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Bob V. Conger, Major Professor

We have read this thesis and recommend its acceptance:

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Carolyn R. Hodges

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To the graduate council:

I am submitting herewith a thesis written by Lisa Michel Bell entitled "Abscisic Acid and Its Relationship to Somatic Embryogenesis in Dactylis glomerata L. (Orchardgrass)." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant and Soil Science.

Bob V. Conger

Bob V. Conger, Major Professor

We have read this thesis and recommend its acceptance:

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Date April 25, 1991

ABSCISIC ACID AND ITS RELATIONSHIP
TO SOMATIC EMBRYOGENESIS
IN *DACTYLIS GLOMERATA* L.

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Lisa Michel Bell

May 1991

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To my son Daniel, for his patience and love.

ABSTRACT

The purpose of this study was to determine if abscisic acid (ABA) was related to genotype differences of orchardgrass (Dactylis glomerata L.) for producing somatic embryos from leaf sections cultured in vitro. Leaf sections of one embryogenic (Embryogen-P) and one nonembryogenic (I-39) genotype were exposed to various concentrations of ABA in the culture medium for differing lengths of time to determine the effect of exogenous application. Endogenous levels of the two innermost leaves were analyzed by high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA) and compared in one embryogenic and two nonembryogenic genotypes. Comparisons were also made between basal (0-3 cm) and distal (3-6 cm) portions of the innermost leaves of one embryogenic and one nonembryogenic genotype.

In the embryogenic genotype, somatic embryogenesis was enhanced by a low level (1 μM) of ABA, while higher levels (10-100 μM) inhibited embryogenesis. Duration of exposure to ABA was also found to be significant, with an initial 3 day exposure period being most effective for enhancing embryo formation.

The nonembryogenic genotype showed no response to ABA application in any of the treatments.

Quantification studies indicated a significant difference in ABA concentrations between the embryogenic and nonembryogenic genotypes. When quantified by immunoassay, the **embryogenic** genotype contained more ABA in basal and distal sections of inner leaves than did the nonembryogenic genotype, I-39; however, the second leaf of the **nonembryogenic** genotype contained more ABA than the embryogenic genotype. High performance liquid chromatography quantification results of combined first and second leaves indicated a significantly higher endogenous level of ABA in the nonembryogenic genotypes than in the embryogenic genotype. There was no significant difference between the two nonembryogenic genotypes.

Abscisic acid immunoassay results indicate that ABA levels decreased as much as 93% in both embryogenic and nonembryogenic genotypes during a 2 week culture period after initial plating.

These results suggest that both concentration and duration of application of ABA influence somatic embryo formation.

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CHAPTER I

INTRODUCTION

Tissue culture techniques for the formation of somatic embryos have been developed for many species (Vasil, 1987; Williams and Maheswaran, 1986). However the factors controlling this process are still chiefly unknown, and most species in the Poaceae have been particularly difficult to manipulate *in vitro* (Vasil,1988).

Factors involved in somatic embryogenesis of *Dactylis glomerata* L. (orchardgrass) have been studied in an attempt to better understand this phenomenon in other Poaceae species. Studies indicate that somatic embryos form directly from mesophyll cells (Conger et al., 1983; Trigiano et al., 1989). Hanning and Conger (1986) found that auxin concentration in the culture medium influenced embryo production. SH medium (Schenk and Hildebrandt, 1972) supplemented with either 30 μM 3,6 dichloro-2-methoxybenzoic acid (dicamba) or 10 μM 2,4-dichlorophenoxyacetic acid produced the most embryos from leaf tissue. The response was also found to be highly genotype dependent. A gradient response was observed in which basal segments of the innermost leaf formed highly embryogenic calli. The occurrence of this type of callus decreased acropetally and

was replaced with direct somatic embryogenesis (formation of embryos with no intervening callus). The gradient response was shifted downward in the second leaf outward.

Endogenous levels of plant growth hormones were also reported to influence somatic embryogenesis in orchardgrass (Wenck et al., 1988). The endogenous level of zeatin and related compounds was significantly higher in nonembryogenic than in embryogenic genotypes, and leaves cultured on SH medium with as little as 0.001 μM of zeatin produced significantly fewer embryos than leaves cultured on medium without zeatin. In addition, the ratio of cytokinins : indole-3-acetic acid (IAA) increased basally to distally in leaves displaying an embryogenic response, indicating a correlation between the decrease in auxin levels and the decrease in embryogenic callus formation, since there was no gradient for cytokinin.

Abscisic acid has been found to have a role in many developmental processes in plants such as growth, abscission, dormancy, senescence and water relations (Addicott, 1983). The purpose of this project was to examine the effect of ABA on the embryogenic response in orchardgrass. The endogenous level of this compound was determined by high performance liquid chromatography (HPLC) and enzyme linked immunosorbent assay (ELISA). ABA levels in leaves were measured to determine if there was a gradient similar to that for IAA.

CHAPTER II

LITERATURE REVIEW

I. Factors Influencing Somatic Embryogenesis

As previously mentioned, Hanning and Conger (1986) noted that genotype and exogenous auxin influenced somatic embryogenesis in orchardgrass. Ammirato (1977) also reported that most plant growth regulators (auxin, ABA, gibberellic acid and cytokinins) and timing of their application influenced somatic embryogenesis in cultured cells of caraway (Carum carvi L.). Zeatin stimulated the growth of aberrant shoots while gibberellic acid affected mostly root formation; however, ABA selectively suppressed abnormal growth.

The gradient response in orchardgrass was accompanied by a higher level of IAA in lower, more responsive portions of the two innermost leaves (Wenck et al., 1988). In napier grass (Pennisetum purpureum L.) the gradient response was accompanied by high levels of IAA and ABA in basal sections (most embryogenic) and low levels in distal sections (Rajasekaran et al., 1987a). In addition, Rajasekaran, et al. (1987b) found that somatic embryogenesis dropped from 18% in leaf explants if ABA was in the medium to 0% if ABA was omitted.

ABA has also been used to modulate development of

embryos in suspension cultures of several species. In orchardgrass, combinations of dicamba (15.0 to 30.0 μM) and ABA (12.5 to 25.0 μM) reduced precocious germination from 40% to less than 10% (Conger et al., 1988). In wheat (Triticum aestivum L.) precocious germination rates were decreased from 80% to 5% by adding ABA at concentrations of 0.25 and 0.5 μM (Qureshi et al., 1989).

II. Isolation and Quantitation of ABA: Physico-chemical Techniques

HPLC

The discovery of ABA and its role in water stress coincided with the early development of physical methods for measuring ABA (Cargile et al., 1979; Durley et al., 1982); therefore, more reliable quantitative data exist for endogenous levels of ABA than for any other plant growth regulator (Neill and Horgan, 1987). Several extensive reviews of analytical methods for quantitation of ABA have been published: (Dorffling and Tietz, 1983; Neill and Horgan, 1987; Saunders, 1978).

Extraction and Purification

Fresh plant material is normally frozen in liquid nitrogen and stored at -20° C if extraction is not immediately performed. Various solvents have been used for ABA extraction. Since ABA may undergo transesterification under alkaline conditions, most of the solvents are a mixture, containing at least one acidic compound. Neill and

Horgan (1987) used 80% acetone in 0.1 M acetic acid whereas others have used different acid acetone mixtures (Boyer and Zeevaart, 1982; Hirai and Koshimizu, 1983). Other solvents have also been used, for example, 95% ethanol (Most, 1971), and 80% ethanol (Tietz et al., 1979). The addition of antioxidants such as BHT (2,6-di-tert-butyl-4-methyl phenol) at concentrations up to 100 mg/l (Zeevaart and Milborrow, 1976; Setter, 1981) has been recommended. However, it has not been shown that these compounds increase the yield of extracted ABA.

Solvent partitioning is also used for purification of ABA and its metabolites. ABA is a weak acid with a pKa of 4.8. At a pH below 3.0, ABA is almost completely undissociated and is soluble in organic substances such as ether. At pH greater than 9.0, ABA is almost completely dissociated and is soluble in water (Neill and Horgan, 1987). Solvent partitioning procedures are outlined in both Yokota et al. (1980) and Neill and Horgan (1987).

The techniques of choice for analysis of ABA are HPLC and gas chromatography with an electron capture detector (GC-ECD). HPLC has several advantages over conventional chromatography: 1) reduced analysis time, 2) far greater resolving power, 3) increased ease of sample recovery, and 4) analysis of nonvolatile samples (Horgan, 1988). Before ABA can be measured by GC it must be methylated with diazomethane, a carcinogenic and explosive compound (Neill

and Horgan, 1987). However, GC-ECD techniques were first used for ABA analysis by Seeley and Powell (1970) and have since become the most commonly used instrument for ABA quantification. ABA can be quantified to 0.3 pg per injection with a GC and the use of capillary columns (Brenner, 1981).

IMMUNOASSAY

Immunochemical techniques for the detection of plant hormones (indole-3-acetic acid and gibberellic acid) were first reported by Fuchs and Fuchs (1969). Immunoassays for ABA were developed soon after by Fuchs et al. in 1972. These early assays relied upon radioactive markers for quantitation of the plant hormone (Walton et al., 1979: Weiler, 1979).

The most recent class of immunoassays available are enzyme-linked immunosorbent assays (ELISA) (Mertens et al., 1983; Perata et al., 1990). These make use of highly specific monoclonal antibodies, which bind to the compound of interest, and of a colormetric indicator, which can be quantified on a spectrophotometer. ELISA's have several advantages over chromatographic methods: 1)high specificity, 2)speed of processing of samples, 3)reduced preparation and cleanup of plant samples and 4)detection of smaller amounts of the compound of interest (Hall et al., 1990). Comparisons between chromatographic and ELISA measurement of ABA indicate that ELISA derived values are somewhat less

than or equal to chromatographically derived measurements (Walker-Simmons, 1987; Spencer and Kitto, 1988; Walker-Simmons et al., 1989; Gupta and Sandhu, 1990; Ross and McWha, 1990). However, the convenience of ELISA may make it the method of choice.

CHAPTER III

MATERIALS AND METHODS

Orchardgrass plants used in tissue culture and ABA quantitation experiments were grown in an environmentally controlled growth chamber on a 16 hour, 22° C light cycle and an 8 hour, 15° C dark cycle.

I. Tissue Culture

For tissue culture experiments, Embryogen-P, (Conger and Hanning, 1991) a genotype that produces somatic embryos when cultured in vitro (embryogenic) and one nonembryogenic genotype, I-39, were used in all experiments.

The basal 3 cm of the two innermost leaves were split along the midvein to form "sister halves". These were surface sterilized by stirring in a 2.6% sodium hypochlorite solution containing approximately 0.1% v/v Triton X-100 surfactant. They were then rinsed three times in sterile water. Each leaf half was transversely cut into six 3 mm sections from the basal end upward. The sections from one leaf half were serially plated onto SH medium containing 30 μM dicamba (SH-30) and denoted as "control", whereas the corresponding sections from the other leaf half were plated onto SH-30 medium containing ABA at concentrations of: 0.001, 0.01, 0.1, 1.0, 10.0, 15.0, 20.0, 40.0, 60.0, 80.0 or 100.0 μM.

Control cultures were maintained in the dark for 28 days at 22 C on the original medium. In experiments I and II, leaf sections were maintained under the same conditions, but were transferred from SH-30 + ABA medium to SH-30 medium after 3 and 7 days, respectively.

In experiment III, treatment cultures were maintained on SH-30 + ABA for the entire 28 day period. In a fourth experiment, treatment cultures were pulsed with ABA the first and third weeks of the four week culture period, i.e. week 1: SH-30 + ABA, week 2: SH-30 alone, week 3: SH-30 + ABA and week 4: SH-30 alone. In experiment V, treatment cultures were transferred after 1 day on SH-30 + ABA medium.

After the 28 day culture period, leaf sections with embryos and/or callus were transferred to SH medium with no growth regulators (SH-0) for germination. At the time of transfer, 10 embryos having a distinct coleoptile notch were also transferred to SH-0 to determine germination rate. Plantlet formation was recorded after 21 days. The number of embryos formed was assessed as the number of plantlets divided by the germination rate (Trigiano et al., 1987). Differences in embryogenesis between control and ABA treatments were determined by a paired t test at the 0.05 level of significance.

II. Quantification

HPLC

The lower 3 cm of the two innermost leaves from tillers

of one embryogenic (Embryogen-P) and two nonembryogenic (I-39 and I-34) lines of orchardgrass were collected and frozen in liquid nitrogen. Long term storage was in an ultralow freezer at -77 C.

Leaf samples were lyophilized and weighed. Approximately 0.5 g of inner leaves and 1.3 g of second leaves were mixed for a total of 1.8 g (dry weight) of leaf tissue. From the total, 3 alliquots of 0.5 g each were taken for HPLC analysis, and 3 alliquots of 0.1 g each were taken for ELISA analysis.

HPLC samples were then ground with a mortar and pestle in 80% methanol (MEOH) to which 0.2 g/l of butylated hydroxytoluene (BHT) was added. A modified extraction procedure developed by Wenck et al. (1988) was followed and is outlined in Fig. 1. After a 12 hour extraction, nonpolar compounds were removed by partitioning with hexane.

Residual hexane was then evaporated under nitrogen. The remaining ABA fraction was slurried with 0.5 g insoluble PVP for 30 minutes to remove phenolics, then filtered.

The filtrate was adjusted to pH 3.5 and eluted through a C-18 Sep-pak cartridge, it was then rinsed with 2 ml of 20% MeOH in 10 mM acetic acid. ABA was eluted with 6 ml 60% MeOH. MeOH was evaporated under vacuum; the aqueous phase was adjusted to pH 3 and partitioned against ethyl acetate (EtOAC) three times. EtOAC was dried under nitrogen, and the residue then dissolved in 50 μl of EtOAC and mixed with

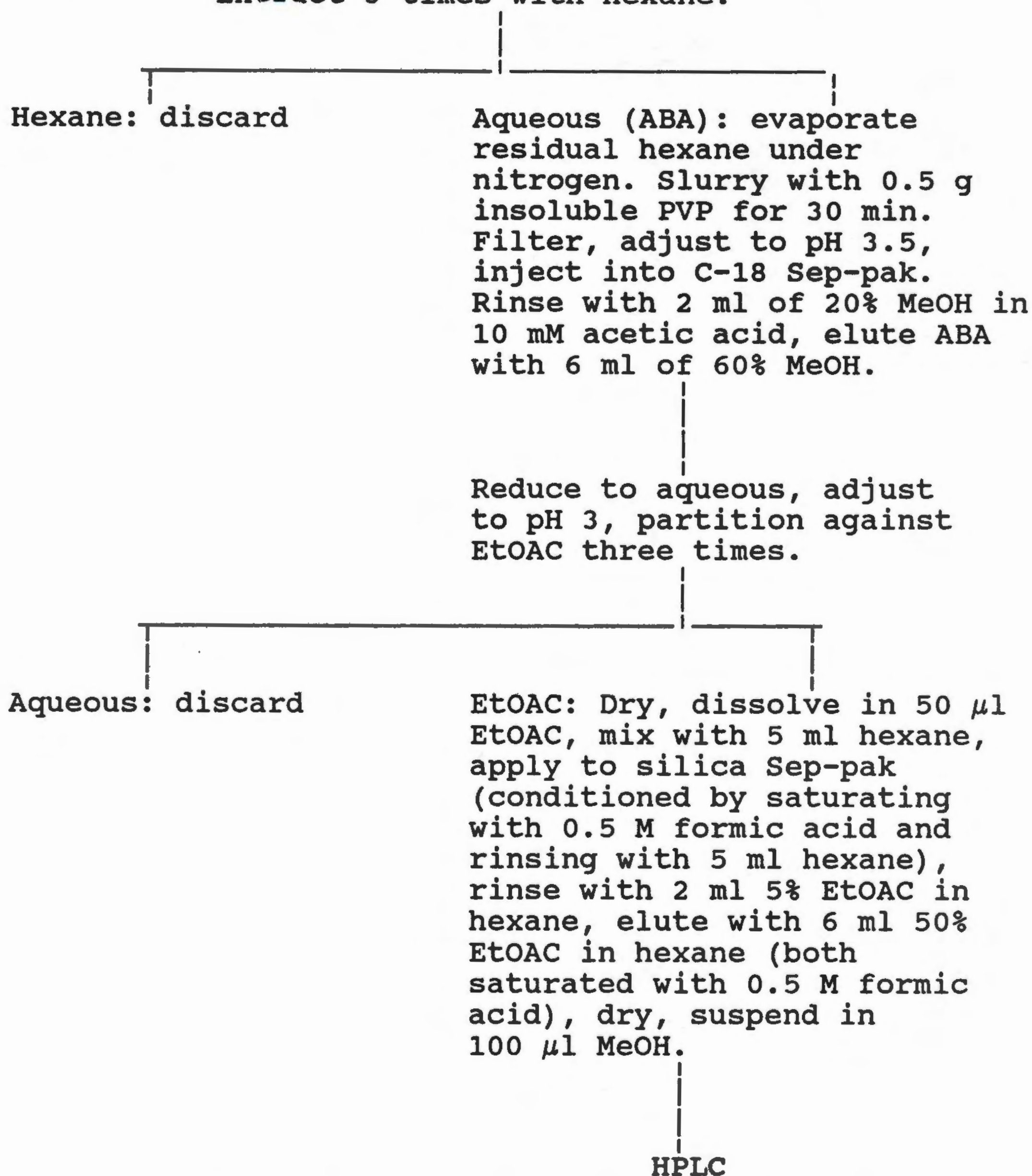

Figure 1. Extraction Scheme for HPLC quantification.

5 ml of hexane. The mixture was then passed through a silica Sep-pak cartridge which had been preconditioned by saturating with 0.5 M formic acid and rinsing with 5 ml hexane. Two ml of 5% EtOAC in hexane was used to rinse the cartridge, and ABA was eluted with 6 ml of 50% EtOAC in hexane. The fraction was dried and resuspended in 100 μl MeOH.

High performance liquid chromatography of ABA was performed using a 25 cm X 4.5 mm Partisil 5-ODS column connected to a Waters HPLC system containing dual pumps, gradient programmer, variable UV detector (set at 254 nm) and data integrator. The solvent was MeOH which was run at a flow rate of 1 ml/min in a gradient with 0.1 mM phosphate buffer (pH 6.6) to which the ion pairing agent, tetrabutylammonium phosphate, was added at a concentration of 0.01 M.

For quantitation, standard ABA concentrations were analyzed. Regression analysis of peak heights was used to produce equations for calculation of plant sample concentrations.

<u>ELISA</u>

Immunoassay samples were ground in liquid nitrogen in 10 ml centrifuge tubes to reduce loss of plant material, and extraction was performed following a modified scheme by Weiler (1984) (Appendix, Fig. 9). After grinding, 10 ml MeOH with 0.1 g/L BHT was added per gram of tissue. After a

16 hour extraction, samples were centrifuged for 5 minutes at 1,000 rpm. The supernatant (containing ABA) was decanted and measured. Alcohol content was adjusted to 70% before filtering through a C-18 Sep-pak. Samples were then dried on a Speedvac concentrator for 4-5 hours.

Pellets were resuspended in 1 ml Tris buffered saline and further diluted 1:3 with buffer. Immunoassay was performed using kits purchased from Idetek, Inc., San Bruno, CA. (Appendix, Fig.10).

Statistical Analyses

All statistical analyses were performed using MSUSTAT (Statistical Analysis Package, Microcomputer version 4.10, developed by Richard E. Lund, Montana State Univ., Bozeman, MT 59717).Numbers generated for ABA were corrected for losses incurred during extraction. These correction factors were determined by performing extraction on known amounts of ABA standard solutions.

CHAPTER IV

RESULTS

A positive effect for embryo formation was obtained only with Embryogen-P. None of the treatments produced an embryogenic response in the nonembryogenic genotype, nor was callus production increased as a result of addition of ABA to the medium.

Embryo production increased in Embryogen-P with 3 day treatments of 0.01, 0.1, 1.0 and 10.0 μM ABA by 1.4, 1.6, 1.8 and 1.8 fold, respectively, (Fig. 2). Higher concentrations of ABA (20-100 μM) caused a significant decrease (4.6-18.5 fold) in the number of embryos formed when compared to controls.

The 1 week ABA treatments showed a significant decrease in the number of embryos formed at concentrations of 1.0 μM and above (Fig. 3). Only the 0.001 and 0.1 μM ABA concentrations showed no significant difference between the treatment and control. Abscisic acid concentrations of .01, 1, 10 and 15 μM caused slight but significant decreases, while higher concentrations of 20, 60 and 100 μM ABA caused even greater decreases of 5.0, 4.1, 5.4 fold, respectively.

Maintaining leaf sections on ABA medium for the entire culture period drastically reduced embryo formation at all concentrations (Fig 4). Those embryos which did form were

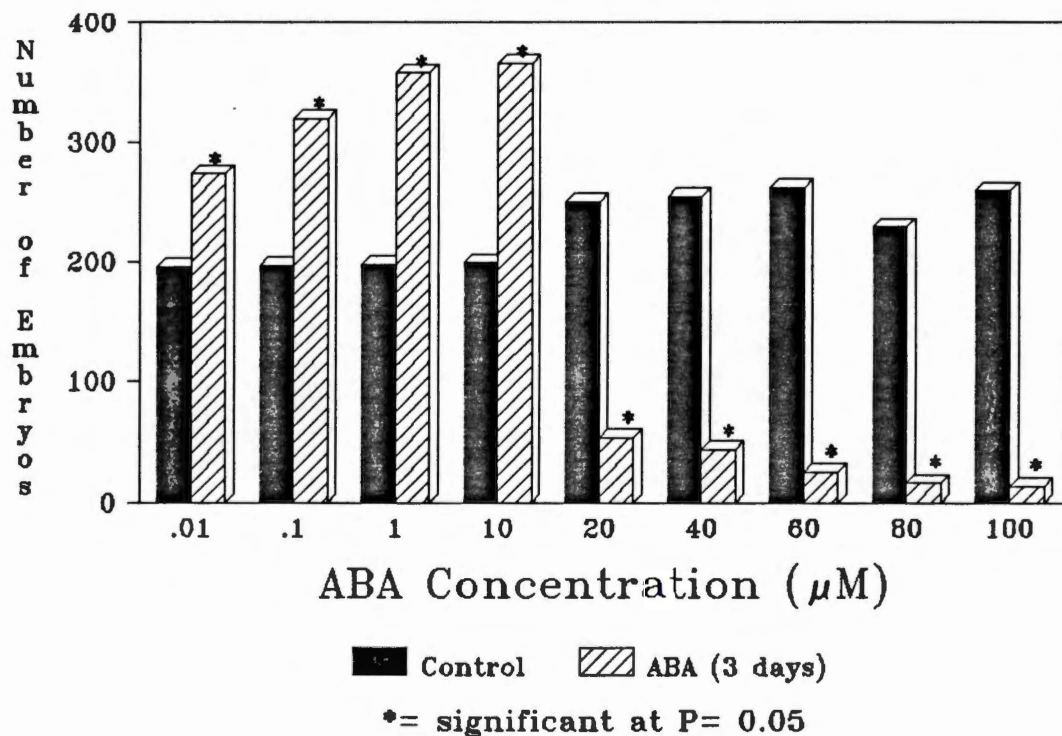

Figure 2. Effects on embryogenesis of 3 day exposure to ABA. Treatment was SH-30 + ABA for 3 days. *Indicates significant difference between treatment and control by a paired t-test at $P<0.05$.

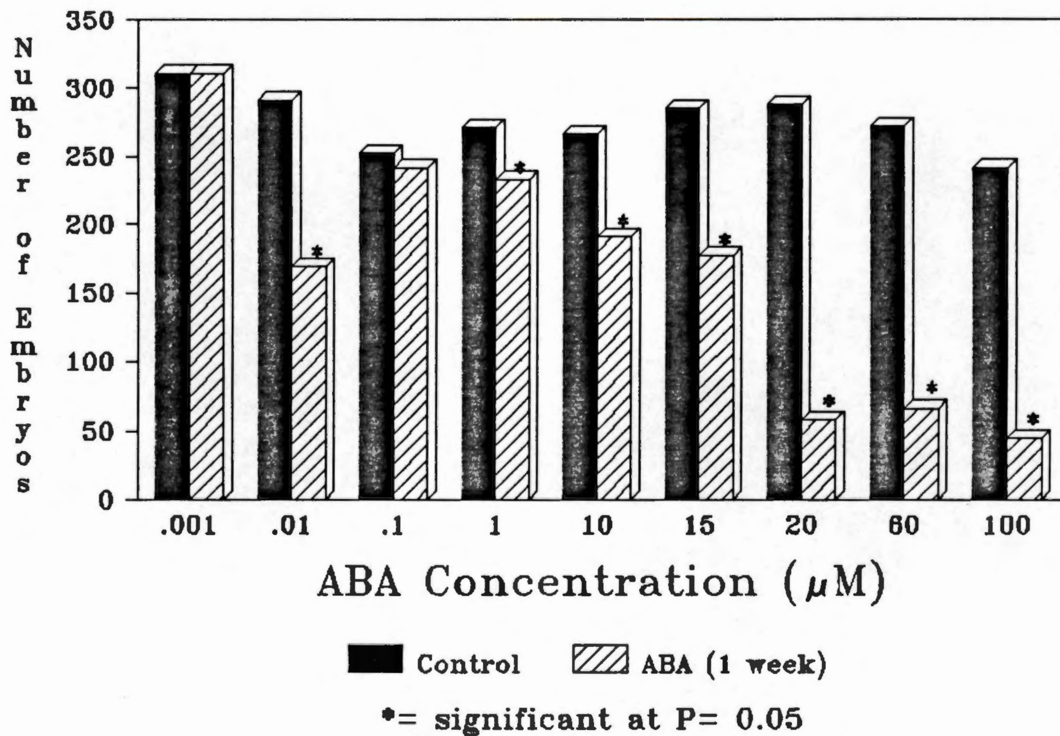

Figure 3. Effects on embryogenesis of 1 week exposure to ABA. Treatment was SH-30 + ABA for 1 week. *Indicates significant difference between treatment and control by a paired t-test at P<0.05.

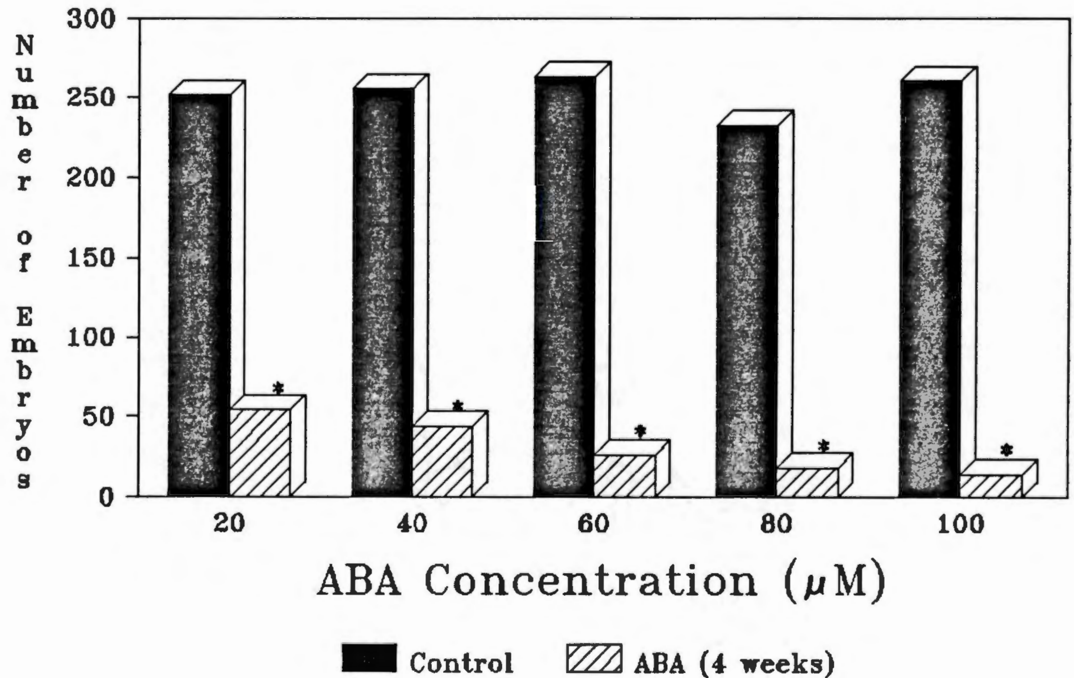

Figure 4. Effects on embryogenesis of 4 week exposure to ABA. Treatment was SH-30 + ABA for the entire culture period. *Indicates significant difference between treatment and control by a paired t-test at $P<0.05$.

slower to develop and had more abnormalities. Pulsing leaf sections with ABA for 1 week intervals also decreased the number of embryos formed (Fig. 5); but less than 4 weeks culture on ABA.

ABA immunoassay of the basal (0-3 cm) and distal (3-6 cm) portions of inner leaves of embryogenic and nonembryogenic (I-39) tillers indicated a significantly greater amount of ABA in the embryogenic genotype (Table 1). However, results for second leaves (0-6 cm) and for inner and second leaves combined showed significantly higher ABA levels in the nonembryogenic genotypes (I-39 and I-34).

Calculated means for all samples are given in Table 1 and are corrected for the recovery rate of each respective test run. Recovery rates were approximately 80% in each case. Standard curves used to calculate values in samples are included in the Appendix (Figures 11-13).

Table 2 contains t-values generated for comparison of sample means. Results showed there were significant differences between both genotypes and leaf tissue within genotypes. The embryogenic genotype contained more ABA in the basal and distal sections of inner leaves than did the nonembryogenic genotype; however a much greater ABA level is present in the second leaves of the nonembryogenic genotype.

Immunoassay of in vitro cultured leaf tissue indicates significant differences between initial endogenous ABA levels and these levels over time. Table 3 contains

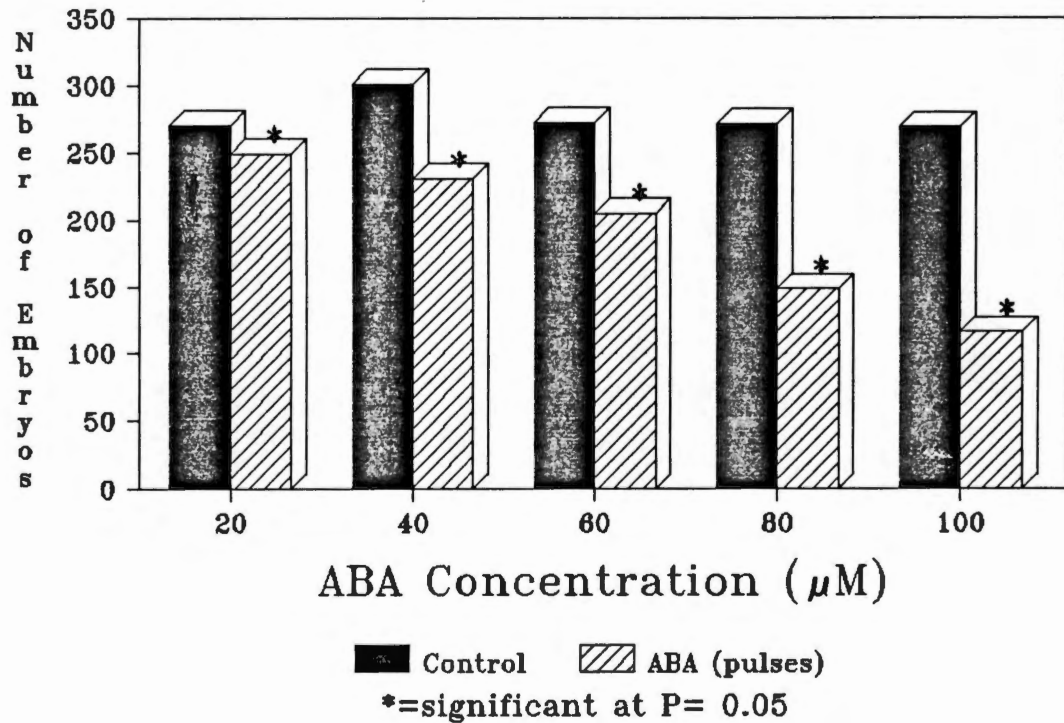

Figure 5. Pulse ABA treatment effects on embryogenesis. Treatment was a pulse of ABA during the first and third weeks of culture. *Indicates significant difference between treatment and control by a paired t-test at P<0.05.

Table 1. Calculated mean values for immunoassay of embryogenic (E-P) and nonembryogenic (I-39, I-34) leaf samples. Means for ABA in the basal area (0-3 cm) of the inner leaf, designated 1b; distal area (3-6 cm) of the inner leaf, designated 1d; second leaf (0-6 cm), designated 2; combined inner and second leaf (0-6 cm), designated c are shown. Means are expressed in pmoles ABA/g of tissue (fresh weight.).

plant material	number of replications	mean ABA
E-P c	4	121.81
E-P 1b	4	91.72
E-P 1d	4	169.64
E-P 2	4	205.97
I-39 c	4	197.30
I-39 1b	4	67.93
I-39 1d	4	139.99
I-39 2	4	288.80
I-34 c	4	231.45

Table 2. t-values generated from contrasts of sample means of leaf cultures. The embryogenic genotype is denoted as E-P and nonembryogenic as I-39 or I-34. Combined inner and second leaf samples are denoted as c; inner leaf, basal portion, as 1b; inner leaf, distal portion, as 1d and second leaf, entire 6 cm as 2.

contrast	degrees of freedom	t-value
E-P c vs. I-39 c	6	2.10 ns
E-P c vs. I-34 c	6	41.76 *
I-39 c vs. I-34 c	6	44.06 *
E-P 1b vs. E-P 1d	6	34.21 *
E-P 1b vs. I-39 1b	6	12.10 *
E-P 1d vs. I-39 1d	6	13.13 *
I-39 1b vs. I-39 1d	6	36.44 *
E-P 2 vs. I-39 2	6	37.66 *

* significant at the 0.01 level

Table 3. Calculated Means for immunoassay of embryogenic (E-P) and nonembryogenic (I-39) tissue cultures. Day 0 (control) measurements = C; day 3 measurements = 3d; day 14 measurements = 14. Treatments are denoted as t. Means are expressed in pmoles ABA/gram of tissue (fresh weight).

plant material	number of replications	mean ABA
E-P 3dC	3	153.12
E-P 3dt	3	83.99
E-P 14dC	3	153.38
E-P 14dt	3	11.23
E-P control mean	6	153.35
I-39 3dC	3	180.05
I-39 3dt	3	16.50
I-39 14dC	3	178.77
I-39 14dt	3	13.31
I-39 control mean	6	179.41

calculated means of these samples. Initial ABA levels are higher in the nonembryogenic genotype; however, after 3 days in culture the ABA level in the nonembryogenic genotype is 5 fold less than that of the embryogenic genotype (Fig. 6) After 14 days in culture, the ABA levels are not significantly different (Table 4).

ABA was identified by HPLC in plant extracts by comparison with retention times of known standards. A chromatogram of ±ABA is shown in Figure 7, and a plant extract is shown in Figure 8. Although no plant samples were spiked with ABA for confirmation of peaks, standards were run periodically to ensure no drift had occurred. Calculated mean values for ABA are given in Table 5. These values were adjusted for a 43% recovery rate.

HPLC analysis of combined inner and second leaves of one embryogenic and two nonembryogenic genotypes indicated an 11-fold lower ABA level in the embryogenic genotype than in the nonembryogenic genotypes. Comparison of means by t-test indicated a significant difference between embryogenic and nonembryogenic lines; however, values for nonembryogenic genotypes were not significantly different (Table 6).

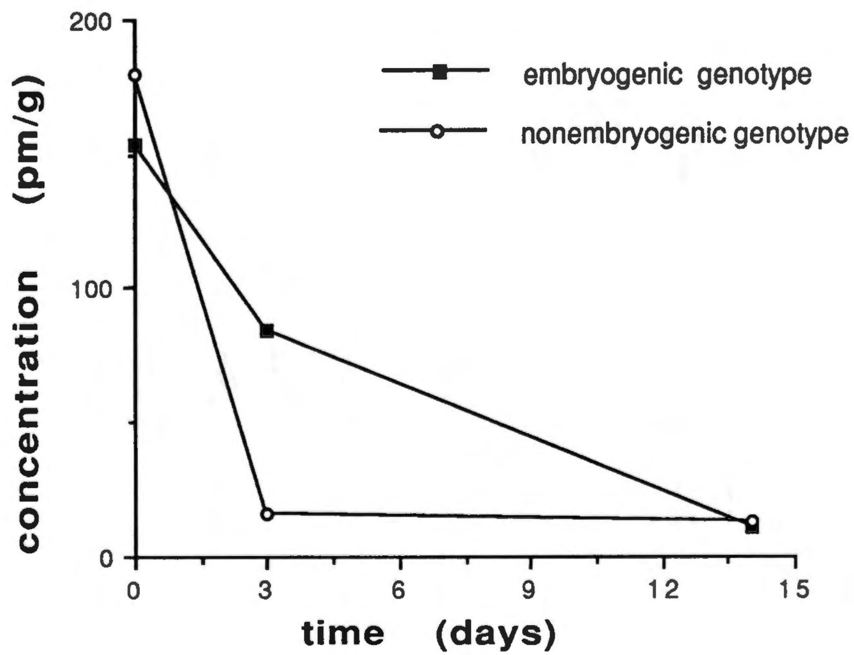

Figure 6. Endogenous levels of ABA in embryogenic and nonembryogenic leaf cultures.

Table 4. t-values generated from contrasts of sample means of tissue cultures. Embryogenic cultures are denoted as (E), nonembryogenic as (NE).

contrast	degrees of freedom	t-value
E 3dC vs. E 3dt	4	42.77*
E 14dC vs. E 14dt	4	52.97*
NE 3dC vs. NE 3dt	4	110.45*
NE 14dC vs. NE 14dt	4	97.24*
E 3dC vs. NE 3dC	4	17.14*
E 14dC vs. NE 14dC	4	16.59*
E 3dt vs. NE 3dt	4	42.45*
E 14dt vs. NE 14dt	4	1.56ns

* significant at the 0.01 level

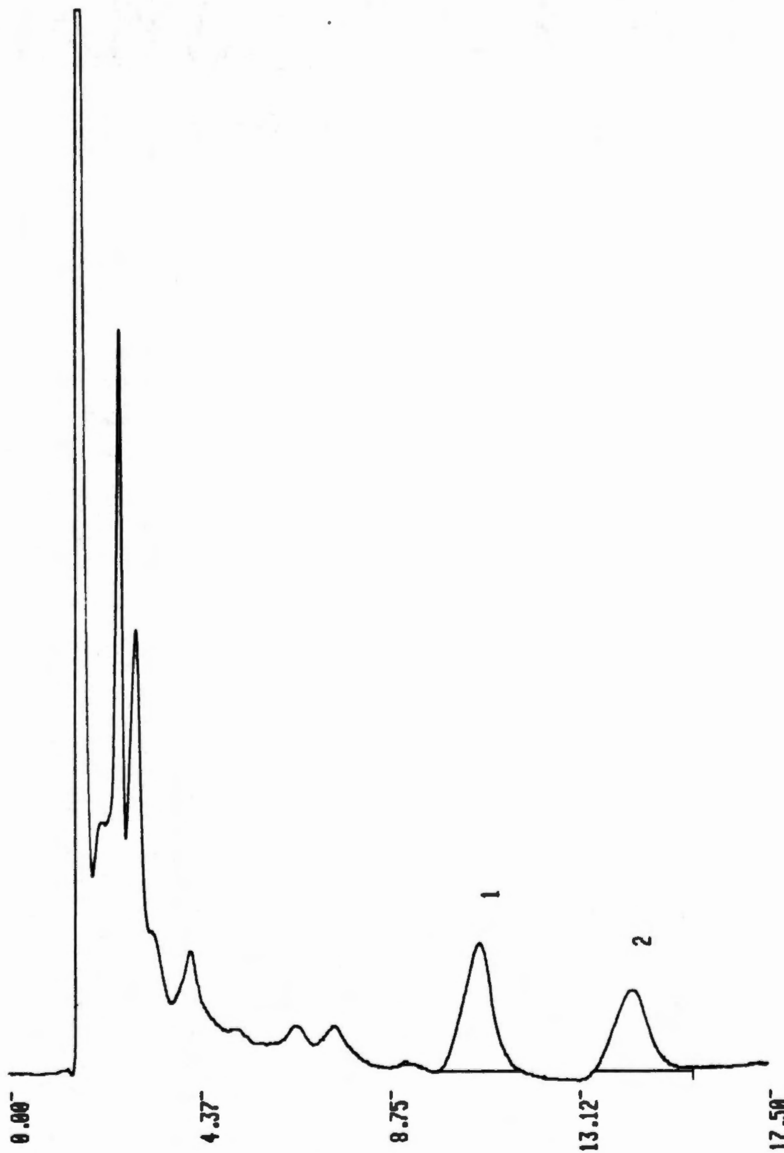

Figure 7. Chromatogram of ± ABA standard.
1= trans-ABA
2= cis-ABA

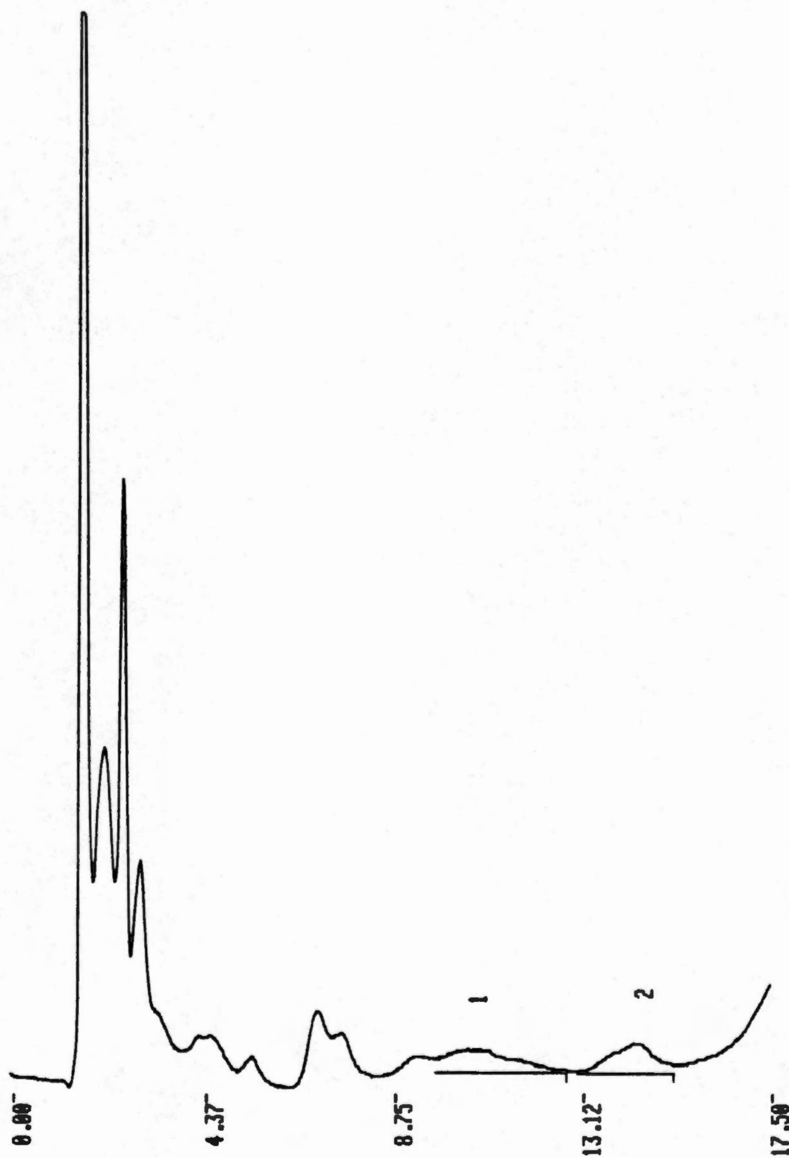

Figure 8. **Chromatogram of a plant extract.**
1= trans-ABA
2= cis-ABA

Table 5. Calculated means for HPLC of embryogenic (E-P) and nonembryogenic (I-39 and I-34) leaf tissue. All samples were a combination of the 0-6 cm basal portion of inner and second leaves. ABA concentration is expressed in nmoles/gram of tissue.

plant material	number of replications	mean ABA
E-P	3	5.53
I-39	3	64.33
I-34	3	61.44

Table 6. t-values generated from contrasts of sample means of leaf cultures. The embryogenic genotype is denoted as E-P and nonembryogenic genotypes as I-39 and I-34.

contrast	degrees of freedom	t-value
E-P vs. I-39	4	11.59 *
E-P vs. I-34	4	18.53 *
I-39 vs. I-34	4	0.66 ns

* significant at the 0.01 level

CHAPTER V

DISCUSSION

Abscisic acid level appears to be associated with somatic embryogenesis from leaf culture in orchardgrass. The youngest, most responsive tissues had less ABA than older, less responsive tissues, when tested by immunoassay. Also, addition of ABA to culture medium in low concentrations (1-10μM) for 3-7 days, increased the number of embryos formed; however, higher concentrations and/or longer times inhibited embryo formation when compared to controls.

Rajasekaran et al. (1987a) found that higher levels of ABA in basal leaf sections of napiergrass were correlated with a greater embryogenic response. Addition of fluridone, an ABA synthesis inhibitor, reduced embryogenesis in that system (Rajasekaran, 1987b). Furthermore, addition of ABA to fluridone-treated plants restored the embryogenic capacity. It appears that an optimum concentration of ABA may be required for an embryogenic response. Embryogenic lines of orchardgrass may have this optimum level while nonembryogenic genotypes do not.

ABA has also been used to modulate embryo development in several species, including caraway (Ammirato, 1977), orchardgrass (Conger et al., 1988) and wheat (Qureshi, 1989). In caraway leaf culture, ABA concentrations of 10^{-6}

to 10^{-7} inhibit abnormal embryo development. Addition of ABA to orchardgrass and wheat suspension cultures synchronized development and reduced precocious germination from 40% to 10% and from 80% to 5% respectively. In the present research with orchardgrass leaf tissue, explants grown on medium containing 1-10 μM ABA, developed more slowly than control cultures, but a greater number produced plantlets upon transfer to regeneration medium, indicating a more normal developmental process. Rajasekaran (1987a) also found that exogenous application of ABA promoted embryogenic callus formation.

A gradient response exists in the embryogenic genotype between ABA level and embryogenic response. As leaves develop morphologically, the ABA level increases, and morphologically older leaves have less competency for embryogenesis. Tissue culture experiments with early stage wheat seed embryos showed a four fold decrease in the ability to produce embryogenic callus when ABA was added to the medium (Qureshi et al., 1989). However addition of ABA to medium stimulated the embryogenic response in late stage seed embryos, possibly because late stage embryos have used their endogenous ABA supply. Orchardgrass tissue culture experiments in which ABA was added to the medium also demonstrated that higher levels of ABA inhibit embryogenesis.

Results also showed a differential loss of ABA when

orchardgrass was cultured in vitro. The lack of embryogenic response in nonembryogenic genotypes may be due to the rapid initial loss of ABA, rather than initial concentrations. Rajasekaran et al. (1987a) obtained similiar results with napiergrass. Embryogenic callus cultures contained approximately 3-4 fold more ABA than nonembryogenic cultures. This trend was maintained in subcultures for more than one year. In fact, ABA levels as well as embryogenic capacity decreased rapidly in cultures that were not maintained by subculturing. Timing and duration of exogenous ABA application had a marked effect on embryo formation and growth, and endogenous ABA levels may have similiar effects.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Endogenous abscisic acid levels may be a factor in the embryogenic response in orchardgrass. When quantified by immunoassay, the embryogenic genotype contained more ABA in the most responsive tissue areas; in addition, the nonembryogenic genotype had a higher concentration of ABA in the second leaf and in samples of inner and second leaves combined, indicating an inhibitory effect. HPLC results also showed higher concentrations of ABA in the nonembryogenic genotypes when samples were mixtures of inner and second leaves. A gradient of ABA was shown in all genotypes. Lower levels of ABA were present in the most responsive portions of the leaves, and the inner leaf contained less ABA than the second leaf outward in both genotypes.

Addition of ABA to culture medium at levels greater than 1 μM, inhibited embryo formation, while lower concentrations enhanced the embryogenic response. Length of ABA exposure during tissue culture was also an important factor. A 3-day period was more effective than a 7-day period. There may be a narrow optimum range of ABA for embryo formation and development which is determined by timing as well as concentration.

Further experiments with orchardgrass are required to determine the role of endogenous ABA in tissue culture. Additional quantification of endogenous levels of ABA and how these levels change during tissue culture would help define the role of ABA. Experiments with ABA inhibitors such as Fluridone might also help to determine the role of ABA in tissue culture.

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APPENDIX

Extraction Scheme for Immunoassay

1. Place plant sample in a 10 ml centrifuge tube.
2. Add enough liquid nitrogen to cover sample.
3. Grind sample until very fine.
4. Add 10 ml 80% MeOH per 1 gram plant tissue.
5. Replace cap on centrifuge tube.
6. Place tubes in rack and shake on rotary shaker at 1,000 rpm overnight at 4° C.
7. Remove tubes and centrifuge at 1,000 rpm for 5 min. to pellet plant tissue.
8. Decant liquid and save. Discard pellet. If necessary, filter supernatant through pre-wetted (80% MeOH) Whatman #1 or #2 filter paper, using a vacuum.
9. Adjust MeOH to 70%.
10. Pass sample through conditioned (70% MeOH) reverse phase C-18 sep-pak. Elute with an additional 1-2 ml 70% MeOH.
11. Place sample in 1 ml alliquots in Eppendorf tubes for drying.
12. Load samples in Speedvac concentrator. Allow to process until sample is completely dry.
13. Resuspend pellet in 1 ml Tris buffered saline.
14. Dilute 1:3 with buffer.

INSTRUCTIONS FOR ABA KIT*

1. Prepare ABA standards and kit reaction solutions.
2. Place 100 μl of standard or sample in each well of multiwell plate.
3. Add 100 μl of diluted tracer to each well, using a multichannel pipette.
4. Mix by tapping the plate.
5. Cover well with plate sealer and incubate at 4 degrees centigrade for three hours.
6. After the 3 hr. incubation, remove the plate from the refrigerator and decant solution.
7. Wash wells by adding 200 μl of the wash solution to each well with a multichannel pepette. Decant wash mixture from the wells. Repeat 2 times.
8. Add 200 μl substrate solution to each well using a multichannel pipette.
9. Cover wells with the plate sealer. Incubate at 37 degrees centigrade for 1 hr.
10. Remove plate from incubator and add 1 drop stopping reagent to each well. Wait 5 minutes.
11. Read color absorbance at 405 nm.
12. Record optical densities.

* Idetek, Inc.

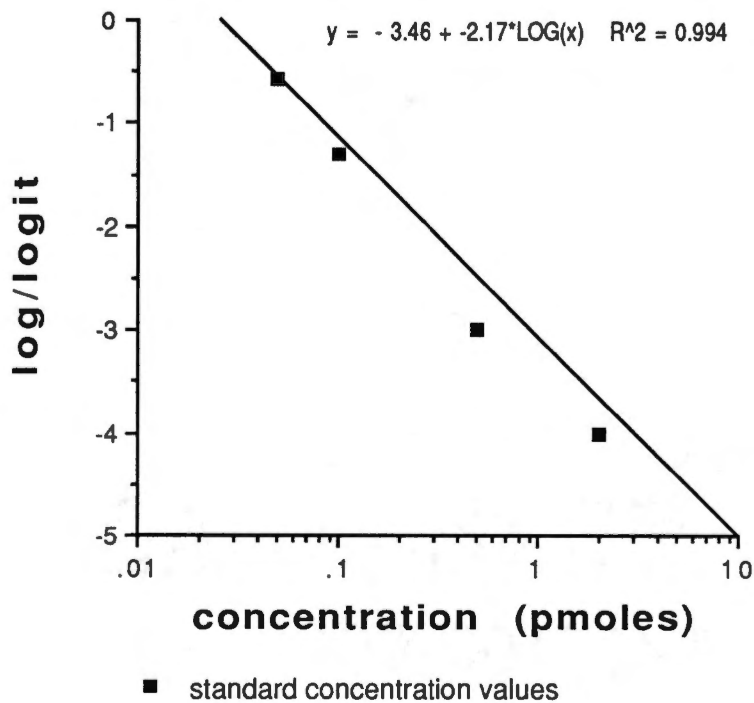

Figure 11. ABA standard curve for test I. Given concentrations were determined by an ABA immunoassay kit purchased from Idetek, Inc.

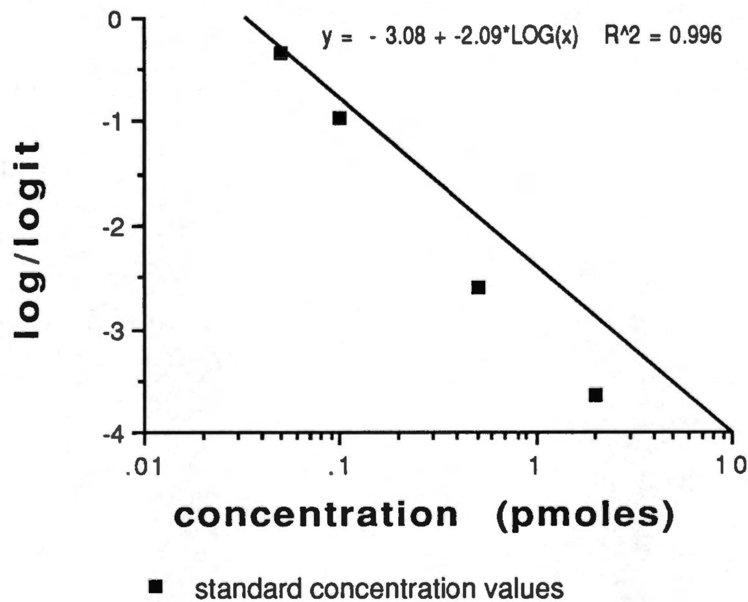

Figure 12. ABA standard curve for test II. Given concentrations were determined by an ABA immunoassay kit purchased from Idetek, Inc.

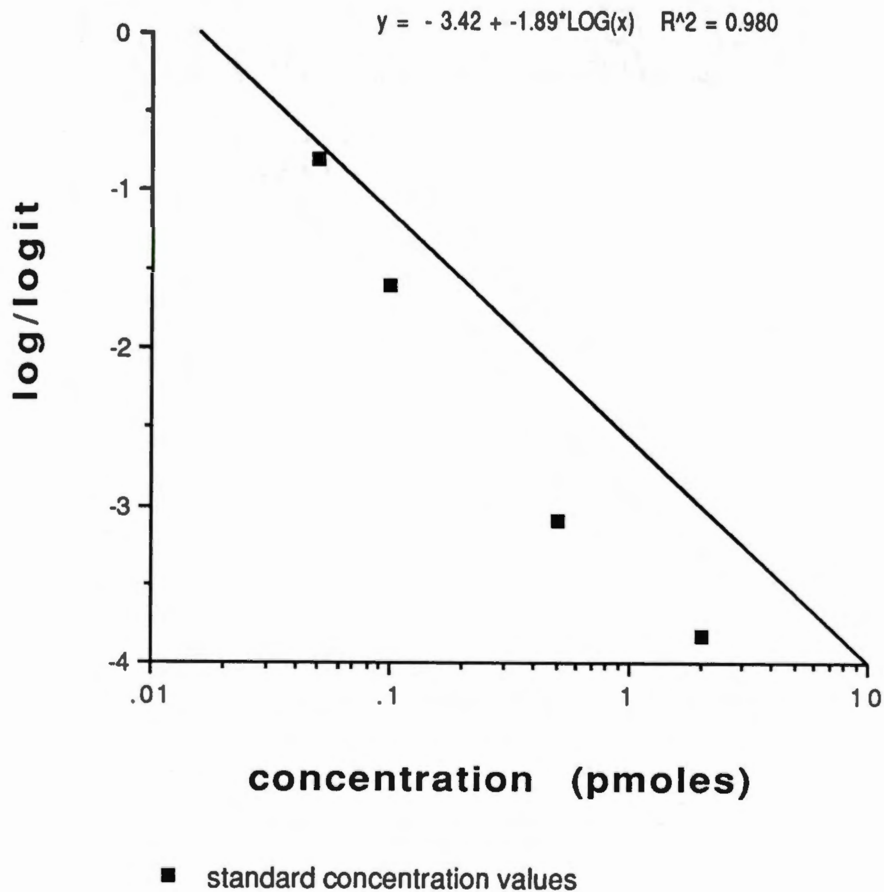

Figure 13. ABA standard curve for test III. Given concentrations were determined by an ABA immunoassay kit purchased from Idetek, Inc.

VITA

Lisa Michel Bell was born in Knoxville, Tennessee in 1960 to Fred and Helen Capps. She graduated from Central High School in 1977 and attended the University of Tennessee. After her marriage and the birth of her son, Daniel, she reentered the university and obtained her Bachelor of Science in Agriculture in 1988. She plans to obtain her Master of Science degree in Plant and Soil Science in May, 1991.

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