3-1986

The effects of boron and in vitro propagation stress on the morphological and physiological development of broccoli

Peter D. Petracek

To the Graduate Council:

I am submitting herewith a thesis written by Peter D. Petracek entitled "The effects of boron and in vitro propagation stress on the morphological and physiological development of broccoli." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant, Soil and Environmental Sciences.

Carl E. Sams, Major Professor

We have read this thesis and recommend its acceptance:

Bob V. Conger, David L. Coffey, Effin T. Graham

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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To the Graduate Council:

I am submitting herewith a thesis written by Peter D. Petracek entitled "The Effects of Boron and In Vitro Propagation Stress on the Morphological and Physiological Development of Broccoli." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant and Soil Science.

Carl E. Sams
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We have read this thesis
and recommend its acceptance:

Effie T. Graham

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Date 12-1985

THE EFFECTS OF BORON AND *IN VITRO* PROPAGATION STRESS ON THE MORPHOLOGICAL AND PHYSIOLOGICAL DEVELOPMENT OF BROCCOLI

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Peter D. Petracek

March 1986

ACKNOWLEDGMENTS

I wish to acknowledge the following people who have provided input and support during my Master's program:

My parents, Doris and Donald Petracek, for their guidance and encouragement.

My advisor at the University of Minnesota, Dr. Robert M. Carlson, for providing me early research opportunities and for sharing his philosophical perspective.

My colleagues, in particular, David D. Songstad and Dr. Dennis J. Gray, for providing valuable conversation.

My committee members, Dr. Bob V. Conger, Dr. David L. Coffey, and Dr. Effin T. Graham, for their useful comments.

Above all, my advisor, Dr. Carl E. Sams, for providing advice, criticism, and encouragement during all stages of my Master's program. I am greatly indebted to him.

ABSTRACT

Boron deficient broccoli plants produce small heads and develop stress symptoms which reduce the marketability of the head. Although the losses in productivity of broccoli due to boron deficiency have been well-documented, the changes in the morphological and physiological development of boron deficient broccoli plants have not been thoroughly examined. The purposes of this project were to examine the influence of boron on the development of broccoli plants and broccoli leaf explants, and to evaluate the potential of using *in vitro* propagation as a means of regenerating broccoli plants for use in commercial production.

Boron deficient broccoli plants developed deformed young leaves which had reduced chlorophyll levels and low stomatal conductance and transpiration rates. Boron deficiency induced stress symptoms which included scaling of the stalk epidermis, increased phenolic compound and fiber content of the head, and pith deformation. Symptoms of boron deficiency were less severe in plants which were more efficient in boron uptake.

Broccoli plants grown in toxic concentrations of boron had chlorotic leaf margins, reduced chlorophyll content, and low rates of net photosynthesis. However, these plants developed larger heads, which may suggest that the boron demand of developing broccoli heads is not met when plants are grown in non-toxic levels of boron.

Boron deficiency enhanced auxin-stimulated ethylene production, while suppressing root initiation of broccoli leaf explants. Ethylene blocker and promoter studies indicated that the reduction in root initiation was not caused by enhanced ethylene production.

Broccoli plants regenerated from buds were smaller and matured sooner than seed grown plants. Concentration of harvest and uniformity of head size were not improved through clonal propagation. Further, the frequent occurrence of somaclonal variation may limit the commercial use of _in vitro_ propagation.

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CHAPTER I

INTRODUCTION

In recent years, the public has become increasingly aware of the effect of daily diet on health. Awareness of the importance of vitamins, minerals and dietary fiber, as well as the possible negative effects of cholesterol and sugar, has resulted in a consumer preference for more nutritious food items. This preference has created an increased demand for fresh vegetables. In order to supply this demand, a vegetable producer must grow a high quality product that meets the expectations of the consumer. One such vegetable is broccoli.

Broccoli (Brassica oleracea var. Italica) has recently found favor among consumers for its high nutrient and fiber content and its appealing taste and appearance. However, quality and marketability of broccoli can be severely reduced by environmental stress. This stress may be induced by drought, extreme temperatures, or mineral deficiency and toxicity. In particular, broccoli quality is greatly reduced by boron deficiency. Since broccoli has a high boron requirement, reduced productivity occurs even in marginally deficient conditions (Gupta and Cutcliffe, 1975; Gupta, 1979). Thus, there is a need to either increase the amount of boron available to the plants or to grow plants which are less susceptible to boron deficiency. Research which leads to an understanding of the mechanisms regulating

boron deficiency could lead to the development of production techniques designed to eliminate this type of mineral stress.

Symptoms of boron deficiency in broccoli are similar to those of other plants (Gupta, 1979). One of the first symptoms of boron deficiency in broccoli is an increased dark green coloration of the cotyledons. Retarded root growth, root tip browning, and reduced formation of lateral roots and root hairs are also early symptoms. Most leaves of plants with severe boron deficiency have narrow leaf blades, thick lamina, uneven chlorosis near the margins, and split petioles and veins from which new lamina tissue can arise. The leaves often curl under at the margin and attachment of the petiole to the stem is often severed. Early foliage droppage, however, may be prevented by a very resinous bond at the leaf axis. Young leaves on mature plants under slight boron stress exhibit symptoms similar to those of severely stressed plants. The main stalk of slightly stressed plants develops epidermal blisters and scales under the attachment point of the youngest leaves during floral initiation while severely stressed plants often form epidermal blisters and scales 4 weeks after germination. Damage to the stalk pith includes hollowing and development of a brown resinous tissue. Floral development is delayed and flower buds do not mature under severe stress. Boron deficient plants produce heads with greater axillary bud development, a higher incidence of foliar growth within the flower cluster, and small chlorotic buds. Flower bud and peduncle tissues from boron deficient plants are bitter. Broccoli plants

grown in boron deficient field conditions develop browning and hollowing of pith tissue which are commonly identified as "brown heart" and "hollow heart," respectively (Gupta and Cutcliffe, 1975; Gupta, 1979).

The effect of toxic levels of boron on broccoli has received less attention due, in part, to the absence of easily recognizable stress symptoms. The most evident symptoms of boron toxicity in broccoli include chlorotic and necrotic leaf margins, a general reduction of chlorophyll throughout the plant, and a depressed growth rate (Gupta, 1979).

The primary scientific goal of studying boron deficiency and toxicity is to clearly define the biochemical role of boron in plant metabolism. If the physiological basis of boron stress symptoms can be determined, then methods of preventing the symptoms or selecting of tolerant plants may be developed.

Boron's metabolic role may be as a regulator of plant growth substances. This may be evidenced by the loss of apical dominance and root initiation in boron deficient plants (Gupta, 1979). In vitro systems which use plant substances such as auxins and cytokinins to stimulate callus formation, organogenesis, and embryogenesis may be ideal systems for determining the interaction between boron and plant growth substances (Ammirato et al., 1983). Study of the in vitro effects of boron may lead to a better understanding of the metabolic role of boron in vitro.

If in vitro systems can select for tissues tolerant to boron deficiency, then plants propagated from these tissues also may be

tolerant to boron deficient conditions. However, the performance of plants propagated through tissue culture has not been thoroughly examined. Thus, a field evaluation of the growth and development of clonal broccoli is necessary to determine if tissue culture is a viable means of propagating broccoli which is tolerant to boron deficiency.

The objectives of the research performed for this thesis were to:

1. Determine the influence of boron stress on greenhouse grown broccoli plants with particular emphasis placed on boron's effect on photosynthesis, transpiration, stomatal conductance, and morphological development.
2. Determine the influence of boron concentration in culture media on root initiation and ethylene production of broccoli leaf explants.
3. Refine techniques for in vitro propagation of broccoli and evaluate the growth of regenerated plants in the field.

CHAPTER II

LITERATURE REVIEW

CHEMICAL PROPERTIES OF BORON

Boron is an element required for plant growth. It is a non-metal classified in Group III B of the periodic table. The loss of bonding energy required by the promotion of electrons from ground state ($1s^1 2s^2 2p^1$) to valence state ($1s^2 2s^1 2p^2$) is compensated for by overlapping orbitals of the valence state. By this mechanism, a stable species of boron covalently bonded to three like atoms can be maintained. Boric acid (H_3BO_3), the predominant form of boron in plants is further stabilized through π bonding resulting from lone electron pairs provided by three oxygen atoms. Borate, a very weak acid, is an electron acceptor. A fourth bond can then be formed when borate accepts electrons from a hydroxyl group, thus filling boron's outer valence shell (Cotton and Wilkinson, 1979; Sharpe, 1980).

Borate may bond with one or two molecules containing 1,2 cis-diol groups (Figure 1). Since only weak ester bonds form, the study of boron's site of biochemical involvement has been difficult. In spite of the inability to isolate borate ester complexes in biological systems, boron's biochemical role as a cis-diol complexing agent has remained most widely accepted because of the availability

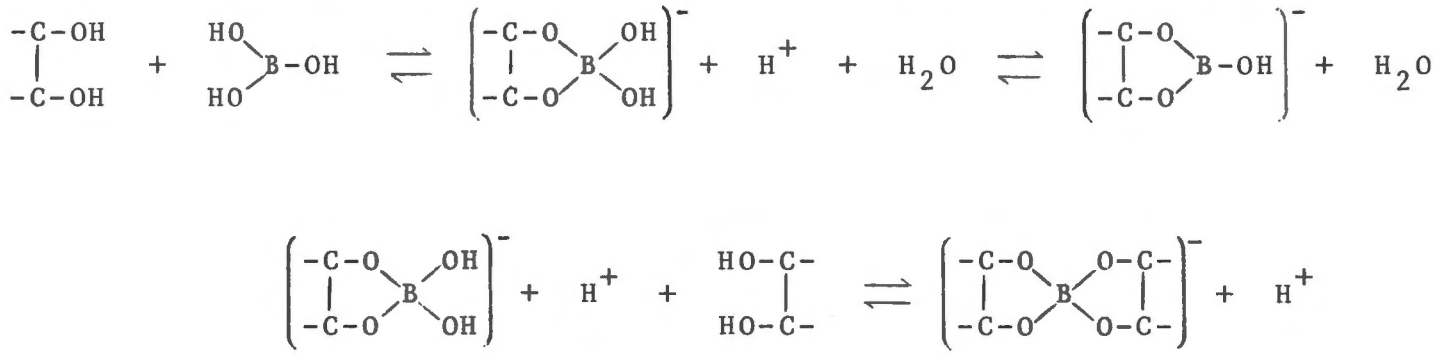

Figure 1. Hypothetical borate ester complexes (from Lewis, 1980b).

of free hydroxyl groups in cell walls and cellular membranes, and because other boron-organic complexes have not been identified (Lewis, 1980b).

BORON REQUIREMENT OF VASCULAR PLANTS

Although the biochemical roles of boron have not been clearly established, physiological effects of boron deficiency in vascular plants were demonstrated as early as 1910 (Agulhon, 1910). Since Warington (1923) established that boron is an essential element for plant growth, boron has been found to be required by some diatoms, pteridophytes, gymnosperms, and angiosperms (Lewis, 1980b). Studies have not conclusively shown that green algae or fungi require boron. and boron requirement studies for bryophytes are incomplete (Lewis, 1980b). Two theories have been postulated in recent years to explain why vascular plants require boron while non-vascular plants apparently do not. Lewis (1980b) suggested that an evolutionary dichotomy developed. In this dichotomy a change in storage carbohydrates, from those containing 1,2 cis-diols to those containing polysaccharides which do not prevent sequestering of borate, occurred. Free borate was thus able to develop a role in enzymatic regulation of lignin production. Plants producing lignin were rigid and developed vascular systems, which in turn allowed the plant to exist outside the aquatic environment. Root development and pollen germination, both boron requiring processes, were changes necessary for terrestrial adaptation. Lovatt (1985) postulated that xylem development was

the cause rather than the effect of the boron requirement of vascular plants and that accumulation of boron at the transpiration terminus may have resulted in a metabolic role in cell division in the apical meristem. Lovatt (1985) further suggested that the essential role of boron may be one of assisting in the maintenance or utilization of pyrimidine nucleotides in dividing cells.

PHYSIOLOGICAL ROLE OF BORON

The physiological role of boron has been periodically reviewed (Gauch and Dugger, 1955; Lewis, 1980b; Pilbeam and Kirkby, 1983; Gupta et al., 1985; and Lovatt, 1985). Boron deficient plants exhibit a depressed growth rate, apical tissue damage, leaf deformation, vascular system disruption, and lack of proper formation of the root system (Gupta, 1979).

Research on the metabolic effects of boron deficiency has yielded inconsistent and, occasionally, opposing conclusions. Several general areas of metabolic involvement, however, have been suggested. The deleterious effects of boron deficiency on membranes include: (1) disruption of the electron transport system and loss of ATPase activity (Pollard et al., 1977), (2) loss of activity of other membrane bound enzymes (Lewis, 1980b), (3) alteration of organic and inorganic molecular transport across the membrane (Goldbach, 1985), and (4) destruction of the contents of membrane bound vesicles (Hudak and Herich, 1976; Hirsch and Torrey, 1980). However, the involvement of boron in membrane maintenance has not

been clearly defined. Boron may maintain systems to protect membranes from destructive compounds, such as free radicals. Alternatively, boron may physically hold the membrane together, perhaps by binding free hydroxyl groups available on oligosaccharides bound to proteins in the membrane. Support of boron binding with membranes was provided by Tanada (1983) who found a major portion of the boron content of mung bean (Vigna radiata L. C.V., Berken) seedlings was localized in the membrane.

The effect of stress due to boron deficiency on membranes and membrane-related functions may be only secondary. Since boron deficiency causes a reduction of cell division (Cohen and Lepper, 1977), the primary effect of boron may occur before the membrane is formed. Researchers have investigated the role of boron in nucleotide metabolism to determine if inhibition of DNA synthesis is the first effect of boron deprivation. The rate of thymidine incorporation in meristematic regions of Cucurbita pepo roots was measured shortly after depriving plants of boron. Incorporation of thymidine by boron-deprived roots was significantly less than that of roots supplied with boron six hours after initiation of boron deprivation (Kreuger et al., 1979). Supplying boron-deprived roots with boron resulted in thymidine incorporation at rates similar to the control (Cohan and Albert, 1974). Thus, inhibition of DNA synthesis by boron deprivation is, perhaps, both rapid and reversible. Blockers of pyrimidine synthesis were shown to induce symptoms of boron deficiency in boron sufficient tissue, while providing

pyrimidine to boron deprived tissue reduced the development of deficiency symptoms (Birnbaum et al., 1977). Other research, however, indicates that the loss of membrane integrity precedes inhibition of nucleotide synthesis or incorporation (Moore and Hirsch, 1983; Goldbach, 1985). The chronology of processes affected by boron deprivation provides only indirect insight into boron's biochemical role and does not necessarily eliminate other metabolic involvement.

Boron may affect the metabolism or the active binding sites of plant growth substances. Although the effect of boron on indole-3-acetic acid (IAA) has often been examined, contradictory results provide no conclusive evidence for the nature of their interaction (Pilbeam and Kirkby, 1983; Fackler et al., 1985). Since some plant responses to boron deficiency are similar to those caused by ultra-high auxin levels (e.g., radial rather than longitudinal cell wall expansion, inhibition of root elongation, root tip browning, and increased lateral root and shoot growth), a stimulation of auxin production in boron deficient plants was suggested (Neales, 1960). Jaweed and Scott (1967) found that boron-deficient plants had abnormally high IAA levels. Coke and Whittington (1968) obtained similar results and found that roots with sufficient boron recovered more quickly from large doses of exogenously applied IAA than did boron deficient roots. Furthermore they noted that previous enzymatic studies showed a decrease in oxidase activity in boron deficient tissue (Dutta and McIlrath, 1964) and that IAA breakdown was inhibited

by dihydroxyphenols (Hare, 1964). Based on this evidence they proposed a model for boron interaction with auxin metabolism (Figure 2). It was suggested that in plants with sufficient boron, catabolic enzymes of IAA are not blocked by dihydroxyphenols because the borate-dihydroxyphenol complex is non-inhibitory to IAA oxidase. In the absence of borate, however, dihydroxyphenols block IAA breakdown, which results in supraoptimal IAA levels in boron deficient plants. Research designed to test this model, generally, has given contradictory results. Bohnsack and Albert (1977) found that IAA oxidase activity in early stages of boron deprivation, in fact, was greater in boron deprived tissue that in that of the boron control. They also found that two dihydroxyphenols common in plants, caffeic acid and chlorogenic acid, did not act as IAA oxidase inhibitors. Furthermore, some researchers found that IAA concentrations are lower in boron deficient plants (Crisp et al., 1976; Smirnov et al., 1977; Fackler et al., 1985). Finally, auxin stimulation of boron deficiency symptoms in in boron-supplied sunflower (Helianthus annuus) roots has been unsuccessful (Hirsch and Torrey, 1980).

Establishing an association between boron deficiency and auxin suppression has been equally difficult. Research which showed that growth in boron-deficient plants was stimulated by auxin was not repeatable (Eaton, 1940; Moinat, 1943; MacVicar and Tottingham, 1946). Hirsch and Torrey (1980) were unable to eliminate root damage induced by boron deficiency through exogenous addition of IAA. Further study showed that IAA levels in boron deficient and

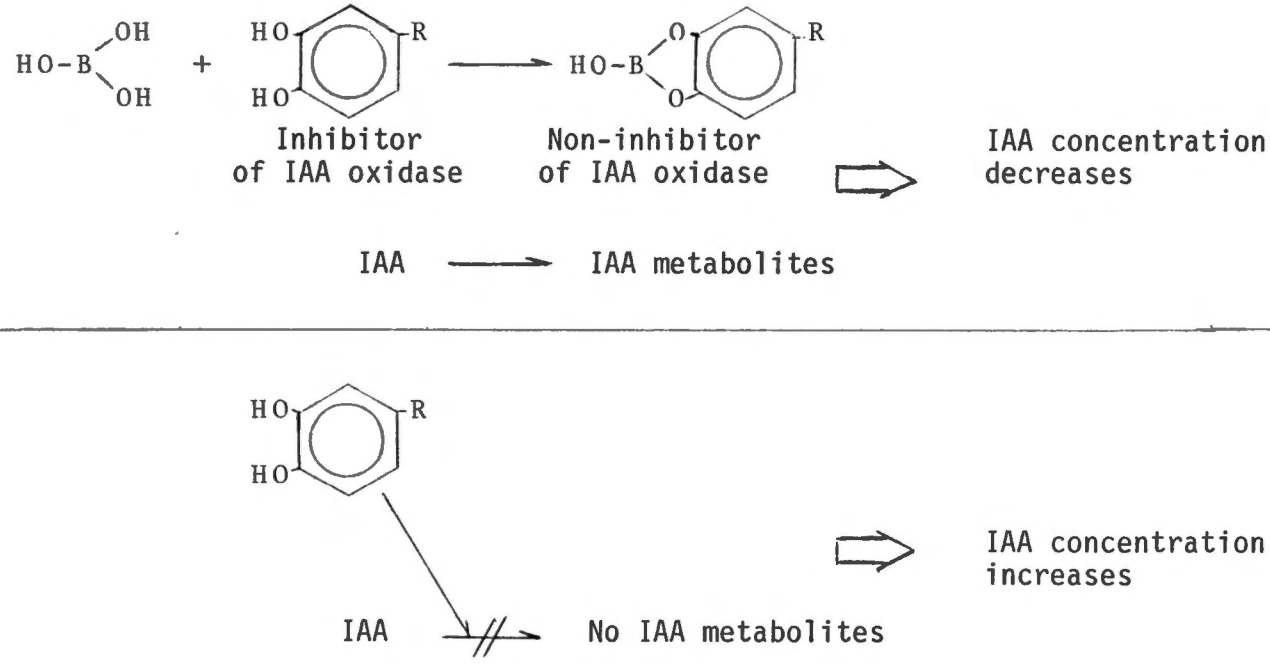

Figure 2. A model for the interaction of boron and IAA (from Coke and Whittington, 1968).

sufficient tissues were similar (Hirsch et al., 1982). Fackler et al. (1985) found that short-term boron deprivation resulted in higher levels of IAA in young roots, whereas, long-term boron deprivation resulted in lower levels of IAA in roots, young leaves, and shoot apices of seedlings.

The interaction between boron and IAA may be indirect. Boron may be required for the development of auxin-initiated response. In a series of experiments studying adventitious rooting of mung bean cuttings, auxin-stimulated root primordia initiation was found to require boron for growth and development (Middleton et al., 1978). Jarvis, Ali, and Shaheed (1983) noted that boron supplements could be delayed 24 hours after auxin treatment without affecting the amount of root initiation. It was also demonstrated that supplementing auxin-treated cuttings with high levels of boron reduced the number of roots formed but enhanced root development Jarvis et al., 1984). These results were interpreted as evidence that boron is necessary for maintaining low auxin levels after root initiation. They may suggest instead that boron is necessary for cell division and development of auxin-stimulated initiated root primordia.

Fackler et al. (1985) found that shoot apices and roots of boron deprived plants had reduced concentrations of abscissic acid (ABA). If boron deficiency symptoms such as reduced cell division, loss of chlorophyll, and loss of membrane integrity actually signify early stages of senescence, then levels of ABA (a senescence-

related hormone) might have been expected to increase (Milborrow, 1984). This finding suggests that another senescence-inducing substance, such as ethylene, may be associated with boron deficiency.

Since high auxin levels can stimulate ethylene production (Abeles, 1973) and boron may affect auxin metabolism, the level of ethylene production may be boron-concentration dependent. Alternatively, since the limiting enzyme for ethylene production may be membrane bound (Yang and Howard, 1984) and boron affects other membrane bound enzymes (Pollard et al., 1977), boron may affect ethylene synthesis by altering the membrane. Lewis (1980a) has suggested that phytoalexin accumulation in boron-deficient plants may be ethylene stimulated. However, auxin-stimulated ethylene emanation from Curcubita pepo roots grown in boron deficient nutrient solution was less than the emanation from those grown in nutrient solutions with sufficient boron.

A fourth possible metabolic role of boron is that of polyamine regulation. Although research concerning the interaction between boron and polyamines is limited, some indirect evidence suggests a possible association. Polyamines, compounds of three or more amine groups connected by short carbon chains, often have growth stimulatory effects (Smith, 1985). Jarvis, Shannon, and Yasmin (1983) found that the polyamine spermine stimulated adventitious rooting with or without the presence of auxin. Spermine-stimulated rooting, like that of auxin (Jarvis, Shannon, and Yasmin, 1983), requires

boron for root initiation. The polyamine inhibitor, methylglyoxal-bis-(guanylhydrazone) (MGBG), reduced the number of roots initiated by both control and auxin-treated stem cuttings in the presence of boron. MGBG also inhibited auxin stimulated spermine production. Thus, boron may be more directly interactive with spermine than with auxin (Figure 3).

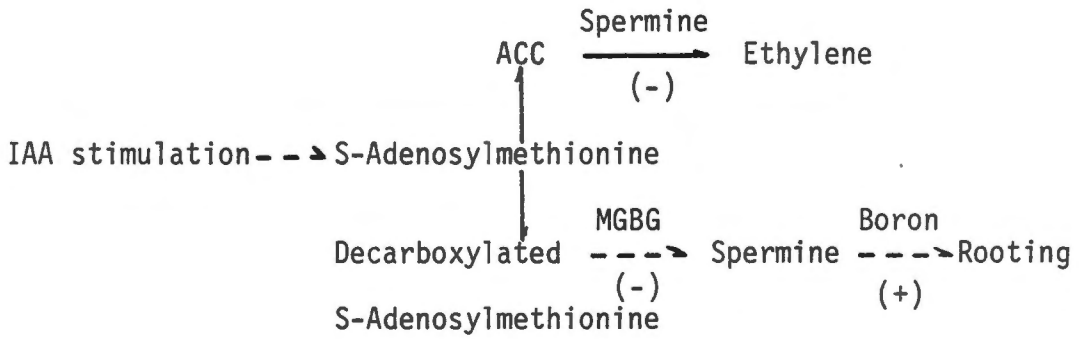

Figure 3. A possible scheme of boron interaction with spermine (from Jarvis, Shannon, and Yasmin, 1983; Smith, 1985).

Polyamines, which are capable of restoring and maintaining membrane stability (Smith, 1985), may be associated with the requirement of boron for membrane integrity (Hudak and Herich, 1976). The effect of boron on polyamine metabolism is uncertain.

BORON TOXICITY

The general characteristics of boron toxicity include loss of chlorophyll, depressed growth rate, and arrested plant development (Gupta et al., 1985). Localized morphological damage attributed to boron toxicity included chlorosis and necrosis of the leaf margin and intervenal regions of the lamina, reduced root elongation, and suppressed lateral root initiation (Gupta, 1979). Inhibition of photosynthesis by plants grown in nutrient solutions containing highly toxic levels of boron is evident by the loss of CO_2 fixation, reduction in stomatal water vapor conductance, and decreased chlorophyll concentration (Lovatt and Bates, 1984). Since boron transport in the phloem is poor, boron tends to accumulate in the older tissues of plants (Raven, 1980). Thus some damage to the photosynthesis systems of older leaves may occur, resulting in a delay in early development. The effect of boron toxicity on the whole plant, however, may be greatly reduced because younger leaves have progressively less boron. Therefore, the ability of some plants to adapt to normally toxic boron conditions may be related to an ability to maintain boron sinks.

The metabolic effects of boron toxicity are uncertain. Perhaps amplified borate complex formation inhibits enzymatic substrate binding or receptor site recognition of hormones. Alternatively, boron complex formation may cause localized acidification through release of hydrogen ions in the formation of borate ester bonds.

BORON STRESS IN CULTURE SYSTEMS

Previous studies of *in vitro* boron stress physiology have offered some explanation of *in vivo* systems. Heller (1949), Koblitz (1955), and Neales (1959) noted that boron deficiency induced a reduction in the growth rates of callus and organs. It was later found that catalase and peroxidase activities of callus and root tissues were slightly lower under boron deficient conditions (Dutta and McIlrath, 1964). More recently it has been shown that carrot (*Daucus carota*) cells in boron deficient suspension culture medium exhibited lower rates of uptake and efflux of phosphate and glucose than did cells in boron sufficient culture medium (Goldbach, 1985). When boron was supplied to the boron deficient medium, transport capabilities were restored within one hour. These results confirm previous hypotheses that boron deficiency directly affects membrane transport of phosphate (Pollard et al., 1977) and glucose (Pilbeam and Kirkby, 1983).

IN VITRO PROPAGATION OF BROCCOLI

Several techniques for *in vitro* propagation of broccoli have been developed. Propagation by organogenesis has been reported using broccoli explants of flower buds (Anderson and Carstens, 1977), stems, leaves, and leaf ribs (Johnson and Mitchell, 1978), and roots (Lazzeri and Dunwell, 1983). A protocol has been established for propagation of plants from callus derived from hypocotyl explants

(Dietert et al., 1982). Embryogenesis from broccoli anthers (Quazi, 1978) and microspores (Keller and Armstrong, 1982) have been reported. Although regeneration of broccoli plants from protoplast has not been clearly documented, regeneration from protoplasts has been reported for cabbage (Brassica oleracea var. capitalata) (Xu et al., 1982) and cauliflower (Brassica oleracea var. botrytis) (Bidney et al., 1983).

Tissue culture has been successfully used to screen for cauliflower plants with higher curd quality (Crisp and Gray, 1979). Similar success may be attained by screening broccoli explants for plants which are tolerant to boron deficient conditions. However, the feasibility of using tissue culture as a means of commercially propagating broccoli has not been established. One important factor that may prohibit the commercial use of tissue culture as a method for propagating broccoli is the high cost of regenerated plants (Anderson et al., 1977). Much of the expense of regenerated plants is due to the extensive handling of plantlets during all stages of early growth. Thus, unless the plants produced by tissue culture are far superior to seed grown plants, the current techniques for in vitro propagation may be economically unfeasible.

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PART I

THE INFLUENCE OF BORON ON THE MORPHOLOGICAL AND PHYSIOLOGICAL DEVELOPMENT OF BROCCOLI PLANTS

CHAPTER I

ABSTRACT

Broccoli (Brassica oleracea var. Italica) plants grown in perlite were supplied with nutrient solutions containing 0.08, 0.41, 0.61, 0.81, 4.06, or 8.11 ppm boron. Plants grown in low (0.08 ppm) or high (8.11 ppm) boron concentrations developed at slower rates than plants in the other boron concentrations. Symptoms of boron deficiency did not appear until shortly before bud initiation. Stomatal conductance and transpiration readings taken during the 45 day period prior to maturity indicated that a reduction in stomatal conductance coincided with the early stages of boron deficiency induced chlorosis and leaf thickening. Chlorophyll concentrations and net photosynthetic (Pn) rates of plants in 0.08 ppm boron were significantly less than those in 0.41, 0.61, and 0.81 ppm boron. Heads produced by plants in 0.08 ppm had small, chlorotic buds, scale covered stalks, and high levels of total phenols and fiber. In addition, the pith of these plants was deformed. Plants grown in high (4.06 and 8.11 ppm) boron concentrations had slightly chlorotic leaves throughout their lifecycle and very chlorotic leaf margins. Stomatal conductance and transpiration rate were not changed by toxic levels of boron in the leaves. Although the chlorophyll content and Pn rates were lower for plants in 4.06 and 8.11 ppm boron than 0.41, 0.61, and 0.81 ppm boron,

head size was slightly greater. This finding suggests that high boron concentrations, which induce boron toxicity symptoms in leaves, may stimulate head development.

CHAPTER II

INTRODUCTION

Although the metabolic role of boron is uncertain (Gauch and Dugger, 1955; Lewis, 1980; Pilbeam and Kirkby, 1983) symptoms of plant stress caused by boron deficiency and toxicity have been well documented (Gupta, 1979; Gupta et al., 1985). Plants grown in either deficient or toxic concentrations of boron commonly show a reduction in growth rate and a loss of chlorophyll. Therefore, the photosynthetic capacity of plants grown in low and high boron conditions may be reduced. Boron deprivation induces leaf thickening, irregular mesophyll cell orientation (Baker, 1956) and chloroplast membrane breakdown (Hudak and Herich, 1976) and inhibits stomatal opening (Roth-Bejerno and Itai, 1983). Plants subjected to very high boron concentrations show a reduction in net photosynthesis (Pn), chlorophyll levels, stomatal conductance, and transpiration 48 hr after treatment (Lovatt and Bates, 1985). The effects of less extreme boron conditions on photosynthesis and water relations over an entire life cycle, however, have not been thoroughly evaluated.

Broccoli plants grown in boron deficient conditions have reduced growth rates and a higher incidence of pith damage (Gupta, 1979). The relationship between parameters which govern the rate of plant growth and development and those which reflect

the extent of damage due to stress may indicate the physiological role of boron in whole plant development.

The objectives of this study were to determine the effects of boron stress on Pn, stomatal conductance, and transpiration of plants grown in deficient and toxic concentrations of boron and to evaluate the relationship between these physiological processes and the morphological and developmental abnormalities associated with boron stress.

CHAPTER III

MATERIALS AND METHODS

NUTRIENT SOLUTION

Hoagland solutions were prepared with 0.08, 0.41, 0.61, 0.81, 4.06, or 8.11 ppm boron. Previous results (data not shown) indicated the occurrence of boron deficiency stress symptoms for plants grown in 0.08 ppm boron and toxicity stress symptoms for plants grown in 8.11 ppm boron. Since some plants in 0.81 ppm boron showed symptoms of boron toxicity, two treatments of lower boron concentrations (0.41 and 0.61 ppm) were added to provide a better estimation of the optimal boron concentration for chloroplast development and photosynthesis. A treatment of 4.06 ppm boron was used to establish boron toxicity stress symptoms less severe than those produced by 8.11 ppm boron. Boron contamination was reduced by using deionized distilled water and plastic vessels throughout the experiment.

PLANT MATERIAL

Broccoli seeds (c.v. Premium Crop) were surface sterilized in 1.0% sodium hypochlorite, rinsed, and soaked in water for 5 hr to allow inbibition. Seeds were planted 5 per 19 liter pot containing hydrated perlite. Pots were spaced 50 cm apart on greenhouse benches and were arranged in a randomized complete block design. Each morning plants were provided with enough water to allow complete rehydration

or perlite and were supplied with 60 ml of nutrient solution. Seedlings were thinned to one per pot upon the emergence of the first leaf. The rate of plant development was monitored by observing the day of emergence of leaves 1-20 and the floral bud. The greenhouse was under 50% shade and temperatures were 30±5°C maximum and 19±2°C minimum.

GAS EXCHANGE MEASUREMENTS

Stomatal conductance, transpiration rates, and PAR (photosynthetically active radiation) were measured within 2 hr of solar noon using a steady-state porometer (Li 1600, LI-COR, Inc., Lincoln, NE). Measurements were taken from leaves 4, 8, 12, and 16 on days 45, 51, 57, 63, 69, and 85 after planting. Readings were taken from the lower third of the leaf 2 cm from the leaf margin. The rate of CO_2 fixation of whole plants was determined using a closed system photosynthesis chamber. Light was provided by two General Electric R 400 watt and one General Electric R 1000 watt multivapor (metal halide) lamps. Lamps were positioned above the chamber such that the PAR at the top of the canopy region of plants inside the chamber was 920 $\mu E\ m^{-2}\ s^{-1}$. Chamber temperature (26±1°C) was controlled by a forced air heat exchanger attached to the base of chamber, which also provided uniform air distribution within the chamber. To minimize CO_2 contribution from root respiration, plastic bags were wrapped around the pots and sealed shut at the base of the plant. Gas samples were taken from the chamber through a septum-covered port by syringe. Samples were taken 0, 5, 10, and 15 min

after sealing the plant in the chamber and were analyzed by an infrared gas analyzer (AR 600 Series, Anarad, Santa Barbara, CA). Pn was calculated from the slope of the line describing the change in chamber CO_2 over time. All plants were sampled within 3 hr of solar noon.

HARVEST DATA

Following Pn determination, all leaves attached to the stalk with areas greater than 10 cm^2 were counted. Areas of leaf 12 and 16, total area of leaves from axillary buds, and total area of all remaining leaves were measured with a leaf area meter (Li 3000, LI-COR, Inc., Lincoln, NE). Heads were cut 15 cm from the top of the inflorescence and head weights were recorded. Stalk diameter (15 cm from the top of the inflorescence) and head diameter were also measured. A 5 cm section of stalk was excised from the head, cut in half, and measured for pith deformation. Pith damage was expressed as the percentage of the total volume of pith that showed browning or hollowing. Tissue for chlorophyll phenol, and fiber analysis was stored at -30°C.

FRESH TISSUE ANALYSIS

Chlorophyll analysis was performed for leaf 12 and 16. Leaf discs (1.0 cm dia.) were excised from intervenal regions at regular intervals along an arc 8 cm from the leaf tip. The discs were weighed to ± 0.1 mg. Chlorophyll was multiple extracted with

80% acetone and analyzed by spectrophotometer (Shimadzu UV-620, Kyoto, Japan) for chlorophyll a and b determination (Inskeep and Bloom, 1985). Total phenol content of stalk and peduncle tissue was analyzed as previously described (Swain and Hillis, 1959) with minor modifications. Phenols were multiple extracted with absolute methanol and were reacted with Folin-Ciocalteau phenol reagent. Total phenol content was expressed as gallic acid equivalent. Determination of fiber content of the stalk was determined by hot water insoluble fiber analysis. Tissue samples (10.00±0.01 g) were weighed and thoroughly homogenized in water. Samples were boiled for 1 hr and were water rinsed for 8 min in a 30 mesh filter. Samples were dried on tared filter paper and weighed.

DRY TISSUE ANALYSIS

Leaves 12 and 16 and the head were dried for 8 hr at 70°C and ground through a 30 mesh screen. Samples were weighed and ashed (8 hr at 470°C). Ash was dissolved in 2 N HCl. Boron was colorimetrically determined with curcumin as previously described (Jackson, 1959).

STATISTICAL DATA

Treatments were replicated six times with one plant per replication and were arranged in a randomized complete block design.

The experiment was performed twice with similar results. Data presented are the results of the second experiment.

CHAPTER IV

RESULTS

A reduction in the growth rate of broccoli plants grown in 0.08 ppm boron, as indicated by the day of leaf emergence, was evident 30 days after planting (Table 1). Scales and blisters of the stalk epidermis of plants grown in 0.81 ppm boron appeared at the 18-20 leaf stage. Scaling began under the attachment point of leaves 15-16 and progressively moved down the stalk to leaf 8-10 by the day of plant maturity. Scale formation of plants in 0.41 ppm boron was initiated at the time of floral bud initiation and remained localized to areas above the axillary buds of leaves 15 and 16. Young leaves of plants in 0.08 ppm thickened and developed irregular chlorosis near the end of the leaf blade by day 69. By day 85 leaf thickening and chlorosis had spread to all leaves and petioles of young leaves were often split and blackened.

The reduction in growth rate of plants in 8.11 ppm boron became evident 48 days after planting (Table 1). Early leaves of these plants were slightly chlorotic. Plants in 4.06 and 8.11 ppm boron developed severe chlorosis of the leaf margins of fully expanded leaves. Tissue analysis revealed that foliar boron concentration increased linearly in leaves 12 and 16 as treatment concentration increased, but the increase in boron content was exponential in heads (Figure 1). Calcium, magnesium, and potassium

Table 1. The influence of the boron concentration of nutrient solutions on the number of days required after planting for the emergence of selected leaves and the floral bud of broccoli plants.

Boron Concentration of Nutrient Solution (ppm)	Leaf Number					
	4	8	12	16	20	Floral Bud
0.08	19.7±0.6[a]	30.2±1.0	40.0±1.0	48.3±1.0	58.3±1.3	68.8±1.1
0.41	19.0±0.7	28.8±0.7	37.5±0.8	45.8±0.7	53.5±1.4	65.7±0.8
0.61	19.3±0.4	28.8±0.5	38.1±0.7	46.1±0.5	53.2±0.6	64.8±0.6
0.81	18.7±0.4	28.2±0.3	36.8±0.6	47.0±0.7	54.8±1.2	66.3±0.7
4.06	19.0±0.6	29.0±0.4	36.5±0.5	45.5±1.0	54.2±1.1	65.5±0.9
8.11	19.0±0.4	28.7±0.2	39.3±1.3	48.3±0.8	56.1±1.0	65.1±1.2
0.08 vs. 0.41, 0.61, and 0.81	NS[b]	*	*	*	*	*
8.11 vs. 0.41, 0.61, and 0.81	NS	NS	NS	*	NS	NS
0.08 vs. 8.11	NS	NS	NS	NS	NS	*

[a]±SE (Standard Error of Mean).

[b]Linear treatment contrast significant at 5% (*) or non-significant (NS).

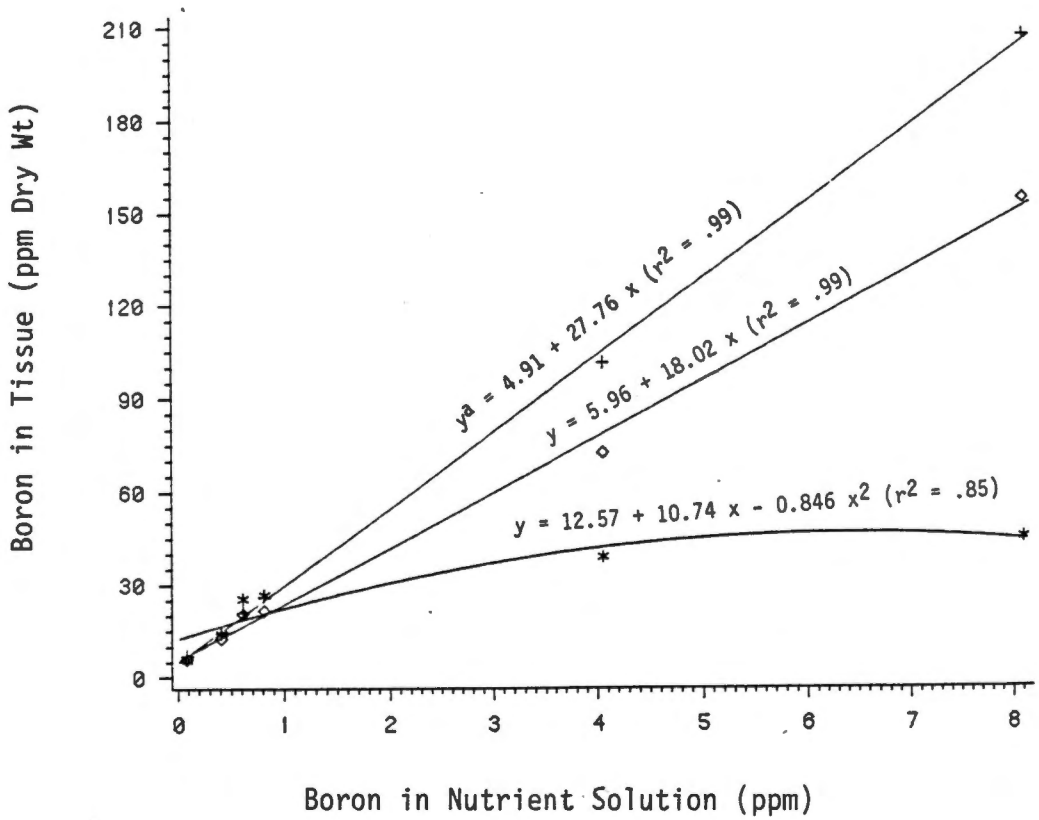

Figure 1. Boron concentration of leaf 12 (+) and 16 (◇) and the head (*) of mature broccoli plants grown in nutrient solution. The points on each line represent from left to right 0.08, 0.41, 0.61, 0.81, 4.06, and 8.11 ppm boron.

[a]y equals the boron content of plant tissue and x equals the boron concentration of the nutrient solution.

concentrations were within the normal range of distribution for broccoli (data not shown).

The photosynthetic rate of broccoli plants (920 μE m^{-2} s^{-1} at 26°C; 80% relative humidity) was significantly reduced under low (0.08 ppm) and high (4.06 and 8.11 ppm) boron concentrations (Table 2). Plants grown in 0.41 ppm boron had the highest Pn rates (20.4 mg CO_2 dm^{-2} hr^{-1}). The reduction of Pn rate of plants in 0.0811 ppm boron coincides with a significant reduction of chlorophyll levels. The chlorophyll levels of plants in 4.06 and 8.11 ppm boron were measurably, but not significantly, less than those of plants in 0.41, 0.61, and 0.81 ppm boron. However, boron toxicity induced the formation of chlorotic bands (0.2 and 0.4 mm for plants in 4.06 and 8.11 ppm boron, respectively) around the perimeter of all fully expanded leaves. Thus, localized chlorophyll loss due to boron toxicity may have resulted in reduced Pn. Chlorophyll a to b ratios ranged from 2.42 to 2.67 but were not significantly different among treatments (Table 2).

Stomatal conductance and transpiration rates were not affected by boron treatment from day 45 through day 63 for leaves 4, 8, 12, or 16 (data not shown). However, stomatal conductance and transpiration rate were both significantly reduced for leaf 16 of plants in 0.08 ppm boron by day 69 (Table 3). This reduction coincided with the initial stages of chlorophyll breakdown and thickening of leaf 16. Stomatal conductance and transpiration rate of leaf 12 were reduced by day 85. Data from a preliminary experiment

Table 2. The influence of the boron concentration of nutrient solutions on the chlorophyll content and net photosynthetic rate of broccoli plants.

Boron concentration of Nutrient Solution (ppm)	Leaf Number	Chlorophyll a ($\mu g\ dm^{-2}$)	Chlorophyll b ($\mu g\ dm^{-2}$)	Chlorophyll a to b Ratio	Total Chlorophyll ($\mu g\ dm^{-2}$)	Net Photosynthetic Rate ($mg\ CO_2\ dm^{-2}\ hr^{-1}$)
0.08	12	101± 6[a]	38±2	2.67±0.10	139± 9	14.4±1.3
	16	96±16	36±6	2.66±0.11	132±22	
0.41	12	117± 8	45±4	2.63±0.10	162±11	20.4±2.1
	16	153± 7	60±3	2.51±0.03	214±10	
0.61	12	116± 8	48±4	2.40±0.07	164±12	19.5±1.5
	16	152± 5	62±2	2.46±0.06	214± 7	
0.81	12	131±10	53±4	2.46±0.06	184±13	19.3±1.5
	16	151± 8	63±3	2.42±0.08	214±10	
4.06	12	117±10	45±5	2.61±0.09	162±15	16.4±1.3
	16	146± 9	55±3	2.66±0.07	201±12	
8.11	12	115± 6	47±3	2.50±0.08	162± 9	18.3±0.8
	16	147± 7	59±3	2.49±0.05	206±10	
0.08 vs. 0.41, 0.61, and 0.81	12	*	*	NS	*	*
	16	*	*	NS	*	
4.06 and 8.11 vs. 0.41, 0.61, and 0.81	12	NS	NS	NS	NS	*
	16	NS	NS	NS	NS	
0.08 vs. 4.06 and 8.11	12	*	*	NS	*	NS
	16	*	*	NS	*	NS

[a]±SE.

[b]Linear treatment contrast significant at 5% (*) or non-significant (NS).

Table 3. The influence of the boron concentration of nutrient solutions on the stomatal conductance and transpiration rate of broccoli plants.

Boron Concentration of Nutrient Solutions (ppm)	Leaf	Stomatal Conductance ($cm\ s^{-1}$) Days After Planting 69	Stomatal Conductance ($cm\ s^{-1}$) Days After Planting 85	Transpiration Rate ($g\ H_2O\ dm^{-2}\ hr^{-1}$) Days After Planting 69	Transpiration Rate ($g\ H_2O\ dm^{-2}\ hr^{-1}$) Days After Planting 85
0.08	12	0.79±0.03[a]	0.64±0.13	3.48±0.15	2.80±0.23
	16	0.75±0.13	0.83±0.13	3.24±0.38	3.55±0.32
0.41	12	0.79±0.05	0.82±0.04	3.45±0.24	3.61±0.13
	16	0.89±0.03	0.95±0.03	3.73±0.06	4.04±0.08
0.61	12	0.80±0.05	0.74±0.02	3.43±0.22	3.25±0.18
	16	0.82±0.06	0.95±0.04	3.74±0.14	4.00±0.69
0.81	12	0.74±0.05	0.73±0.03	3.37±0.25	3.20±0.21
	16	0.86±0.08	0.90±0.02	3.78±0.20	3.84±0.06
4.06	12	0.72±0.03	0.81±0.05	3.00±0.15	3.58±0.21
	16	0.80±0.02	0.92±0.04	3.27±0.07	3.92±0.09
8.11	12	0.77±0.05	0.82±0.34	3.49±0.05	3.58±0.23
	16	0.88±0.06	0.93±0.06	3.69±0.14	3.93±0.15
0.08 vs. 0.41, 0.61 and 0.81	12	NS[b]	NS	NS	NS
	16	*	NS	*	NS
4.06 and 8.11 vs 0.41, 0.61 and 0.81	12	NS	NS	NS	NS
	16	NS	NS	NS	NS
0.08 vs. 4.06 and 8.11	12	NS	*	NS	*
	16	*	NS	NS	NS

[a]±SE.

[b]Linear treatment contrast significant at 5% (*) or non-significant (NS).

confirm that stomatal conductance and transpiration rates are reduced for plants in 0.08 ppm boron at maturity (data not shown). High boron concentrations (4.06 and 8.11 ppm) did not induce suppression or stimulation of stomatal conductance or transpiration (Table 3).

Plants grown in 0.08 ppm boron had significantly more leaves at maturity than plants from other treatments (Table 4). The leaves of plants in 0.08 ppm boron were smaller and thicker (as evidenced by a greater weight per area of the intervenal tissue), than those of plants grown in higher boron concentrations. The total leaf area was not affected by boron concentration. Leaves from axillary buds of plants in 0.08 ppm boron accounted for about 8% of the total leaf area, but axillary bud development of plants in other boron concentrations did not contribute to the total leaf area.

Although plants in 8.11 ppm boron had fewer leaves and lower Pn rates than did plants in 0.41, 0.61, and 0.81 ppm boron, yields of plants in the highest boron treatment were higher than those in lower boron concentrations (Table 5). In contrast plants grown in 0.08 ppm boron had small chlorotic or necrotic floral buds. Further, stalk diameters of these plants were relatively large due to thickening caused by epidermal scale formation.

Pith damage (browning and hollowing) was observed in plants from the three lowest boron concentrations (Table 6). Total phenol accumulation in stalk and peduncle tissue was greatest for plants grown in 0.08 ppm boron although some plants in 0.41 and 0.61 ppm boron also had high levels. The level of hot water insoluble fiber

Table 4. The influence of the boron concentration of nutrient solutions on the leaf area, intervenal tissue weight of leaf 12 and 16, total number of leaves attached to the main stem, area of leaves from axillary buds, and total leaf area of mature broccoli plants.

Boron Concentration of Nutrient Solution (ppm)	Leaf Area (cm^2)		Leaf Weight ($mg\ cm^{-2}$)		Total Number of Leaves[b]	Leaf Area of Axillary Growth (cm^2)	Total Leaf Area (cm^2)
	Leaf 12	Leaf 16	Leaf 12	Leaf 16			
0.08	330±14[c]	318±20	163±5	210±21	14.3±0.6	352±33	4218±138
0.41	428±18	356±22	145±4	166±10	13.5±0.8	0	4847±215
0.61	442±14	350±19	130±8	148± 9	13.2±0.5	0	4484±244
0.81	441±13	309±11	151±6	149± 9	12.1±0.4	0	4383±219
4.06	414±26	338± 5	146±4	155±20	13.0±0.7	0	4834±205
8.11	470±16	338±21	142±7	155± 8	12.2±0.7	0	4625±208
0.08 vs. 0.41, 0.61, and 0.81	*[d]	NS	*	*	*	*	NS
4.06 and 8.11 vs. 0.41 0.61, and 8.11	NS	NS	NS	NS	NS	NS	NS
0.08 vs. 4.06 and 8.11	*	NS	*	*	*	*	*

[a]The fresh weight of intervenal leaf tissue 8 cm from the leaf tip.

[b]Total of leaves attached to the main stem with leaf areas >25 cm^2 at maturity.

[c]±SE.

[d]Linear treatment contrast significant at 5% (*) or non-significant (NS).

Table 5. The influence of the boron concentration of nutrient solutions on the stalk diameter, head diameter, and head weight of broccoli plants.

Boron Concentration of Nutrient Solutions (ppm)	Stalk[a] Diameter (mm)	Head Diameter (mm)	Head[b] Wt. (g)
0.08	26.0±1.5[c]	46.2±3.7	86.5± 5.3
0.41	24.7±1.2	77.2±2.7	138.7±11.6
0.61	24.0±1.1	82.7±3.9	153.7± 9.0
0.81	24.0±1.5	80.3±1.9	134.2± 7.8
4.06	24.8±1.0	73.5±4.2	150.2± 5.5
8.11	25.7±0.8	84.7±3.7	168.0± 7.9
0.08 vs. 0.41, 0.61, and 0.81	NS[d]	*	*
4.06 and 8.11 vs. 0.41, 0.61, and 8.11	NS	NS	*
0.08 vs. 4.06 and 8.11	NS	*	*

[a]Diameter of the stalk 15 cm below the top of the head.

[b]Weight of the head cut 15 cm below the top of the head.

[c]±SE.

[d]Linear treatment contrast significant at 5% (*) or non-significant (NS).

Table 6. The influence of boron concentration of nutrient solution on the occurrence of boron deficiency related stress symptoms of broccoli.

Boron Concentration of Nutrient Solutions (ppm)	Pith[a] Damage (%)	Total Phenols (µg Gallic Acid g^{-1} Fresh Wt.) Stalk	Total Phenols (µg Gallic Acid g^{-1} Fresh Wt.) Peduncle	Hot Water Insoluble Fiber (mg g^{-1} Fresh Wt.)
0.08	12.0±3.4	31.0±6.1	65.2±4.0	206±12
0.41	5.6±1.3	19.8±1.6	54.8±4.4	42± 3
0.61	1.8±0.7	24.0±2.2	55.3±5.0	44± 2
0.81	0.0	18.3±1.2	52.7±5.5	40± 1
4.06	0.0	18.8±1.7	45.5±1.5	37± 1
8.11	0.0	18.3±2.4	51.0±2.7	39± 1
0.08 vs. 0.41 and 0.61	*[c]	*	*	*
4.06 and 8.11 vs. 0.41 and 0.61	*	*	*	NS
0.81 vs. 4.06 and 8.11	*	*	*	*

[a]Pith damage is the percentage of the total volume of cortex showing browning or hollowing.

[b]±SE.

[c]Linear treatment contrast significant at 5% (*) or non-significant (NS).

was about five times higher in 0.08 ppm boron plants than in plants from any other treatment.

CHAPTER V

DISCUSSION

Boron deficiency symptoms were evident in mature broccoli in 0.61 ppm boron, while plants in 4.06 ppm boron showed boron toxicity symptoms. Within this range of boron in solution the boron content of leaf 12 was estimated to fall between 21 ppm and 100 ppm boron (Figure 1, page 38). This range was comparable to, though somewhat narrower than, a previous estimate of the optimal boron content for broccoli plants (Gupta and Cutcliffe, 1975).

The morphological symptoms of boron deficiency in broccoli may be explained, in part, by the physiological roles of boron. Plants in 0.08 ppm boron have a reduced rate of new leaf emergence (Table 1, page 37). This occurrence may be related to the requirement for boron in cell division (Cohen and Lepper, 1977). Alternatively boron deprivation has been shown to induce chloroplast membrane breakdown (Hudak and Herich, 1976) and inactivation of membrane-bound ATPase (Pollard et al., 1978). The loss of chloroplast membrane integrity could result in decreased Pn and less assimilates for growth. Therefore, the reduction of growth rate, loss of chlorophyll, and reduction of Pn rates (Table 2, page 40) in boron deficient plants may be related to the role boron plays in membrane maintenance.

Broccoli plants grown in boron deficient conditions tasted bitter and developed off flavors. This bitterness may be associated with the increased phenolic content in boron deficient tissues (Table 6, page 45). This finding agrees with previous work which suggested boron is required for the polymerization of phenols to lignin (Lewis, 1980). The high level of hot water insoluble fiber in boron deficient plants is not easily explainable. An increase in fiber formation should be accompanied by an increase in complex carbohydrate synthesis. However, complex carbohydrate synthesis has been shown to decrease in boron deficient conditions (Pilbeam and Kirkby, 1983). Thus, the absence of boron may not have directly stimulated synthesis of fiber but may have induced stress in one part of the plant which caused the fiber accumulation in the stalk.

The interrelationships among boron deficiency symptoms are not clearly understood. However, plants grown in 0.08 ppm boron, which delayed development of epidermal scaling, had relatively high Pn rates, chlorophyll levels, and day 85 stomatal conductance and transpiration rates. Perhaps the tolerance mechanisms to boron deficiency-induced damages to leaves are related to those in the stalk epidermis. The phenol content of peduncles of plants grown in 0.08 ppm boron is negatively correlated with the boron content of the head ($r=-.64$). Similarly, but to a lesser degree, other stress symptoms such as fiber accumulation and pith deformation decrease as the boron content of the tissue increases. The data may indicate that these plants which are more tolerant to low boron conditions in the root zone are better able to take up and transport boron.

Plants grown in high (4.06 and 8.11 ppm) boron concentrations had lower chlorophyll levels and Pn (Table 2, page 40), but stomatal conductance was not reduced (Table 3, page 41). Previous work has shown that boron toxicity induces reduction of stomatal conductance as well as chlorophyll levels and Pn (Lovatt and Bates, 1984). However, boron toxicity did not cause a reduction in transpiration rate (Table 3, page 41). This finding indicates that the reduction in Pn of plants in high boron is related more to a loss of chlorophyll than to a reduction in stomatal function.

The reduction in Pn of plants grown in 4.06 and 8.11 ppm boron did not cause a reduction in leaf area (Table 4, page 43). The severity of boron toxicity may be mitigated by the localization of ultra high boron concentrations (Raven, 1980). Boron is transported by the transpiration stream to the outer perimeter of the leaves (Kohl and Oertli, 1961). This occurrence is demonstrated in broccoli grown in high boron concentrations by severe chlorosis of the leaf margin. The reduction in Pn caused by boron toxicity may reflect both a slight reduction in photoassimilation capacity due to the loss of chlorophyll throughout the leaf and a more extensive loss of photoassimilation in the severely chlorotic leaf margin.

In spite of the reduction of Pn caused by boron toxicity, yields of plants grown in high (4.06 and 8.11 ppm) boron concentrations were high (Table 5, page 44). The high levels of boron may be beneficial during head formation. It has been shown that boron is required for meristematic cell division (Cohen and Lepper, 1977).

The rate of cell division during head formation may cause the demand for boron to exceed its availability. The amount of boron available to the developing buds of plants in 4.06 and 8.11 ppm boron may be sufficiently large enough to reduce a boron deficit during head formation and thereby increase the head size.

From a practical perspective, boron deficiency causes stress symptoms that severely reduce the size and marketability of broccoli heads. Boron toxicity in broccoli causes a reduction in the Pn rate but may stimulate head development. It may be advantageous to develop techniques that would favor partitioning of boron directly into developing broccoli heads rather than the leaves.

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APPENDIX

APPENDIX A

MINERAL CONTENT OF BROCCOLI LEAVES

Table 7. The influence of the boron concentration of nutrient solutions on the potassium (K), calcium (Ca), and magnesium (Mg) content of leaf 12 and 16 and the head of broccoli.

Boron Concentration of Nutrient Solution (ppm)	K (% Dry Wt.)			Ca (% Dry Wt.)			Mg (% Dry Wt.)		
	Leaf			Leaf			Leaf		
	12	16	Head	12	16	Head	12	16	Head
0.08	1.61	1.57	3.15	1.62	1.06	0.58	0.199	0.152	0.202
0.41	1.71	1.60	2.46	1.67	1.23	0.43	0.191	0.174	0.221
0.61	1.55	1.52	2.23	1.68	1.21	0.43	0.211	0.187	0.227
0.81	1.87	1.51	2.36	1.70	1.18	0.42	0.239	0.194	0.249
4.06	1.77	1.51	2.26	1.66	1.21	0.49	0.224	0.187	0.248
8.11	1.84	1.38	2.27	1.69	1.26	0.47	0.250	0.207	0.247
0.08 vs. 0.41, 0.61, and 0.81	*[a]	NS	*	NS	*	*	NS	*	*
4.06 and 8.11 vs. 0.41, 0.61, and 0.81	NS	NS	NS	NS	NS	*	*	NS	*
0.08 vs. 4.06 and 8.11	NS	NS	*	NS	*	*	*	*	*

[a]Linear treatment contrast significant at 5% (*) or non-significant (NS).

Note: Dry leaf and head tissue of broccoli plants grown in six concentrations of boron were weighed (0.5000±0.0002g), ashed (470°C for 8 hr), and dissolved in 2 N HCl. Samples were diluted and analyzed by atomic absorption for K, Ca, and Mg.

PART II

THE INFLUENCE OF BORON ON AUXIN-STIMULATED ETHYLENE PRODUCTION AND ROOT INITIATION OF BROCCOLI LEAF EXPLANTS

CHAPTER I

ABSTRACT

In vitro auxin-stimulated ethylene production of broccoli leaf explants was promoted by B deficiency and reduced by B toxicity. Root initiation and chlorophyll concentration of leaf explants after 21 days in culture were less in both B deficient and B toxic media than in B control medium. Ethylene blocker and promoter studies indicate that enhancement of auxin-stimulated ethylene production caused by boron deprivation does not directly cause the reduction in auxin-stimulated root initiation. Similar ethylene blocker and promoter studies of tissues under B toxicity show that root initiation can be stimulated by enhancement of ethylene production. This occurrence suggests that the suppression of ethylene synthesis due to B toxicity causes reduced root initiation.

Abbreviations: B = boron, IAA = indol-3yl-acetic acid, AVG = aminovinylethoxyglycine, ACC = 1-amino-cyclopropane-1-carboxylic acid, SE = standard error of the mean.

CHAPTER II

INTRODUCTION

Although B clearly plays an essential role in root growth and development, the metabolic role of B is uncertain. Neales (1959) found that deficient and toxic levels of B *in vitro* inhibited root growth. Similarly, Cohen and Lepper (1977) reported that B deprivation resulted in a decrease in root cell division and elongation. Further, it has been demonstrated that auxin-stimulated adventitious root initiation requires B for root primordium formation (Middleton et al., 1977).

The hypothesis that B deficiency causes increased IAA production (Jaweed and Scott, 1967; Coke and Whittington, 1968) has not been substantiated (Crisp et al., 1976; Hirsch et al., 1982). Fackler et al. (1985) found that B deprivation generally did not result in increased auxin levels. However, root tissues in the early stages of B deprivation root tissues accumulated relatively high auxin levels.

While auxin stimulation of ethylene production and root initiation have been clearly demonstrated, the influence of ethylene on auxin-stimulated root initiation is uncertain (Abeles, 1973). Chadwick and Burg (1969) reported ethylene inhibition of root elongation. Thus, ethylene appears to be negatively associated with root development. The similarity between the effects of B deficiency

and ethylene toxicity on root development suggest a possible metabolic relationship between B and ethylene. We have conducted experiments to determine the possible nature of such a relationship.

CHAPTER III

MATERIALS AND METHODS

CULTURE MEDIA

Liquid culture media consisted of Schenk and Hildebrandt (1962) salts supplemented with 1.0 g l^{-1} inositol, 30 g l^{-1} sucrose, 0 or 46 µm IAA, 0.58 µm kinetin, and 0.0 to 8.1 mM B. Explant discs (10 mm dia.) were excised from intervenal regions of mature leaves of broccoli which were grown in reduced B nutrient solution. Explants were surface sterilized with 0.5% sodium hypochlorite for 12 min, rinsed three times with deionized distilled water, and plated. Explants were plated onto plastic petri plates (100 x 15 mm) containing 12 ml liquid medium and 6 g washed perlite. Cultures were stored in growth chambers at 18° and 22°C (for 10 and 14 hr, respectively) under 60 µE m^{-2} s^{-1} fluorescent light for 14 hr daily. Boron contamination was reduced by using deionized metal distilled water, plastic vessels, and perlite as a tissue support medium.

TIME COURSE

Cultures with media containing 0.0 (deficient), 0.081 (control), or 8.1 (toxic) mM B and 0 or 46 µm IAA and a control consisting of deionized distilled water were plated. Analyses of explants were performed 0, 1, 2, 5, 10, and 25 days after plating. Ethylene

production was determined by sealing two explants in a 25-ml erlenmeyer flask with a septum for 1 hr and then taking gas samples. Samples of the flask atmosphere were taken with a needle and syringe and were analyzed by gas chromatography (Porosil alumina column at 70°C). Explants were hand dried of free-standing moisture and were weighed by analytical balance for fresh weight determination. Roots were counted under a stereo microscope.

BORON CONCENTRATION

Media with six concentrations of B (0.0, 0.00081, 0.0081, 0.081, 0.81, and 8.1 mM) were prepared by serial dilution and cultures were plated as described above. Explant ethylene production and root number were analyzed after 21 days in culture. Chlorophyll was multiple extracted with 80% acetone and spectrophotometrically analyzed for total chlorophyll and chlorophyll a to b ratio determination (Inskeep and Bloom, 1985).

ETHYLENE BLOCKAGE AND PROMOTION

Explants were soaked 1 hr in 10^{-3} M AVG, deionized distilled water, or 260 ppm ethephon and were plated onto medium containing either a deficient, control, or toxic level of B. The effect of explant treatment on auxin-stimulated ethylene synthesis was determined by analyzing ethylene production 2 days after plating. Ethylene production was measured and roots were counted 25 days after plating.

STATISTICAL ANALYSIS

All treatments were replicated 4-8 times with three samples/replication and two explants/sample. All experiments were performed 2 or 3 times. Linear contrasts were used to compare B treatment effects on second day ethylene production in the time course study and on root initiation in the ethylene blocker and promoter study ($\alpha = 0.05$).

CHAPTER IV

RESULTS

The time course study showed a 10 to 20 fold stimulation of ethylene production on the second day in culture due to the presence of IAA (Figure 1). Second day auxin-stimulated ethylene production by explants on B deficient medium was found to be significantly greater than that of explants on the B control. Auxin-induced root primordia became visible around the perimeters of explants on B control medium after 5 days in culture while explants on B deficient and toxic media developed root primordia 3 to 5 days later. Six days after plating a greater loss of chlorophyll was noticeable for explants on auxin-containing B deficient and toxic media than the auxin-containing B control medium. Explants on B deficient medium with IAA developed short, brown roots with few root hairs and brown callus around the perimeter of the explant. Explants on B toxic medium with IAA formed fewer, but relatively long roots and little callus. In contrast, explants on B control medium with IAA developed numerous roots with many root hairs and callus with little browning. Explants on media without IAA did not produce high levels of ethylene 2 days after plating, formed no roots or callus, and suffered greater losses of structural integrity.

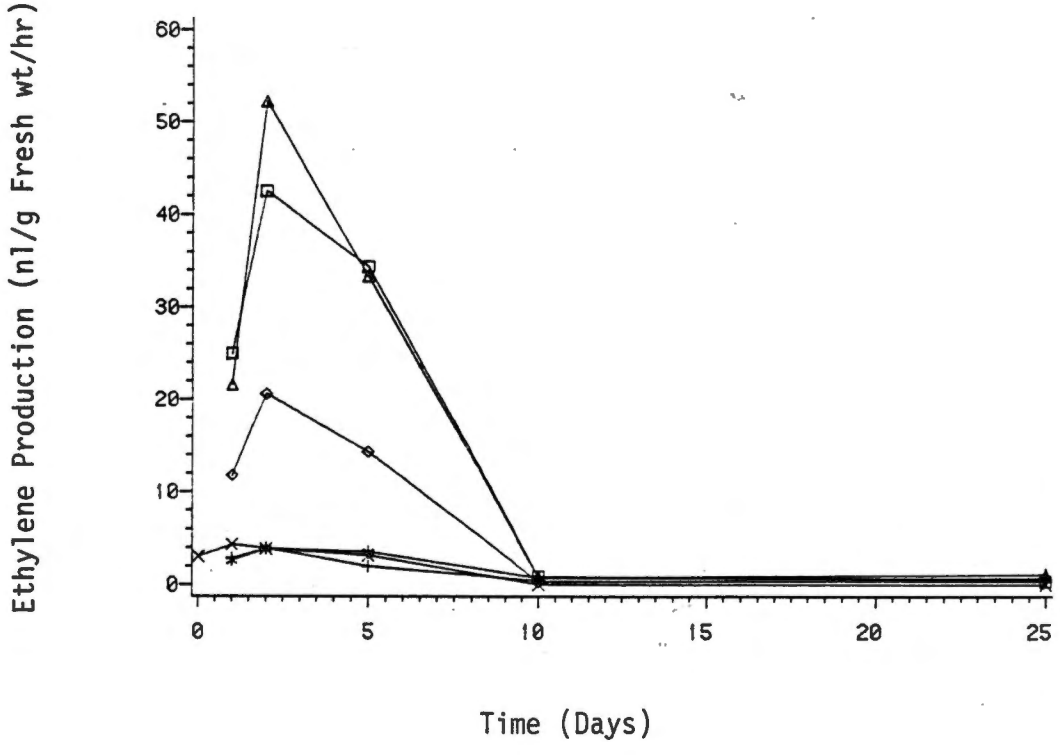

Figure 1. The influence of B and auxin on ethylene production of leaf explants over a 25-day time course. Culture medium contained 0.0 mM B with 46 µM IAA (△) or with no IAA (*), 0.081 mM B with 46 µM IAA (□) or with no IAA (+), or 8.1 mM B with 46 µM IAA (◇). Explants were also plated on deionized distilled water (x).

Twenty-one days after plating, ethylene production of explants was negatively correlated with the B concentration of the medium ($r = -.71$) (Figure 2). The amount of adventitious root initiation (Figure 3) and chlorophyll content (Figure 4) were reduced under extreme B concentrations. The ratio of chlorophyll a to b was abnormally high for explants on medium with the highest B concentration (Figure 5).

Ethylene blocker and promoter studies were performed to determine the nature of the influence of ethylene and boron on root initiation. Preliminary work with ethylene blockers showed 10^{-3} M AVG to be a more effective blocker of auxin-stimulated ethylene production than 10^{-6} M silver nitrate, 10^{-3} M sodium benzoate, or 10^{-4} M AVG (data not shown). In the presence of auxin, ACC enhanced ethylene production in the first hours after treatment, but did not stimulate ethylene production 2 days after plating. However, we were able to enhance ethylene production with ethephon.

Ethylene production of explants was significantly decreased by AVG and increased by ethyphon for all B treatments (Table 1). Root initiation of explants treated with AVG and ethephon was significantly altered only under toxic B conditions (Table 2). Ethylene production 25 days after plating (Table 3) appears to be more dependent on B treatment than preplating treatment.

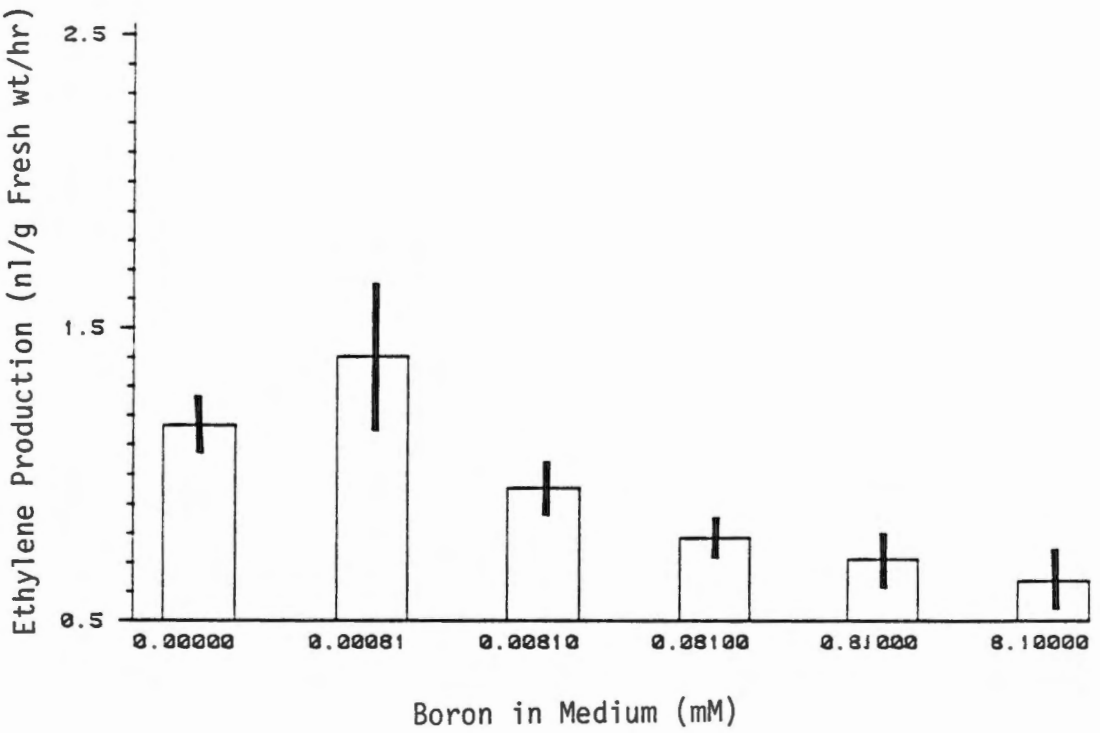

Figure 2. The influence of B concentration of medium on ethylene production of leaf explants after 21 days in culture (bar equals ± SE).

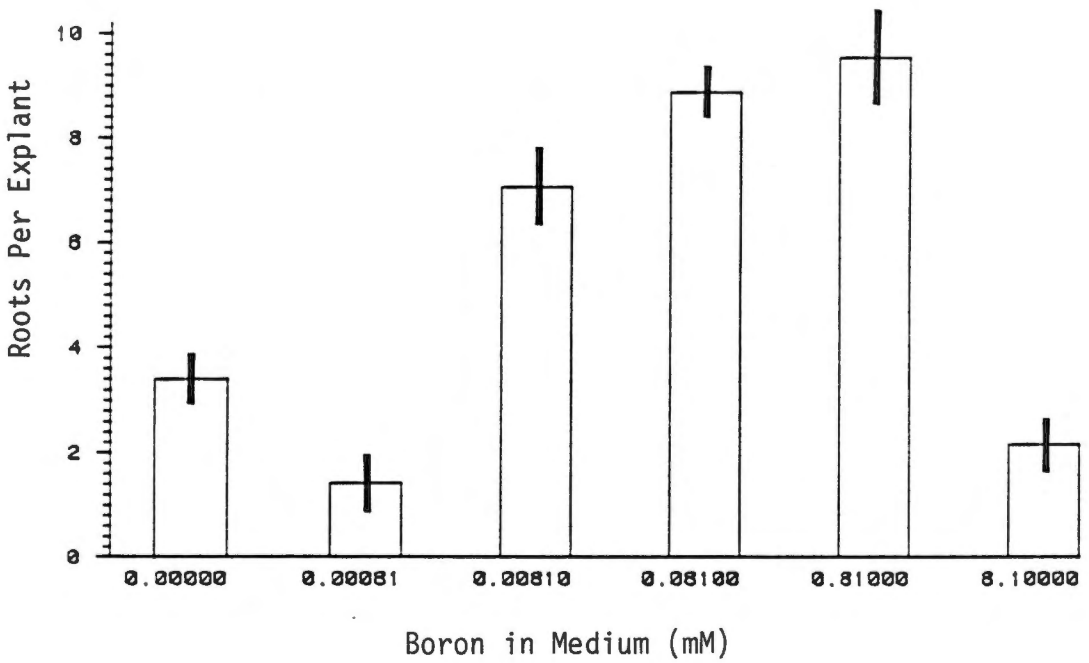

Figure 3. The influence of B concentration of medium on root initiation of leaf explants after 21 days in culture (bar equals ± SE).

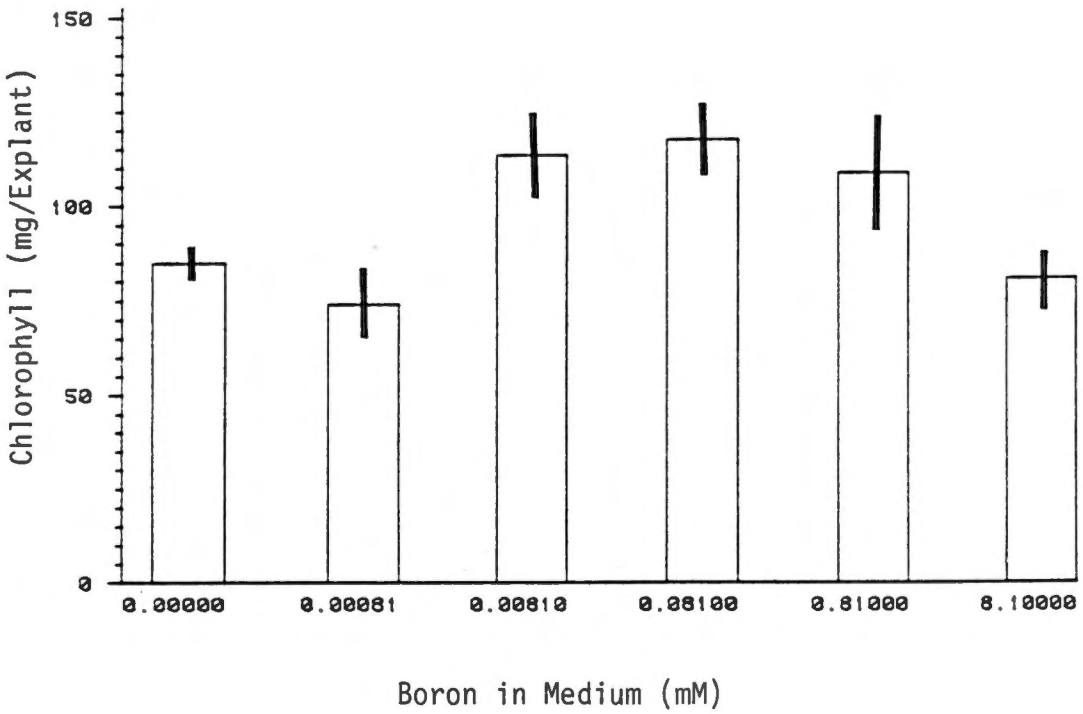

Figure 4. The influence of B concentration of medium on chlorophyll content of leaf explants after 21 days in culture (bar equals ± SE).

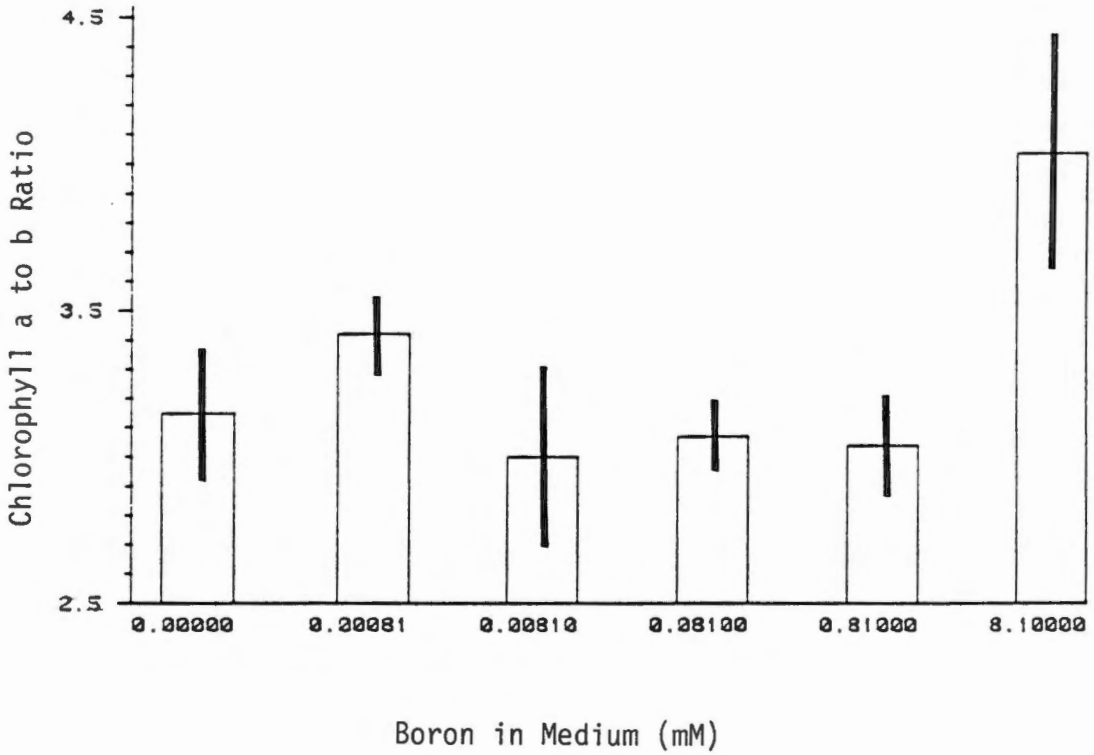

Figure 5. The influence of B concentration of medium on chlorophyll a to b ratio of leaf explants after 21 days in culture (bar equals ± SE).

Table 1. The effect of AVG and ethephon on ethylene production of broccoli leaf explants 2 days after plating onto medium containing 0.0 (deficient), 0.081 (control), or 8.1 (toxic) mM B.

Boron Content of Medium (mM)	Ethylene Production (nl/g Fresh Wt/hr) Preplating Treatment of Explants		
	AVG (10^{-3} M)	Deionized Distilled Water	Ethephon (260 ppm)
0.0	2.1*[a]	19.2	23.2*
0.081	0.9*	14.7	24.4*
8.1	1.0*	4.3	13.0*

[a]Linear contrast with deionized distilled water treatment and same boron treatment significant at 5% (*).

Table 2. The effect of AVG and ethephon on root initiation of broccoli leaf explants 25 days after plating onto a medium containing 0.0 (deficient), 0.081 (control), or 8.1 (toxic) mM B.

Boron Content of Medium (mM)	Number of Roots Initiated Per Explant Preplating Treatment of Explants		
	AVG (10^{-3} M)	Deionized Distilled Water	Ethephon (260 ppm)
0.0	2.9	4.1	4.0
0.081	20.5	21.9	25.3
8.1	0.2*[a]	0.8	1.5*

[a]Linear contrast with deionized distilled water treatment and same boron treatment significant at 5% (*).

Table 3. The effect of AVG and ethephon on ethylene production of broccoli leaf explants 25 days after plating onto a medium containing 0.0 (deficient), 0.081 (control), or 8.1 (toxic) mM B.

Boron Content of Medium (mM)	Ethylene Production (nl/g Fresh Wt/hr) Preplating Treatment of Explants		
	AVG (10^{-3} M)	Deionized Distilled Water	Ethephon (260 ppm)
0.0	0.95*[a]	1.72	0.99*
0.081	0.70	0.67	0.89
8.1	0.18*	0.40	0.48

[a]Linear contrast with deionized distilled water treatment and same boron treatment significant at 5% (*).

CHAPTER V

DISCUSSION

The reduction or enhancement of ethylene production in the presence of high auxin levels did not significantly alter auxin-induced root initiation of explants in B deficient or control medium (Table 2). This finding suggests that, under these conditions, the rate of ethylene production does not determine the extent of root initiation. Thus, enhancement of auxin-stimulated ethylene production and suppression of auxin-stimulated root initiation are, perhaps, two separate effects of B deprivation.

Results presented in this report support those by Middleton et al. (1978) which indicate that B is required for auxin-induced root initiation. The nature of a B-auxin interaction, however, is unclear. Coke and Whittington (1968) proposed that the absence of B causes accumulation of auxin through blockage of auxin catabolism. Our results, which show that B deprivation enhances auxin-induced ethylene production, support this model assuming that hyperethylene levels were induced by hyperauxin levels.

Alternatively, B may affect the action, not metabolism, of auxin. Jarvis et al. (1983) have shown that auxin stimulates production of the polyamine, spermine, and that adventitious root initiation is induced by spermine, but only in the presence of boric acid. The influence of boron on auxin-stimulated rooting may be as a

regulator of the metabolism or action of spermine. Furthermore, since auxin can induce production of both ethylene (Abeles, 1973) and spermine (Jarvis et al., 1983), and since ethylene and spermine share S-adenosyl methionine as a precursor (Smith, 1985), B may play a role in regulating their biosynthesis.

The reduced chlorophyll levels of explants under B deprivation (Figure 4) support previous work which showed B deprivation induces breakdown of chloroplast membranes (Hudak and Herich, 1976). Membrane destabilization induced by B deprivation may affect the membrane-associated ethylene forming enzyme (EFE) (Yang and Hoffman, 1984). It has been shown that spermine is a membrane stabilizing agent (Smith, 1985). The effect of spermine on membrances may be reduced under high auxin, low B conditions, and, in turn, the activity of EFE may be stimulated.

Auxin-stimulated ethylene production in the presence of toxic B is less than one-third that of the B control (Table 1). Root initiation is severaly inhibited under toxic B, but can be slightly promoted by increasing ethylene levels with ethephon or be further reduced by blocking ethylene synthesis with AVG (Table 3). Thus, inhibition of toxic B root initiation may be due, in part, to suppression of ethylene biosynthesis.

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APPENDIXES

APPENDIX A

PRELIMINARY ETHYLENE BLOCKER STUDY

Table 4. The effect of silver nitrate, sodium benzoate, and aminovinylglycine (AVG) on ethylene production and root initiation of broccoli leaf explants.

Explant Treatment	Ethylene Production (nl/g Fresh Wt/hr) Time After Plating		Root Initiation After 8 Days in Culture (Number of Roots/Explant)
	2 Days	8 Days	
Deionized distilled water	37.8	5.8	5.2
Silver nitrate (10^{-6} M)	27.8	4.4	12.4
Sodium benzoate (10^{-3} M)	22.7	3.4	13.9
AVG (10^{-4} M)	14.6	4.4	16.1
AVG (10^{-3} M)	2.7	2.9	8.0

Note: Leaf discs (10 mm dia.) were excised from intervenal regions of mature broccoli leaves and were soaked for 2 hr in deionized distilled water, silver nitrate (10^{-6} M), sodium benzoate (10^{-3} M) or AVG (10^{-4} or 10^{-3} M). Plating of leaf explants and analysis of ethylene and root initiation were performed as described in the previous paper. The data indicate that some compounds which reduce auxin-stimulated ethylene production may also stimulate root initiation.

APPENDIX B

PRELIMINARY ETHYLENE PROMOTER STUDY

Table 5. The effect of 1-aminocyclopropane-1-carboxylic acid (ACC) on ethylene production of broccoli leaf explants.

Explant Treatment	Infiltration Method	Ethylene Production (nl/g Fresh Wt/hr) Time After Plating				
		2 hr	7 hr	1 day	2 days	4 days
Deionized distilled water	Soak	3.0	13.2	9.2	24.7	15.7
ACC (10^{-3} M)	Soak	38.8	74.9	7.7	21.4	17.4
ACC (10^{-2} M)	Soak	53.0	101.1	7.9	20.9	20.6
Deionized distilled water	Vacuum	5.6	12.6	11.4	36.3	21.5
ACC (10^{-3} M)	Vacuum	26.9	71.5	11.2	28.0	15.0
ACC (10^{-2} M)	Vacuum	60.1	103.3	6.7	33.4	18.8

Note: Leaf discs (10 mm dia.) were excised from intervenal regions of mature broccoli plants. Explants were infiltrated with deionized distilled water, 10^{-3} M or 10^{-2} M ACC by soaking the tissue in solution for 1 hr or vacuum (0.5 atm for 5 min). Discs were plated and was ethylene production analyzed as described in previous paper. The data show that ACC, a precursor to ethylene, stimulates ethylene production soon after plating (2-7 hr), but causes a slight reduction in auxin-stimulated ethylene production (2 days after plating). Since ACC did not enhance auxin-stimulated ethylene production, an ethylene-releasing reagent, ethephon was used as the ethylene promoter in the experiments described in the previous paper.

PART III

GREENHOUSE AND FIELD PERFORMANCE OF PLANTS PROPAGATED THROUGH TISSUE CULTURE

CHAPTER I

ABSTRACT

Broccoli (*Brassica oleracea* var. *Italica*) plants propagated from buds and seeds were evaluated for growth and development in the greenhouse and field. Regenerated plants produced fewer and smaller leaves and yielded heads that were 15 to 40 percent smaller than seed plants. Average harvest date for regenerated plants was 8 to 13 days earlier than for seed grown plants of similar age. Uneven bud maturation, leaf deformation, and multiple shoot development were more common in regenerated plants. The variation in head size and the time required for head maturation was not decreased through *in vitro* propagation.

CHAPTER II

INTRODUCTION

In vitro propagation techniques have been reported for broccoli (Anderson and Carstens, 1977; Johnson and Mitchell, 1978; Keller and Armstrong, 1983; Lazzari and Dunwell, 1984; Quazi, 1978), and the economic feasibility of propagating broccoli by tissue culture has been estimated (Anderson et al., 1978). Commercial broccoli producers would benefit from *in vitro* propagation if regenerated plants had greater yields and/or more concentrated dates of harvest. The purpose of this study was to compare the growth, development, and productivity of plants regenerated by *in vitro* propagation with plants grown from seed.

CHAPTER III

MATERIALS AND METHODS

The procedure used for tissue culture propagation was modified from a method previously reported (Anderson and Carstens, 1977). Regeneration medium consisted of Schenk and Hildebrandt inorganic salts (Schenk and Hildebrandt, 1972) supplemented with 30 g sucrose, 1.0 g inositol, 80 mg adenine sulfate dihydrate, 2.1 mg kinetin, and 0.5 mg indoleacetic acid (IAA) per liter. Liquid medium was adjusted to pH 5.8, autoclaved with 6 g prepurified agar per liter, and dispensed 20 ml per plastic petri plate (100 x 15 mm).

All plants grown from seed or used as an explant source were of the broccoli cultivar Premium Crop. Mature flower buds were excised from the peduncle such that 1-3 mm of pedicel remained attached to the flower. Buds were surface sterilized in 0.5% sodium hypochlorite and 0.1% surfactant for 12 min. They were then rinsed three times in sterile deionized distilled water, and plated 10 buds per plate with the cut end of the pedicel placed into the agar medium. Plates were stored in an incubator (14 hr at 22°C and fluorescent light intensity of 600 $\mu E\ m^{-2}\ sec^{-1}$ and 10 hr at 18°C and no light) for 30 days. Shoots with 2 to 4 leaves were excised from the pedicel base and were transferred to a medium (as described above, but lacking adenine sulfate, kinetin, and IAA) for root initiation. After 20 days shoots which formed roots

were planted in hydrated peat plugs, sealed in clear plastic bags, and placed in the greenhouse. To provide slow acclimation of the young leaves to lower humidity, plastic bags were opened 5 days after planting and were removed 2 days later. Plantlets were then set in a cold frame for 10 days. To provide seed propagated plants of the same developmental stage as the regenerated plants, seeds were planted in hydrated peat pellets on the day shoots were transferred for root initiation. Seedlings were transferred to a cold frame on the same day as the regenerated plants. Seedlings and regenerated plants at the 4 to 5 leaf stage were planted in the field in a 30 cm x 90 cm spacing and provided with 225 kg/hA N, 135 kg/hA P and 135 kg/hA K. Plants grown for greenhouse studies were propagated as described above and planted in Pro-Mix BX in 19 liter pots after regenerated plants were removed from plastic bags. Plants were placed on greenhouse benches in a 60 cm x 60 cm spacing and provided with 20 g of Peter's 20-20-20 liquid fertilizer in solution weekly.

Stomatal conductance and transpiration were measured with a steady state porometer (LiCor Li 1600) within 2 hr of solar noon on clear days (1630±250 $\mu E\ m^{-2}\ sec^{-1}$ in the field and 380±60 $\mu E\ m^{-2}\ sec^{-1}$ in the greenhouse) 1 day before initial harvest. The number of leaves at harvest was determined by summing the number of nodes where leaves had abscissed and leaves with areas greater than 25 cm^2. Leaf area, weight of axillary bud growth, number of epidermal scale clusters, pith disfiguration, head diameter, stalk diameter

15 cm from the top of the inflorescence, and weight of the head (cut 15 cm from the top of the inflorescence) were analyzed at harvest.

Plants regenerated from seed grown plants (first generation regenerated plants) have been labeled R_1. Regenerated plants of R_1 have been labeled R_2 and so on. Plants used for explant material were selected to be representative of their generation. Regenerated plants in spring field and greenhouse experiments were propagated from plants grown during the previous fall. Plants grown in the fall field experiment were propagated from plants grown in the spring field experiment.

CHAPTER IV

RESULTS AND DISCUSSION

The results from the two field studies (Tables 1 and 2) and the greenhouse study (Table 3) show that all generations of regenerated plants are smaller and mature earlier than seed grown plants. Stomatal conductance was measured to determine if regenerated plants were smaller due to reduced gas exchange capability of regenerated plant leaves. Stomatal conductance of regenerated plants was not significantly different from that of seed grown plants (Table 4). Since the stomatal conductance of a leaf indicates its capacity for CO_2 assimilation (Farquhar and Sharkey, 1982), it is suggested that the CO_2 uptake capacities are the same for leaves of regenerated and seed grown plants.

Reduced overall plant growth of regenerated plants may be induced by *in vitro* protocol. To insure that regenerated and seed grown plantlets would be at the same leaf stage at planting, shoots of regenerated plants had to be initiated 10-20 days before the appearance of the first seedling leaf. During the 20 days of root initiation, leaf initiation and leaf growth are greatly diminished (data not shown). Perhaps developmental time is lost during this lag period. This may cause the lack of formation of 3 to 5 leaves. Thus, the reduction of leaf area and yield of regenerated plants may be due to reduced total assimilation capacity caused by the

Table 1. Performance of regenerated and seed grown broccoli in the spring, 1985, field experiments.

Plant Type	Time from Planting to Maturity (Days)	Diameter (mm)		Head Weight (g)
		Head	Stalk	
Seed	66a[z] (4)*	123a	30a	266a (72)
R_1	55b (9)	79c	20c	133b (44)
R_2	58b (8)	85bc	23b	137b (69)
R_3	58b (8)	93b	22b	145b (52)

[z]Mean separation by Duncan's Multiple range test at 5% level of significance.

*S.D. (Standard deviation) in parentheses.

Table 2. Performance of regenerated and seed grown broccoli in the spring, 1985, greenhouse experiment.

Plant Type	Time from Potting to Maturity (Days)	Number of Leaves	Leaf Area (cm^2)	Axillary Bud Growth (g)	Diameter (mm) Head	Diameter (mm) Stalk	Head Weight (g)
Seed	70a[z] (7)*	21a	4013a	3a	105a	21a	119a (23)
R_1	58b (1)	6b	1999b	5a	76b	17b	88a (19)
R_2	61b (7)	14b	2430b	11a	81b	18ab	107a (14)
R_3	62b (3)	6b	3644a	4a	74b	17b	102a (17)

[z]Mean separation by Duncan's multiple range test at 5% of level of significance.

*S.D. in parentheses.

Table 3. Performance of regenerated and seed grown broccoli in the fall, 1985, field experiments.

Plant Type	Time from Planting to Maturity (Days)	Number of Leaves	Leaf Area (cm^2) Leaf 8	Leaf 12	Leaf 16	Axillary Bud Growth (g)	Diameter (mm) Head	Stalk	Head Weight (g)
Seed	61a[z] (2)*	19a	543[a]	583a	295a	45ab	132a	31a	298a (95)
R_1	53b (4)	16bc	483ab	359b	71bc	33b	98b	28ab	190b (16)
R_2	48c (3)	15c	272c	94c	28c	23b	100b	23b	186b (13)
R_3	52b (4)	16bc	396b	370b	74bc	64a	104b	28ab	194b (80)
R_4	50bc (2)	17b	526a	364b	89b	43ab	114ab	29ab	233b (40)

[z]Mean separations by Duncan's multiple range test at 5% level of significance.

*S.D. in parentheses.

Table 4. Stomatal conductance and transpiration rates of mature regenerated and seed grown broccoli in the spring, 1985, greenhouse and the fall, 1985, field experiments.

Plant Type	Greenhouse (Spring, 1985)				Field (Fall, 1985)			
	Stomatal Conductance ($cm\ s^{-1}$)		Transpiration Rate ($g\ H_2O\ dm^{-2}\ s^{-1}$)		Stomatal Conductance ($cm\ s^{-1}$)		Transpiration Rate ($g\ H_2O\ dm^{-2}\ s^{-1}$)	
	Leaf 8	Leaf 12	Leaf 8	Leaf 12	Leaf 8	Leaf 12	Leaf 8	Leaf 12
Seed	0.22b[z]	0.56a	1.53a	3.16a	0.65b	0.65a	2.93a	2.99a
R_1	0.40ab	0.39a	2.72a	2.53a	0.62b	0.62a	2.90a	2.84a
R_2	0.44ab	0.42a	2.92a	2.78a	0.81a	0.80a	3.31a	3.68a
R_3	0.48a	0.54a	2.29a	3.15a	0.55b	0.70a	2.58a	3.28a
R_4	--	--	--	--	0.55b	0.70	2.57a	3.28a

[z]Mean separation by Duncan's multiple range test at 5% level of significance.

absence of these early leaves. Furthermore, the loss of early leaf development may result in regenerated plantlets with 4-5 leaves that are developmentally equivalent to seedlings with 7-10 leaves. This, in turn, may explain the shortened period between the 4-5 leaf stage and plant maturity observed for regenerated plants.

Alternatively, early maturation may be due to premature floral bud initiation induced by *in vitro* stress. Plant hormones which stimulate shoot formation may also stimulate premature formation of bud primordia.

Symptoms of environmental stress in broccoli such as scaling of the main stalk epidimis, pith damage occurred at the same or greater frequency in regenerated plants as in seed grown plants (Table 5). Transpiration data indicate that water movement in regenerated plants and seed grown plants in similar (Table 4).

The most common atypical morphological characteristic of regenerated broccoli was that of uneven bud maturation. About 15% of all regenerated plants developed florets with large outer buds that matured much sooner than the inner buds. Leaf deformation (abnormal thickening irregular palisade layers, irregular venation, dentate leaf margins, and oblate-shaped leaves) was found in about 10% of the regenerated plants. Other atypical characteristics at low frequencies included elongated internodes, loss of the apical meristem, formation of heads with very small buds (0.5 mm dia.) and floret formation from all axillary buds on the upper

Table 5. Frequency of stress symptoms of regenerated and seed grown broccoli under field conditions.

Plant Type	Percentage of Plants with Stress Symptom			
	Field (Spring, 1985)		Field (Fall, 1985)	
	Epidermal Scaling	Pith Damage	Epidermal Scaling	Pith Damage
Seed	36	36	7	20
R_1	57	43	44	19
R_2	43	2	0	43
R_3	86	57	27	27
R_4	--	--	50	0

half of the plant. In comparison, about 5% of seed grown plants had uneven bud maturation and less than 1% had leaf deformations as described for regenerated plants. Other atypical traits reported for regenerated plants were not found in seed grown plants.

In general, the variation of head weight and the time required for head maturation was not decreased in regenerated plants (Tables 1-3). Furthermore, plant uniformity was not increased through successive regenerations. Variation in the time required for shoot initiation in culture may have increased variation in harvest date of regenerated plants. Since regenerated plants were developed from shoots whose days of initiation varied by as much as 10 days, harvest dates may also have been caused to vary. Plant size and date of maturation did not decrease further after successive regenerations. This occurrence indicates that reduced plant size and early maturation of regenerated plants is due to a factor in tissue culture protocol which is common to all generations rather than a factor, such as genetic change, that can be successively increased. Modifications of tissue culture protocol which would eliminate the developmental lag in shoot growth during root initiation and increase the uniformity of the time required for shoot initiation may improve the yield and concentrate the harvest date of regenerated broccoli. Without such improvements in protocol the commercial utility of regenerated broccoli plants will be limited.

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