THE EFFECT OF EXOGENOUS ABA, DEHYDRATION AND OSMOTIC STRESS ON THE SYNTHESIS OF THE ALPHA-AMYLASE INHIBITOR PROTEIN IN YOUNG SEEDLING TISSUE OF HORDEUM VULGARE L.

BY

DOUGLAS JOHN MUNRO

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THE EFFECT OF EXOGENOUS ABA, DEHYDRATION AND OSMOTIC STRESS ON THE SYNTHESIS OF THE ALPHA-AMYLASE INHIBITOR PROTEIN IN YOUNG SEEDLING TISSUE OF HORDEUM VULGARE L.

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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Doug Munro

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ABSTRACT

Munro, Douglas John. M. Sc., The University of Manitoba, March, 1994. The Effect of Exogenous ABA, Dehydration and Osmotic Stress on the Synthesis of the Alpha-amylase Inhibitor Protein in Young Seedling Tissue of Hordeum vulgare L.. Major Professor; Dr. R.D. Hill.

An indirect enzyme-linked immunosorbant assay (ELISA) was developed to quantify the amount of the endogenous alpha-amylase inhibitor protein in barley (Hordeum vulgare cv. Bonanza) seedling tissue.

The majority of the inhibitor content was found in residual embryonic tissue, with trace amounts in roots and coleoptile. The accumulation of the inhibitor in residual embryonic tissue reached a maximum at 10 μM exogenous ABA. In roots and coleoptile, it was not discernible if the change in the inhibitor was the result of new inhibitor synthesis or a fresh weight change.

In germinating kernels, the inhibitor increased in the embryo in the first 24 hours, then decreased, whereas the ABA content was initially high in the mature embryo, then decreased upon the start of imbibition and germination. With dehydration stress the increase in the inhibitor paralleled the increase in ABA in residual embryonic tissue. Upon rehydration both the inhibitor and ABA content decreased.

Within the roots, the inhibitor content increased initially with stress, then decreased as the stress continued. The ABA content increased throughout the stress period. Upon rehydration, both the ABA and inhibitor content decreased. In the coleoptile, the inhibitor increase paralleled the ABA increase. Upon rehydration both the inhibitor and ABA content decreased.

With osmotic stress, the inhibitor and ABA increased in residual embryonic tissue in the first few days of stress then levelled off. With roots the results were variable. The inhibitor content did increase slightly during the first few days of stress along with the ABA content, then both levelled off. In the coleoptile, the inhibitor and ABA both increased initially then decreased as the stress continued. With these experiments it seems the seedling adapted to the osmotic stress.

In the residual embryonic tissue the increase in inhibitor was paralleled by an increase in ABA, so there was no direct evidence that ABA mediated inhibitor synthesis. Because of the trace amounts of inhibitor in roots and coleoptile it was difficult to draw any conclusions.

1. INTRODUCTION

It is estimated that about half the annual barley production in the United States and about 15 to 20% of that in Canada is used for the production of malt. During the germination of barley, which is a key component of the malting process, hydrolytic enzymes are synthesized and secreted by the aleurone layer and the scutellar tissue to degrade and mobilize endosperm material for absorption by the embryo (Gram, 1982). One of the major enzymes synthesized is alpha-amylase, which is responsible for the breakdown of starch in the endosperm (Varner and Chandra, 1964).

The alpha-amylase enzyme in fact consists of a complex mixture of isozymes. The high pI group (alpha-amylase 2) is the predominant form synthesized by the aleurone layer of germinating barley. The low pI group (alpha-amylase 1) is found during both development and germination. The third group (alpha-amylase 3) proved to be an interesting one. It seems when alpha-amylase extracts from malting barley were heat treated there was a simultaneous decrease in alpha-amylase 3 and an increase in alpha-amylase 2 (MacGregor and Ballance, 1980). Alpha-amylase 3 was shown to be a complex between alpha-amylase 2 and an inhibitor protein specific for alpha-amylase 2 (Weselake et al. 1983a; Mundy et al. 1983). Both alpha-amylase and the inhibitor protein are regulated by plant

growth hormones. Alpha-amylase synthesis is promoted by gibberellic acid (GA) and inhibited by abscisic acid (ABA) (Chrispeels and Varner, 1967b), whereas the inhibitor protein synthesis is the opposite, ABA promotes and GA inhibits (Mundy, 1984). The suggested function of the inhibitor protein is that it attenuates the germination process by slowing down starch hydrolysis by forming a complex with alpha-amylase (Hill et al., 1987).

The plant hormone ABA is involved in plant stress responses. Plants exposed to stress undergo adaption to the stress by means of physiological and biochemical changes. Synthesis of unique sets of stress induced proteins have been shown to be mediated by ABA and these proteins may be involved in adaptation to the stress. ABA also regulates embryo development and inhibits embryo germination, in these cases unique sets of proteins have been induced (Skriver and Mundy, 1990). The alpha-amylase inhibitor protein has been shown to be induced by ABA in late embryogenesis in vivo, and by water stress in immature embryos and seedlings (Robertson et al., 1989; Robertson, 1989).

The objectives of this study were: 1) to further examine the induction of the alpha-amylase inhibitor protein in young seedlings by stress, focusing on the different tissue types in the seedling, 2) to examine if induction was similar in the residual embryonic tissue, roots and coleoptile.

2. LITERATURE REVIEW

2.1. Cereal Grain Embryo Structure and Germination

The fruit of cereals is referred to as the caryopsis and has three major parts: the embryo, the endosperm which includes the aleurone layer, and the pericarp.

Anatomically, the mature embryo consists of: the root system showing rootlets covered by the coleorhiza; the scutellar node between the root and shoot axis; the acrospire which includes the coleoptile and the enclosed foliar shoot. Lateral to the root-shoot axis is the scutellum. The enclosed aleurone layer is next to the endosperm (Palmer, 1989).

In the mature caryopsis the development of the embryo has been temporarily arrested until conditions are favourable for germination and growth of the embryo can resume. Dry arrested embryos are characterized by low water content and a low metabolic activity (Swift and O'Brien, 1972a). However, the potential for active metabolism is retained, providing there is sufficient water, oxygen and a suitable temperature. Enzymes are then activated, the respiration rate rises rapidly and the breakdown of the reserve materials is initiated (Abdul-Baki, 1969).

Structurally, the embryo is a fully developed plant which is capable of growth when isolated from the endosperm because

it contains adequate food reserves. To develop, however, into a seedling in the climatic regions in which it has evolved the embryo requires the extra reserves stored in the endosperm.

A large number of biochemical changes occur in the embryo during germination. For example, sucrose, raffinose, protein and lipid are hydrolysed rapidly in order to supply the embryo with initial energy and building material for growth (Macleod and Palmer, 1966; Swift and O'Brien, 1972b). Mature embryos also contain active ribosomes and preformed mRNA that are capable of synthesizing protein during germination (Brooker et al., 1977 and 1978). The embryo also produces gibberellins, plant hormones, which are secreted into the endosperm and induce enzyme production in, and secretion from the aleurone layer (Radley, 1967; Cohen and Paleg, 1967). The aleurone-produced enzymes, such as alpha-amylase, endo-beta-glucanase and endo-proteases, are known to be responsible for the breakdown of the starchy endosperm (Briggs et al., 1981; Palmer, 1988).

2.1.1 The Scutellum

The scutellum is also important during germination as it absorbs the endosperm breakdown products that are transported to the embryo where they are resynthesized into compounds necessary for growth. Some evidence suggests that the scutellum can produce alpha-amylase for secretion into the endosperm (MacGregor and Matsuo, 1982; Briggs and Macdonald,

1983; Gibbons, 1981). Ranki (1990), using immuofluorescence, showed that alpha-amylase in the scutellum was located in the epithelial cells, but no label was found in the parenchyma cells. It was also shown that the release of alpha-amylase was arrested by a golgi inhibitor, monensin, which caused accumulation of alpha-amylase in the scutellum instead of the incubation medium. There is also evidence against this because of the structural association between the aleurone and the scutellum (Smart and O'Brien, 1979; Palmer, 1982) and therefore the capacity of excised scutellum to produce alpha-amylase might be related to the scutellum being contaminated naturally with aleurone cells (Mundy and Munck, 1985; MacGregor and Marchylo, 1986; Palmer, 1988).

The three most obvious changes in the scutellum during germination are the degradation of protein bodies, the loss of phytin and the accumulation of starch (Swift and O'Brien, 1972b). Protein bodies supply nitrogen and phytin supplies phosphorus to the embryo before mobilization of the reserve endosperm. During germination there is an increase, followed by a decrease, in the amount of starch in the scutellum. Three days after germination begins, there is abundant starch in the scutellum but, after seven days there is little starch left in the scutellum (Swift and O'Brien, 1972b). The ultimate source of the starch that forms in the scutellum during germination has been variously proposed as the endosperm (Brown and Morris, 1890) and as the lipid reserves of the scutellum

itself (Swift and O'Brien, 1972a, 1972b). But, since triglycerides are only slowly broken down (Holmer et al., 1973) and the excised scutellum amasses starch during germination (Macleod and Palmer, 1966), it seems neither of these explanations is adequate. It is more likely that at least the early starch originates from the high soluble sugar content in the scutellar cells before germination (Dubois et al., 1960). It is also possible that later on the rapid starch and sucrose synthesis in the scutellum is a result of the transfer of glucose and maltose from the enzymatically modified starch of the endosperm (Macleod and Palmer, 1966). It takes approximately 12-24 hours for gibberellic acid to induce the aleurones to produce and secret alpha-amylase into the starchy endosperm (Palmer, 1989).

2.1.2. The Roots

During germination, the coleorhiza guides and protects the rootlets through the covering layer (Palmer, 1989). Germination is complete when the chit has emerged from the husk. Root emergence from the chit initiates the seedling growth phase of embryo development. At this time the embryo develops a complex vascular transport system (Palmer, 1989).

2.1.3 The Coleoptile

In normal (husked) barley, the acrospire lifts the pericarp-testa as it forces its way fully protected, over the

dorsal surface of the grain, before emerging at the distal end of the grain (Palmer, 1989). In barley, the coleoptile elongates towards the soil surface. The coleoptile stops growth when it breaks through the soil surface into the sunlight, and the first foliar leaf it was protecting emerges through the coleoptile tip (Palmer, 1989; Swift and O'Brien, 1972b).

2.2. Alpha-amylase Isozymes and the Alpha-amylase Inhibitor Protein

2.2.1. Alpha-amylase Isozymes

As mentioned, during the germination of cereals, hydrolytic enzymes are synthesized and secreted by the aleurone and possibly the scutellar tissue to degrade and mobilize endosperm material for absorption by the embryo (Gram, 1982). One of the major enzymes synthesized is alpha-amylase, which is responsible for the breakdown of starch in the endosperm (Varner and Chandra, 1964).

The alpha-amylase enzyme, in fact, consists of a complex mixture of isozymes which can be separated by isoelectric focusing. The alpha-amylases are endo-enzymes which hydrolyse starch to oligosaccharides of six glucosyl units and above. They hydrolyse alpha-1,4 linkages in the starch polymers, amylose and amylopectin (Bamforth and Quain, 1989). The high pI group called alpha-amylase 2 is the predominant form

synthesized by the aleurone layer of germinating barley. The isoelectric point of the alpha-amylase 2 group is 6.2 in barley (Silvanovich and Hill, 1977). The low pI (4.8-5.0) group (alpha-amylase 1) is found during both development (in developing kernels called green alpha-amylase) and germination (MacGregor and Ballance, 1980). The third group, alpha-amylase 3 (pI=6.9) proved to be an interesting one. It seems when alpha-amylase extracts from malted barley were heat treated there was a simultaneous decrease in alpha-amylase 3 and an increase in alpha-amylase 2 (MacGregor and Ballance, 1980). Alpha-amylase 3 was shown to be a complex between alpha-amylase 2 and a inhibitor protein specific for alpha-amylase 2 (Weselake et al., 1983a; Mundy et al., 1983).

2.2.2. Alpha-amylase Inhibitor

The inhibitor is a heat labile factor (Weselake et al., 1983a). This accounts for the heat facilitated conversion of alpha-amylase 3 to alpha-amylase 2 during the kilning process (MacGregor and Daussant, 1981).

This endogenous protein inhibitor of barley malt alpha-amylase has also been found to inhibit the bacterial protein subtilisin (Mundy et al., 1983). The protein is now referred to as the barley alpha-amylase subtilisin inhibitor (BASI). The molecular weight of the inhibitor protein based on its amino acid sequence is 19,865 for barley (Svendson et al., 1986) and 19,641 for wheat (Maeda, 1986). The amino acid

sequences of the barley and wheat inhibitors show a homology of greater than 90%. Interestingly, the amino acid composition shows similarities to a subtilisin inhibitor from barley reported by Yoshikawa et al. (1976). The inhibitor has an isoelectric point of 7.3 and it has a pH optimum for inhibition of 6.5-7.0 (Weselake et al., 1985b). The inhibitor:alpha-amylase 2 binding ratio has been determined to be 2:1. The binding affinity is pH dependant, being 7 fold greater at pH 7 than at pH 5.5 (Halayko et al., 1986). The difference in binding affinity corresponds to the observed difference in inhibitory ability, suggesting that complex formation is the mechanism of inhibition (Weselake et al., 1983b). Salt concentration affects complex dissociation; a concentration of 0.3M NaCl reduces the association by 50-fold (Halayko et al., 1986)

The inhibitor protein inhibits the bacterial protein subtilisin at a 1000-fold greater affinity than its binding affinity to alpha-amylase 2. The inhibitor also appears to have two binding sites available as the anti-amylase activity is not affected by the presence of subtilisin in assay solutions (Mundy et al., 1983). Weselake et al. (1985a) found the inhibitor in the kernel extracts of barley, wheat, rye, and triticale, but were unable to detect it in oats, rice, millet, corn or sorghum. In fact, the inhibitor protein has only been found in caryopses that are capable of synthesizing alpha-amylase 2 (Hill and MacGregor, 1988).

2.2.3. Regulation of Alpha-amylase Synthesis

Regulation of alpha-amylase synthesis is important during germination of barley, an important consideration in the malting industry. The topic, therefore, has been researched extensively. The site of enzyme synthesis is in the aleurone layer and possibly the scutellum (Varner and Chandra, 1964; Chrispeels and Varner, 1967a; Gibbons, 1981). In the aleurone, GA promotes, while ABA inhibits synthesis of alpha-amylase (Chrispeels and Varner, 1966 and 1967b). Jacobsen and Higgins (1982) have shown that alpha-amylase 2 is the isozyme induced by gibberellic acid in the aleurone. Induction of the enzyme occurs after an initial lag, then is followed by a linear increase (Jacobson, 1973; Moll and Jones, 1982). A shorter lag occurs for the message (Chandler et al., 1984; Higgins et al., 1982). Secretion of the enzyme is dependant on Ca^{2+} being present (Moll and Jones, 1982) which also has a regulatory affect on alpha-amylase synthesis. Translation of alpha-amylase 2 also requires calcium (Bush et al., 1986; Jones and Jacobsen, 1983). When detected, scutellar production appears largely unaffected by GA, at least in the case of exogenous hormone treatment (Gibbons, 1981). In the aleurone, the increase of amylase activity is due to de novo synthesis (Jacobson and Knox, 1974). GA stimulates transcription of the genes encoding alpha-amylase; but this stimulation of the transcription can be blocked by abscisic acid (ABA) (Higgins et al., 1982). Therefore, GA stimulates alpha-amylase 2

secretion and ABA inhibits it in the aleurone layer.

2.2.4. Regulation of Inhibitor Protein Synthesis

In contrast to the pattern of synthesis for alpha-amylase, ABA promotes the synthesis of the inhibitor and GA inhibits in mature aleurone layers (Mundy, 1984). Furthermore, while alpha-amylase is secreted by the aleurone layers when treated with GA most of the inhibitor protein remains in the tissue when aleurone layers and embryos are treated with ABA (Mundy, 1984; Mundy and Rogers, 1986)

2.2.5 Inhibitor Protein Synthesis During Kernel Development

The inhibitor comprises 5% of the buffer-soluble protein in the mature endosperm and approximately 0.001% of the buffer soluble protein in the mature embryo (Hill *et al.*, 1990). Although inhibitor synthesis is induced by ABA in mature aleurone layers, no inhibitor message was detected in immature layers at 20 and 30 DPA (days post-anthesis) or in mature aleurone layers. The synthesis of the inhibitor appears to be localized in the starchy endosperm during kernel development (Mundy *et al.*, 1986; Mundy and Rogers, 1986). Tissue localization by immunohistochemical methods indicates the association of the inhibitor around the starch granules within the endosperm (Lecommandeur *et al.*, 1987). No positive presence of the inhibitor has been detected in the aleurone layers, using the same method. During whole kernel

development, inhibitor accumulation increases logarithmically at 20 to 30 days after pollination (Munck et al., 1985). The inhibitor is detectable at 14 DPA and its abundance increases up to 44 DPA (Mundy and Rogers, 1986). Robertson and Hill (1989) found inhibitor synthesis (in Hordeum vulgare cv. Bonanza) within 7 days of fertilization, the most active accumulation was between the second and third weeks PA, and was almost complete by the fourth week. The messenger RNA was detected 4 DPA and was in abundance 13 to 28 DPA and then declined towards maturity (Hill et al., 1990). Hill et al. (1990) also studied the accumulation of the storage protein hordein, which had a higher messenger RNA level but followed a similar time profile as the inhibitor. Even though the hordein mRNA is 5000 fold more abundant than the inhibitor mRNA the differences in protein levels is about 50 fold suggesting the inhibitor mRNA is more efficiently translated. Similar patterns of accumulation of the inhibitor have been reported for other cultivars (Rasmussen et al., 1988).

Even though inhibitor synthesis is induced by ABA, the level of the inhibitor was not effected by treatment of barley spikes with ABA or fluoridone even though the compounds had a visible effect on the spike (Hill et al., 1990). In mature barley kernels, inhibitor contents range from 20 to 38 µg/kernel (Rasmussen et al., 1988), 13 µg/kernel (Lecommander et al., 1987), and 180 to 450 mg of inhibitor per 1 kg of grain (Munck et al., 1985). The ranges in values represents

the variation in factors including cultivar, crop year, location and also assay procedure.

2.2.6 Inhibitor Synthesis in Developing Embryos and Germinating Seedlings

The inhibitor protein in the embryo increased as development proceeded from 14 DPA to 50 DPA (Robertson et al., 1989). Culturing immature embryos with ABA or applying dehydration stress to isolated embryos, also increased inhibitor content over in vivo embryos (Robertson et al., 1989). The shoots (residual embryonic tissue and coleoptile) of two day germinated seedlings contained inhibitor and this amount could be increased with dehydration stress (Robertson et al., 1989). Leah and Mundy (1989) did not find inhibitor mRNA in five day old roots or shoots: they also did not find mRNA in leaves or roots from ABA-treated or desiccated barley plants. The age of these plants was not stated.

2.2.7. Possible functions of the Alpha-amylase Inhibitor

The alpha-amylase inhibitor inhibits bacterial subtilisins, therefore one function is that it possibly protects the kernel in the soil from bacterial pathogens. But, considering the fact that it also inhibits the germination specific, alpha-amylase 2 enzyme, it probably has other and more important functions.

Hill et al. (1987) suggested that the inhibitor protein,

which is found throughout the germination process, possibly attenuates the degradation of starch granules by alpha-amylase upon germination. The inhibitor binds to the synthesised alpha-amylase 2, but as germination proceeds the amylase levels and pH of the tissue likely minimize the effects of the inhibitor on starch breakdown. If conditions become unfavorable to germination, pH changes may result in increased inhibition.

The possibility that the inhibitor protein may have a role in preventing preharvest sprouting has been studied (Munck et al., 1985; Abdul-Hussain and Paulson, 1989) but, no heritable relationship between inhibitor production and sprouting has been found. In one study on preharvest sprouting, Audette (1990) found that kernels from wheat cultivars grown during drought conditions had considerably more inhibitor protein than ones from non-drought conditions. This along with the fact that ABA and dehydration increases the amount of the inhibitor in barley seedlings (Robertson et al., 1989) suggests that the inhibitor may have a role in stress resistance.

2.3. Physiological and Biochemical Responses to Abscisic Acid in Plants

Abscisic acid (ABA) was originally considered a growth inhibitor, but it has also been called a stress hormone, since it enhances adaptation to various stresses. The parallel physiological effects of water deficit and applied ABA (Zeevart and Creelman, 1988), added to the observation that water deficits lead to an increase in endogenous ABA content (Zeevart and Creelman, 1988), suggests ABA may be a primary agent in a number of plant responses to dehydration. ABA has also been found to enhance adaptation to other stresses: freezing tolerance (Mohapatra et al., 1988; Chen and Gusta, 1983): cold hardening (Lalk and Dorffling, 1985): heat stress (Heikkila et al., 1984): salinity (Ramagopol, 1987) and reduction in osmotic potential (Reid and Wample, 1985). It is now known that ABA has multiple roles during the life cycle of plants. Initially, ABA was considered a general inhibitor of RNA and protein synthesis (Walton, 1980), but it is now evident that in certain tissues ABA can induce the formation of specific proteins (Skriver and Mundy, 1990). The physiological responses to ABA range from very rapid to long-term, suggesting that ABA has different modes of action and different receptors (Zeevart and Creelman, 1988; Reid and Wample, 1985).

2.3.1 Physiological Responses of ABA

ABA has been shown to decrease the growth of coleoptiles in maize. It also inhibited the growth induced by auxin. In these cases this effect was due to an inhibition of cell wall loosening (Kutschera and Schopfer, 1986a, 1986b). Water deficit has been shown to decrease the growth of hypocotyls in soybean. When the lowest growth rate occurred, the greatest ABA content was found (Benson et al., 1988). The turgor pressure in the elongating hypocotyl in the water-limited seedlings did not change. It seems cells maintained turgor by osmotic adjustment in the stressed elongating region of the hypocotyl. Applied ABA also caused slower growth rates in soybean hypocotyls. Therefore, it would appear that induced or applied levels of ABA do not effect turgor pressure but may alter cell wall extensibility (Mason et al., 1988).

The reports of ABA effects on root growth are contradictory. It seems ABA can inhibit as well as promote root growth (Mulkey et al., 1983; Pilet and Rebaud, 1983). Pilet and Saugy (1987) found the effect of exogenous ABA on root growth in maize depended on the initial elongation of the root.

There are several lines of evidence that indicate ABA prevents precocious germination in developing embryos. Exogenous ABA prevented germination of immature embryos of several species; in maize (Neill et al., 1987), in wheat (Triplett and Quatrano, 1982), in soybean (Ackerson, 1984a and

1984b), in barley (Morris et al., 1985; Robertson et al., 1989).

Many different environmental stresses have been shown to increase ABA content in plant tissue and exogenous ABA has been shown to enhance adaptation to various stresses. Cold hardening can be increased by ABA in plants; in potato (Chen et al., 1983), in winter wheat (Lalk and Dorffing, 1985). It can also increase cold hardening in cell culture; in alfalfa (Mohapatra et al., 1988) and winter wheat and bromegrass (Chen and Gusta, 1983).

There have been a number of reports of abscisic acid inhibiting transpiration in plants. Stomates close and ABA levels increase in water stressed plant tissue (Zeevart and Creelman, 1988). Exogenous ABA will initiate stomatal closure when applied to intact leaves or isolated strips. This has led to the hypothesis that ABA is involved in regulating stomatal apertures in water-stressed plants, thus regulating water loss by transpiration. The fact that wilty tomato plants contain low levels of ABA and that their tendency to wilt can be reduced by application of exogenous ABA lends further evidence that ABA is involved in stomatal closure under water stress conditions (Bray, 1988). It appears now that it is an ABA-dependant increase in cytosolic Ca^{2+} in the guard cells that leads to rapid stomatal closure by cation and anion effluxes (McAinsh et al., 1990).

2.3.2. Biochemical Responses to ABA

Salinity stress can cause specific salt effects in plants as well as reducing water availability. Barley responds to salinity stress by expressing stress-induced proteins specific to cell, tissue and genotype. Ramagopal (1987), using a salt-tolerant variety and a salt-sensitive variety of barley, showed that in the roots the salinity effects are identical in both varieties. Salinity modulated the synthesis of two sets of proteins, one which was elevated, the other depressed. Second, six new proteins were induced in the roots. In contrast to the roots, salinity induced cultivar specific shoot proteins. Five new shoot proteins were induced in the salt sensitive cultivar, only two of these proteins were found in the salt-tolerant cultivar. In cultured tobacco cells, induction of the salinity protein, osmotin, seems to be mediated by ABA as both ABA and NaCl solutions induce its synthesis (Singh et al., 1987 and 1989).

Heat-shock proteins, hsp 27 in soybean (Czarnecka et al., 1984) and hsp 70 in maize (Heikkila et al., 1984), can also be induced by exogenous ABA and polyethylene glycol-applied water stress. Harrington and Alm (1988) have shown that salt shock results in the synthesis of several polypeptides which are similar in size on SDS gels to known HSPs in cultured tobacco cells.

All environmental stress, be it heat, cold or salt, stress the plant in a unique way but they also affect the

water availability of the plant. Stress by dehydration and by osmotic change are well studied. The use of controlled systems of dehydration and osmotic stress are preferable because it allows for precise control of the experimental system and also allows monitoring of the magnitude of the stress.

The role of ABA in dehydration has been studied using ABA mutants of tomato (Bray, 1988). A set of proteins is synthesized in wild type tomato leaves by dehydration. In the mutant plant the same set of proteins is induced but to lesser extent under dehydration. Upon ABA application to the mutant the proteins are induced to a greater extent. The results indicate that the elevated ABA levels induced by dehydration are required for the induction of this set of proteins (Bray, 1988).

The "slender" mutant of barley resembles a normal barley plant when treated with gibberellic acid. Expression of ABA regulated mRNA was studied by Chandler (1988) in the roots of the "slender" mutant and the wild type. In both "slender" and wild type seedlings the level of mRNA hybridizing with the DNA clone increased substantially above the controls, following dehydration or exogenous ABA, indicating that gene expression is being regulated by ABA in the "slender" seedling as it is in the wild type. Chandler et al. (1988) also found a family of mRNAs which can be induced in the roots and shoots of barley plants under dehydration. In Brassica napus a tissue specific induction of proteins occurs in the roots during

dehydration stress. The roots also undergo a morpholgical change for better adaptation to the stress (Damerval et al., 1988). Dehydration of maize leaves which increases the ABA levels induces a mRNA for a glycine rich protein. This protein is also induced by wounding which also increases ABA levels (Gomez et al., 1988) Mundy and Chua (1988) have found a rice gene, which is also rich in glycine, which is induced in ABA-treated shoots, roots and embryos and dehydrated shoots. Raikhel et al. (1986) showed that the lectin, wheat germ agglutinin (WGA) in wheat roots can be induced by exogenous ABA and that fluoridine treatment, an inhibitor of ABA synthesis, decreased the levels of WGA when compared to the controls. Further to this Cammue et al. (1989) have shown that WGA can be increased in wheat roots by dehydration and osmotic stress when using mannitol solution or polyethylene glycol. These increases in WGA were preceded by ABA increases. It seems the stress-induced increase of WGA is under ABA control.

Interestingly, alpha-amylase 1 activity increases in barley leaves during dehydration stress in coordination with increases in ABA and a decrease in water potential (Jacobsen et al., 1986). The regulation of alpha-amylase synthesis, by ABA and dehydration stress in the leaves, is opposite to the regulatory pattern in the aleurone layer in the seed. This indicates that different control mechanisms occur in a plant dependent on the tissue type.

2.3.3. Late Embryogenesis Abundant Proteins (lea)

Upon maturation, the seed generally becomes dehydrated, and its embryo becomes dormant. Therefore, the embryo may express dehydration-induced genes in the later stages of seed development. The inhibitor protein is induced later in barley kernel development and is preceded by an increase in ABA (Robertson et al., 1989). In fact, some of the other dehydration-induced proteins in vegetative tissues are present in the mature seed (Chandler et al., 1988; Gomez et al., 1988; Mundy and Chua, 1988).

Probably the most extensively studied lea protein is the Em protein from wheat. Em begins to accumulate in wheat embryos 21 days post anthesis. Embryos removed from the maturing kernels and cultured in ABA, both the Em mRNA and the protein are rapidly synthesized and accumulated (Williamson et al., 1985). Marcotte et al. (1988 and 1989) have shown with the use of a full-length cDNA clone of Em gene, the promoter region of the gene, inducible by ABA, in a transient expression assay using rice protoplasts. The ABA response of the Em promoter expressed in the rice protoplast is similar to ABA regulation of the natural gene in the wheat embryo, both with respect to dose of ABA and the initiation of RNA transcripts. Other lea type proteins have been found in cotton (Baker et al., 1988), in barley and corn (Close et al., 1989), 7S globulin in wheat (Williamson and Quatrano, 1988) and dormant wheat (Morris et al. 1991). All lea proteins are very

hydrophilic. Although lea proteins share similar characteristics, they can be divided into three groups based on amino acid sequence similarity (Dure III et al., 1989). It has been proposed that the lea proteins function in desiccation survival of seeds (Baker et al., 1988).

3. MATERIALS AND METHODS

3.1. Chemicals and Materials

1. Anti-rabbit Ig biotinylated species specific whole antibody (from donkey), Streptavidin-biotinylated horseradish peroxidase complex were obtained from Amersham Canada Ltd., Oakville, Ontario.

2. Protein Assay Dye Reagent, Goat-anti-rabbit horseradish peroxidase (GAR-HRP) and it's substrate, 4-chloro-1-naphthol, were obtained from Bio-Rad Laboratories [Canada] Ltd., Mississauga, Ontario.

3. Immulon 2 flat bottom plates were obtained from Dynatech Laboratories Inc., Chantilly, Virginia. Titertek Flat bottom plates were obtained from Flow Laboratories Inc., McLean, Virginia.

4. (+/-)Abscisic acid (ABA), o-phenylendiamine, p-nitrophenyl phosphatase and Rabbit antimouse alkaline phosphatase conjugate were obtained from Sigma Chemicals Co., St. Louis, Missouri.

5. Monoclonal antibody (Mab) to free (+/-)ABA was obtained from Idetek, Inc., San Bruno, California.

6. Nitrocellulose was obtained from Schleicher and Schuell, Keene, New Hampshire.

7. All other chemicals were reagent grade unless indicated.

3.2. Plant Material

3.2.1. 21 DPA and 35 DPA Embryos

Barley, Hordeum vulgare L. cv.Bonanza, was grown in growth cabinets under 16 hour photoperiod with 18/14°C day/night temperature. Seeds were planted in either an artificial planting mix (Metro-mix 220, W. R. Grace and Co. of Canada Ltd. Ajax, Ontario) in 1L milk cartons or in a mixture of 2 parts loam, 1 part sand and 1 part peat moss in 1L pots. After emergence the seedlings were thinned to 2 plants per pot or carton. The plants were fertilized with a 20-20-20 fertilizer once a week until maturity. Each head was marked at mid-anthesis and was harvested at the required time intervals.

3.2.2. Young Seedlings

Young seedlings were obtained by germinating barley seeds, Hordeum vulgare L. cv. Bonanza. Seeds were surface sterilized with 1% hypochloric acid and 0.1% Tween 20 for 20 minutes and then rinsed with copious amounts of sterilized distilled water. The seeds were then germinated by three different methods depending on the experiment: 1) 100 seeds were placed on two sheets of round 3 MM Whatman paper with 6 mLs distilled water in plastic petri plates and the plates were sealed with parafilm, 2) many seeds on two layer thick 3 MM Whatman paper in a square glass pan with saran wrap over

the top and with 6 mLs of distilled water per 100 seeds, 3) seeds were placed on sterilized nylon mesh screens which were placed in Tupperware containers with enough sterilized water to come in contact with the seeds on the mesh. The tops were then snapped in place. For all methods the seeds were placed in the dark, at room temperature, for two days.

3.3. Experiments

3.3.1. Effect of ABA on Inhibitor Synthesis in Cultured 21 and 35 Days Post-anthesis Embryos

The effect of ABA on inhibitor protein synthesis in barley embryos was studied with *in vitro* embryo culture in a nutrient medium with varying ABA treatments.

Embryonic stages were determined from marked heads of barley plants at mid-anthesis according to Rogers and Quatrano (1983). Immature embryos were isolated aseptically from whole kernels. The kernels were first surface sterilized with 1% hypochloric acid and 0.1% Tween 20, then rinsed with distilled water. The embryos were placed scutellum-side down either individually in a well of a 96 well Corning culture plate with 50 µL of medium or 10 embryos in a 35 X 10 mm Corning culture petri plate with 500 µL of medium. Plates were sealed with parafilm. ABA concentrations used were 1, 10, 100 µM. Embryos were cultured in the dark for 5 days and then analyzed for germination and inhibitor protein content. Embryos were also

analyzed for inhibitor content at the time of harvest.

The medium used was Murashige and Skoog (MS) (1962) with the following changes: KH_2PO_4 was increased from 170 to 1200 mg/L to satisfy the K requirement in the absence of KNO_3 (Donovan and Lee, 1977). The vitamins (myo-inositol 100 mg/mL and thiamine 0.8 mg/mL) and sucrose concentration (230 mM) were derived from the conditions for optimum growth of cultured grains as found by Gifford and Bremmer (1981). Casein hydrolysate (which contains around 14 amino acids) at 20 mg/mL and glutamine at 0.9 mg/mL were used instead of NH_4NO_3 and KNO_3 or a mixture of amino acids and seemed to satisfy the needs of the isolated embryo for growth.

3.3.2. Effect of Exogenously Applied ABA on Inhibitor Synthesis in Seedling Tissue

Sterilized seeds were germinated on 3 MM Whatman paper with 6 mLs distilled water per 100 seeds in parafilm sealed petri plates. After two days, measurements were made of coleoptile and root length . One set of seedlings (2 day - starting point control) was examined for inhibitor protein content of the residual embryonic tissue (RET), roots and coleoptile (COL). The other sets of seedlings were then transferred to new petri plates with 3 MM Whatman paper; which were then saturated with 5 mLs of ABA solutions (1 μM, 10 μM, or 100 μM) or distilled water and allowed to grow a further 2 days. The seedlings were then remeasured for coleoptile and

root growth. The seedlings were then dissected (roots, coleoptile, and residual embryonic tissue) and each part analyzed for inhibitor protein content.

3.3.3. Inhibitor Synthesis During Unstressed Seedling Tissue Development - (Germination Experiment)

Sterilized seeds were placed on two layer 3 MM Whatman paper in glass pans with 6 mLs of distilled water per 100 seeds. Saran wrap was stretched over top. The seeds were randomly removed to study the change in embryo inhibitor protein content as the seed germinated. The beginning of sterilization process was used as the start of imbibition. Seeds were removed at 3, 6, 12, 24, 30, and 36 hours after the start of imbibition and the embryos removed from the kernels and inhibitor protein and ABA content measured. Continuing on at 2, 3, 4, and 5 days after imbibition, the seedlings were dissected into roots, coleoptile, residual embryonic tissue and these sections were also measured for inhibitor protein and ABA content. The seedlings were also watered after 2 days of germination at a rate of 3 mLs per 100 seedlings.

3.3.4. Inhibitor Synthesis in Seedling Tissue During Dehydration Stress

Two day-old seedlings which were germinated on 3 MM Whatman paper in sealed petri plates were placed on new dry petri plates without 3 MM Whatman paper and then placed in a

desiccator. One set of seedlings was used as a starting point control at two days and the inhibitor protein and ABA content of the residual embryonic tissue, roots and coleoptile measured. The desiccator had been equilibrated with 25% (v/v) glycerol solution at 20°C (Chandler, 1988). The 20°C was achieved by keeping wet paper towels around the desiccator and blowing air on the desiccator with a small fan. The glycerol was kept in solution with a stirring bar. The calculated relative humidity for this chamber is 91% (Chandler, 1988). Every day for five days seedlings were removed from the chamber and the seedlings were dissected into three parts: the roots, the coleoptile and the residual embryonic tissue. After 5 days of dehydration stress the remaining seedlings were rehydrated on wet 3 MM Whatman paper for two days and seedlings were dissected into the three parts. These parts were then analyzed for inhibitor protein and ABA content.

3.3.5. Inhibitor Synthesis in Seedling Tissue Under Osmotic Stress

Seedlings which were germinated for two days on nylon mesh screens in distilled water were transferred to solutions of mannitol (0.5 M or 1 M). The roots would then hang in the mannitol solution. The mannitol was kept in solution by a stirring bar. Every day, for 5 days, seedlings would be removed and dissected into parts (residual embryonic tissue, roots, coleoptile) and the inhibitor protein and ABA content

analyzed. After 5 days in mannitol solution the seedlings were placed back in the distilled water for two days, the parts were dissected and the inhibitor protein and ABA content measured.

3.4. Analytical Methods

3.4.1. Buffer Soluble Proteins - Extraction

The embryos and seedling parts were homogenized using a teflon/glass homogenizer with 10 mM phosphate-buffered-saline (PBS : 10 mM KH_2PO_4/K_2HPO_4 at pH 7.0, 145 mM NaCl), 0.1% (w/v) Triton X-100, and 100 mM N-acetylglucosamine) or they were homogenized using a polytron with 50 mM Tris (pH 7.5) and 1 mM NaCl. Both methods were equally effective in extracting the inhibitor protein. The homogenates were kept on ice for an hour with occasional shaking or vortex mixing, then centrifuged and the supernatant drawn off.

3.4.2. Buffer Soluble Proteins - Determination

The concentration of the buffer soluble proteins was determined using the Bio-Rad Protein assay (Bradford, 1976) performed in 96-well microtitre plates (Titretek). 200 µL of diluted dye reagent (1:4) was added to the wells. A standard curve was preformed on each plate, by adding 10 µL of several dilutions (between 0.2 and 1.44 mg/mL) of gamma-globulin protein standard. 10 µL of dilute sample was then added to

appropriate wells. The absorbance at 590 nm was measured with the EL308 microplate reader (Mandel Scientific Company Ltd., Guelph, Ontario). Absorbance at 590 nm was plotted versus the concentration of the standards, a standard curve was plotted and the concentrations of the unknowns was determined. Each sample was analylzed in triplicate.

3.4.3. Protein Gel Electrophoresis

The protein samples were separated by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% (T) gels by the Laemmli (1970) method, as modified by Maniatis et al. (1982) for one-dimensional electrophoresis. A silver stain (Merril et al., 1981) was used to visualize the protein bands.

3.4.4. Western Blot of Protein Electrophoresis Gels

Protein SDS-PAGE gels were electroblotted onto nitrocellulose in a LKB Transfer Electroblotting Unit (LKB-Pharmacia) according to the manufacturer's instructions. The alpha-amylase inhibitor antigen band was visualized using a specific rabbit antisera raised against the purified inhibitor protein (Weselake et al., 1985), GAR-HRP conjugate and 4-chloro-naphthol as the substrate according to Bio-Rad's instructions. The inhibitor content of the samples at various protein loads was quantified visually against purified antigen standard of known amounts on the same gel. Samples giving band

intensities outside the standards range were appropriately diluted and the analysis repeated.

3.4.5. Indirect ELISA for Inhibitor Content Determination

Protein samples from the various tissue parts obtained from the various experiments were analyzed by indirect ELISA to obtain more reliable and reproducible results than obtained with the band visualization and comparison method using Western blots.

The assay protocol relies on the formation of an antigen-antibody complex which is fundamental to all immunological assays and uses the biotin-streptavidin system for detection.

Assay Reagents

Carbonate buffer : 0.2 M, pH 9.6

- sodium carbonate solution was added to sodium bicarbonate solution until pH 9.6 was reached.

Phosphate buffered saline (PBS) : 0.05 M pH 7.2

- monobasic sodium phosphate solution was added to dibasic sodium phosphate solution until pH 7.2 was reached.

Phosphate buffered saline with Tween 20 (PBS-Tween) : 0.05 M pH 7.2 (wash solution)

- as PBS solution, with addition of Tween 20 at 0.05% v/v.

Blocking solution PBS-BSA :

- PBS solution with 1% bovine serum albumin (BSA).

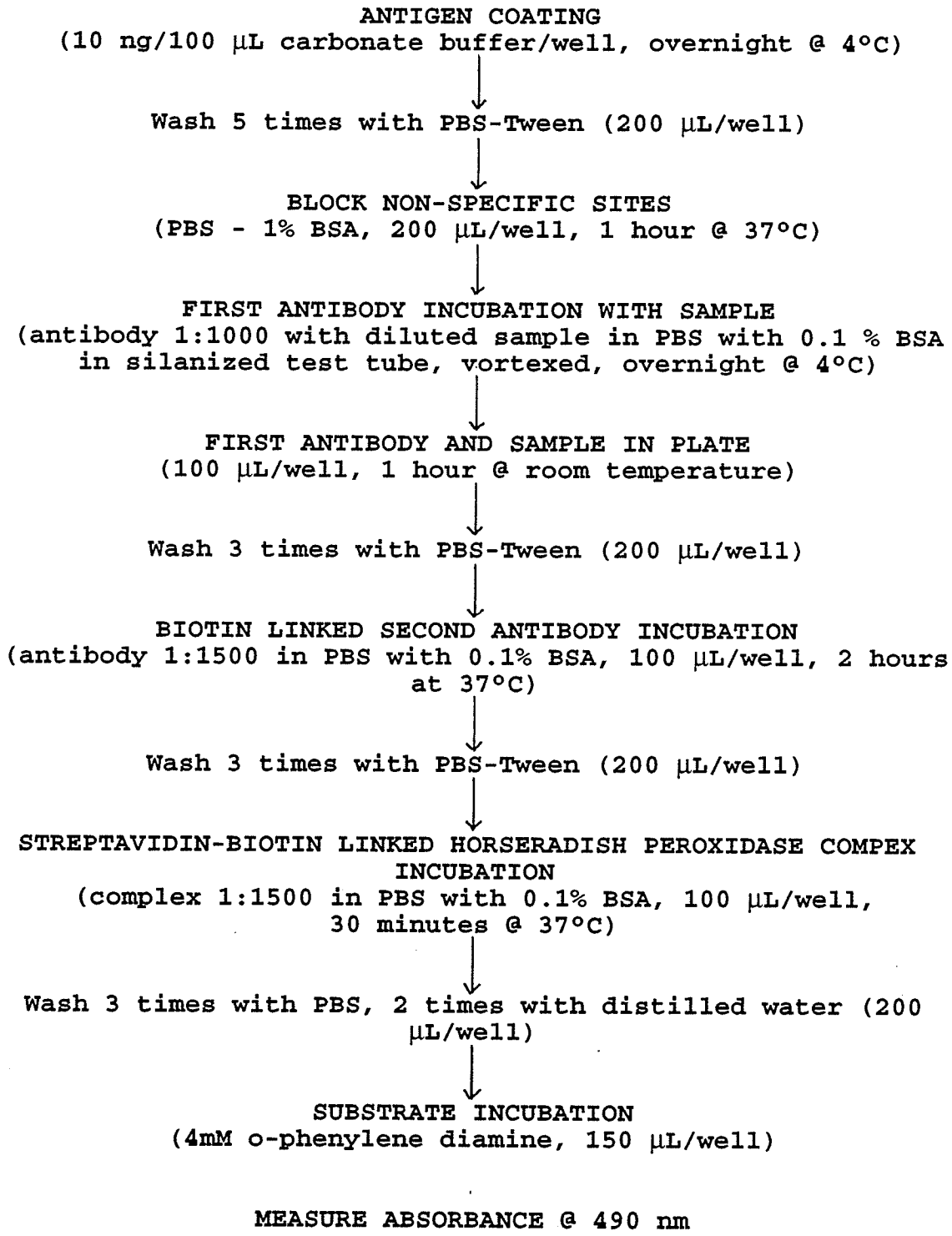
Citric acid (0.02 M) sodium phosphate (0.05 M), pH 5.0 :

- citric acid solution was added to sodium phosphate solution until pH 5.0 was reached.

Assay Procedure

A flow chart of the indirect ELISA procedure developed for the alpha-amylase inhibitor quantitation is illustrated in Figure 1. The ELISA was performed in Immulon 2 96-well polystyrene plates (Dynatech). The samples and standards were incubated with the primary antibody in silanized 5 mL glass test tubes. Samples and standards were analyzed in triplicate per plate. The top row (A) was used as the blank and either the primary antibody was omitted or the antibody for alpha-amylase was used instead.

Figure 1. Outline of indirect ELISA for the alpha-amylase inhibitor protein.


ANTIGEN COATING
(10 ng/100 µL carbonate buffer/well, overnight @ 4°C)
Wash 5 times with PBS-Tween (200 µL/well)
BLOCK NON-SPECIFIC SITES
(PBS - 1% BSA, 200 µL/well, 1 hour @ 37°C)
FIRST ANTIBODY INCUBATION WITH SAMPLE
(antibody 1:1000 with diluted sample in PBS with 0.1 % BSA
in silanized test tube, vortexed, overnight @ 4°C)
FIRST ANTIBODY AND SAMPLE IN PLATE
(100 µL/well, 1 hour @ room temperature)
Wash 3 times with PBS-Tween (200 µL/well)
BIOTIN LINKED SECOND ANTIBODY INCUBATION
(antibody 1:1500 in PBS with 0.1% BSA, 100 µL/well, 2 hours
at 37°C)
Wash 3 times with PBS-Tween (200 µL/well)
STREPTAVIDIN-BIOTIN LINKED HORSERADISH PEROXIDASE COMPEX
INCUBATION
(complex 1:1500 in PBS with 0.1% BSA, 100 µL/well,
30 minutes @ 37°C)
Wash 3 times with PBS, 2 times with distilled water (200
µL/well)
SUBSTRATE INCUBATION
(4mM o-phenylene diamine, 150 µL/well)
MEASURE ABSORBANCE @ 490 nm

1. Antigen coating

Each well was coated with 10 ng of purified inhibitor protein. This was done by adding 10 ng of inhibitor in 100 μL of carbonate buffer to each well. Carbonate buffer deprotonates the amino and the carboxyl groups of the protein, which allows the protein to undergo ionic binding with the positively charged polystyrene plates. A standard curve was created by allowing the antibody to first mix with known amounts of purified inhibitor (range 0 to 2 ng). Then the mixture was added to the plates. Therefore an inverse curve of inhibitor amount to absorbance is obtained. eg. 0 ng inhibitor gives the highest absorbance, whereas 2 ng of inhibitor gives an absorbance of approximately zero.

2. Washing

PBS-Tween was used for all the washing steps except the last one. A saline concentration of 1.5% was used because this level was found to be the best at reducing non-specific binding of the antibodies. The last washing steps of PBS and distilled water were preformed to remove the PBS and Tween residues before the substrate was added, so it would not interfere with the colour development.

3. Blocking

Blocking is performed to reduce non-specific binding of antibodies to free binding sites in the wells. These free binding sites exist due to the fact there is not enough of the inhibitor protein to completely bind to every site. After the

blocking solution is removed from the plates, the plates can be stored a with parafilm cover in a bag at 4°C until needed.

4. First Antibody and Sample Incubation

Samples were diluted to a certain amount of total buffer soluble protein per 100 µL of PBS and 0.1% BSA and then diluted again with 100 µL of 1:500 inhibitor antibody in PBS and 0.1% BSA, which in turn diluted the antibody to 1:1000. This was done to have enough sample so triplicate analysis could be done on the plate. All sample and antibody mixtures were made in silanized glass test tubes and allowed to incubate overnight at 4°C before adding to the plates.

3.4.6. Indirect ELISA for ABA Content Determination

Dissected embryos and seedling parts were frozen, lyophilized, and then powdered in a glass mortar by a pestle. The samples were extracted in either cold methanol as described by Walker-Simmons (1987a; 1987b) or by a hot water treatment also described by Walker-Simmons (personal communication). Both methods gave reliable results. The ABA-4'-BSA conjugate was prepared according to Weiler (1979). ABA content of the extracts was measured by indirect ELISA also developed by Walker-Simmons (1987a; 1987b).

4. RESULTS AND DISCUSSION

4.1. Effect of ABA on Inhibitor Synthesis in Cultured 21 and 35 Days Post-anthesis Embryos

This introductory experiment was used to determine how closely the results obtained with the indirect inhibitor ELISA would compare with the results published in Robertson et al. (1989). In Robertson et al. (1989) immature embryos were isolated from developing kernels of Hordeum vulgare cv. Bonanza and cultured in ABA for five days. Using the Western blot technique, the inhibitor protein level was visually estimated against a set of purified inhibitor protein standards on the same blot and the inhibitor protein per embryo was determined. Table 1 shows the comparison. The inhibitor ELISA results were similar to the results derived from the Western blot technique. For both cases, the 21 DPA and 35 DPA embryos, the 1 μM ABA concentration effect on inducing the inhibitor protein is small, whereas, the 10 μM and 100 μM ABA concentration had a significant effect on inducing the inhibitor protein. At the 10 μM ABA level the effect appears to be maximal. Embryos cultured without ABA, germinated and the inhibitor level declined. Comparing the embryos taken directly from developing seeds, inhibitor protein increased as the embryo matured from 21 to 40 DPA.

Table 1.Comparison of Inhibitor Levels in ABA-treated Immature Embryos by Western Blot and ELISA

Embryo Sample	Western Blot avg.(ng) per embryo*	ELISA avg.(ng) per embryo	ELISA st.dev. per embryo
21 d postanthesis trial			
Direct from seed			
(21 DPA)	36.2	41.0	5.3
(26 DPA)	69.2	76.2	11.1
Cultured (5 days)			
without ABA (germinated)	95.0	34.4	15.8
1 μM ABA (arrested)	299.0	191.2	18.4
10 μM ABA (arrested)	713.0	684.9	42.4
100 μM ABA (arrested)	835.0	765.6	41.3
35 d postanthesis trial			
Direct from seed			
(35 DPA)	225.0	296.8	46.8
(40 DPA)	376.0	342.4	19.2
Cultured (5 days)			
without ABA (germinated)	113.0	180.3	34.4
1 μM ABA (arrested)	521.0	466.4	54.9
10 μM ABA (arrested)	1001.0	935.3	49.9
100 μM ABA (arrested)	1328.0	1105.1	113.4

* Robertson et. al. (1989)

Robertson et al. (1989) showed that the inhibitor content increased throughout embryo development.

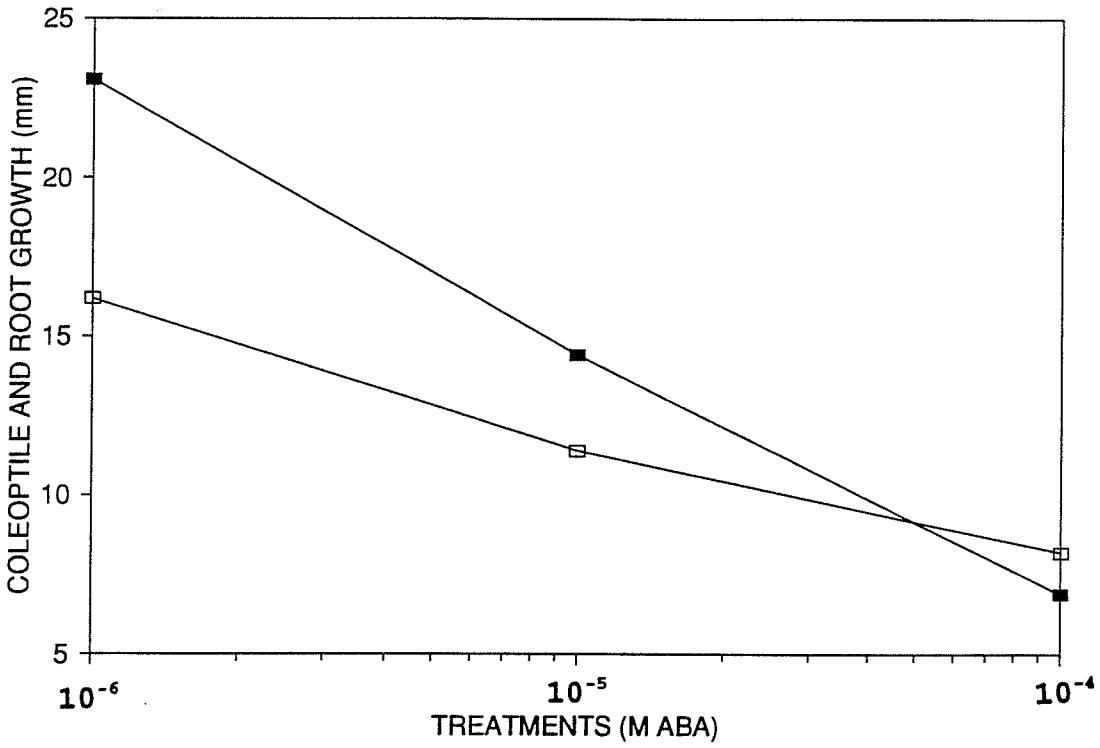
4.2. Effect of Exogenously Applied ABA on Inhibitor Synthesis in Seedling Tissue

After showing that exogenously applied ABA induced the inhibitor protein in cultured immature embryos, young seedlings were studied to see if inhibitor could be induced in the roots, the coleoptile and the remaining embryo axis-scutellar node tissue (hereafter referred to as residual embryonic tissue). Two day-old seedling tissue was chosen as a starting point because roots and the coleoptile have developed to a point where they can be removed from the residual embryonic tissue intact. Two day-old seedlings were given one of four treatments: 5 mL of water, 5 mL of 1 μM ABA solution, 5 mL of 10 μM ABA solution, or 5 mL of 100 μM ABA solution. The seedlings were allowed to grow a further two days. The seedlings from each treatment were dissected into: root tissue, coleoptile tissue and residual embryonic tissue. Since the tissue was growing and there was a substantial change in weight of the tissue it was decided to report the inhibitor content in terms of fresh weight. The assumption was that certain tissues would have similar water contents, since moisture was abundant.

Figure 2 shows the results of the coleoptile and root

Figure 2. The effect of various concentrations of ABA (10^{-6}, 10^{-5}, 10^{-4} M) for a period of 2 days on root and coleoptile growth in seedlings which initially were germinated in water for 2 days.

—■— COLEOPTILE —⊟— ROOT


25
20
15
10
5
COLEOPTILE AND ROOT GROWTH (mm)
10⁻⁶
10⁻⁵
10⁻⁴
TREATMENTS (M ABA)

growth in terms of length after 2 days in the ABA treatments. Both the coleoptile and root show an inverse logarithmic response to the ABA treatment. The greater amount of ABA the lesser the growth. The water treatment gave the greatest amount of growth (21.3 mm for the roots and 26.6 mm for the coleoptile).

Figure 3 shows inhibitor content and fresh weight of the residual embryonic tissue. The fresh weight of the residual embryonic tissue stayed relatively constant with all treatments. The inhibitor content in the residual embryonic tissue increased with ABA treatments. As with the immature embryos there seemed to be a maximal effect with the 10 μM ABA treatment, although none of the ABA treatments were significantly different from one another. The inhibitor content increased slightly from the 2 day-old water starting point to the 4 day-old water treatment but, it was not significant. The residual embryonic tissues were checked for remaining endosperm and aleurone layer in this time course and all other experiments due to the fact that the endosperm contains the majority of the seed inhibitor protein (Robertson and Hill, 1989) and the inhibitor is also found in the aleurone layer (Mundy, 1984). If endosperm or aleurone material was found, it was removed.

Figure 4 shows inhibitor content and fresh weight of the roots. The fresh weights of the ABA-treated roots were similar to each other. This occurred even though the root growth

Figure 3. Inhibitor content and fresh weight of the residual embryonic tissue from seedlings germinated for two days on water (starting point), then grown with various ABA concentrations (1, 10, 100 μM) or water for another 2 days.

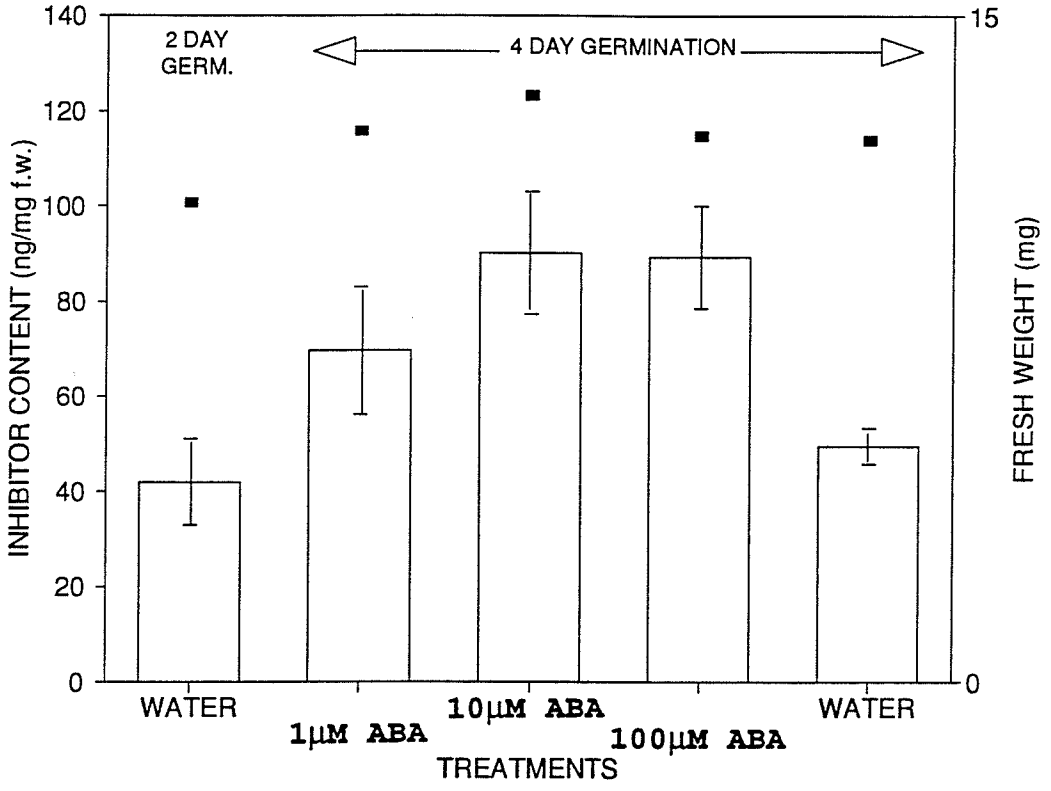
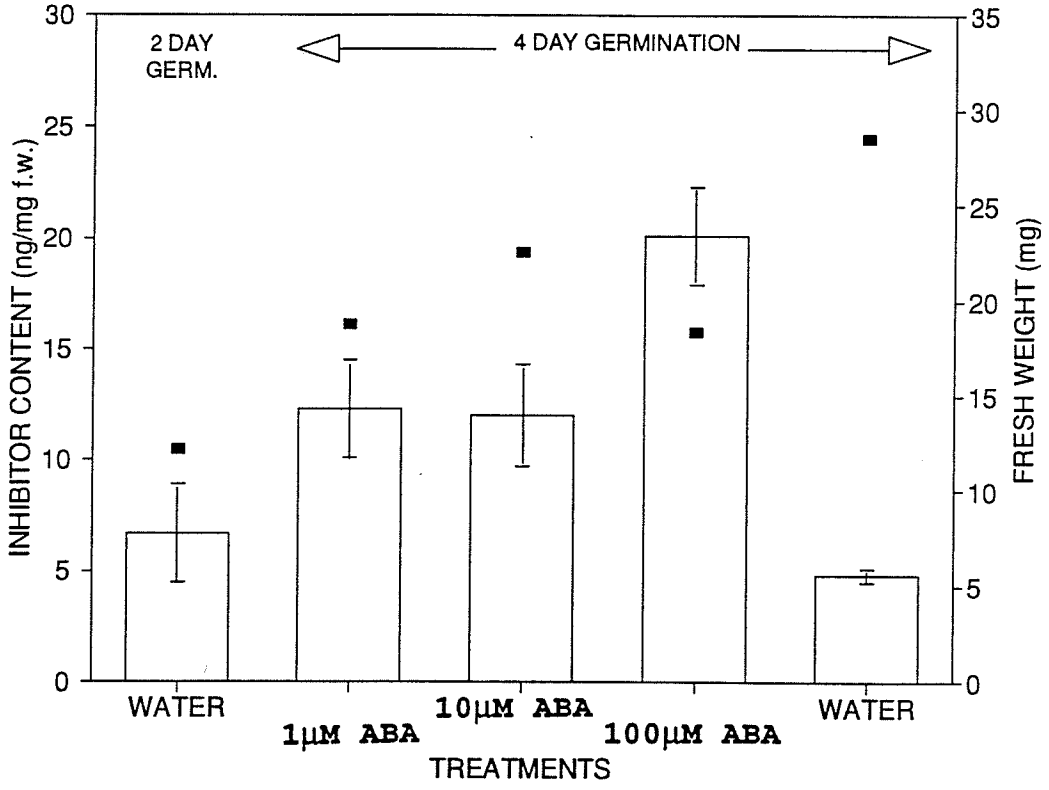

140
120
100
80
60
40
20
0
15
0
2 DAY
GERM.
4 DAY GERMINATION
INHIBITOR CONTENT (ng/mg f.w.)
FRESH WEIGHT (mg)
WATER
1µM ABA
10µM ABA
100µM ABA
WATER
TREATMENTS

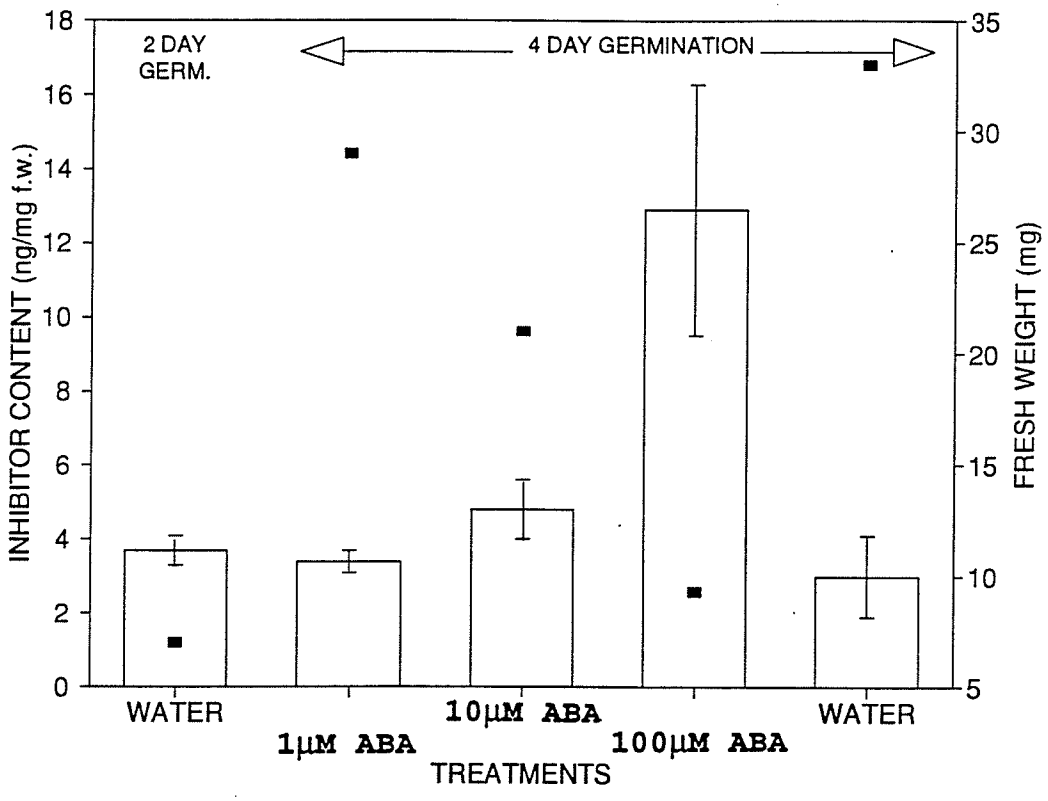
Figure 4. Inhibitor content and fresh weight of the roots from seedlings germinated for two days on water (starting point), then grown with various ABA concentrations (1, 10, 100 μM) or water for another 2 days.


2 DAY
GERM.
4 DAY GERMINATION
INHIBITOR CONTENT (ng/mg f.w.)
FRESH WEIGHT (mg)
30
25
20
15
10
5
0
35
30
25
20
15
10
5
0
WATER
1µM ABA
10µM ABA
100µM ABA
WATER
TREATMENTS

(length) was effected by ABA treatments (Fig.2). It seems increased ABA shortened and thickened roots but did not affect fresh weight gain. The inhibitor content of the roots increased with the ABA treatments but, there was not a significant difference between the values. Unlike the residual embryonic tissue maximum inhibitor content was found at 100 μM ABA. The inhibitor level stayed approximately the same in the roots for the 2 day water treatment and the 4 day water treatment.

Figure 5 shows inhibitor content and fresh weight of coleoptile tissue. The fresh weight of the coleoptile, like the growth (length) is affected by the ABA treatment (Fig.2); increased ABA decreased fresh weight. The inhibitor content of the coleoptile did not change with the 1 μM ABA treatment but, the inhibitor content did increase slightly with 10 μM ABA and significantly with the 100 μM ABA. When expressed as inhibitor content (ng) per coleoptile however, the three ABA treatments and the 4 day water treatment all contained approximately 100 ng of inhibitor. The two day starting point control had roughly 25 ng. Therefore, the difference between the trials is probably a reflection of the change in fresh weight in the coleoptile. This did not occur with the residual embryonic tissue or the roots as the fresh weights for the ABA trials were similar to each other and the inhibitor content (ng) per tissue showed an increase with the ABA.

Figure 5. Inhibitor content and fresh weight of the coleoptile from seedlings germinated for two days on water (starting point), then grown with various ABA concentrations (1, 10, 100 μM) or water for another 2 days.


2 DAY
GERM.
4 DAY GERMINATION
INHIBITOR CONTENT (ng/mg f.w.)
FRESH WEIGHT (mg)
0
2
4
6
8
10
12
14
16
18
5
10
15
20
25
30
35
WATER
1µM ABA
10µM ABA
100µM ABA
WATER
TREATMENTS

4.3. Effect of Dehydration and Osmotic Stress on Inhibitor Synthesis in Seedling Tissue

The previous experiment provided evidence that exogenous ABA induced the synthesis of the inhibitor protein in residual embryonic tissue and root tissue of young seedlings, and possibly the coleoptile tissue. To gain more evidence that inhibitor synthesis may be mediated by ABA, treatments that increase endogenous ABA levels in the seedling tissue were examined to see if there was a corresponding increase in the inhibitor protein. To begin with, an experiment in which seeds were allowed to germinate for five days under normal conditions (no stress) was examined for a control. Next, an experiment where the seedlings experienced dehydration stress was examined and finally, two experiments where the seedlings experienced two different osmotic stresses were examined. Due to the fact that a change in fresh weight will occur in the seedling with the dehydration and osmotic treatments, the inhibitor and ABA content were expressed in units per mg dry weight of the tissue.

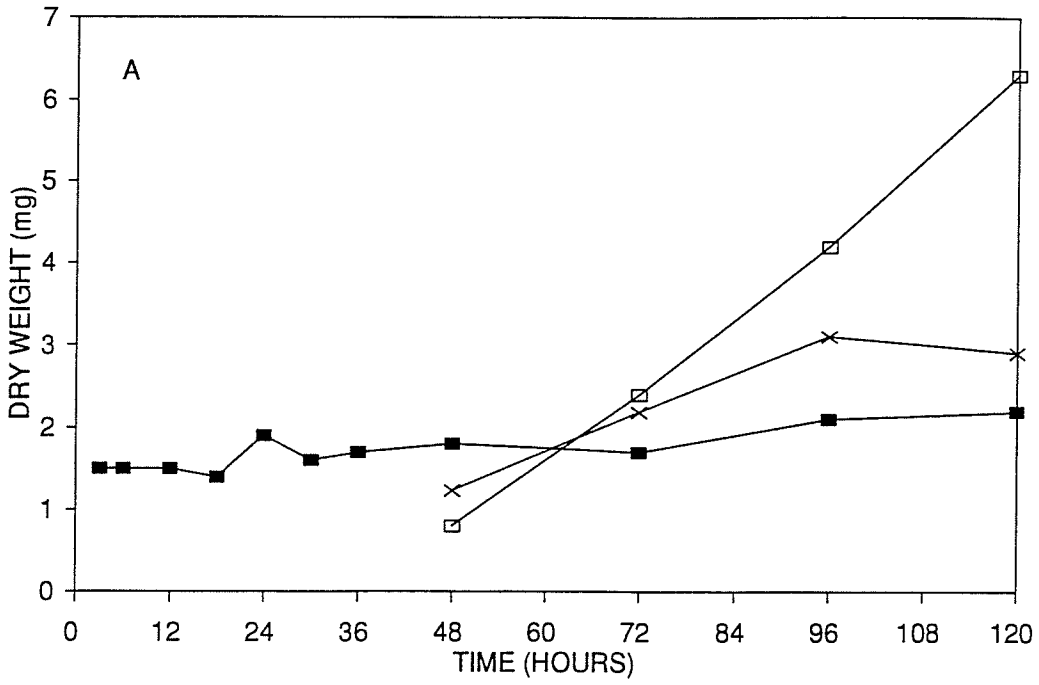
4.3.1. Inhibitor Synthesis During Unstressed Seedling Tissue Development - (Germination Experiment).

The objective of this experiment was to quantify the amounts of the inhibitor and ABA that would be found in seedling tissue during normal (unstressed) germination. This

experiment will be referred to as the germination experiment. A time course was started at the time the seeds were first imbibed (at the beginning of sterilization) to study the inhibitor content and ABA content in germinating embryos. From 3 hours after imbibition to 36 hours the embryo was left intact for inhibitor analysis. Sampling was done more frequently at the beginning of the time course because preliminary experiments had shown a high degree of variability in inhibitor content of embryos after imbibition, it was decided to see if a pattern existed in the first 48 hours of germination. Starting at 48 hours to 120 hours the young seedling was dissected into roots, coleoptile, and residual embryonic tissue.

Figure 6 shows dry weight (A) and water content (B) of the seedling tissue. The dry weight of the residual embryonic tissue stays relatively constant throughout the time course, whereas the water content of the residual embryonic tissue increased rapidly in the first 36 hours after imbibition and then stayed relatively constant throughout the rest of the trial. The dry weight of the roots increased up to the 96 hour of germination. The roots increased in water content up to 72 hours after imbibition and then stayed constant. The dry weight of the coleoptile increased throughout the trial, and the coleoptile gained water up to 96 hours after imbibition then stayed constant.

Figure 6. Dry weight (A) and water content (B) of embryos and seedling parts of seeds germinated without stress. Intact germinating embryos were considered as residual embryonic tissue (RET) up to 36 hours after imibition, after this time seedling were dissected into residual embryonic tissue (RET), roots and coleoptile (COL) up to 120 hours after imibition.


A
DRY WEIGHT (mg)
0
1
2
3
4
5
6
7
0
12
24
36
48
60
72
84
96
108
120
TIME (HOURS)

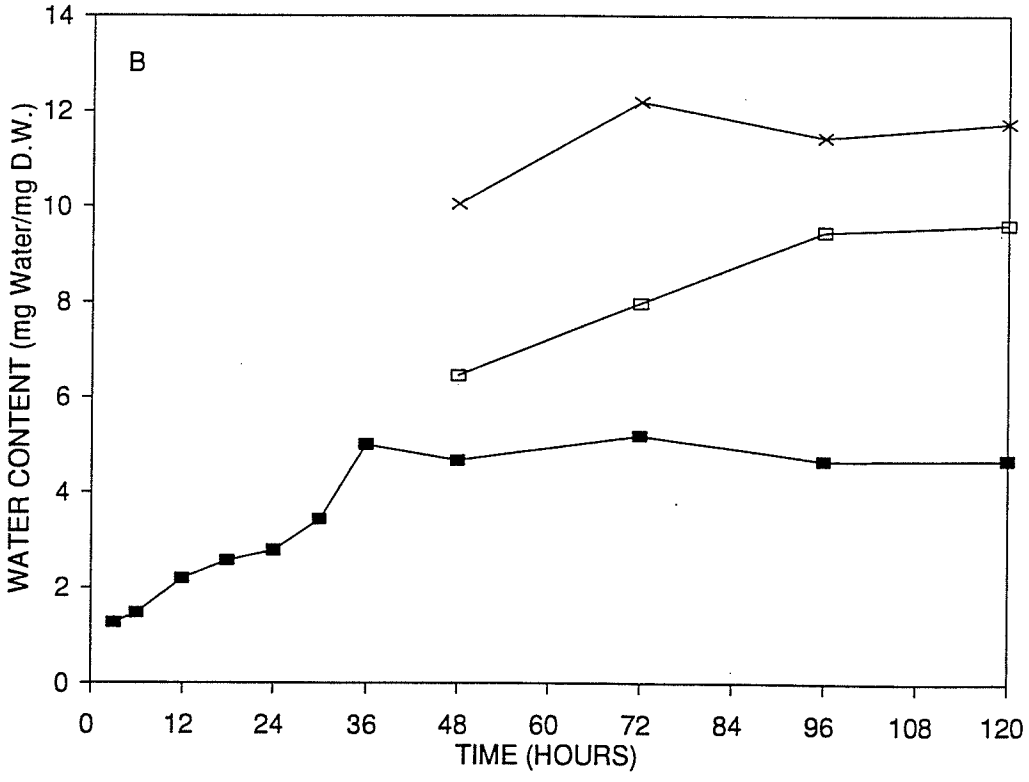

B
WATER CONTENT (mg Water/mg D.W.)
0
2
4
6
8
10
12
14
0
12
24
36
48
60
72
84
96
108
120
TIME (HOURS)

Figure 7 shows inhibitor and ABA content of embryo and residual embryonic tissue of young seedlings during germination. At the beginning (3 hours after imbibition) the ABA content in the embryo was at its highest level (149 pg/mg d.w.). This was 210 pg of ABA/embryo which is close to 250 pg of ABA/embryo for a mature quiescent embryo found by Robertson et al. (1989). The ABA content then decreased as germination continued. At around 30 hours after imbibition the ABA content stayed constant at about 40 pg/mg d.w.. The inhibitor protein content of the residual embryonic tissue at 3 hours after imbibition was 150 ng/mg d.w., this is about half of the amount found by Robertson et al. (1989) in mature quiescent embryos. The inhibitor protein increased in content to 250 ng/mg d.w. at 24 hours after imbibition and stayed relatively constant until 48 hours after imbibition. After 48 hours the inhibitor content then decreased to around 175 ng/mg d.w. at 120 hours (5 days) after imbibition.

Figure 8 shows inhibitor and ABA content of root tissue during germination. The ABA content was between 15 and 30 pg/mg d.w. for the period of 2 to 5 days after imbibition. The inhibitor content also stayed relatively constant, between 20 and 30 ng inhibitor/mg d.w. for the 4 day period.

Figure 9 shows inhibitor and ABA content of coleoptile tissue during germination. The ABA content was 35 pg/mg d.w. at 2 days and increased up to around 100 pg/mg d.w. at five days after imbibition. This increase in ABA was not

Figure 7. Inhibitor and ABA content of germinating embryos and residual embryonic tissues from seeds germinated without stress. From 3 to 36 hours the germinating embryo was left intact and considered residual embryonic tissue. From 48 to 120 hours roots and coleoptile were removed from the residual embryonic tissue.

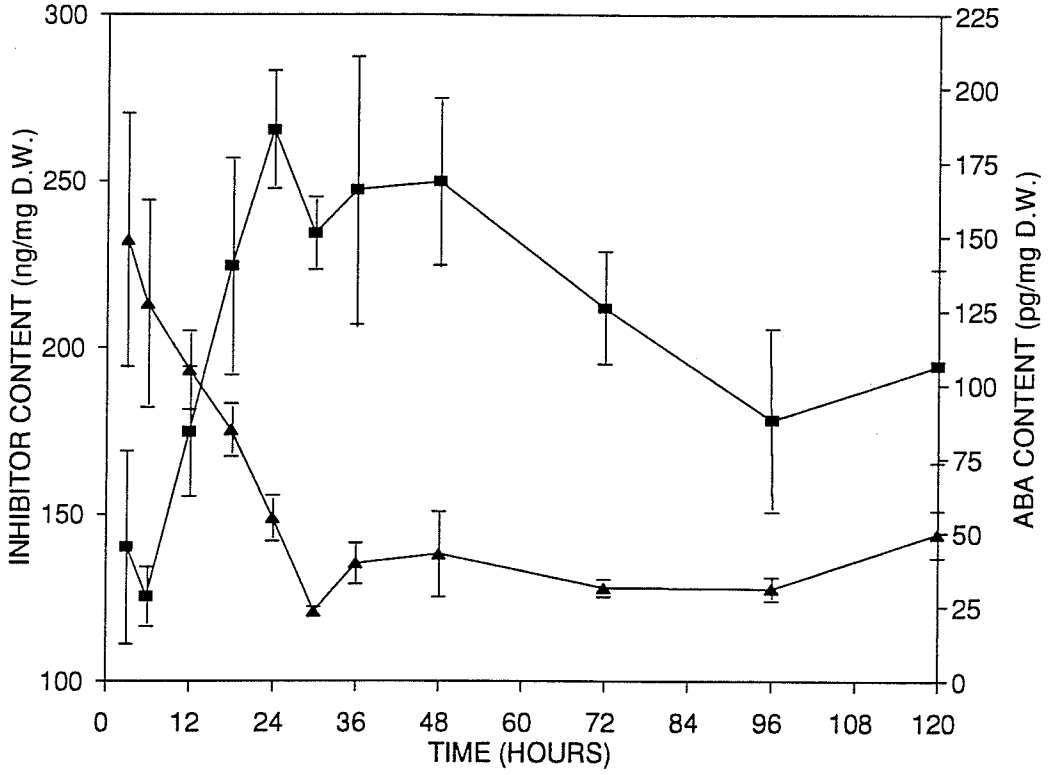

300
250
200
150
100
INHIBITOR CONTENT (ng/mg D.W.)
225
200
175
150
125
100
75
50
25
0
ABA CONTENT (pg/mg D.W.)
0
12
24
36
48
60
72
84
96
108
120
TIME (HOURS)

Figure 8. Inhibitor and ABA content of roots from unstressed seedlings. After 2 days of germination and up to 5 days of germination, roots were removed from seedlings for analysis.

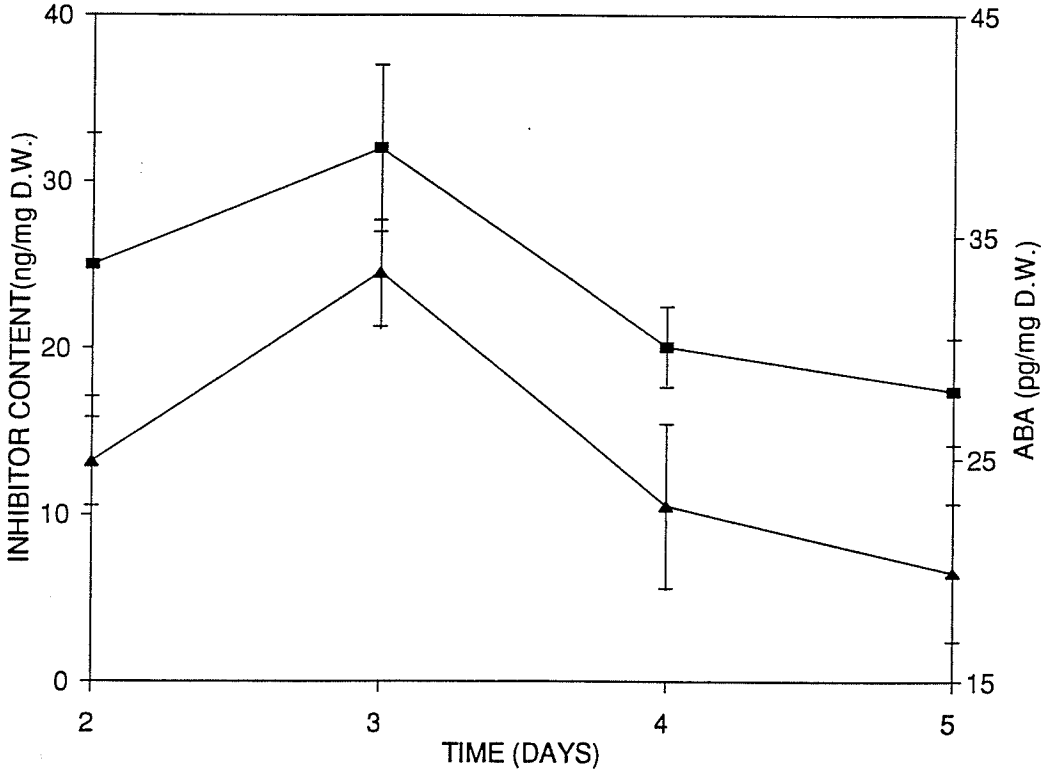
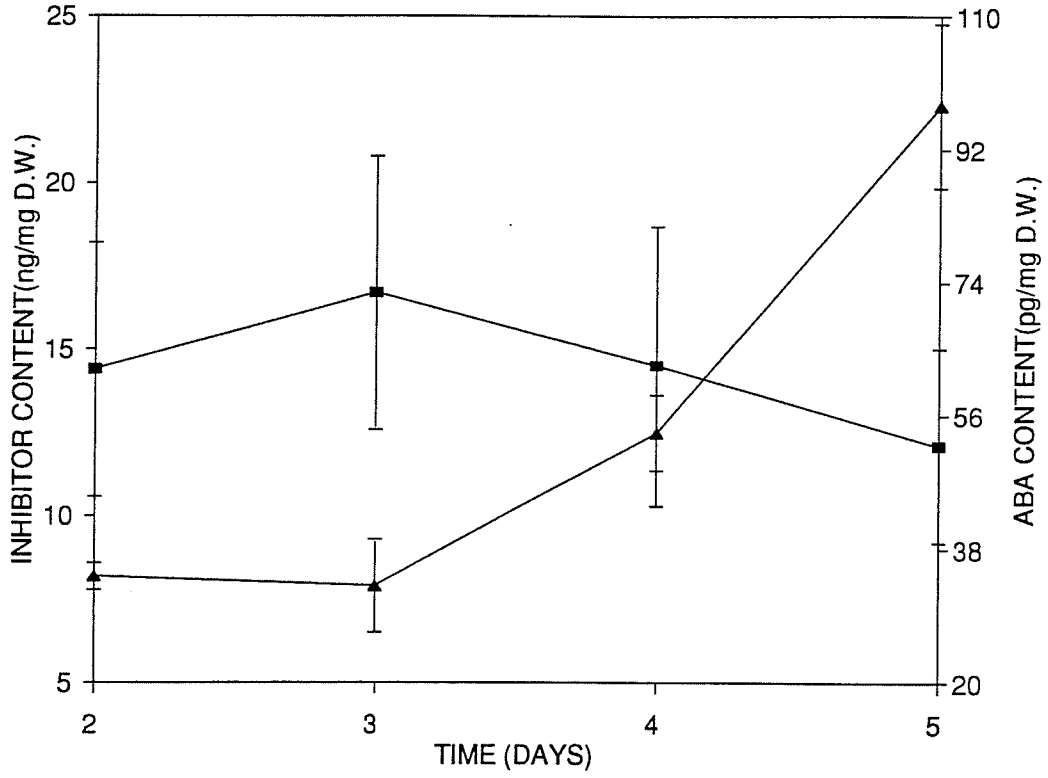

40
30
20
10
0
45
35
25
15
2
3
4
5
INHIBITOR CONTENT(ng/mg D.W.)
ABA (pg/mg D.W.)
TIME (DAYS)

Figure 9. Inhibitor and ABA content of coleoptiles from unstressed seedlings. After 2 days of germination and up to 5 days of germination, coleoptiles were removed from seedlings for analysis.


25
20
15
10
5
INHIBITOR CONTENT(ng/mg D.W.)
110
92
74
56
38
20
ABA CONTENT(pg/mg D.W.)
2
3
4
5
TIME (DAYS)

accompanied by an increase in inhibitor protein. The inhibitor stayed constant at 10-15 ng/mg d.w. from 2 days to 5 days.

4.3.2. Inhibitor Synthesis in Seedling Tissue During Dehydration Stress

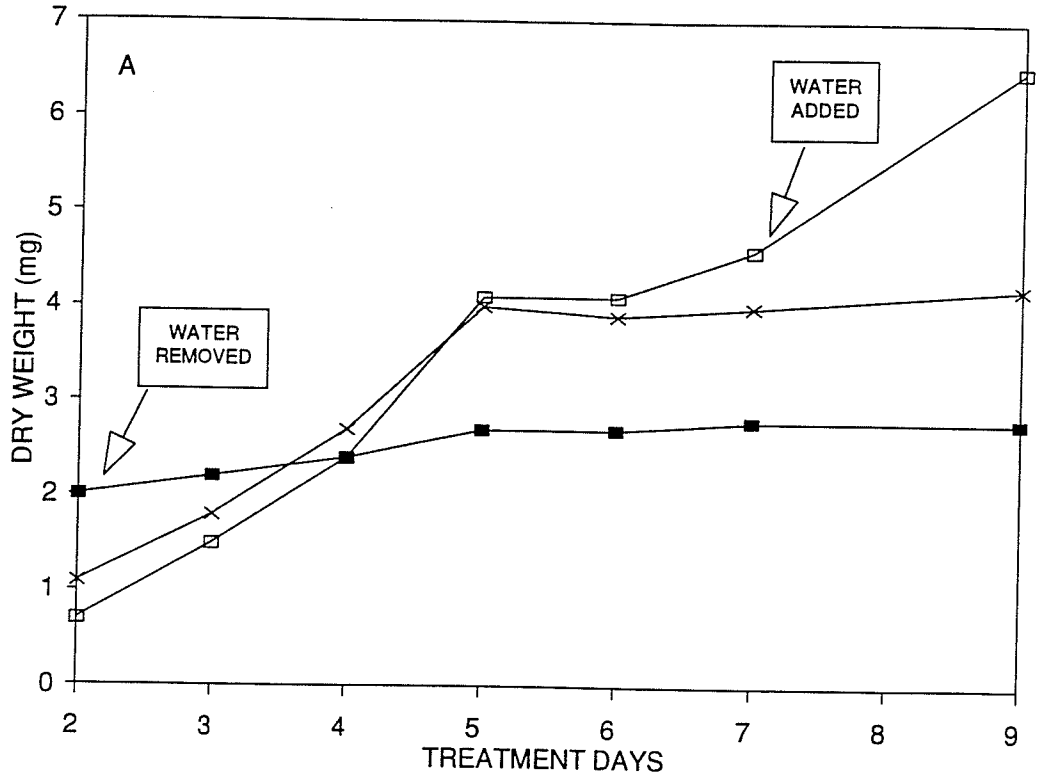
During dehydration stress, plants try to adapt to the condition by physiological and biochemical responses. The rapid accumulation of ABA in the plant tissue during dehydration has been extensively studied (Reid and Wample, 1985). In this experiment, dehydration stress was used to increase ABA to see if it would lead to induction of the inhibitor protein.

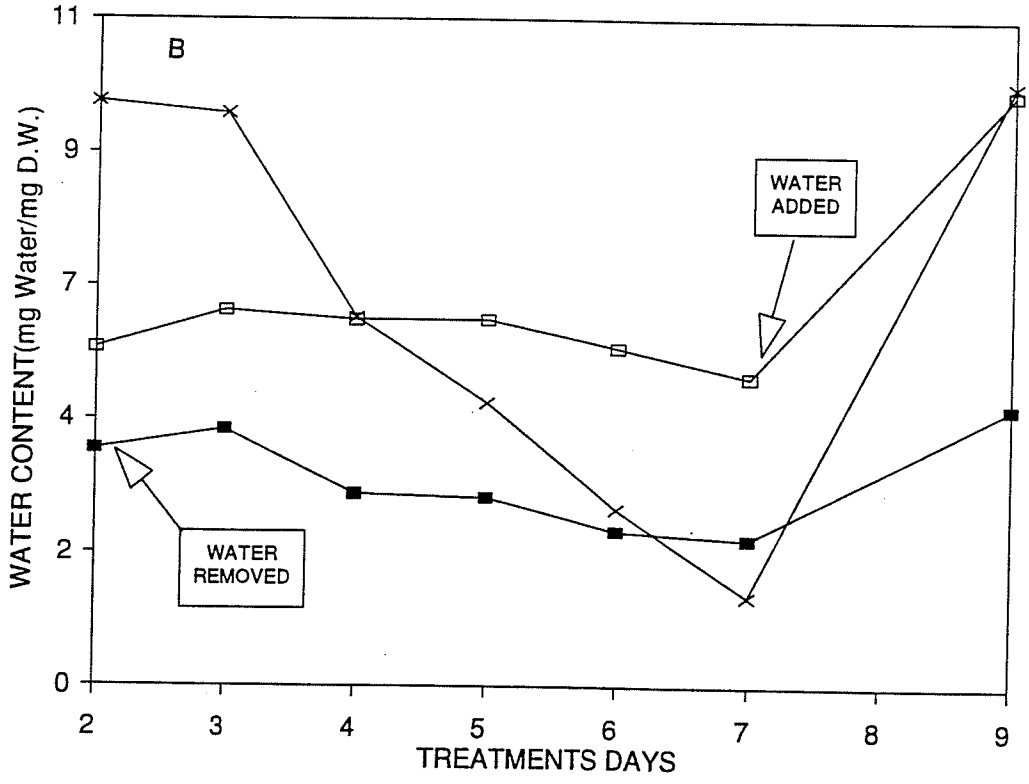
In the desiccator, water was slowly lost from the seedlings. After one day the fresh weight loss of the seedlings was approximately 6.5%, after two days 11.5%, after three days 13%, after four days 14.5%, and finally after five days 20%.

Figure 10 shows dry weight (A) and water content (B) of the tissues as the trial continued. The dry weight of the residual embryonic tissue slightly increased throughout the trial. The water content of the residual embryonic tissue started to decrease after the first day in the desiccator and gradually decreased as the dehydration continued. The dry weight of the roots increased the first three days in the desiccator and then stayed constant for the fourth and fifth days. The roots did not gain dry weight on rehydration. The

Figure 10. Dry weight (A) and water content (B) of seedling parts from seedlings that experienced dehydration stress. Seedlings germinated for 2 days (starting point) were placed in a desiccator and sampled for 5 days. After 5 days the seedling were rehydrated for 2 days. The seedlings were dissected into residual embryonic tissue (RET), roots and coleoptile (COL).

RET ROOTS COL

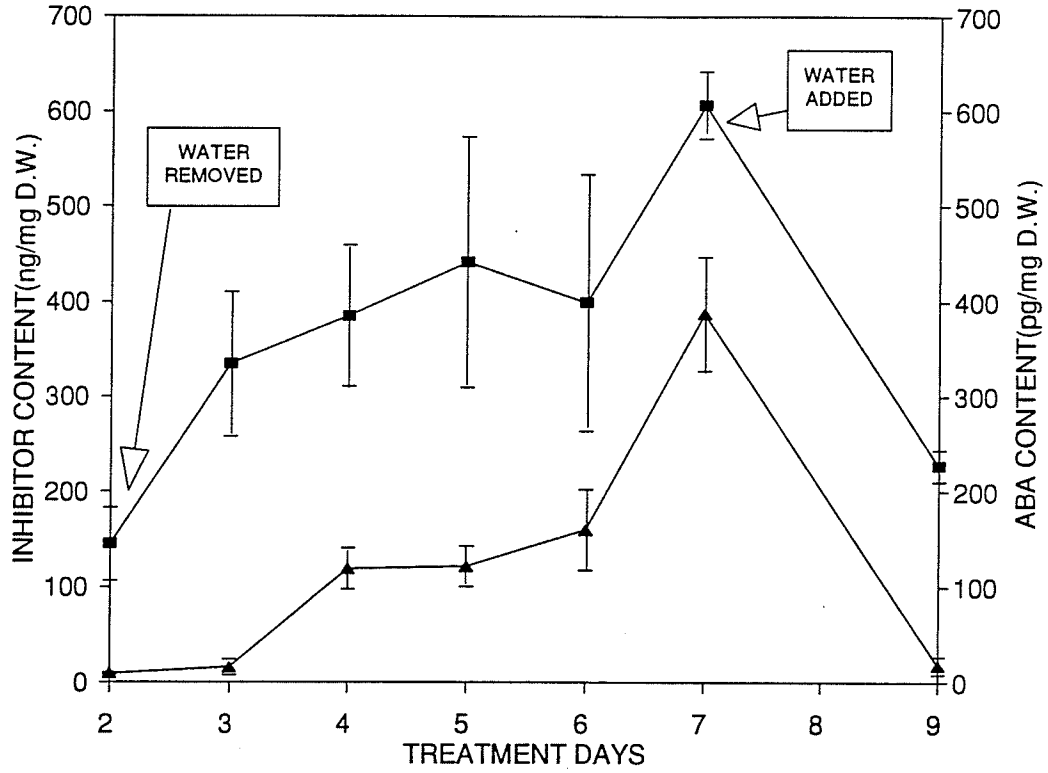

A
WATER ADDED
WATER REMOVED
DRY WEIGHT (mg)
TREATMENT DAYS
0
1
2
3
4
5
6
7
2
3
4
5
6
7
8
9


B
WATER ADDED
WATER REMOVED
WATER CONTENT(mg Water/mg D.W.)
TREATMENTS DAYS
0
2
4
7
9
11
2
3
4
5
6
7
8
9

water content of the roots also stayed constant the first day of the dehydration stress, but in the second day, it decreased significantly. The dry weight of the coleoptile followed a similar pattern to the roots, the weight increased the first three days, and then staying constant until rehydration. The water content of the coleoptile only decreased slightly during the stress period.

Figure 11 shows inhibitor and ABA content of residual embryonic tissue through the dehydration experiment. At the start of the dehydration treatment (2 day) the ABA content was 9.6 pg per mg d.w.. This is significantly lower than what was obtained in the germination experiment for 2 day germination which was 43 pg ABA per mg d.w. residual embryonic tissue (Fig.7). It seems that during the germination experiment the seedlings experienced some stress. The ABA content after 1 day in the desiccator was still lower (16 pg/mg d.w.) than the equivalent time in the germination experiment. After 2 days in the desiccator the ABA content increased dramatically. The ABA content then increased slightly the next two days then increased significantly on the fifth and final day in the desiccator. When the seedlings were rehydrated for two days the ABA content decreased to 18 pg/mg dry weight, which was around the original level. The inhibitor protein content at 2 day germination was 145 ng/mg d.w., this is also less than found in the germination experiment at the equivalent time (Fig. 7 - 250 ng/mg d.w.). The inhibitor protein increased as

Figure 11. Inhibitor and ABA content of the residual embryonic tissue from dehydrated seedlings. Seedlings germinated for 2 days (starting point) were placed in a desiccator and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The seedlings were dissected to remove the residual embryonic tissue.


700
600
500
400
300
200
100
0
INHIBITOR CONTENT(ng/mg D.W.)
ABA CONTENT(pg/mg D.W.)
WATER REMOVED
WATER ADDED
2
3
4
5
6
7
8
9
TREATMENT DAYS

the stress period was applied, ending up around 600 ng/mg d.w. residual embryonic tissue. Upon rehydration for two days the inhibitor content decreased to 230 ng/mg d.w.. There is a linear relationship (r^2 = 0.79) between the ABA content and the inhibitor content in the residual embryonic tissue during a slow desiccation.

The results for the dehydration of the residual embryonic tissue was not what was expected. It was expected the ABA would rise initially followed by an increase in inhibitor, in this experiment both ABA and inhibitor rose together, therefore there was no correlation between ABA inducing the inhibitor. In Robertson et al. (1989), they examined the complete shoot portion of the seedling (residual embryonic tissue and coleoptile), the ABA content increased in this tissue 20 fold in 12 hours during dehydration stress. The inhibitor did increase in this period, but it only doubled. In this experiment a 20 fold increase in the shoot region (the residual embryonic tissue and coleoptile together) did not occur until 48 hours in the desiccator. In 24 hours the ABA only increased 2.5 times while the inhibitor doubled. There were probably subtle differences in the stress regimes between the two experiments even though the desiccator set up was similar. The inhibitor content of the shoots found in this experiment were similar to what was found by Robertson et al. (1989).

Figure 12 shows inhibitor and ABA content of root tissue through the dehydration experiment. The ABA content in the roots did not increase until the second day of stress then it increased dramatically, by the fifth day there was a 20-fold increase. The first day in the desiccator the inhibitor content was similar to what was found in the germination experiment at 3 days. On the second day in the desiccator the inhibitor content increased matching when the ABA first increased, this also parallels the first substantial water content decrease. As the experiment continued the inhibitor content decreased even though the ABA still was increasing. There might be some point during the dehydration trial where the root tissue is not functional in protein synthesis but, ABA is still produced. Upon rehydration the roots seemed healthy and functional but, there was adventitious root growth. Unlike the residual embryonic tissue there does not seem to be a linear relationship in the roots between inhibitor and ABA content (r^2 = 0.00003).

Figure 13 shows inhibitor and ABA content of coleoptiles through the dehydration experiment. In the unstressed two day old coleoptile tissue no ABA was found, this is different from the germination experiment (Figure 9) in which 34 pg/mg d.w. coleoptile was found. After the first day of stress only 10 pg/mg d.w. was found, this slowly increased and then after the fourth day in stress, it rose to 350 pg/d.w. of ABA. The inhibitor protein in the 2 day unstressed coleoptile was 7

Figure 12. Inhibitor and ABA content of the roots from dehydrated seedlings. Seedlings germinated for 2 days (starting point) were placed in a desiccator and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The roots were removed from the seedlings for analysis.

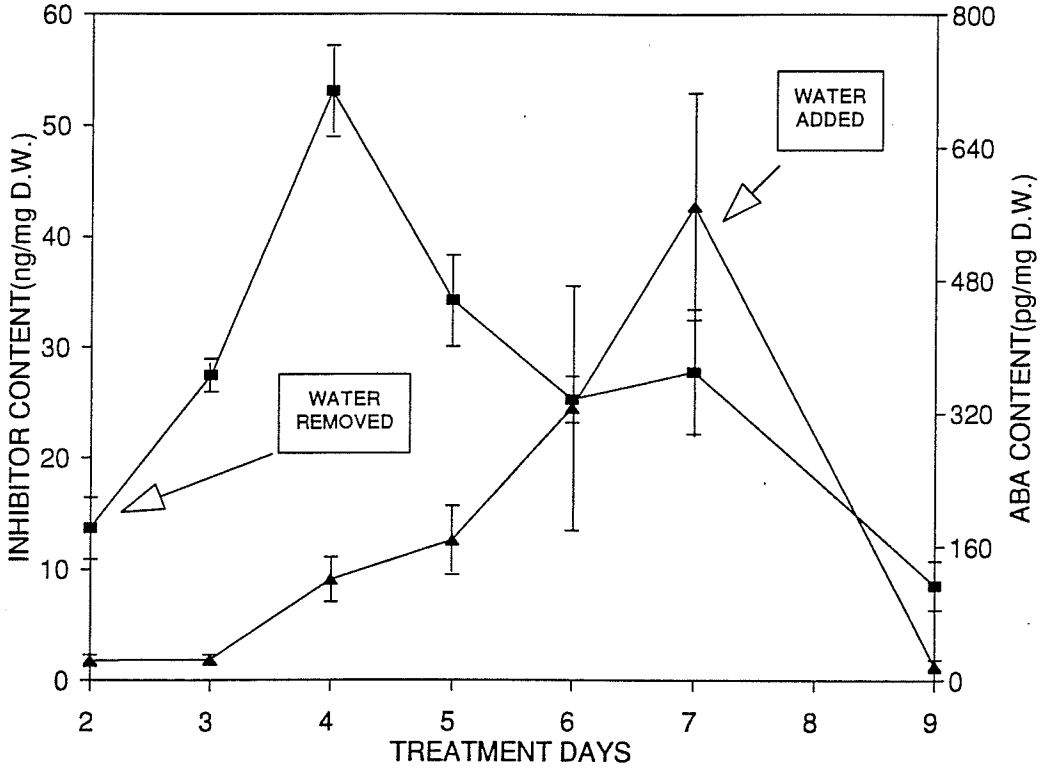
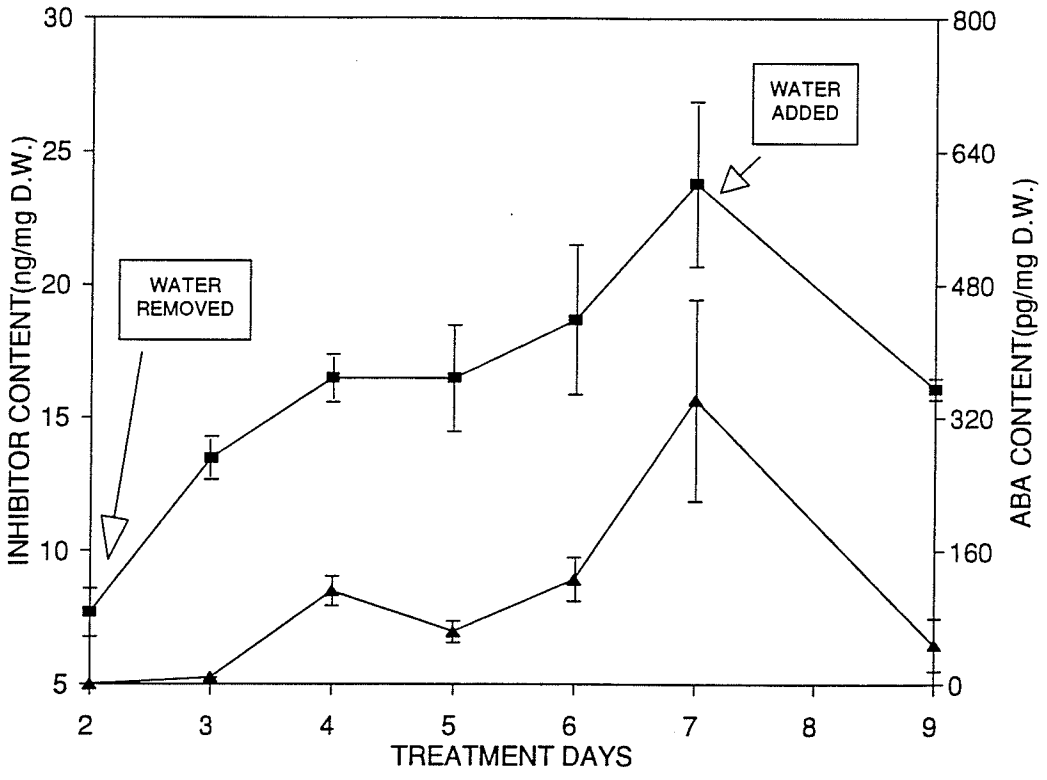

INHIBITOR CONTENT(ng/mg D.W.)
ABA CONTENT(pg/mg D.W.)
TREATMENT DAYS
WATER ADDED
WATER REMOVED
0
10
20
30
40
50
60
0
160
320
480
640
800
2
3
4
5
6
7
8
9

Figure 13. Inhibitor and ABA content of the coleoptile from dehydrated seedlings. Seedlings germinated for 2 days (starting point) were placed in a desiccator and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The coleoptiles were removed from the seedlings for analysis.


30
25
20
15
10
5
800
640
480
320
160
0
2
3
4
5
6
7
8
9
INHIBITOR CONTENT(ng/mg D.W.)
ABA CONTENT(pg/mg D.W.)
TREATMENT DAYS
WATER REMOVED
WATER ADDED

ng/mg d.w.. As the stress period continued the inhibitor increased up to 24 ng/mg d.w. In comparison with the germination experiment the inhibitor level stayed around 15 ng/mg dry weight coleoptile throughout the trial. There is a linear relationship between the inhibitor protein content and the ABA content in the coleoptile (r^2 =0.80).

With the dehydration experiment, the ABA content in all three tissues significantly increased. The inhibitor content in the residual embryonic tissue also increased significantly from 150 to 600 ng/mg d.w., with the roots and the coleoptile the inhibitor protein started at low levels and increased with dehydration but, the inhibitor levels were still very low.

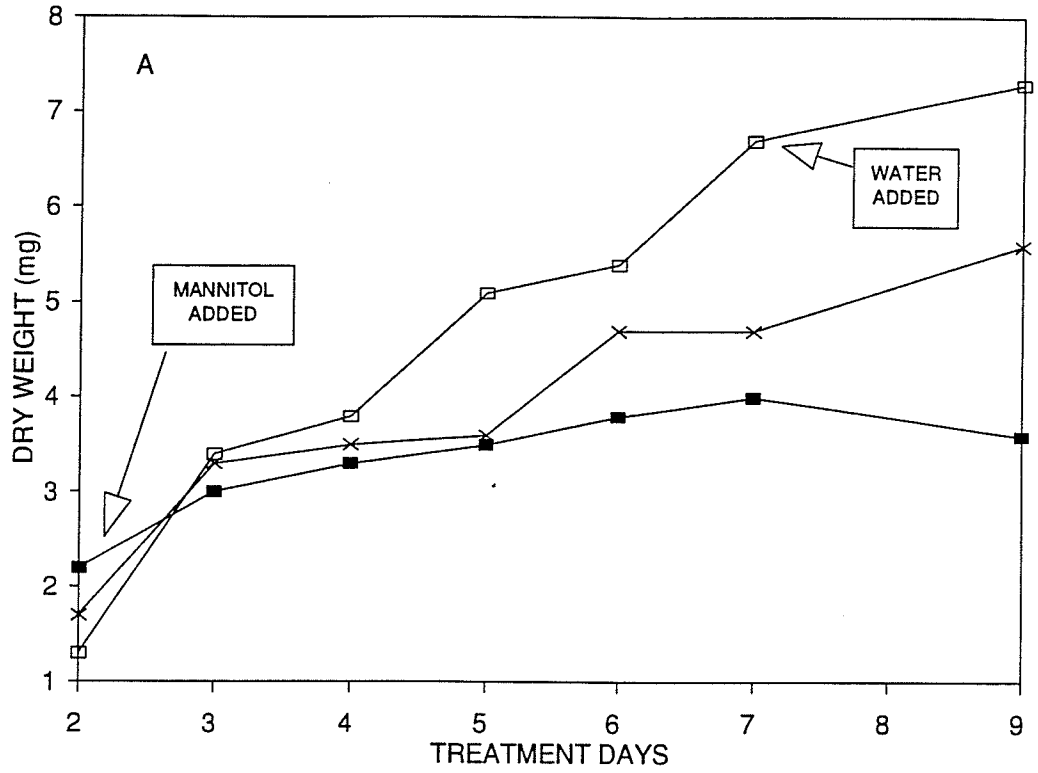
4.3.3. Inhibitor Synthesis in Seedling Tissue Under Osmotic Stress With 0.5 M Mannitol Solution

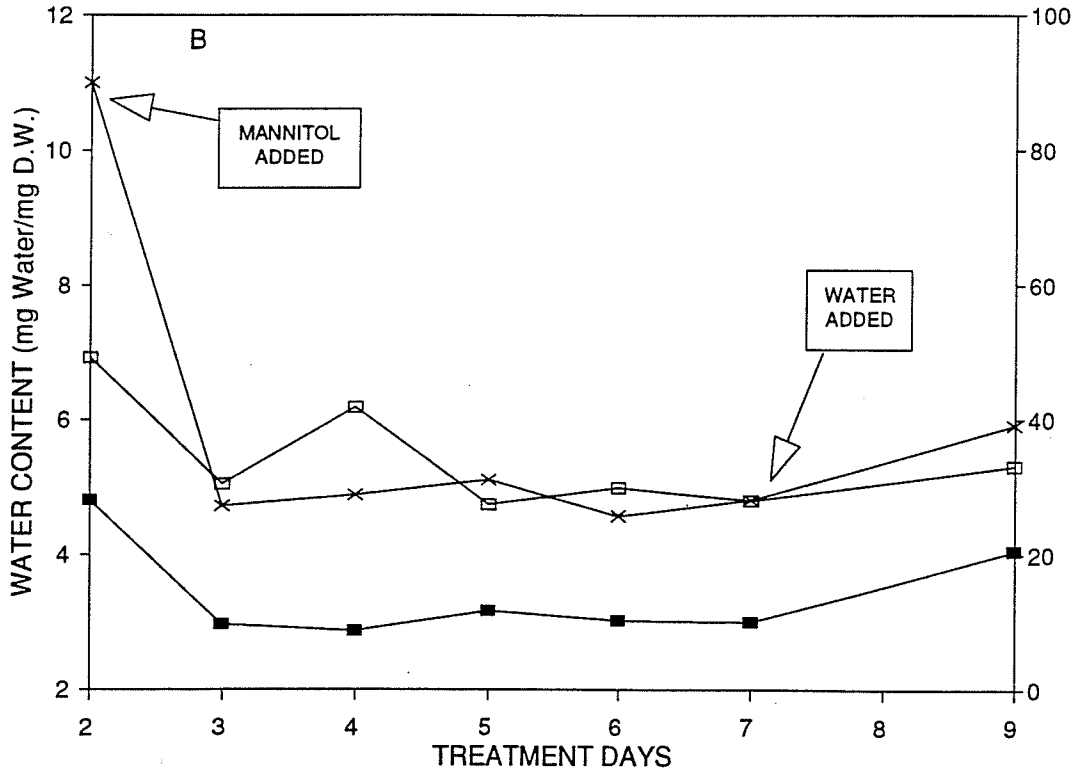
Plant tissue under osmotic stress also rapidly accumulates ABA. It was decided to examine osmotic stress effects on ABA and the inhibitor protein in the seedling tissue. The dehydration stress seemed to damage the roots too quickly, limiting physiological and biochemical responses.

Figure 14 shows dry weight (A) and water content (B) of the seedling parts throughout the treatment with 0.5 M mannitol. All three tissues increased in dry weight throughout the experiment. The water content of all three tissues decreased significantly the first day in the mannitol and then stayed relatively constant throughout the trial and all

Figure 14. Dry weight (A) and water content (B) of seedling parts from seedlings that experienced osmotic stress. Seedlings germinated for 2 days (starting point) were placed in a solution of 0.5 M mannitol and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The seedlings were dissected into residual embryonic tissue (RET), roots and coleoptile (COL).

RET ROOTS COL


A
DRY WEIGHT (mg)
TREATMENT DAYS
MANNITOL ADDED
WATER ADDED
1
2
3
4
5
6
7
8
2
3
4
5
6
7
8
9


B
WATER CONTENT (mg Water/mg D.W.)
TREATMENT DAYS
MANNITOL ADDED
WATER ADDED
2
4
6
8
10
12
0
20
40
60
80
100
2
3
4
5
6
7
8
9

increased slightly on rehydration. There was some adventitious root growth on rehydration.

Figure 15 shows inhibitor and ABA content of residual embryonic tissue during the experiment. On the first day in the osmoticum the ABA content increased slightly. The ABA content then fluctuated around 20 to 30 pg/mg d.w. throughout the rest of the trial. This was significantly less than found in the dehydration trial. The inhibitor content in the 2 day starting point control was higher than that found in the previous experiments. As the osmoticum trial continued the inhibitor protein content gradually increased. The increase of the inhibitor in this experiment was not as pronounced as it was in the dehydration experiment.

Figure 16 shows inhibitor and ABA content of roots during the experiment. The ABA content increased the first two days in the osmoticum. The rest of the trial the ABA content stayed around 25 pg/mg dry weight. The dehydration experiment induced 25x more ABA than the 0.5 M mannitol osmoticum experiment. The inhibitor content in this experiment is between 20 and 40 ng/mg d.w.. Again the inhibitor content for the 2 day starting point control was higher than other experiments.

Figure 17 shows inhibitor and ABA content of the coleoptile tissue during the experiment. The ABA content increased as the stress period continued but the inhibitor content only increased slightly the first day, then levelled out for the rest of the trial. The ABA induced in the

Figure 15. Inhibitor and ABA content of the residual embryonic tissue from osmotically stressed seedlings. Seedlings germinated for 2 days (starting point) were placed in a solution of 0.5 M mannitol and sampled for 5 days. After 5 days the seedling were rehydrated for 2 days. The seedlings were dissected to remove the residual embryonic tissue.

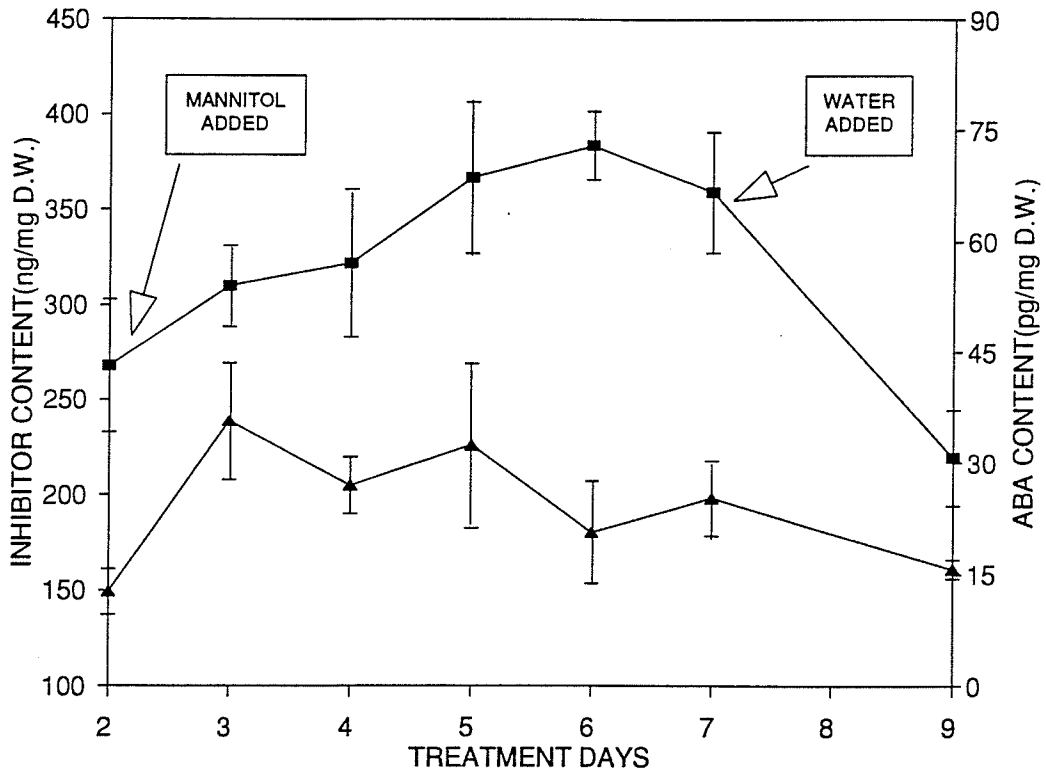

MANNITOL ADDED
WATER ADDED
INHIBITOR CONTENT(ng/mg D.W.)
ABA CONTENT(pg/mg D.W.)
TREATMENT DAYS
450
400
350
300
250
200
150
100
90
75
60
45
30
15
0
2
3
4
5
6
7
8
9

Figure 16. Inhibitor and ABA content of the roots from osmotically stressed seedlings. Seedlings germinated for 2 days (starting point) were placed in a solution of 0.5 M mannitol and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The roots were removed from the seedlings for analysis.

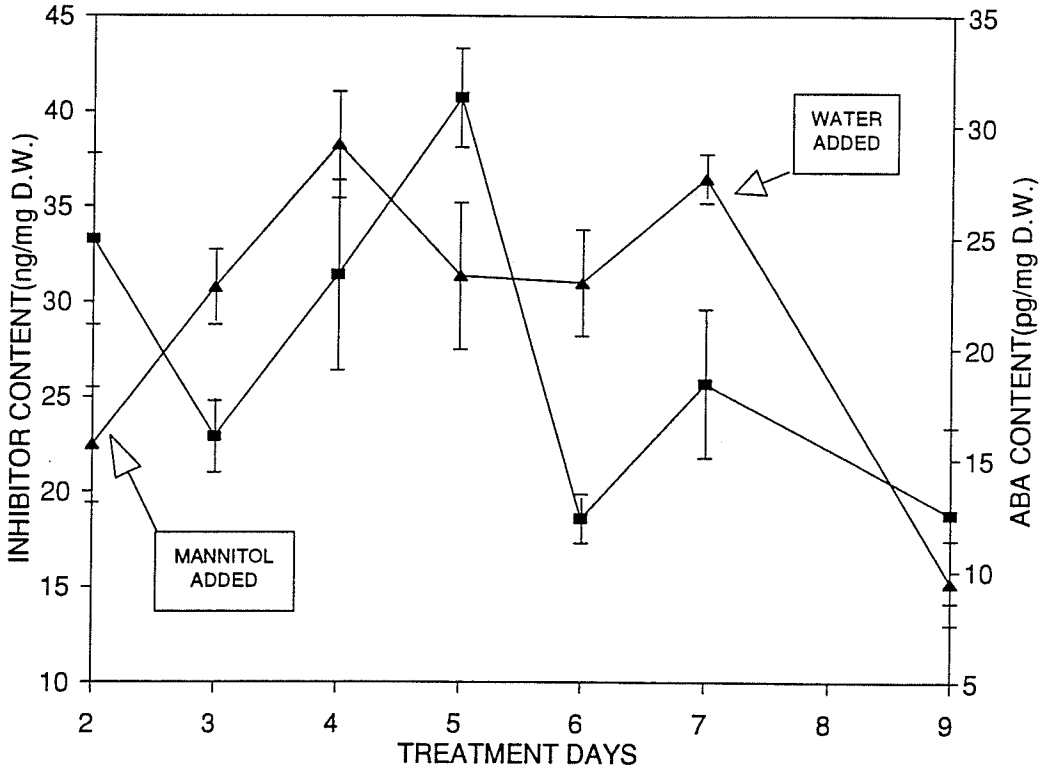
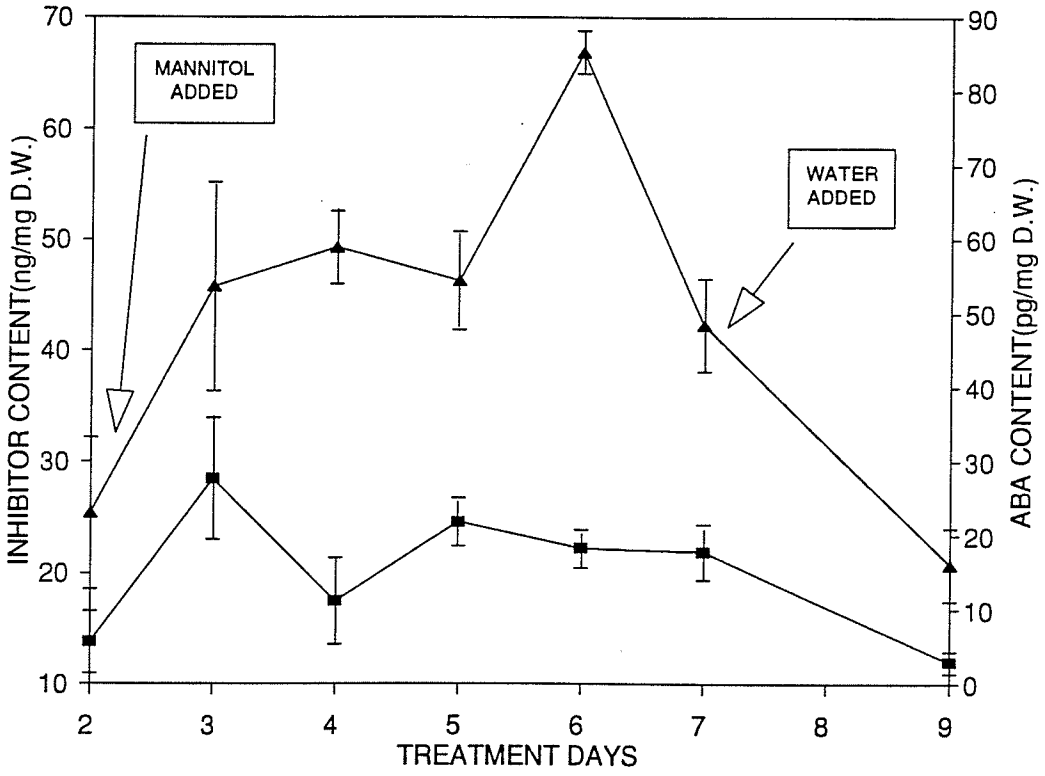

INHIBITOR CONTENT(ng/mg D.W.)
ABA CONTENT(pg/mg D.W.)
TREATMENT DAYS
WATER ADDED
MANNITOL ADDED
45
40
35
30
25
20
15
10
35
30
25
20
15
10
5
2
3
4
5
6
7
8
9

Figure 17. Inhibitor and ABA content of the coleoptile from osmotically stressed seedlings. Seedlings germinated for 2 days (starting point) were placed in a solution of 0.5 M mannitol and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The coleoptiles were removed from the seedlings for analysis.


MANNITOL ADDED
WATER ADDED
INHIBITOR CONTENT(ng/mg D.W.)
ABA CONTENT(pg/mg D.W.)
TREATMENT DAYS
70
60
50
40
30
20
10
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9

coleoptile in this experiment was considerably less than that induced in the dehydration experiment.

It seems the seedling adapted quite well to growth in the 0.5 M mannitol solution.

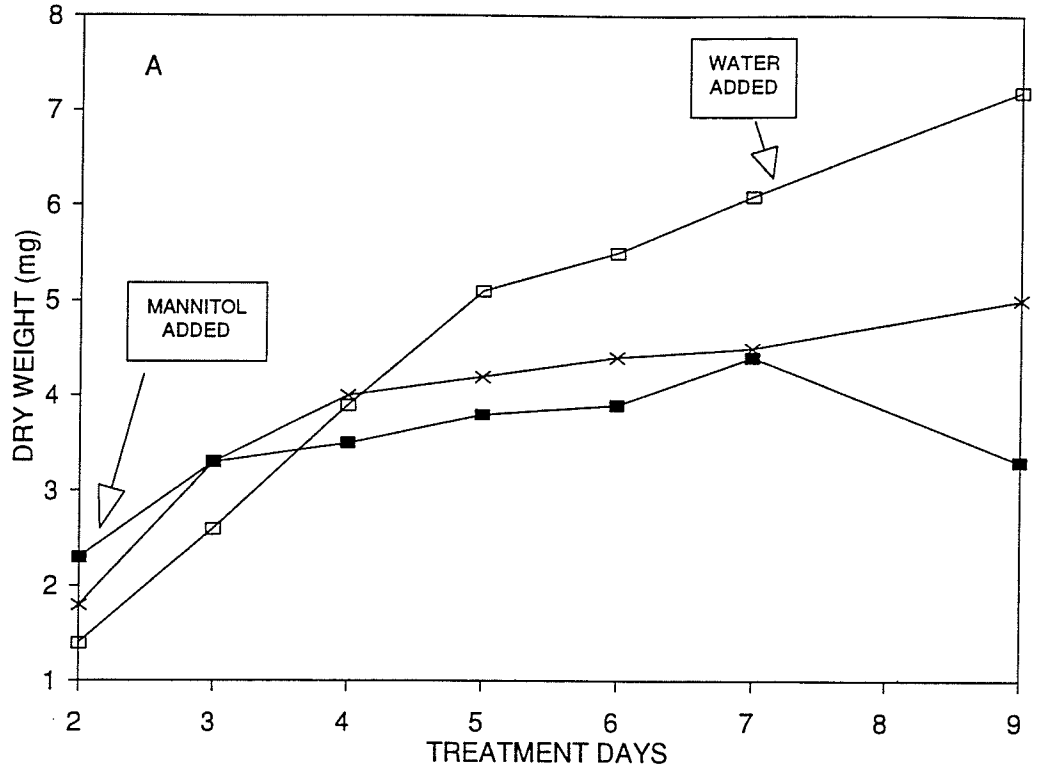
4.3.4. Inhibitor Synthesis in Seedling Tissue Under Osmotic Stress With 1.0 M Mannitol Solution

This experiment is the same as the 0.5 M mannitol experiment except 1.0 M mannitol solution was used. The 1.0 M mannitol solution was used to try to apply a greater osmotic stress to the seedlings.

Figure 18 shows dry weight (A) and water contents (B) of the seedling parts during the trial. With the residual embryonic tissue the dry weight increased throughout the trial. The water content decreased during the first day in the mannitol then stayed constant. The dry weight of the roots increased throughout. The water content of the roots also decreased the first day in the mannitol then stayed constant. The dry weight of the coleoptile increased throughout the trial and the water content decreased the first day and then stayed constant.

Figure 19 shows inhibitor and ABA content of residual embryonic tissue during the trial. The ABA increased significantly the first day in the mannitol from 6 pg to 370 pg per mg d.w.. The ABA then decreased throughout the trial. The 0.5 M mannitol trial ABA pattern is similar to this but,

Figure 18. Dry weight (A) and water content (B) of seedling parts from seedlings that experienced osmotic stress. Seedlings germinated for 2 days (starting point) were placed in a solution of 1.0 M mannitol and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The seedlings were dissected into residual embryonic tissue (RET), roots and coleoptile (COL).


A
WATER ADDED
MANNITOL ADDED
DRY WEIGHT (mg)
TREATMENT DAYS
1
2
3
4
5
6
7
8
2
3
4
5
6
7
8
9

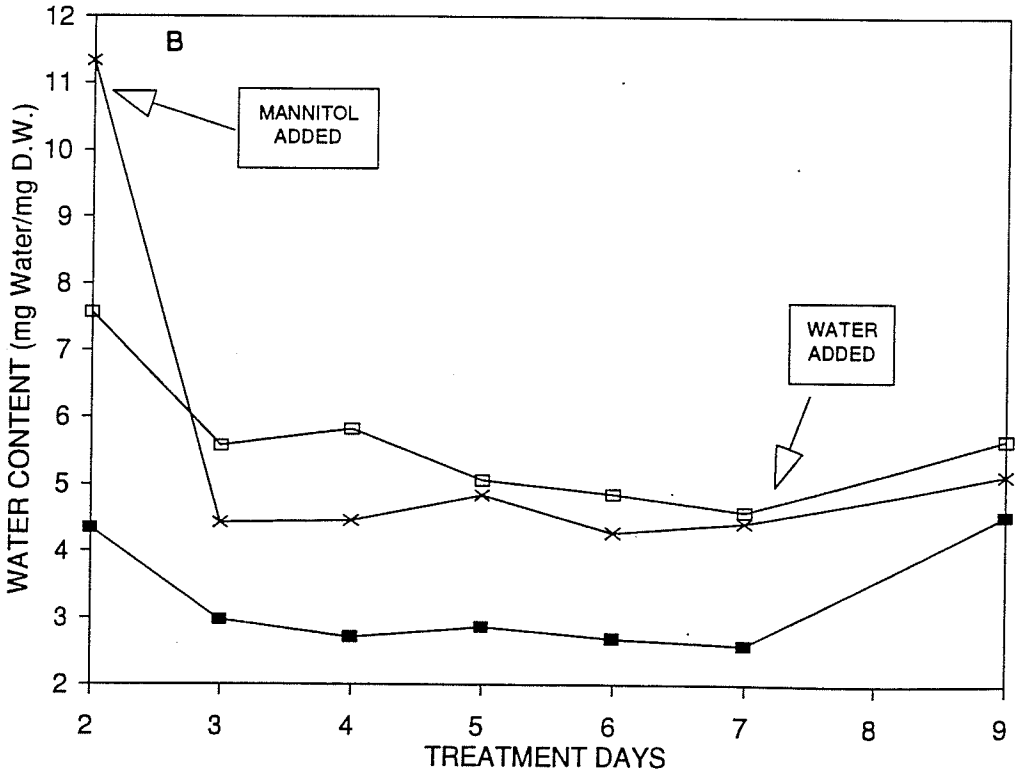
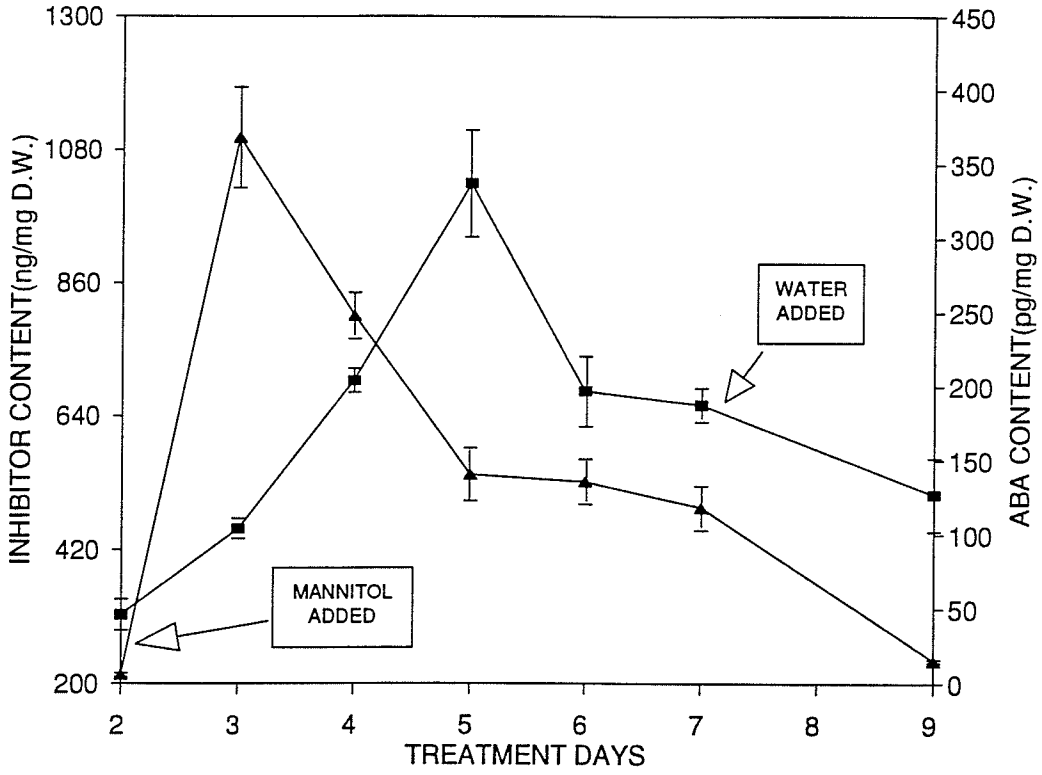

B
MANNITOL ADDED
WATER ADDED
WATER CONTENT (mg Water/mg D.W.)
TREATMENT DAYS
2
3
4
5
6
7
8
9
10
11
12
2
3
4
5
6
7
8
9

Figure 19. Inhibitor and ABA content of the residual embryonic tissue from osmotically stressed seedlings. Seedlings germinated for 2 days (starting point) were placed in a solution of 1.0 M mannitol and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The seedlings were dissected to remove the residual embyronic tissue.


1300
1080
860
640
420
200
INHIBITOR CONTENT(ng/mg D.W.)
450
400
350
300
250
200
150
100
50
0
ABA CONTENT(pg/mg D.W.)
WATER ADDED
MANNITOL ADDED
2
3
4
5
6
7
8
9
TREATMENT DAYS

not as dramatic. The inhibitor content for the control (2 day germinated) was higher than the starting point controls for the other experiments, but was similar to the 0.5 M mannitol experiment. The method used to germinate the seedlings in the osmoticum experiments might be supplying an effect, but this does not reflect an ABA response. The inhibitor content increased up to 1 μg per mg dry weight on the third day in the mannitol. This was the highest amount found in any tissue of any experiment. After three days the inhibitor decreased. On rehydration the inhibitor content decreased to around 500 ng/mg d.w. for the residual embryonic tissue.

Figure 20 shows inhibitor and ABA content of the roots during the experiment. The ABA response in the roots mirrors the residual embryonic tissue where the ABA increased the first day in the mannitol from 12 pg to 55 pg per mg d.w. and then decreased slowly during the trial. The inhibitor pattern again in the roots is not as clear. In this experiment the inhibitor content began quite high, 60 ng/mg d.w.. This was the highest amount found in two day starting point control root tissue. The inhibitor content then stayed constant for two days then increased to 120 ng/mg d.w. on the third day in the mannitol. This amount was the highest amount found in any experiment for the root, but the increase was similar to that found in the dehydration experiment.

Figure 21 shows inhibitor and ABA content of the coleoptile during the 1.0 M mannitol experiment. Similar to

Figure 20. Inhibitor and ABA content of the roots from osmotically stressed seedlings. Seedlings germinated for 2 days (starting point) were placed in a solution of 1.0 M mannitol and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The roots were removed from the seedlings for analysis.

—■— INHIBITOR CONTENT —▲— ABA CONTENT

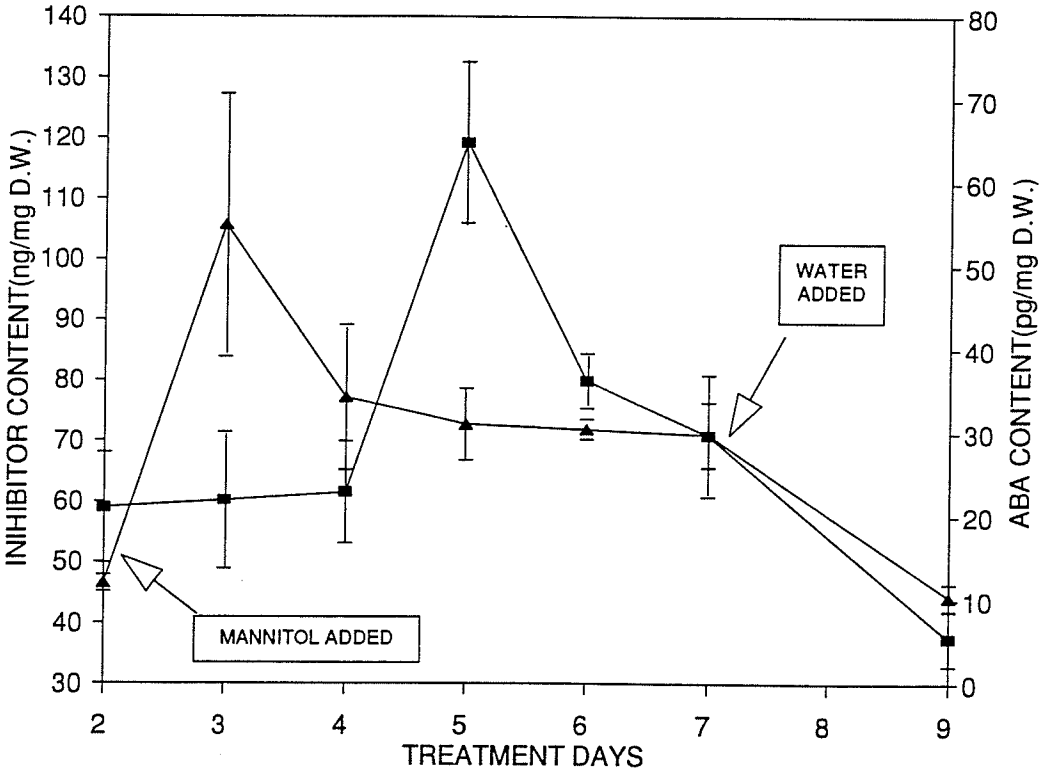
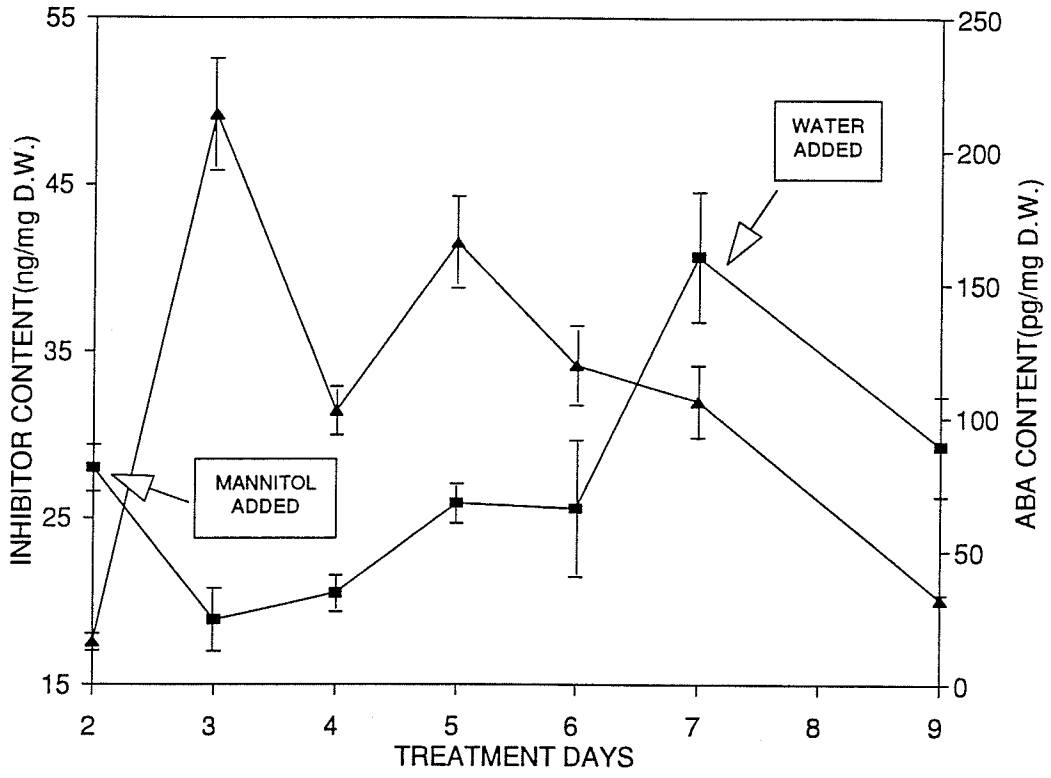

INIHIBITOR CONTENT(ng/mg D.W.)
ABA CONTENT(pg/mg D.W.)
TREATMENT DAYS
WATER ADDED
MANNITOL ADDED
140
130
120
110
100
90
80
70
60
50
40
30
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9

Figure 21. Inhibitor and ABA content of the coleoptile from osmotically stressed seedlings. Seedlings germinated for 2 days (starting point) were placed in a solution of 1.0 M mannitol and sampled for 5 days. After 5 days the seedlings were rehydrated for 2 days. The coleoptiles were removed from the seedlings for analysis.


55
45
35
25
15
INHIBITOR CONTENT(ng/mg D.W.)
250
200
150
100
50
0
ABA CONTENT(pg/mg D.W.)
WATER ADDED
MANNITOL ADDED
2
3
4
5
6
7
8
9
TREATMENT DAYS

the residual embryonic tissue and the roots the ABA content in the coleoptile increased dramatically the first day in 1.0 M mannitol, going from 16 pg to 200 pg per mg d.w.. The ABA content then also deceased during the rest of the trial, and decreased significantly on rehydration. The two day inhibitor content was very high in this experiment, 28 ng/.d w. This is similar to the 2 day root tissue in both the 0.5 M and 1.0 M mannitol experiment which was also high in comparison with the other experiments. The inhibitor increased slowly up to the fourth day in mannitol and then increased substantially on the fifth day and then decreased upon rehydration.

With the 1.0 M mannitol solution the seedlings adapt to the stress after the first day; there was the initial ABA induction on the first day in the mannitol, but after this the seedlings seemed healthy in the mannitol. This experiment would probably be more revealing by slowly increasing the mannitol concentration as the experiment continued.

5. GENERAL DISCUSSION AND CONCLUSIONS

Alpha-amylase inhibitor content in the embryos of mature kernels is about 1% of what is found in the whole kernel (Robertson and Hill, 1989). Therefore, embryos were carefully examined when the young seedlings were removed from the remaining endosperm to make sure contamination by the endosperm was minimized. Alpha-amylase inhibitor was found in all three seedling tissues examined, but in both the roots and the coleoptile only trace amounts were found. The majority was found in the residual embryonic tissue. It generally had more than 10X the amount found in the other tissue and also inhibitor induction was more responsive in the residual embryonic tissue. Trace amounts in roots and coleoptile were also found by Gubbels (1992) using immunocytochemistry on tissue sections.

In the ABA-treated seedlings, all the ABA treatments increased the inhibitor per fresh weight in the residual embryonic tissue, under conditions where the fresh weight of the tissue remained constant. ABA also increased the inhibitor per fresh weight in the roots and coleoptile, but here the fresh weight of the tissue changed. Therefore, there is some question on whether the induction did occur or was it simply a change in water content showing the increase.

Dehydration stress increased the inhibitor content and

ABA content in the residual embryonic tissue and the coleoptile. Here inhibitor and ABA increased at the same time. There was no direct evidence, that ABA mediated inhibitor synthesis, as with Robertson et al. (1989) where ABA increase preceded inhibitor synthesis. In the roots during the first few days of dehydration both the inhibitor and the ABA increased, but as the stress continued the inhibitor decreased. It seems that stress on the roots is too great and protein translation might be affected. With the osmotic stress treatments, the seedlings increased in inhibitor and ABA initially, then both declined, the seedlings seemed to adjust to the stress. Cammue et al. (1989) also noticed that WGA and ABA of roots in 0.5 M mannitol solution increased initially then declined. The osmotic stress experiment results suggest that it would probably be better to increase the osmoticum as the stress continues to keep the seedlings from adjusting to the stress.

Leah and Mundy (1989) found no inhibitor message in five day old shoot and roots, but there were trace amounts of the inhibitor protein in the roots and shoots, so it seems this protein is formed early on in the germination process for some reason. It would be beneficial to probe early seedling tissue which has been stressed for the message. The inhibitor message (Leah and Mundy, 1989) and protein (Robertson et al. 1989) also were not found in stressed mature leaves and roots. Induction of the inhibitor by stress seems to be limited to

embryonic tissue.

In the endosperm, the inhibitor is found around starch granules (Lecommandeur et al., 1987). There also seems to be correlation in the embryonic tissue between starch granule accumulation and the inhibitor (Gubbels, 1992). Gubbels (1992) showed with immunocytochemistry that inhibitor increased as starch accumulation occurred in the scutellum during germination, then decreased as the germination continued and the starch in the scutellum decreased. This might be why there was an increase in inhibitor in the embryo during early germination in the unstressed seedlings because starch granules are forming in the scutellum. Then as germination proceeded the inhibitor declined, as the starch granules would decrease in the embryo. Also ABA is present at the beginning of germination and then declines so it might induce the increase in inhibitor when the embryo begins germination.

Inhibitor control is unlike RAB (responsive to ABA) genes which are not organ specific or the late embryogenic-abundant proteins (Skriver and Mundy, 1990). The inhibitor protein is interesting because it forms during development of the kernel (Robertson and Hill, 1989) and can also be induced during germination by stress (Robertson et al., 1989) and is turned off at some point when the seedling reaches at certain age or maturity (Robertson et al., 1989; Leah and Mundy, 1989). This all suggests that the prime role of the inhibitor is that it attenuates the degradation of starch upon germination as

hypothesized by Hill et al. (1987) and if the seedlings experience stress not only is the alpha-amylase 2 transcription blocked (Higgins et al., 1982), inhibitor is produced to stop existing alpha-amylase degradation of starch. The seedling can then wait for better germination conditions to resume before all its storage material is consumed.

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