OH^-. Ca can be removed from exchange sites and stored in vacuoles or apoplasm as a salt for osmoregulation to balance cation-anion ratio during nitrate reduction (Franceschi and Horner, 1980). Calcium oxalate, for example, has been detected in the vacuoles of soybean leaf (Kausch and Horner, 1982). Thus, nitrate reduction in plant cells has an important role to play in removing Ca from the binding sites in the apoplasm to affect its movement and accumulation. Nitrate reduction is catalyzed by nitrate reductase enzyme (NR). Although we could not measure the NR activity because of the contamination problem of radioactivity, it has been known that soybean leaves produce three types of this enzyme. The inducible NR enzyme is active in leaves and roots of soybean only when growth media contain nitrate. The constitutive NR enzymes (c1NR, c2NR) are active only in leaves of soybean that grow without nitrate in the medium (Nelson *et al.*, 1983) as was done in our experiments. Li and Gresshoff (1990) reported that the top leaf has more cNR activity than the bottom leaves. Thus, Ca accumulation in leaves and petioles at NN8 of LD-NS treatment could be explained in terms of more cNR activity in top leaves. This activity acts as a Ca sink and force its movement to the top. In old leaves, cNR activity is diminished, and Ca is removed from binding sites in the apoplasm resulting in less Ca. Ca distribution pattern of LD-NS was similar to those reported earlier (Sorooshzadeh *et al.*, 1995).

Light, water stress, temperature, and mineral nutrition could influence the activity of nitrate reductase (Campbell and Smarrelli, 1986), and NR enzymes could be

inactivated (Sinha and Nicholas, 1981; Aryan et al., 1983; Kaiser and Förster, 1989). Reduction in Ca concentration in leaves at NN8 in SD-NS, and SD-ST compared with LD-NS, and LD-ST treatments may be due to the reduction in the activity of NR enzymes.

LD-ST, and SD-ST reduced Ca concentration in some plant parts, and produced more uniform distribution of Ca in tissues compared with LD-NS, and SD-NS treatments. The reduction in Ca concentration for both photoperiods with water stress may be due to the inactivation of nitrate reductase enzyme (Fig.4.3 and Fig.4.4). However, LD-ST treatment did not affect Ca content in the bottom leaves. This may be explained in terms of cNR activity which depend on the photoperiod and light intensity (Cantliffe, 1972; Harper and Paulsen, 1986; Hageman and Flesher, 1961) via the mechanism of synthesis/degradation of the enzyme protein (Remmler and Cambell, 1986) and by providing reducing power (reduced ferredoxin) and ATP (Corzo and Niell, 1992). The proposed mechanisms for Ca regulation by nitrate reduction need to be confirmed by further experiments.

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LINKAGE BETWEEN CHAPTER 4 AND CHAPTER 5

Ca could be remobilized from vegetative plant parts to the developing seed when Ca stress prevails. Also, Ca could be translocated from the pod to the vegetative plant parts through the xylem when a large difference in the water status occurs between leaf and pod. Chapters 3 and 4 demonstrated that water stress during the seed-filling stage reduced Ca concentration in all plant parts which could be attributed to retranslocation. Chapter 3 considered Ca-feeding through injection, Chapter 4 through the root system, and Chapter 5 will deal with an immersion method of feeding. The results of Chapter 5 were published: A. Sorooshzadeh and Barthakur, N.N. 1995. Moisture stress and calcium absorption from immersions during the seed-filling stage of soybean. Communications in Soil Science and Plant Analysis. 26:2309-2318.

CHAPTER 5

MOISTURE STRESS AND CALCIUM ABSORPTION FROM IMMERSIONS DURING THE SEED-FILLING STAGE OF SOYBEAN

ABSTRACT

Calcium (Ca) uptake was studied by immersing the central tip of a trifoliate leaf in various concentrations of $^{45}CaCl_2$ solutions and under moisture stress conditions during the seed-filling period of soybean. Beta-ray gauging and the diurnal leaf temperature variation showed similar characteristics for leaf water status. The activities of ^{45}Ca were significantly higher ($P < 0.0001$) at 5, 10, 20, and 30 mM concentrations for water stressed and non-stressed leaves compared with the control. Calcium (^{45}Ca) activities at 5, 10, and 20 mM Ca concentrations between stressed and non-stressed leaves were not significant, but the difference in their mean values at 30 mM Ca concentration was significant ($P = 0.0159$). The relationship between ^{45}Ca uptake and Ca concentration was parabolic for both stressed ($R^2 = 0.77$) and non-stressed ($R^2 = 0.81$) leaves. Autoradiographs indicated Ca movement through the mid-rib and veins of the tip-immersed trifoliate leaf but showed no activity in other plant parts. An activity gradient developed between seeds when a pod-tip was immersed in the radioactive solution.

5.1 INTRODUCTION

5.1.1 Importance of Ca-deficiency in Agriculture:

The supply to meet the demand of calcium for plant growth is adequate in most agricultural soils, yet 35 Ca-deficient disorders have been reported in various crops (Shear, 1975; Bangerth, 1979). The poor germination and slow seedling growth rate of Ca-deficient soybean seeds due to water stress during the seed-filling stage, could reduce yield especially in the semi-arid climate, where scant precipitation is the major prohibiting factor for crop production. Understanding Ca uptake and translocation mechanisms could alleviate the Ca-deficiency problem.

5.1.2 Ca Translocation in Plant:

There are reports of Ca absorption and transport via passive diffusion and exchange reactions in conducting tissues (Palzkill and Tibbitts, 1977; Van de Geijn and Petit, 1979). Ca movement through the apoplastic pathway in apple fruits is mediated by a combination of cation exchange and diffusion processes (Harker *et al.*, 1988; Harker and Ferguson, 1988). Ca transport from roots to the shoot apex occurs with the transpiration stream, and its reverse transfer from old to young tissues is negligible since only minute quantities of Ca have been detected in the phloem sap (Clarkson, 1984). Thus, the relative phloem immobility of Ca causes its deficiency in plant parts with low

transpiration rates (Limani and Lamaze, 1991). However, Ca does move from leaf to other tissues if a concentration gradient and transport promoting substances are applied (Millikan and Hanger, 1965). Moreover, moisture stress during the reproductive stage reduces Ca content in stems, leaves, pods, and seeds of soybean (Batchelor *et al.*, 1984; Smiciklas *et al.*, 1989). The seeds from water stressed plants do not germinate well. The germinability of seeds from water stressed soybean plants during seed-filling might be improved through seed Ca application (Smiciklas *et al.*, 1989). Foliar Ca application may be a practical method to increase seed Ca concentration during seed-filling. However, the use of this method requires a knowledge of how Ca moves from leaf to seed and how Ca is absorbed by the pod. Ca could also be remobilized to the developing seeds when Ca stress develops (Keiser and Mullen, 1993). Ca translocation and moisture stress studies during the seed-filling stage ideally require simultaneous monitoring of both with sensitivity and accuracy.

5.1.3 Monitoring Ca Movement and Water Status in Plant:

The radiotracer technique in plant nutrient uptake studies provide both sensitivity and accuracy of measurements (Vose, 1980). The measurement of plant water status is complex, and the methods used for horticultural crops have been thoroughly reviewed and the shortcomings in the performance of the two most popular instruments—the thermocouple psychrometer and the pressure chamber—discussed (Spomer, 1985).

5.1.4 Objectives:

Thus, the objectives of the present study were to: (i) explore the beta-ray gauge and leaf temperature as measures of plant water status, and (ii) assess Ca movement from the foliar application of radiocalcium (^{45}Ca) as a function of moisture stress and concentration during the seed-filling period of soybean.

5.2 MATERIALS AND METHODS

5.2.1 Cultivar and Culture:

Soybean *(Glycine max* L. Merr.) seeds of determinate cultivar Chuusei kuro daizu (PI 416835) were obtained from USDA Soybean Germplasm Collection, Urbana, Illinois. Seeds were sown in 20-cm diameter plastic pots filled with 1:1:1 (v/v/v) sand-soil-peat mixture. The substrate and plant growth conditions used were similar to those recommended elsewhere (Djekoun and Planchon, 1991). Twenty g of commercial lime was added per pot, and the pots were given 250 mL of 20-20-20 (N-P-K) fertilizer twice weekly.

5.2.2 Experimental Setup:

All plants were treated identically until the seed-filling period, phenologically classified as R5 (Fehr *et al.*, 1971), was attained. At the beginning of R5, a group of

plants was irrigated (each pot with 300 mL of water daily), while the other group received 300 mL of water every fourth day and was designated non-irrigated.

5.2.2.1 Experiment 1: Leaf Immersion

The treatments were arranged in a factorial complete randomized experiment and Ca concentrations of 5, 10, 20, and 30 mM used each with four replications. The central tips of a trifoliate leaf from the upper part of the plant were immersed in 5 mL 30 mM $CaCl_2$ containing 22.2 KBg/mL ^{45}Ca and diluted 5, 10, 20 mM solutions. Samples were taken from the leaf bases of the immersed leaf and seeds of the same node and one below. Great care was taken to cut off the immersed tip portion before sampling to avoid contaminating other plant parts. Figure 5.1 shows the leaf immersion arrangement used in this study.

5.2.2.2 Experiment 2: Pod Immersion

In a second experiment, a pod-tip was immersed to study Ca movement from pod to other tissues under water stress. The pod tips were immersed for two weeks in 5 mL water with 130 KBq ^{45}Ca. Samples were taken from the pod and seeds of the immersed pod after removing the tip. Figure 5.1 shows the pod immersion arrangement.

5.2.3 Sampling and Measurements:

The radioactive material with 99% radionuclide purity was purchased from ICN Biomedicals, Inc. Irvine, California. Plants were harvested at the end of the seed-filling period and samples digested in a 0.75 mL solution of a 2:1 mixture by volume of 60% perchloric acid and 30% hydrogen peroxide in an oven at 65°C for two hours. In view of the relatively small pulse sizes from ^{45}Ca beta spectrum with 0.257 MeV maximum energy, it was important to prevent interference from chemiluminescence which was achieved by storing the samples for 24 h at 30°C in the dark before actual counting. The storage allowed the chemiluminescence pulses to be completely decayed. Counting was done with a liquid scintillation spectrometer (LKB, 1219 Rackbeta, Turku, Finland) and expressed as disintegration per minute (DPM/mg) of fresh weight after correcting for chemical quenching using a standard curve. The spectrometer automatically corrected for color quenching.

Macro-autoradiographs were taken for localization studies using Kodak no-screen X-ray films. Leaf temperatures were continuously monitored with a 0.035 cm diameter copper-constantan thermocouple.

The beta-ray gauge (BRG) system was described in detail elsewhere (Barthakur

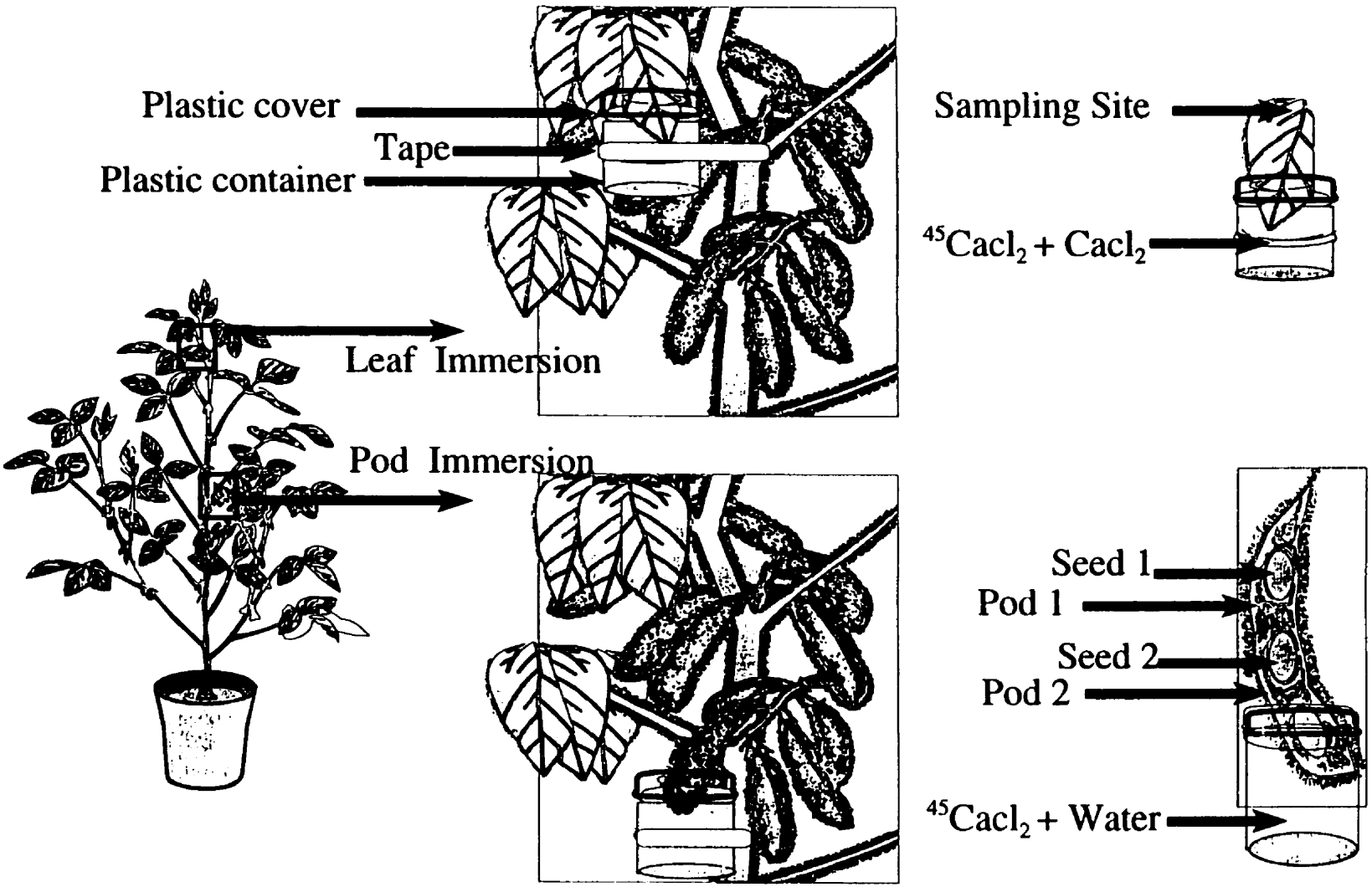


Figure 5.1 Leaf and Pod Immersions

and Tomar. 1987). Briefly, the experimental leaf was held fixed 2 cm above the mica window of the cylindrical Geiger-Mueller (GM) detector (Fig. 5.2). A radioactive point source of thallium-204 (^{204}Tl) of 185 KBq activity, a pure beta-emitter of 0.763 MeV maximum energy, was installed one cm above the leaf. The beta-ray transmission (T) was continuously monitored with a ratemeter-recorder system using a mature leaf as the absorber within a constant source-detector geometry. T can be approximately expressed in Equation [1] as:

$$T = e^{-\mu m} D \approx 1-\mu m D \qquad (1)$$

where: μm = mass absorption coefficient in m^2/kg and **D** = mass thickness, kg/m^2 which is a product of linear thickness and density of the leaf. The μm value is assumed to be independent of beta-ray energy and the nature of absorbers. Thus, the BRG was used to monitor water status in mature leaves without calibration as our purpose was not to determine the leaf water content. Since the dry matter of a mature leaf remains almost constant, T in Equation [1] becomes an indicator of its water status.

5.3 RESULTS AND DISCUSSIONS

5.3.1 Monitoring Water Status in Plant:

The diurnal variation of beta-ray transmission (T) with local time for a non-

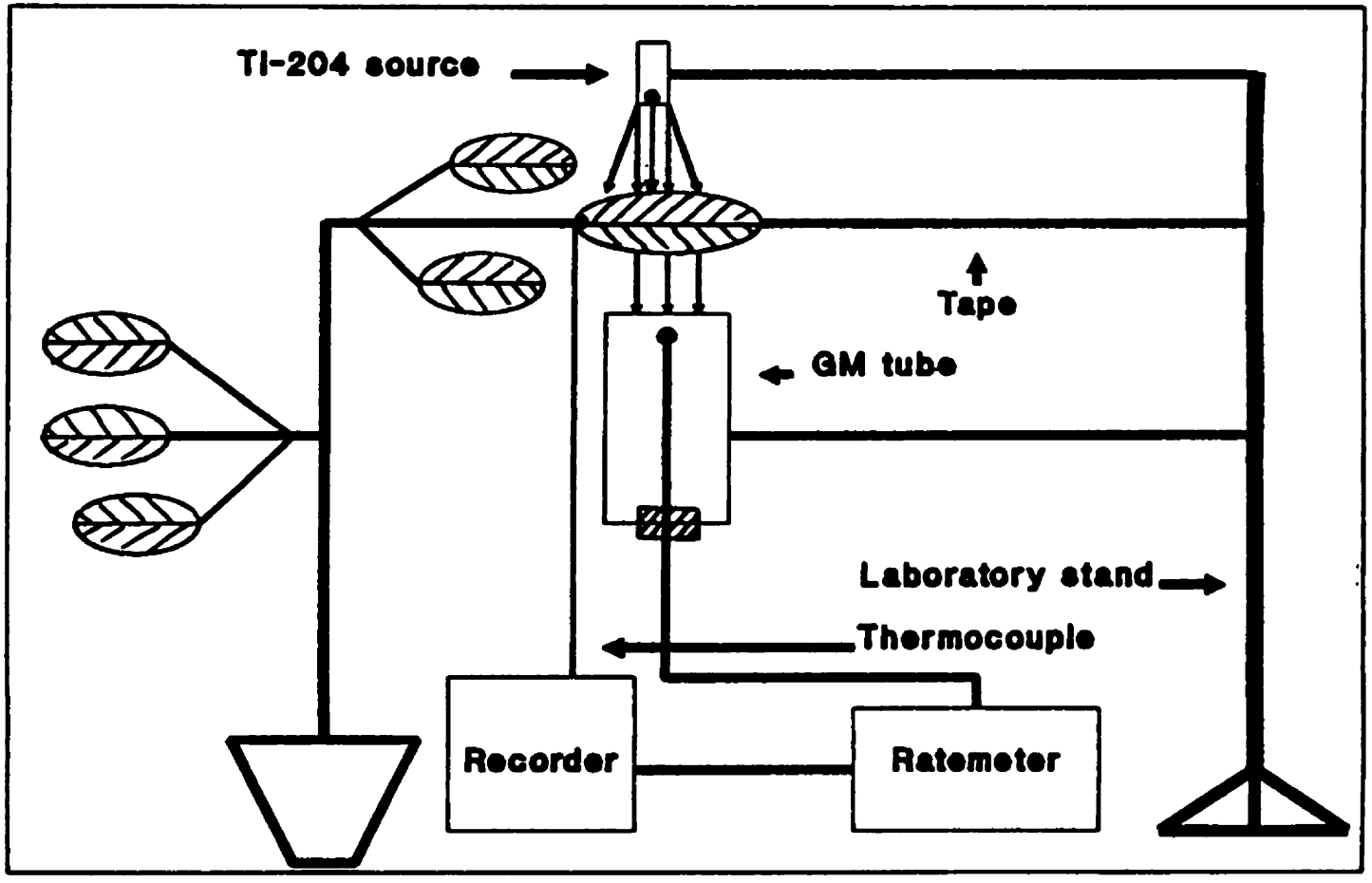


Figure 5.2 The experimental set-up

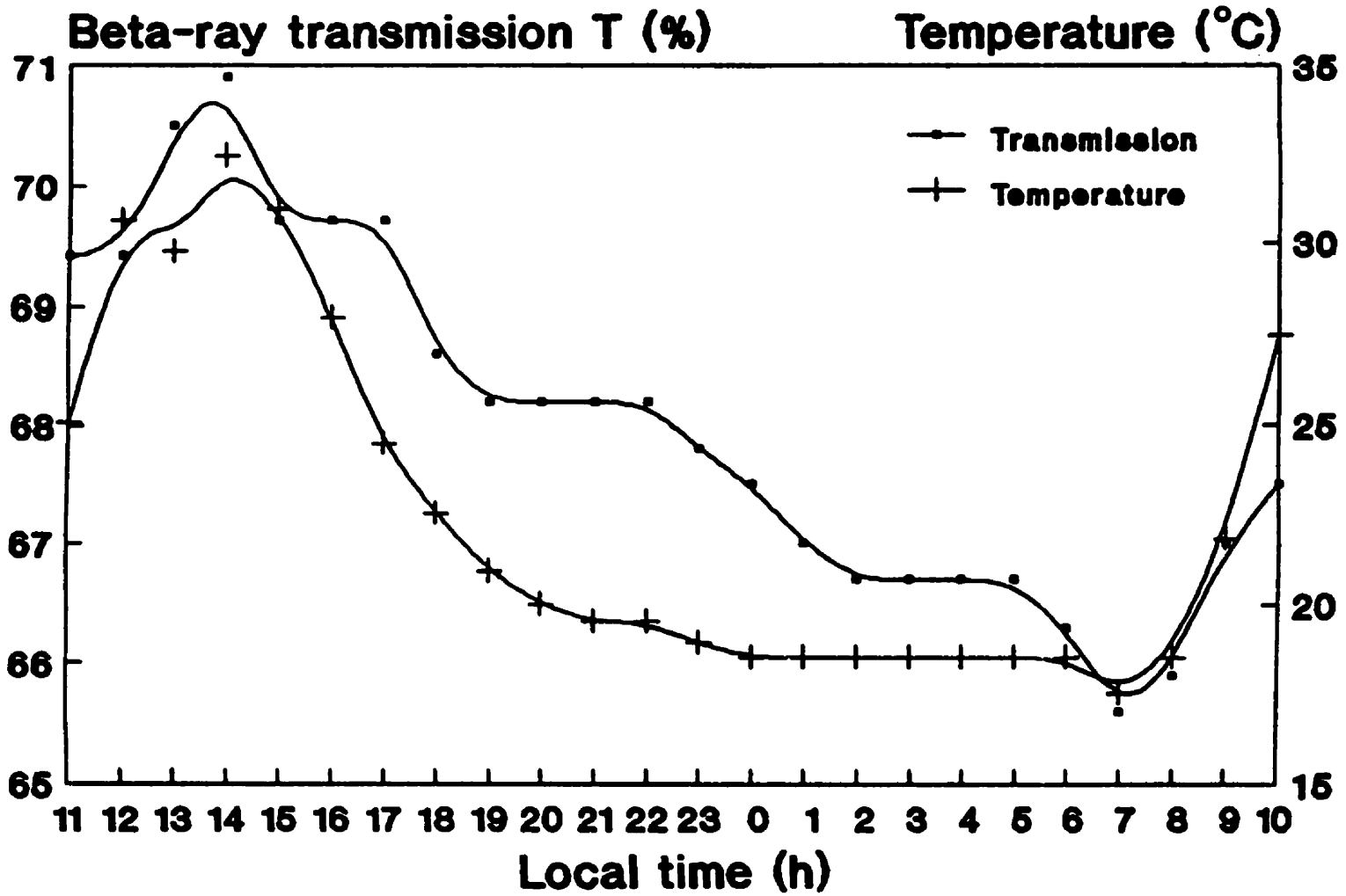


Figure 5.3 Diurnal temperature variation and transmission

irrigated plant on a sunny day corresponded well with that of leaf temperatures (Fig. 5.3). The maxima and minima of T and leaf temperature coincided at about 1400 and 0700 hours local time, respectively. Leaf temperature increased during the daytime and since any water loss from transpiration could not be replenished quickly enough because of soil moisture stress, T increased commensurately reflecting a low leaf water status. Leaf temperature decreased at night and with stomatal closure, water status improved somewhat and consequently T decreased. This pattern of behavior was observed everyday during the seed-filling period. Unlike the daily maximum leaf temperature which fluctuated depending on the weather of the day, T increased each day for non-irrigated plants except when watered (Fig. 5.3). A stress-degree-day concept was developed (Idso *et al.*, 1981) to measure water stress by using foliage-air temperature differential which was affected by cloud cover. Therefore, the monotonic increase in T with time showed a realistic indicator of water status, and the non-destructive nature of the BRG gave it a distinct advantage over the psychrometer and the pressure chamber. Since the leaf-source-detector geometry was constant and no visible leaf shrinkage occurred, increase in T kinetics indicated progressively worsening plant water status. For a daily irrigated plant, T was known to remain constant within the Poisson distribution of random emission of radioactivity. Thus, the BRG could be used to provide an indication of plant water status particularly during a drought situation.

Thermocouples generally provide $+0.5^{\circ}C$ accuracy, the error of beta-ray gauge

from the pen-and- ink recordings can be estimated from Equation [2]:

σ = **fractional standard deviation** = $1/\sqrt{N_c t}$ [2]

where: N_c = count rate in CPM and t = observation time. Thus, the maximum fractional standard deviation in T (Fig. 5.3 and Fig. 5.4) was estimated at 0.004.

The beta-ray transmission of 61% of the BRG (Fig. 5.4) for the soybean leaf at the start of the experiments was equivalent to -1.1 MPa leaf water potential measured by a thermocouple psychrometer (Model HR-33T, Wescor, Utah). The minimum was reached at about -2 MPa by harvest time.

5.3.2 Monitoring Ca Movement:

The activities of ^{45}Ca were normalized and expressed in DPM/mg fresh weight which increased significantly ($p < 0.0001$) at 5, 10, 20, and 30 mM Ca concentrations for both stressed and non-stressed plants compared with the control (Fig. 5.5). The relationship between the foliar uptake of ^{45}Ca and Ca concentration was parabolic in nature for stressed ($R^2 = 0.77$) and non-stressed ($R^2 = 0.81$) plants. The differences in the means of ^{45}Ca activities at 5, 10, and 20 mM Ca concentrations were not significant, but at 30 mM Ca concentration Student t-test showed the difference between the mean absorption values for water stressed and non-stressed plants to be significant ($P = 0.0159$). Although ^{45}Ca translocated from foliar application, activities were below

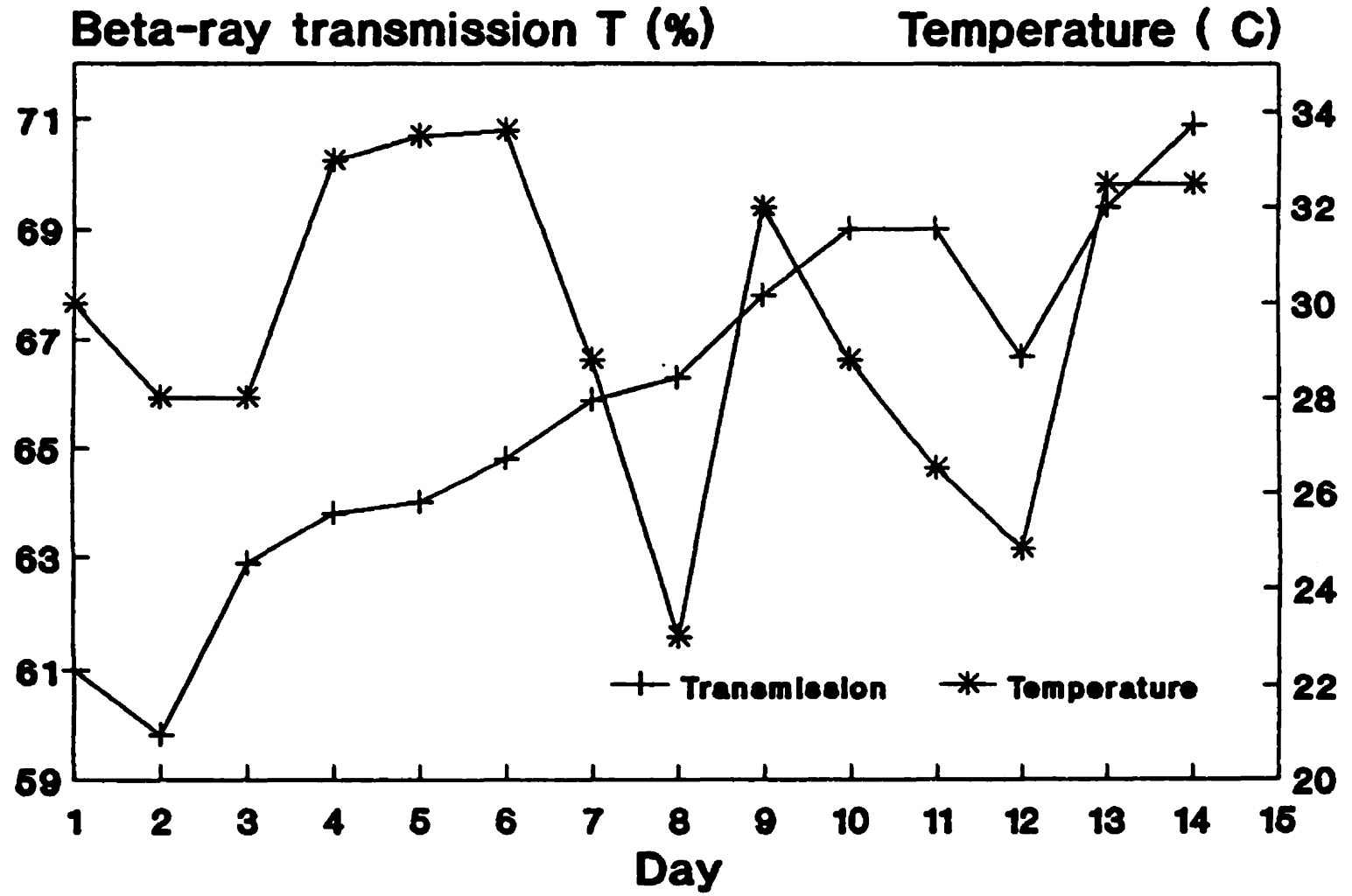


Figure 5.4 Beta-ray transmission and temperature versus day

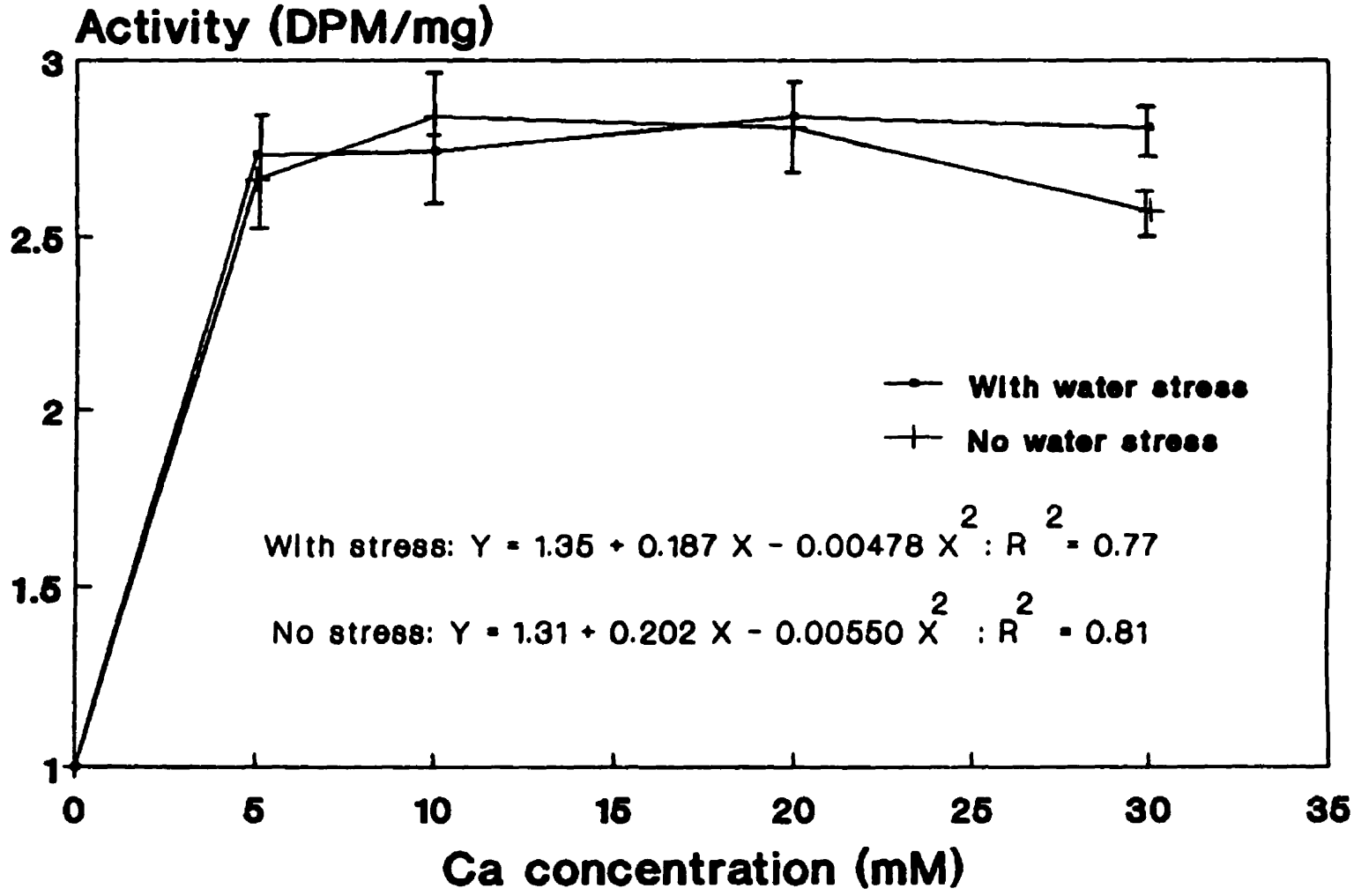


Figure 5.5 Calcium-45 uptake and Ca concentration

3 DPM/mg. Since Ca may be remobilized to developing seeds under Ca stress conditions (Keiser and Mullen, 1993), it may be that stress was not sufficiently developed in our experiments to affect Ca movement to the filling seeds.

Pod-tip immersion produced a large biological variability in ^{45}Ca uptake between individual plants. Since the distribution pattern was the same for all, typical ^{45}Ca activities are shown (Fig. 5.6) using results from one pod. The error in radioactivity measurements was evaluated (Horrocks, 1974) from Equation [3]:

$$\sigma = (\sqrt{N_s/t_s + N_b/t_b})/(N_s-N_b) \qquad [3]$$

where: t_s = sample counting time, N_b = background counting rate, and t_b = background counting time. The error bars in Fig. 5.6 showed standard deviations calculated using Equation [3]. Pod tissue near (pod 2) the tip absorbed considerably more Ca than the far sample (pod 1) for both irrigated and non-irrigated plants (Fig. 5.6). Similarly, the near seed (seed 2) contained more ^{45}Ca than the far seed (seed 1). Pod tissue samples showed higher ^{45}Ca activities than the seeds at the same sites. Our results agreed with an earlier report of lower Ca content in seeds and the signified endocarp than in other pod layers (Smal *et al.*, 1989). Seeds and pods of irrigated plants absorbed more ^{45}Ca than the non-irrigated ones. Calcium could be translocated from the pod to the leaf through xylem when a large difference in water status of leaves and pods occur (Ziegler, 1963). Our results, however, showed no Ca movement from pod to leaf under water stress.

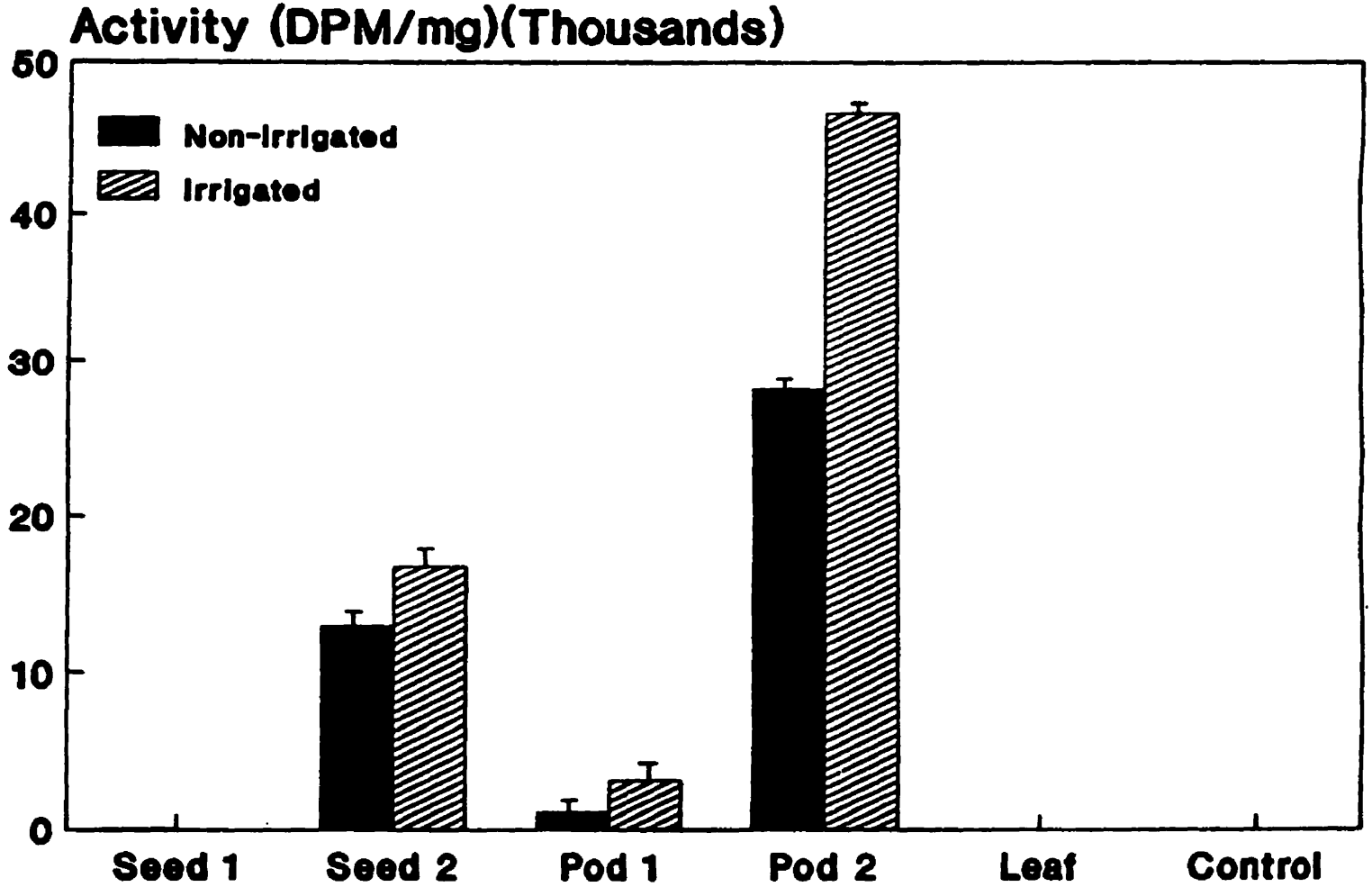


Figure 5.6 Calcium-45 uptake from pod-tip immersion

5.3.3 Macro-autoradiographs:

^{45}Ca localizations in plant parts were studied by autoradiography, and for easy comparative purposes all autoradiograms are placed together in this section. The macro-autoradiographs of the longitudinal sections of stems of plants (Fig. 5.7) fed with Ca through the root system (Chapter 4) clearly showed that Ca was mostly deposited (dark areas) around the central channel in the stem. The central channel is developed in the lower part of soybean stem before the flowering stage when the central pith cells of the stem collapsed (Carlson, 1987). Although it was hard to separate the Ca deposit in the xylem from that in the phloem from Fig. 5.7, it has been well-known that Ca is not transported in the phloem. However, some radiotracer studies have shown that Ca may enter the phloem (Hanger, 1979).

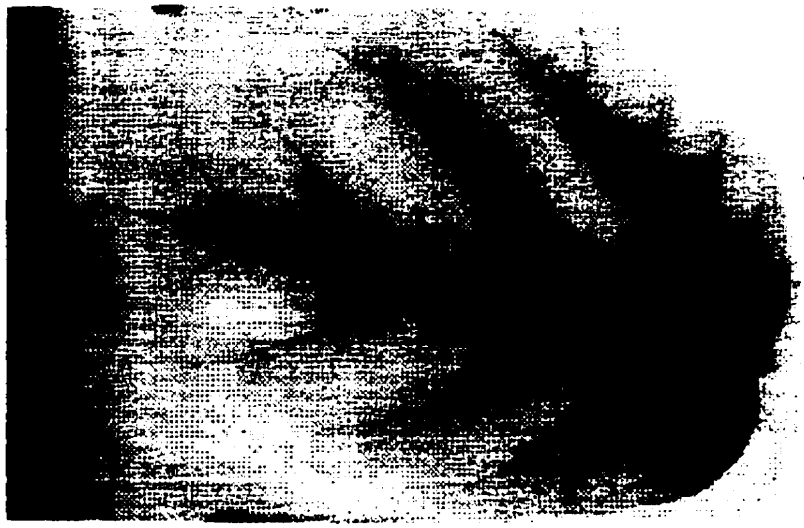
The macro-autoradiograms of top leaves in the injection experiment (Chapter 3) (Fig. 5.8) showed the pathways of Ca in the leaf to be the mid-rib, secondary, and tertiary veins. The effect of water stress on leaf Ca content can be seen very clearly when Fig. 5.8a (water stressed) and Fig. 5.8b (non-stressed) are compared. The control autoradiogram of a leaf from a non-injected plant did not leave any trace of radioactivity on the film. Ca translocation in plant mostly occurs in the xylem with the transpirational stream, and any reduction in transpiration rate could reduce Ca concentration in the plant. The results agreed with the quantitative counting of radioactivity.

The tissue macro-autoradiographs clearly demonstrated that ^{45}Ca activity remained on the trifoliate leaf and did not spread to other parts of the plant in the immersion

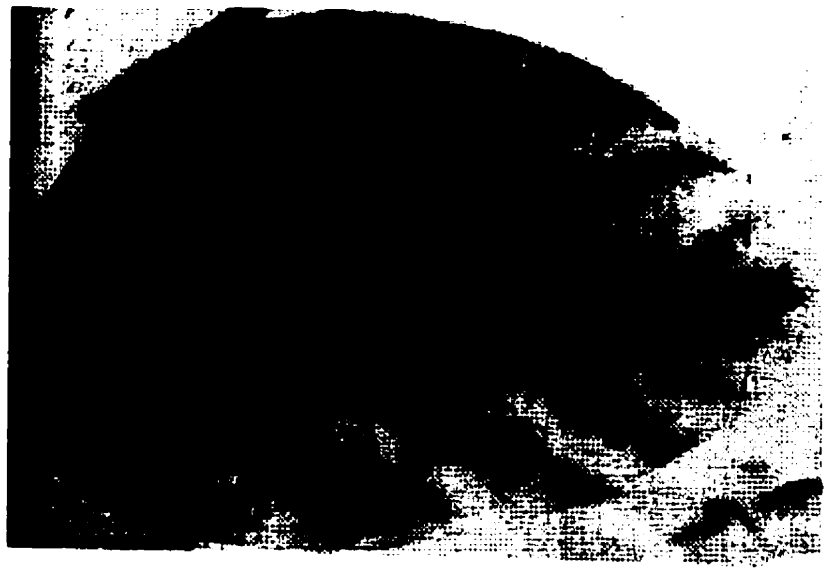
experiment. But by increasing Ca concentration from 10 to 30 mM Ca (Fig. 5.9a and Fig 5.9b) the backward movement (in contrast to the forward movement in Fig. 5.8) of Ca improved considerably which was in agreement with Hanger (1979). The mid-rib was the dominant channel for Ca transport and radioactivity converged and moved as a sharp front towards the leaf base in a conical fashion when Ca concentration was relatively low. The primary and secondary veins also transported ^{45}Ca, and intervein activities were faintly visible especially in low Ca concentration due to less radioactivity. The tadpole-shaped autoradiogram (Fig. 5.10a) did not show the leaf boundary because of heavy and faster movement through the mid-rib and much less at the border. In the original negative autoradiogram a faint silhouette of the leaf boundary was barely visible. Figure 5.10a and Figure 5.10b showed Ca movement during tip immersion for water stressed and non-stressed plants, respectively. The rate of ^{45}Ca movement was affected by water stress and concentration gradient. At 30 mM concentration, ^{45}Ca moved at a speed of 0.55 cm/day in stressed compared with 0.28 cm/day in non-stressed leaves. The average speed of movement was estimated at 0.4 cm/day for other concentrations studied. Ca might enter leaf tissue from immersion region by injuring the cells, since Ca uptake is passive and its high concentration inside will destroy the cell and then enter the mid-rib. Back flow of water in the leaf has been considered as the main pathway of Ca export to other plant parts for foliar application (Hanger, 1979). The radioactivity counting and macro-autoradiograph showed that in the case of foliar application, Ca was translocated in the leaf, particularly, when Ca concentration was increased during water stress. However,

the movement was very slow and Ca was not detected in the seeds. Thus, Ca penetration in the pod is very important for Ca foliar application especially under water stress condition during the seed-filling stage which causes Ca reduction in seeds. The macro-autoradiograms of the pod immersion experiment (Fig. 5.11) showed Ca penetration into the pod. The macro-autoradiograms of pod immersion and injection experiments (Fig. 5.11 and Fig. 5.12) showed Ca entrance into the pod through the dorsal suture of pod and then diffused outside the pod surface. The macro-autoradiograms of seeds in these experiments (Fig. 5.13) also showed that Ca mostly accumulated on the seed coat (dark part). The seed cotyledon did not absorb much Ca (light part of the seeds)

Figure 5.7 Autoradiograms of longitudinal sections of soybean stems (3 samples) from root-feeding



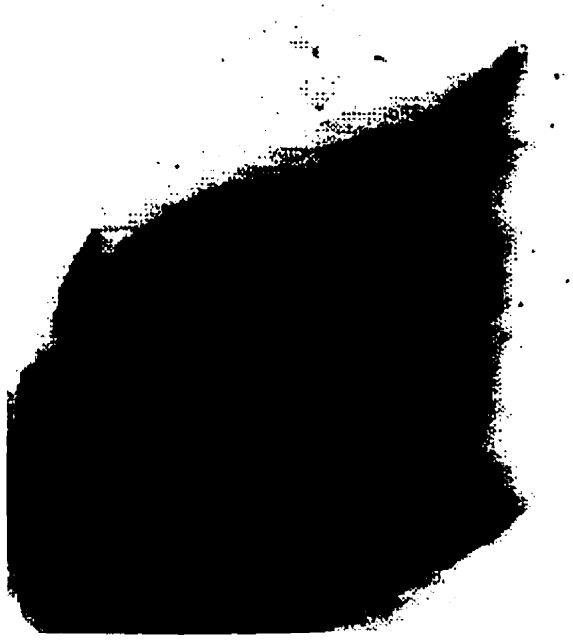
(a)



(b)

Figure 5.8 Autoradiograms of top leaves of injected soybean (a) water stressed, (b) non-stressed

(a)



(b)

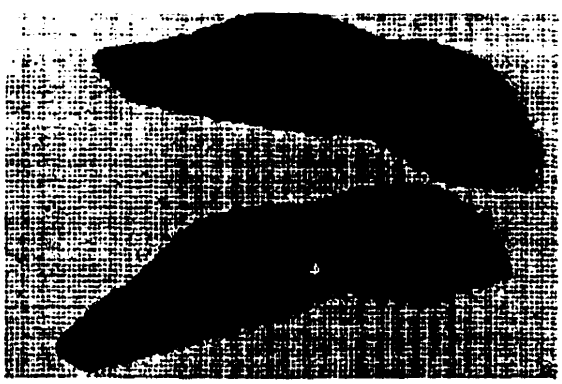
Figure 5.9 Autoradiograms of immersion leaves of soybean (a) 10 mM, and (b) 30 mM concentrations

(a)

(b)

Figure 5.10 Autoradiograms of immersion leaves of soybean (a) water stressed, and (b) non-stressed

(a)



(b)

Figure 5.11 Autoradiograms of pod-tip immersion of soybean plants (a) water stressed, and (b) non-stressed

(a)

(b)

Figure 5.12 Autoradiograms of pods from injected soybean plants (a) water stressed, and (b) non-stressed

(a)

(b)

Figure 5.13 Autoradiograms of soybean seeds (a) from immersion pod-tips, and (b) from injected plants. Notice the dark areas of seed coats

5.4 CONCLUSIONS

Calcium translocated slowly but significantly towards the leaf base when the tip was immersed in $^{45}CaCl_2$ solutions of various concentrations. The mid-rib was the principal transport conduit and the primary and secondary veins also transported ^{45}Ca. The near seed absorbed considerably more Ca than the far seed in a pod-tip immersion trial. Calcium was not translocated from pods to leaf under water stress conditions during seed-filling. The beta-ray gauge could provide a realistic indication of plant water status during drought conditions than leaf temperatures which fluctuated depending on the weather.

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CONTINUITY OF THE THESIS TOPIC BETWEEN CHAPTER 5 CHAPTER 6

Ca concentration and distribution were studied after Ca was administered to soybean plants through leaf-tip and pod-tip immersions in Chapter 5. The idea for yet another way of administering Ca came from previous Chapters. Ca was fed through a branch of soybean in combination with some known compounds that affect Ca translocation in plants. This work is descibed in Chapter 6. A manuscript on work in Chapter 6 has been submitted for publication.

CHAPTER 6

WATER STRESS AND CALCIUM CONCENTRATION DURING THE SEED-FILLING STAGE OF SOYBEAN AFFECT SENESCENCE

ABSTRACT

Solutions of ruthenium red (RR, 0.01 mM), Ethylene Glycol-bis-(β-aminoethyl ether)-*N,N,Ń,Ń*-Tetraacetic Acid (EGTA, 0.1 mM), calcium (Ca, 1 mM), and double distilled water (control) were fed through a bottom branch of soybean with (ST) and without (NS) water stress at the seed-filling stage. The volume absorptions and transpiration rates were significantly higher for NS than ST plants and decreased almost linearly with time for all treatments. The transpiration rates of Ca-feeding ST plants and the control overlapped while the NS plants approached the same rate of transpiration by the third week. Ca was implicated in stomatal closure for the reduction in the transpiration rates. The relative amounts of chlorophyll decreased with time but chlorophyll was least affected for Ca-absorbing plants for both ST and NS plants. The use of RR (Ca transport blocker), and EGTA (Ca chelator) indicated the role of intracellular Ca concentrations on stomatal closure and foliar senescence at the end of the season.

Key words: chlorophyll content, calcium, soybean

6.1 INTRODUCTION

6.1.1 Role of Ca in Leaf Senescence:

Senescence not only constitutes a key plant developmental phase but also serves as an adaptation mechanism for survival in a changing environment (Noodén, 1980). For example, plants are known to respond when droughts occur during the reproductive stage by enhancing senescence which prematurely shortens plant life (Pell and Dann, 1991; Duncan, 1994). Although the exact mechanism for the onset of senescence is unknown, efforts are being made at an understanding from the perspectives of molecular biology involving genes (Buchanan-Wollaston, 1997), and biochemical reactions involving calcium (Ca) as a second messenger (Poovaiah, 1988; Leshem, 1987).

Ca may have a dual role in that it could either delay (Poovaiah and Leopold, 1973; Poovaiah, 1988), or promote (Leshem et al., 1982, 1984, 1986) senescence depending upon its intracellular or extracelluar concentrations. A relatively high extracellular concentration of Ca (1-5 mM) is necessary for maintaining the structural integrity of both membranes and the cell wall when stresses develop particularly from low pH, salinity, toxicity, and nutrient imbalance (Hanson, 1983). An increased apoplastic Ca concentration is known to inhibit senescence by preventing both disintegration of membranes and subsequent leakage of solutes (Poovaiah, 1988). However, a low intracellular Ca concentration (10^{-5} - 10^{-3} mM) is also important for it to act as a second messenger in response to most environmental stimuli (Poovaiah, 1985; Reddy, 1995), An increased cytoplasmic Ca concentration is reported to stimulate

phospholipase activity which in turn accelerates leaf senescence by degrading membrane structure and releasing fatty acids (Leshem et al., 1984; Leshem, 1987). Water stress during the seed-filling stage of soybean growth reduced foliar concentration of Ca (Batchelor et al., 1984; Sorooshzadeh et al., 1995; Sorooshzadeh and Barthakur, 1997), and promotes leaf senescence (Sionit and Kramer, 1977, Cure et al., 1983, Meckel et al., 1984; Cortes and Sinclair, 1986). However, the role of intracellular and extracellular concentrations of Ca on the relationship between water stress and senescence has not been explored.

6.1.2 Objective:

Thus, the objective of this paper was to examine the effects of Ca content in leaves and water stress during the seed-filling stage on senescence in soybean plants.

6.2 MATERIALS AND METHODS

6.2.1 Seed Culture and Plant Growth:

Soybean (*Glycine max* L. Merr) seeds were obtained and germinated as described in detail elsewhere (Sorooshzadeh and Barthakur, 1997). The seedlings were transferred to 20-cm diameter plastic pots which were filled with 1:1.5:1.5 (v/v/v) sand, vermiculite, and peat. The plants were grown in the same soil environment until the seed-filling stage was reached as defined by Fehr *et al.* (1971).

6.2.2 Imposing Treatments:

Plants were then selected on the basis of their uniformity in growth, and a factorial complete randomized experiment used with two factors (irrigation level and feeding solutions) and four replicates. The procedure for maintaining two irrigation levels as described by Dornbos et al. (1989) was followed. The plants with no water stress (NS) received water to saturation at the interval of every two days whereas the stressed (ST) plants received 50% of NS.

Feeding solutions were double distilled water, one mM concentration of $CaCl_2$, 0.01 mM ruthenium red (RR), and Ethylene Glycol-bis-(β-aminoethyl ether)-*N,N,Ń,Ń*-Tetraacetic Acid (EGTA) of 0.1 mM concentration. RR and EGTA solutions contained 10^{-5} mM $CaCl_2$. The pH of all solutions was adjusted to 6.5. EGTA and RR were purchased from Sigma (Oakville, Ontario). A branch of 25-30 cm in length from the bottom of a plant was selected for the feeding. The leaves of the branch were removed and its tip cut under water to prevent air entry. The cut-end was then immersed through a narrow neck of a glass container that fitted the branch snugly to minimize the evaporative loss which was monitored in a parallel experiment. Four identical containers were filled each with one of the above fluids, which were changed every two days, and a freshly cut tip was reimmersed. The total volume of fluid absorbed was measured with time after correcting for the evaporative loss. The experiment lasted for three weeks.

6.2.3 Measurement of Parameters:

The transpiration rate was measured with a portable, steady-state LI-6200 photosynthesis system (LI-COR Inc., Lincoln, NE, USA). Leaf greenness on the uppermost fully expanded leaves were measured weekly with a chlorophyll meter developed by the Soil-Plant Analyses Development (SPAD-502) section of Minolta Camera Company (Ramsey, NJ) which provided the relative amount of chlorophyll present based on light transmittance by a leaf at 650 nm (not affected by carotene) and 940 nm regions of the spectrum. The SPAD values were measures of chlorophyll concentrations obtained from the ratio of transmittances through the leaf tissue at the two wavelengths. The SPAD-502 is a hand-held chlorophyll meter that provides an estimate of extractable chlorophyll in leaves non-destructively (Earl and Tollenaar, 1997). Virtanen and Peltonen (1996) cited 15 different plant species on which the relative amount of leaf chlorophyll were measured with SPAD-502 by 15 research groups in widely different geographical areas.

At the end of the final chlorophyll measurements, the leaves were harvested and their Ca concentrations were determined by using atomic absorption spectrometer (Perkin-Elmer, Model 2380, Norwalk, CT, USA).

The data were analyzed statistically by the repeated measurement method using the SAS software (SAS Institute, Cary, NC, USA).

6.3 RESULTS

The volume absorptions of Ca, EGTA, and RR solutions by the NS plants decreased almost linearly as the number of weeks increased (Fig. 6.1a) except that the water uptake (control) decreased sharply from the second to the third week. Ca uptake was significantly ($P < 0.001$) lower compared with the RR and EGTA absorptions and the absorption characteristics of the latter solutions were very similar. The water and Ca absorptions were significantly different ($P < 0.001$) for the first two weeks but by the third week the absorptions approached almost the same value. The uptake volumes decreased significantly for ST compared with NS plants (Fig. 6.1b). The absorption kinetics remained almost linear with greater negative slopes than the corresponding NS plants. The RR and EGTA absorptions were again very similar and each was significantly higher than those of Ca and water. Water and Ca absorptions were almost the same except at the second week when a significant difference appeared between them.

The transpiration rate of the NS plants decreased with time for all treatments (Fig. 6.2a). Ca transpiration was significantly lower than the control for the first two weeks but by the third week reached about 50% of its initial rate. RR and EGTA transpiration rates were significantly higher than those of Ca. The transpiration rates of the ST plants (Fig. 6.2b) decreased almost linearly with time, and were significantly lowered from those of the NS plants for all treatments. However, unlike the NS plants, the transpiration rates of control and Ca overlapped. The transpiration rate was significantly higher for RR compared with Ca and the control plants. Significant differences in the transpiration rates were observed between EGTA, Ca, and control for only the first two

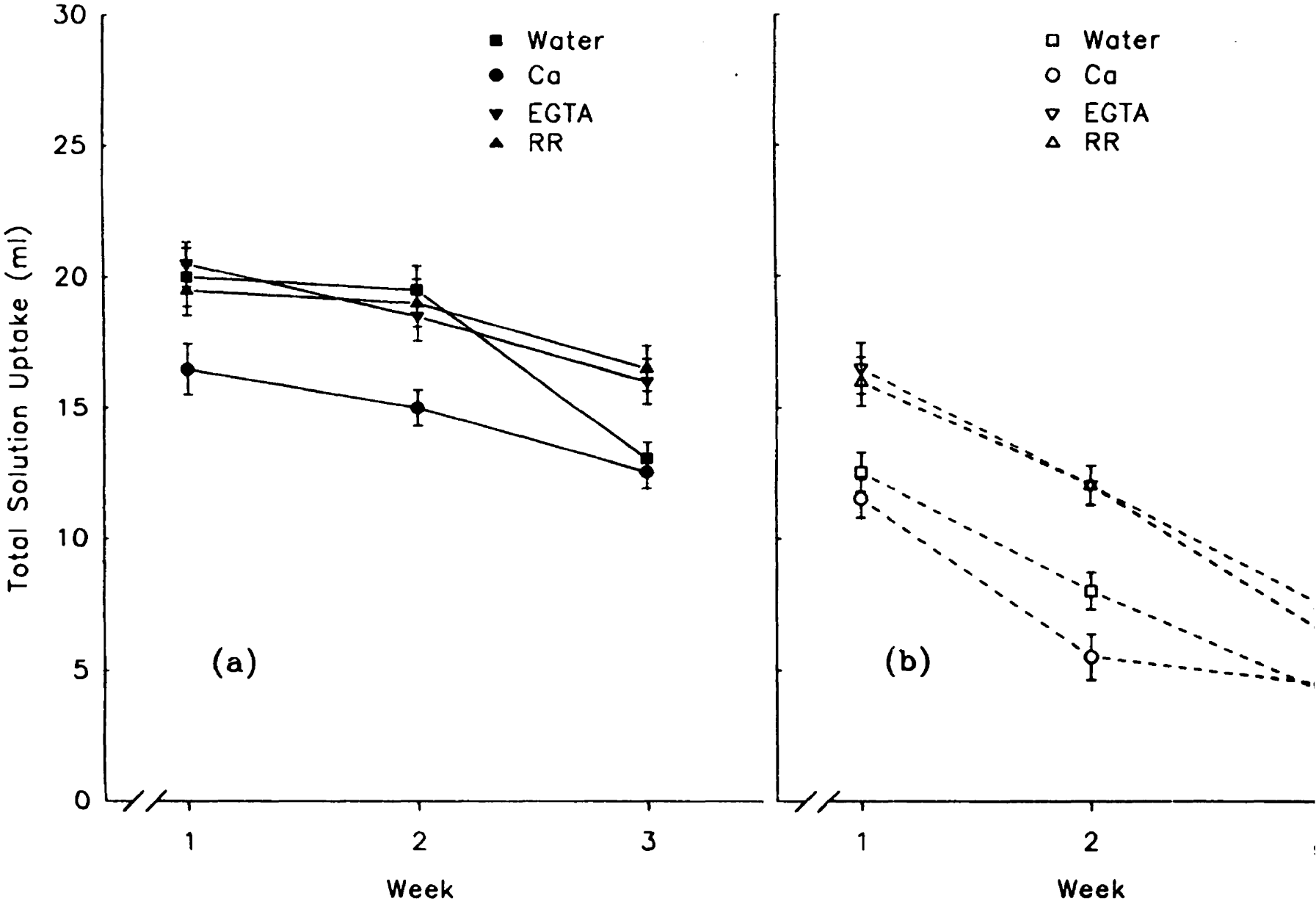

Figure 6.1 Mean cumulative solution uptake per week without (a) water stress (NS), and (b) with water stress (ST) for soybean plants. Each point represents mean of four replicates and the error bars indicate SD

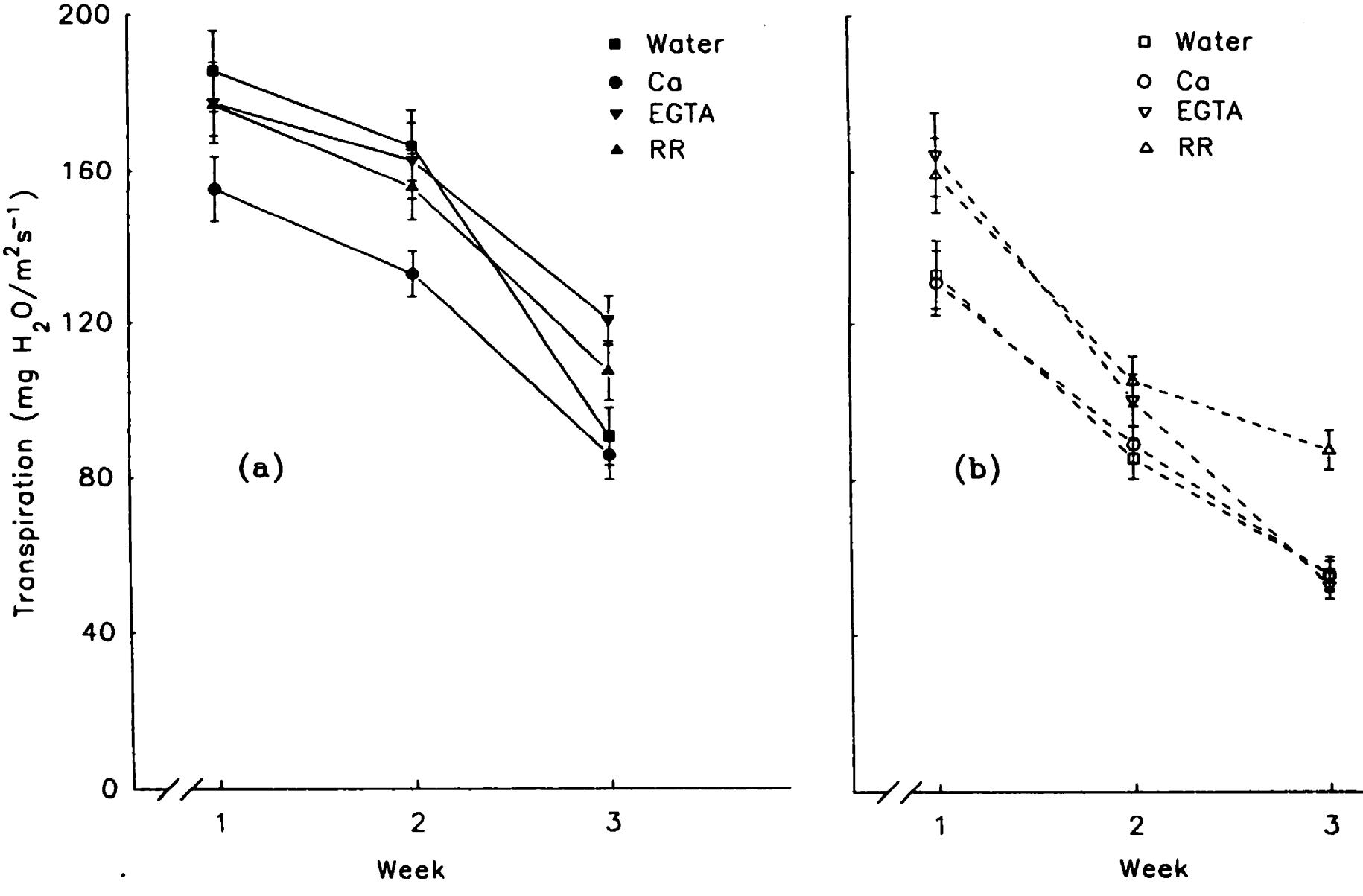

Figure 6.2 Mean transpiration rate kinetics without (a) water stress (NS), and with (b) water stress (ST) for soybean plants.

Each point represents mean of four replicates and the error bars indicate SD

weeks. RR and EGTA transpiration rates were not significantly different except at the third week.

The relative amounts of chlorophyll decreased almost linearly with increased number of weeks for all treatments (Fig. 6.3a) on NS plants. There was a significant difference between treatments at the third week. Chlorophyll was least affected for plants absorbing Ca. Chlorophyll also decreased linearly with time for ST (Fig. 6.3b) but with greater negative slopes compared with the NS plants. Significant differences were observed between treatments for the second and third weeks. Chlorophyll was again least affected for plants absorbing Ca. The present experiments clearly showed that Ca can delay senescence in NS plants by at least one week and two for ST.

Under both irrigation levels Ca concentrations in Ca-feeding plants were 4-5 times (Table 6.1) higher than the control. Ca concentrations in EGTA and RR treatments were similar to the control for ST and NS plants. Water stress reduced Ca in all feeding treatments by approximately 50% compared with NS.

6.4 DISCUSSIONS

The control plants absorbed in three weeks an average of 54 ml of water which compared favorably with 49-52 ml of stem infusions reported by Grabau et al. (1986) for two soybean cultivars. The close agreement between the two methods indicated that our feeding technique with no external force was satisfactory compared with the infusion method. The difference in the two methods was the infusion force which could change the distribution pattern of the feeding solution in the plant. In a radiotracer experiment,

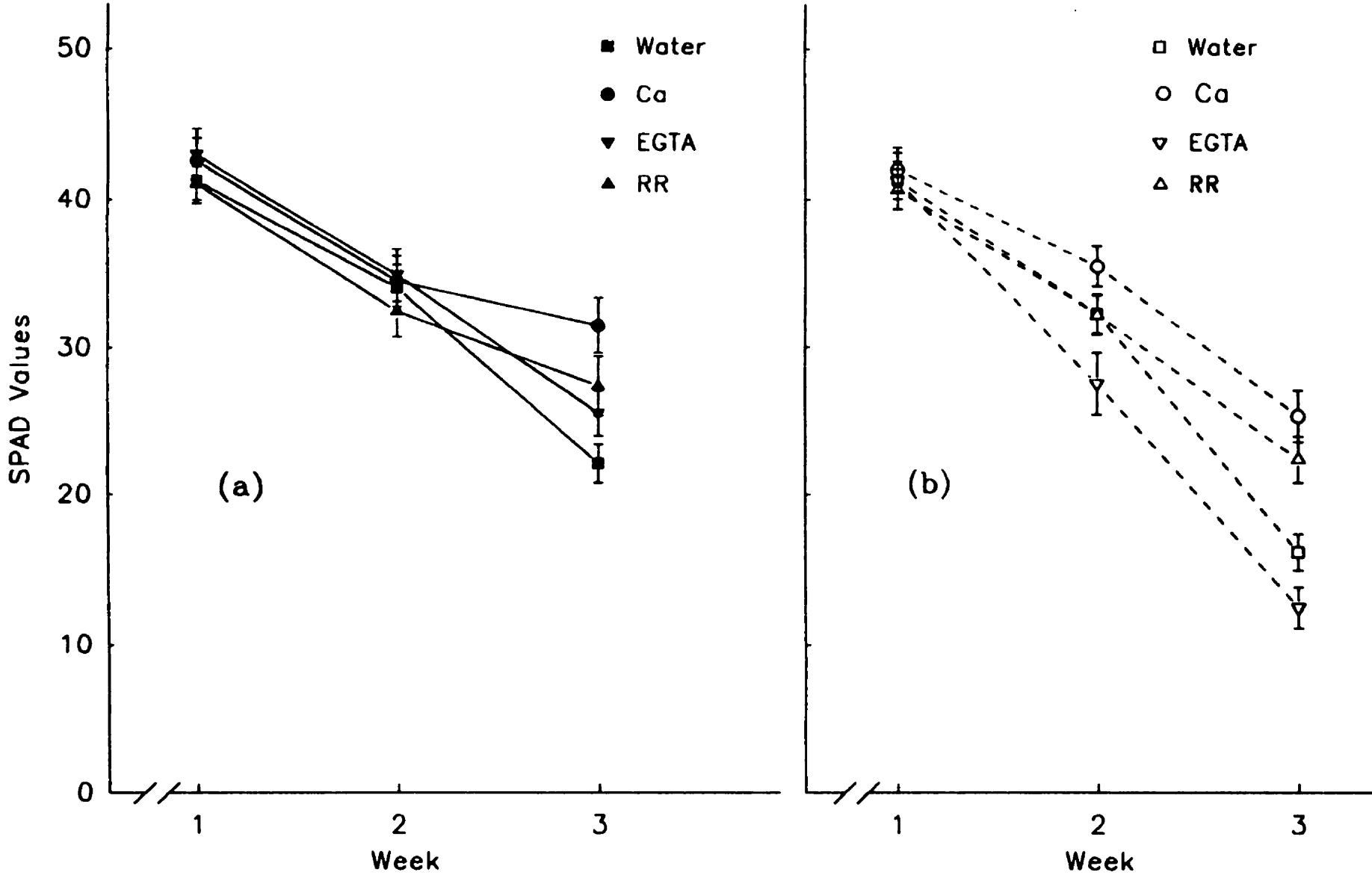

Figure 6.3 Mean SPAD values (chlorophyll mater) with time without (a) water stress (NS), and with (b) water stress (ST) for soybean leaves. Each point represents mean of four replicates and the error bars indicate SD

Table 6.1 Ca concentration percentage on moisture-free basis for the uppermost fully expanded soybean leaf when fed with calcium (Ca), EGTA, RR, and water. Mean values on four replicates are shown with their SD

	NS	ST
Ca	10.83±1.3	6.36±0.8
EGTA	2.48±0.4	1.35±0.3
RR	2.17±0.54	1.75±0.33
WATER	2.71±0.3	1.23±0.41

the presence of radiocalcium below the point of infusion indicated the effect of the feeding force (Sorooshzadeh et al., 1995).

The decreasing solution uptake kinetics may be explained in terms of transpiration rates because of their similar patterns. The guard cells control stomatal movement, and the production of abscisic acid (ABA) in response to water stress produces a signal that triggers the guard cells into closing the stomata (McAinsh et al., 1990). Although these authors have shown that the ABA signal is transduced through increased Ca in the cytoplasm of guard cells, yet the ABA mechanism for stomatal closure has not been fully understood. Also, the effect of cytoplasmic Ca on stomatal closure at the whole plant level is unknown because experiments have been performed on isolated epidermis (De Silva, 1994). The cytoplasmic Ca is known to be regulated precisely by the cell and environmental changes (Bush, 1995). It is regulated via the influx and efflux of Ca^{2+} from the extracellular (apoplastic) through the plasma membrane or from the intracellular pools (mitochondria, vacuole, endoplasmic reticulum). Intracellular and extracellular sources of Ca^{2+} have been reported for increased cytoplasmic concentration of Ca in guard cells in response to ABA production (Schroeder and Hagiwara, 1990; Gilroy et al., 1991). EGTA (Ca chelator), Ca ionophore (A23187)(Ca antagonist), ruthenium red (Ca transport blocker) were used to study the effects of increasing or decreasing concentrations of the cytoplasmic Ca on cell metabolic activities in a changing environment (Poovaiah and Reddy, 1987; Huang *et al.*, 1990).

EGTA depletes Ca from the cytoplasm by removing it from the cell wall, and preventing its influx into the cell (Tlalka and Gabrys, 1993). Ca ionophore and ruthenium

red (RR) increase Ca in the cytoplasm (Huang *et al.*, 1990), although the exact mechanism for RR action has not been fully investigated (Subbaiah *et al.*, 1994). Experiments were performed with EGTA and the Ca ionophore, which showed stomatal closure of detached epidermis with increased cytoplasmic Ca concentration (De Silva et al., 1985; Schwartz, 1985). However, Inoue and Katoh (1987) reported that RR had no effect on stomatal movements on isolated epidermis of *Commelina communis* when Ca was not present in the incubation medium. These investigators observed that in presence of Ca, RR produced an inhibitory effect on stomatal opening. Also, a pretreatment of epidermis with RR prevented Ca to act on stomatal opening which indicated the inability of Ca to penetrate into the guard cells after the treatment. Effects of Ca, EGTA, and RR on transpiration in our experiment might be viewed in light of the above discussions.

Since Ca concentrations were measured at the third week subsequent discussions will refer to this time. The higher transpiration rates of RR plants compared with the control under both irrigation levels were perhaps caused by Ca blocking. RR increased Ca in cytoplasm (Huang *et al.*, 1990), but a new theory indicated that RR might block Ca transport through plasma membrane independent of and in addition to organellar fluxes (Subbaiah et al., 1994). Thus, RR blocks the internal store of Ca and reduces the influx capacity resulting in an inhibition of Ca influx and efflux. Wilkinson and Duncan (1993) reported $^{45}Ca^{2+}$ uptake inhibition by sorghum roots in presence of 0.01-0.1 mM of RR. A relatively high transpiration rate for RR feeding plants compared with the control may be explained in terms of the inhibitory effect of RR on Ca uptake by guard cells for ST and NS plants.

EGTA depletes cytoplasmic Ca and reduced transpiration rate for NS but ST plants were not affected compared with the control. Why ST plants were not affected remains to be investigated.

The difference in Ca concentrations between the Ca-feeding and the control plants with no difference in their transpiration rates for both irrigated levels indicated the source of Ca may be intracellular. Atkinson et al. (1990) also reported stomatal closure when a high concentration of Ca (8-16 mM) was fed via the xylem vessels of *Commelina communis* L. and *Triticum aestivum* (wheat) leaves which agree with our results prior to the third week for NS plants. Swietlik and Miller (1987) reported a reduction in stomatal conductance and transpiration rates when $CaCl_2$ (60-100 meq l^{-1}) was foliarly applied on water-stressed apple trees. The difference between our and the results of these authors could be attributed to a direct Ca uptake by guard cells when applied foliarly.

Loss of membrane integrity and cell membrane stability from water stress in soybean have been reported (Senaratna and McKersie, 1983, Krishnamurti et al., 1984). Premachandra *et al.*, (1990) showed that Ca concentrations in leaf tissue and cell sap of soybean were negatively correlated with the percentage of injury of cell membrane in a PEG (polyethylene glycol) solution. Ca binds to the negative charge of the plasmalemma and can protect the membranes from lipid degradation and maintain stability of cell membranes (Cheour et al., 1992). Thus, a relatively high extracellular concentration of Ca is required to maintain structural integrity of membrane and cell wall subjected to water stress (Hanson, 1983). Experiments have shown water stress during the seed-filling stage of soybean reduced Ca concentration in leaves (Batchelor et al., 1984;

Sorrooshzadeh et al., 1995; Sorooshzadeh and Barthakur, 1997) and hastened leaf senescence (Sionit and Kramer, 1977, Cure et al., 1983, Meckel et al., 1984; Cortes and Sinclair, 1986). Leaf senescence is delayed by a high extracellular concentration of Ca due to its action on cell wall and plasma membrane stability (Ferguson 1984). However, an increased concentration of Ca in cytoplasm of leaf cells caused leaf senescence in pea plants (Leshem et al., 1982, 1984, 1986), while retarding senescence in detached rice leaves (Huang *et al.*, 1990). Thus, it seems Ca has a dual role on leaf senescence depending on its intracellular and extracellular concentrations, and may act differently in different plant species.

Water stress during the seed-filling stage of soybean hastened senescence by 7-10 days (Sionit and Kramer, 1977; Cure *et al.*, 1983; Meckel *et al.*, 1984; Cortes and Sinclair,1986). However, plants on Ca-feeding had less yellowness compared with the control plants which indicated the role of Ca in delaying leaf senescence. The Ca-feeding plants had more chlorophyll at third week than the control for NS and ST plants which may explain the effect of Ca in improving the cell membrane stability as discussed above.

Increased chlorophyll contents in EGTA and RR relative to the control plants under NS condition may be explained in terms of their ability to reduce cytoplasmic Ca and inhibit Ca uptake by leaf cells. EGTA enhancement of leaf senescence of the ST plants might be due to a partial de-stabilization of cell wall and cell membrane resulting from the removal of Ca (Virk and Cleland, 1990). This produces an additional effect on plasma membrane stability for ST plants. Our chlorophyll experiments in general

supports the role of intracellular Ca on leaf senescence enhancement while the extracellular Ca delays senescence.

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CHAPTER 7

A Holistic View at this Thesis with Suggestions for Future Research on the Subject

7.1 An Overall Survey of the Thesis:

Plant growth and development are limited by environmental stresses, and as such studies of plant responses to the stress stimuli are very important for improving agricultural production. Physiological and biochemical studies during the last 17 years show Ca to have a crucial influence in plant responses to the environment. An astonishingly large number of cellular processes are regulated by the Ca homeostat in plants. The Ca homeostat and how it regulates stimulus-response relationships in plants are highly complex. However, today it is a subject of intensive research on plant responses to the environment at the cellular level for both animals and plants.

One of the most common environmental stresses is drought, which occurs most frequently in the arid and semi-arid regions, although droughts are not uncommon in other climatic regions as well. The role of Ca under water stress has been known at the cellular level, and during drought ABA causes stomatal closure by increasing Ca concentration in the cytoplasm of guard cells. Ca serves in the signal transduction process to leaves in order for the foliage to sense soil water status, and thus regulates plant

growth and development when water stress prevails. The part Ca plays in plant adaptation to drought at the cellular level has been demonstrated, but the function of Ca at the whole plant level is still under investigation. Studies of Ca concentration and distribution in plants exposed to environmental stresses constitute a preliminary step to a better understanding of Ca involvement in plant response.

In this thesis an attempt has been made to understand how Ca concentration and distribution in soybean plants are affected by water and light stresses. The seed-filling stage of soybean has been the main theme of this thesis because water stress during this stage reduces seed weight, depresses seed germinability, enhances senescence, and lowers seed Ca concentration. The question of why water stress during the seed-filling stage of soybean promotes senescence still remains unknown. The main hypothesis of this thesis is that the reduction in leaf Ca concentration from water stress contributes towards promoting leaf senescence. Figure 7.1 illustrates a simplified model of this hypothesis. Ca moves in the soils by mass flow and enters via apical zones of roots along with water uptake followed by its movement in the plant through transpiration. Thus, as the soil moisture stress develops plant water uptake and Ca absorption are reduced by the root system; consequently, Ca concentration in the xylem sap is also reduced. This results in a reduction in Ca concentration in the apoplasm of cells of leaf tissue. On the other hand, an increased ABA level in the leaf arising from water stress causes an influx of Ca from the extracellular and intracellular pools. Simultaneously, there is an efflux of K^+ ions

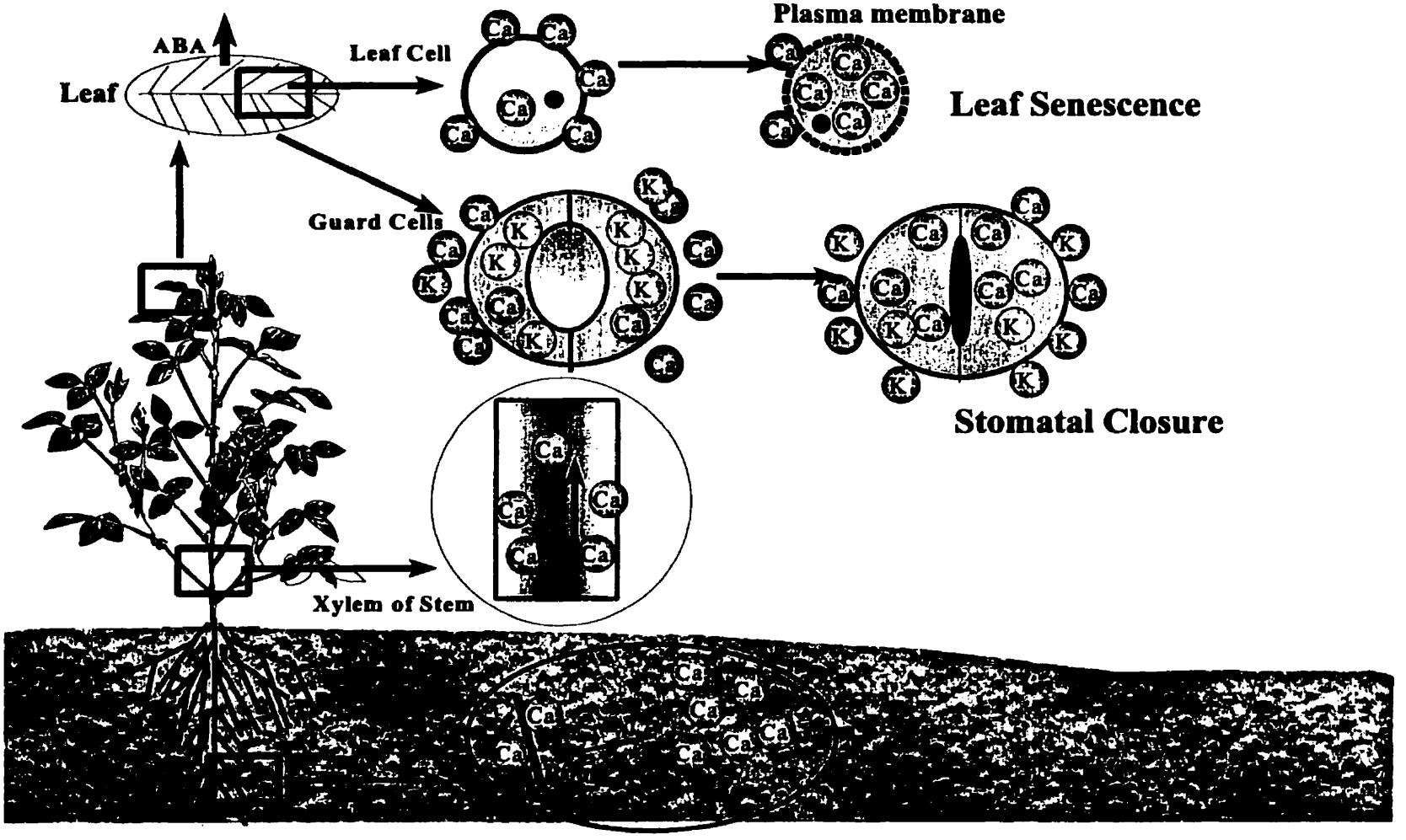

Figure 7.1 A simple model for the hypothesis of the thesis

which induces stomatal closure. Also, an increased cytoplasmic Ca concentration in leaf cells by ABA can stimulate phospholipase activity which degrades the membrane structure (broken circle). The reduction in Ca concentration in the apoplasm around cell walls due to water stress is conducive to the breakdown of the cell membrane structure which is an early feature of senescence.

7.2 Suggestions for Future Research on the Subject:

The present thesis could not cover a comprehensive study of the key roles Ca play in regulating various plant processes as the environmental stresses change in degree and nature. The subject is simply too vast to cover all aspects in a thesis like this. The molecular biology of Ca was not the main component of this thesis. Ca homeostasis does call for research on both cellular and the whole plant levels. However, the type of research presented in this thesis is expected to be increasingly important, particularly, in the context of the impending global warming phenomenon that a majority of scientists anticipate. The rapid rise of atmospheric CO_2 concentrations together with pollutant anthropogenic gases like methane, chlorofluorocarbons, nitrous oxide are enhancing the 'greenhouse effect' on earth. The plant response to the climate change could be profound and remains largely unknown. The photosynthetic pathway (C_3, C_4, CAM), water-use efficiency, growth due to temperature elevation or depression, drought and freezing tolerances, rooting depth are expected to be affected by the climate change. Reproductive

rate, plant lifespan, seed size, and seed number may also be adversely affected. In this scenario, one may well ask the question whether Ca as a macro-nutrient and a regulator of cell processes could ameliorate the deleterious effects of global warming on plant life. The following topics may be cited as research projects for the future.

1) Investigate whether Ca could modify, particularly, delay the process of senescence with increased temperature and precipitation changes predicted by various climate models in existence. Delaying senescence by even a few days will have a considerable economic impact.

2) Determine the onset of senescence and the role of Ca in it. Environmental stresses, such as droughts and radiation, may very well initiate senescence in plants.

3) Study the patterns of gene expression during foliar senescence. Several senescence-enhancing genes have already been identified, and research in this area has been active. Ca as a second messenger may play an important role in this type of research.

4) Find the optimum concentration of Ca for foliar application of irrigated and non-irrigated soybean field crops to increase seed yield.

5) Explore signal transduction pathways in guard cells of soybean by using mutants and transgenic plants, since these have been used successfully as tools in Ca and plant hormone-related research.

6) Develop an imaging technique suitable for plant cells to shed new light on Ca influxes and effluxes. The imaging technique with sophisticated cameras and computers has been highly successful in Ca research on human and animal cells.

7) Include other environmental stresses, such as soil salinity, to determine the degree of enhanced tolerance of soybean plants when Ca availability is increased. This research is especially important since a considerable portion of the agricultural land in the developing world is saline. Soybean is probably more a calcicole than a calcifuge species.

CHAPTER 8

SUMMARY AND CONCLUSIONS

8.1 Summary:

The motivation to undertake the research culminating in the present thesis originated from reports of findings that Ca has a crucial role in plant cell responses to changes in the abiotic environment. The idea of a similar role for Ca at the whole plant level took root at the time of starting the work on the thesis. Basic information on a possible role of Ca in delaying the senescence process when water stress occurred during the seed-filling stage of soybean formed a cornerstone of this thesis. The relationships between water stress and Ca concentration and distribution during the seed-filling stage were examined, since the literature review revealed conflicting reports on the subject. The contradictory reports in the literature might be, at least partly, explained in terms of the variations introduced by the root system. Therefore, the effects of water stress on Ca concentration were explored by both non-root and root feedings of radiocalcium (^{45}Ca) in this thesis. The first was accomplished by using a stem injection method in conjunction with the radiotracer methodology which provided both sensitivity and accuracy in the determination of Ca distribution and concentration. The potential application of the method in plant physiological studies to monitor the distribution of other mineral nutrients can be anticipated. The foliar concentration of Ca was reduced for soybean plants in water stress independent of the feeding technique used. A long photoperiod also reduced

the effect of water stress on Ca concentration in the upper leaves. The infusion method, however, involved force-feeding which might influence the distribution pattern of Ca.

A technique of leaf-tip and pod-tip immersions in radioactive $CaCl_2$ solution was used to study Ca distribution and translocation in soybean plants suffering from droughts. Ca appeared in pod tissue and reached the seeds. No Ca translocation from leaf to seed occurred and its penetration into pod tissue was a slow process. Thus, foliar application of Ca may improve its concentration in leaves, but may not substantially increase its concentration in seeds.

An method of branch feeding was developed to investigate the effect of increased Ca concentration in the xylem of whole soybean plant in response to droughts during the seed-filling stage. An increased xylem concentration of Ca can affect stomatal movement and leaf senescence depending on water stress.

8.2 Conclusions:

The Ca distribution pattern in soybean during the seed-filling stage demonstrated a significant reduction in the concentration of the nutrients in stem, leaves, and seeds for moderately water-stressed compared with non-stressed plants. Thus, Ca deficiency may be anticipated in soybean cultivation in semi-arid regions. The radiotracer technique combined with stem infusion provided an appropriate methodology to monitor Ca movement in plants.

Ca translocated slowly but significantly towards the leaf base when the tip was immersed in $^{45}CaCI_2$ solutions of various concentrations. The mid-rib was the principal

transport conduit and the primary and secondary veins also transported ^{45}Ca. The near seed absorbed considerably more Ca than the far seed in a pod-tip immersion trial. Ca was not translocated from pods to leaves under water stress during the seed-filling stage. The beta-ray gauge could provide a realistic indication of plant water status during droughts than leaf temperatures which fluctuated depending on the weather. Both root and non-root feedings of Ca showed its concentration in stem, leaves, seeds of soybean significantly reduced if droughts prevailed during the seed-filling stage.

Photoperiod influenced Ca concentration and distribution pattern, and delayed soybean senescence and altered stomatal dynamics when moisture stress occurred during the seed-filling stage.

THE END