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ENDOGENOUS AUXIN AND CYTOKININ CONCENTRATIONS AND THEIR RELATIONSHIP TO SOMATIC EMBRYOGENESIS IN *DACTYLIS GLOMERATA* L. (ORCHARDGRASS)

A Thesis

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Degree

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ABSTRACT

The purpose of this study was to determine if indole-3-acetic acid (IAA) and cytokinins were related to genotypic and gradient responses of orchardgrass (*Dactylis glomerata* L.) leaf sections in vitro to produce somatic embryos. Plant growth regulator (PGR) concentrations of leaves were analyzed by high performance liquid chromatography (HPLC) and compared in basal sections (lower 3 cm) of the two innermost leaves of two embryogenic subclones and two nonembryogenic genotypes. Plant growth regulator concentrations were also determined and compared between basal and distal (3 cm to 6 cm) leaf sections of embryogenic plants. The ratio of cytokinins/IAA was also compared.

The cytokinins, zeatin riboside (ZR), dihydrozeatin riboside (DHZR), zeatin (Z) and dihydrozeatin (DHZ) were identified and quantified in leaves and summed to yield a value for total cytokinins. Leaves of the two nonembryogenic genotypes contained significantly greater amounts of total cytokinins than those from the two embryogenic subclones on a $\mu g\ g^{-1}$ dry weight basis (10.74 and 8.06 vs. 2.08 and 3.05 respectively). The concentrations of ZR and DHZ, were greater in the nonembryogenic genotypes (7.13 and 4.96 vs. 1.05 and 1.74 for ZR; 0.92 and 0.86 vs. 0.19 and 0.08 for DHZ). Levels of Z and DHZ were not significantly different between nonembryogenic and embryogenic genotypes (0.89 and 0.88 vs. 0.33 and 0.81 for DHZR; 1.81 and 1.28 vs. 0.51 and 0.36 for Z). Cytokinin concentrations in basal leaf sections of the embryogenic genotype were not significantly different from those found in distal sections. Cytokinin

concentrations for distal sections ($\mu g\ g^{-1}$ dry weight) were 1.07 and 1.68 of ZR, 1.12 and 0.17 of DHZR, 2.15 and 0.17 of Z and 0.19 and 0.03 of DHZ respectively for the two subclones. There were no significant differences between the two embryogenic subclones or between the two nonembryogenic genotypes. One embryogenic subclone and one nonembryogenic genotype were analyzed on a whole tiller basis and contained 2.83 vs. 4.26 of ZR, 0.45 vs. 0.74 of DHZR, 0.24 vs. 0.75 of Z, 0.01 vs. 0.45 of DHZ, and 3.53 vs. 7.35, respectively of total cytokinins. Concentrations of total cytokinins was significantly higher in the nonembryogenic genotype than in the embryogenic subclone.

Indole-3-acetic acid levels ($ng\ g^{-1}$) were not significantly different between basal leaf sections of the embryogenic subclones and the nonembryogenic genotypes (18.32 and 20.93 vs. 22.91 and 26.25 respectively). Distal leaf sections of the embryogenic subclones contained significantly less IAA (9.47 and 2.66) than basal sections. Tillers of the embryogenic genotype contained 16.39 $ng\ g^{-1}$ of IAA; whereas, the nonembryogenic genotype contained 12.11 $ng\ g^{-1}$. This difference was not significant. The ratio of cytokinins/IAA was significantly different both between basal and distal sections and between basal sections of leaves of embryogenic and nonembryogenic genotypes.

In the embryogenic genotype, embryogenesis was inhibited by exogenously added cyotkinins. Zeatin levels as low as 0.001 μM reduced somatic embryo formation from cultured leaf segments.

These results suggest possible relationships between PGR levels and genotypic and gradient specific responses of orchardgrass leaves in in vitro culture. In particular, cytokinin levels seem to influence genotypic specific responses.

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CHAPTER I

INTRODUCTION

Somatic embryogenesis from cultured leaf explants of orchardgrass (*Dactylis glomerata*) was first reported by Hanning and Conger (24). Since then, somatic embryogenesis has also been reported to occur directly from leaves (14), anthers and pistils (63) and in suspension cultures (21, 22) of this species. These systems are influenced by several factors including genotype, medium constituents and ontogenetic stage of the explant (14, 21, 22, 24, 25, 45, 62). The embryogenic response is limited to younger portions of leaves from a few, selected genotypes (24, 45). The most basal (meristematic) sections of the innermost leaf produced embryogenic callus, while sections more distal to the base produced embryos directly from mesophyll cells. This gradient was shifted toward the base in the second leaf outward (14). Sections even more distal to the base or tissue from older, more differentiated leaves did not respond in culture. Exogenous auxin concentration in the media was also a contributing factor to embryogenesis (24). Greatest embryo production was obtained with a modified Schenk and Hildebrandt (SH) medium (59) supplemented with either 15 to 30 μM 3,6-dichloro-o-anisic acid (dicamba) or 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Cytokinins added to the medium had an inhibitory effect on

embryogenesis in anther and ovule cultures (62).

Production of somatic embryos in orchardgrass is highly developed for two aspects: direct embryo formation from mesophyll cells (14) and the production of fully developed embryos capable of germinating entirely within one liquid medium (22). These aspects make orchardgrass among the most advanced of tissue culture systems in the *Poaceae* and a potential model for further study.

The orchardgrass leaf culture system provides an opportunity for studying the basic physiology of somatic embryogenesis because of differences between genotypes (24, 45) and the differential gradient response of leaves on solid media (14, 24). Nonembryogenic plants can be used in comparison studies with embryogenic plants to help elucidate factors responsible for the embryogenic trait. The objective of this study was to determine and compare the endogenous concentrations of auxins and cytokinins in orchardgrass leaves from embryogenic and nonembryogenic genotypes and from basal and distal portions of leaves of embryogenic subclones.

CHAPTER II

LITERATURE REVIEW

Factors Influencing Somatic Embryogenesis

Plant tissue culture systems are mediated by several factors. One of the most important is plant growth regulator (PGR) levels both in vitro and in vivo. In the classic work by Skoog and Miller (61), auxin and cytokinin concentrations were shown to influence tobacco (*Nicotiana tobacum*) tissue culture response. Roots, shoots or callus were produced soley by manipulating the auxin to cytokinin ratio in the medium. Researchers have used this general theme for manipulation of plants in tissue culture to achieve plant regeneration through production of shoots, roots and somatic embryos (69).

Factors influencing somatic embryogenesis were of particular interest for this research. Auxin and/or cytokinin type and quantity have been shown to influence somatic embryogenesis (4, 17, 24, 42, 54, 68, 72). Cytokinins stimulated embryo production at certain concentrations but were inhibitory at higher concentrations when used in carrot (*Daucus carota* L. [19, 20]), bean (*Phaseolus coccineus* [9]), celery (*Apium gravedens* L. [3]) and anise (*Pimpinella anisum* L. [16]) cultures. In the cases of napiergrass (*Pennisetum purpureum* Schum. [53]), carrot (19,20) and celery (3), auxins at high levels or at

certain times during culture were found to inhibit embryogenesis.

Genotype has also been shown to be a factor in somatic embryogenesis. Differential genotypic responses were reported in petunia (*Petunia hybrida* [55]), wheat (*Triticum aestivum* L. [12, 40, 41, 77]), maize (*Zea mays* L. [8, 31]), rice (*Oryza sativa* L. [1]), the legumes alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), peanut (*Arachis hypogaea* L.), yellow sweet clover (*Melilotus officinalis* L.) and broad bean (*Vicia faba* L. [51]) and orchardgrass (24, 45).

Two important aspects in the embryogenic response are the physiological and the developmental state of the explant (72, 73, 80). In grasses, embryogenesis is limited to meristematic regions of leaves (basal regions) or other actively dividing tissues (24, 72, 73, 77, 78, 79). In these regions of napiergrass leaves, Rajasakeran et al. (54) reported high endogenous levels of indole-3-acetic acid (IAA) and abscisic acid (ABA) when compared to more distal, nonresponsive regions of the leaf. Isopentyl adenine, 2IP, (the only cytokinin detected in leaves) levels were higher in distal portions than in basal portions. Higher levels of the cytokinins zeatin riboside (ZR), dihydrozeatin riboside (DHZR) and 2IP were found in nonembryogenic callus suspension cultures of napiergrass than in embryogenic cultures.

Besides the factors mentioned above, specific ammonium concentrations were reported to stimulate orchardgrass (RN Trigiano,

BV Conger, RA May, unpublished data) and alfalfa embryogenesis in suspension cultures (74). Casein hydrolysate and the amino acids proline and serine were shown to stimulate embryogenesis in orchardgrass (21, 70). Gibberillic acid (GA) increased abnormal embryo developement in caraway (*Carum carvi* L. [4]). Other variables such as length of time on a particular medium and pH have been reported to influence somatic embryogenesis with optimum values for each of these factors (43).

Isolation and Quantitation of PGRs: Physico-Chemical Techniques

Brenner (11) defined the criteria for isolation and quantitation methods for PGRs. They must be sensitive, selective and precise; sampling must be uniform and limited to the plant part being studied. Furthermore, all extraction and partitioning procedures need to achieve high, repeatable yields. The traditional bioassay does not fit this criteria since it may be sensitive, but is not very selective for specific chemical structures. Therefore, a bioassay is not normally used when the structure and properties of the chemical are known.

Plant growth regulators are most commonly extracted in methanol (MEOH), ethanol (ETOH) or acetone and water solvents and partitioned against other organic or aqueous solvents to separate various groups. A basic aqueous extract can be separated into an IAA containing fraction and a cytokinin containing fraction by partitioning with ethyl acetate (ETOAC). Shindy and Smith (60) used this

technique to analyze IAA, GA, ABA and cytokinin concentrations in the same cotton ovule sample.

The analytical techniques of choice are high performance liquid chromatography (HPLC) with ultraviolet (UV) and/or fluorescence detectors and gas chromatography coupled with mass spectrometry (GC-MS), a flameless nitrogen phosphorus detector (NPD) or an electron capture detector (ECD). HPLC has several advantages over GC. It is fast, easy and inexpensive to use. Solvent systems and/or columns may be easily changed to help resolve co-chromatographing peaks. Furthermore, PGRs may be analyzed by HPLC without prior chemical modification. Of the known PGRs, only ethylene can be analyzed by GC without being modified. Modification (methylation, trimethylsilynation, trifluoroacetylation) of a compound often forms unstable products or causes side products. Also, 100% of the compound in the sample may not be modified. An advantage of GC-MS over HPLC is that there is very little ambiguity as to a compound identity once its mass spectrum is obtained since each compound has a unique MS fractioning pattern. Also, GC detectors are more sensitive and selective for several PGRs. The most common HPLC detector, UV, is not very selective nor sensitive below the ng range.

Cytokinins

Cytokinins are most commonly extracted in an 80% MEOH or ETOH solution. Organic phase partitioning is used to separate

cytokinins from undesirable or interfering compounds (28, 29). Further purification is often accomplished with thin layer chromatography (TLC) or liquid (column) chromatography (LC) (10, 11, 39, 50, 52, 65). Columns used have been polyvinyl pyrrolidone (PVP), sephadex LH-20 and DEAE-cellulose.

Recently, prepacked LC columns containing various packing materials have become available commercially. One type, Sep-pak C18 (Waters Assoc.) was used by Hashizume et al. (27) to purify cytokinins. These columns are inexpensive, easy to use, require less solvent than other LC columns and are efficient.

High performance liquid chromatography of cytokinins was reviewed by Challice (13) and Horgan and Kramers (33). Horgan and Kramers (33) used several columns and solvent systems for HPLC and concluded that reverse phase chromatography yielded the best separation of cytokinin mixtures. The best column was an octadecyl silica type (C18) and the best eluent was a mixture of acetonitrile and water. Ethanol and MEOH eluents gave inferior separations. Also, a pH of 7 was required to achieve the best separation of cytokinin mixtures. As low as 10 ng of zeatin (Z) was detectable using this HPLC system coupled with a UV detector set at 270 nm.

GC-MS is commonly used to identify cytokinins and is often coupled with HPLC prepurification (11). Cytokinins have been trimethylsilynated, trifluoroacetylated and permethylated for GC use

(11). The two latter methods were used to detect 1 pg of cytokinins by employing an ECD (44) and a NPD (83).

Auxin

Indole-3-acetic acid is most commonly extracted with an 80% MEOH or ETOH mixture for time periods of 1 to 24 hours at ambient (≈25^{o}C) or refrigerated (≈4^{o}C) temperatures (11, 48). Several purification procedures are used to concentrate IAA in the extract. McDougall and Hillman (47) used several TLC and LC types for purification and concluded that PVP and DEAE-cellulose columns both gave good yields and separations of IAA. Recently, silica gel and C-18 type Sep-pak columns have been incorporated into IAA purification schemes (2, 38).

Stoessl and Venis (64) used a specific acetic anhydride reaction of IAA to form indole-∝-pyrone detectable both fluorometrically and photometrically. As low as 10 ng of IAA was detectable; however, the endogenous plant products 4-chloro-IAA and 5-hydroxy-IAA were found to also react thereby giving erroneous results. Iino and Carr (34) and Iino et al. (35) utilized this method to obtain results that were comparable to those obtained by GC-MS. However, in a study that compared the indole-∝-pyrone method to a HPLC method Sandberg and Dunberg (57) concluded that the latter was more efficient, equally sensitive and as accurate as the former.

IAA can be detected by UV, fluorescence or electrochemical detectors (11). Sweetser and Swartzfager (66) and Wright and Doherty (82) used fluoresence and electrochemical detectors for IAA detection with HPLC separation. Although the electrochemical detector could detect as low as 5 pg of IAA it was more difficult to use than the fluorescence detector which could detect less than 1 ng.

Jensen (37) used different solvent systems and columns with HPLC and concluded that a water-acetonitrile eluent used with a C18 type column gave the most effecient separation of indole containing compounds. Ion pair chromatography was found to be superior to ion suppression chromatography for IAA (49, 58). With ion pair chromatography, the pH of the eluent is increased such that IAA is in an ionized state. The carboxyl ion of IAA is "paired" with a quaternary ammonium compound containing four lipophilic groups. Using ion pair chromatography with HPLC, better separations of IAA from other compounds were obtained with lower eluent viscosity and lower pressure.

Gas chromatography coupled with mass spectrometry detection and GC coupled with other detectors have also been used to determine IAA concentrations (11). IAA is usually methylated with diazomethane to make it more stable and volatile for GC use. Using a GC with a fused silica capillary column and a NPD, Jensen et al. (36) were able to detect picogram quantities of IAA methyl ester.

Isolation and Quantitation of PGRs: Immunological Techniques

Immunoassay of PGRs was first used by Fuchs and Fuchs (18) to determine IAA and GA concentrations. Since then, both radioimmunoassays (RIA) and enzyme linked immunosorbant assays (ELISA) have been developed for IAA and several of the cytokinins (for review see 75).

Immunoassays for IAA have been found to be very specific and sensitive even when performed with crude extracts. Weiler (76) produced antibodies to the methyl ester of IAA which were highly specific and detected as little as 3 to 4 pg. Significant cross reactivities occured only with indole-3-acetone and 1-naphthyl acetic acid.

Immunological assays for cytokinins have been utilized that can detect as little as 50 pg of isopentyl adenosine (IPA) and ZR (7). Cytokinin antibodies, however, have not been found to be highly selective when used to analyze crude extracts; therefore, some form of prepurification (most commonly HPLC) is required before immunoassays can be performed (6, 26, 32, 46).

The use of antibodies in PGR analysis has been receiving more attention recently not only as an analytical tool but also as a tool for purification of compounds by immunoaffinity chromatography (IC). Sandberg et al. (56) bound IAA antibodies to a Glutardialdehyde-activated affinity adsorbent and used it to make an IC column. A plant extract was passed through this column and the IAA was eluted.

purification of compounds by immunoaffinity chromatography (IC). Sandberg et al. (56) bound IAA antibodies to a Glutardialdehyde-activated affinity adsorbent and used it to make an IC column. A plant extract was passed through this column and the IAA was eluted. Using this technique, Sandberg et al. (56) were able to delete three purification steps and achieved a IAA peak with only one other minor peak when injected into an HPLC.

More researchers may start using immunoassays and IC for PGRs because antibodies are now available commercially. Ideally, immunoassay or purification techniques can be used in conjunction with physico-chemical techniques for the detection, isolation and identification of PGRs. The influence of endogenous PGRs on somatic embryogenesis has been analyzed by both of these techniques in several studies (17, 53, 54, 68).

CHAPTER III

MATERIALS AND METHODS

Plant Tissue

Orchardgrass plants used for tissue culture and extraction procedures were grown in an environmentally controlled growth chamber on a 12 hour day/night cycle at temperatures of 22^{o}C day and 15^{o}C night. Two subclones of an embryogenic genotype designated II-10-10 and II-10-16 and two nonembryogenic genotypes designated I-34 and I-39 were assessed for embryogenesis response as described below.

Tissue Culture Techniques

The basal 30 to 40 mm of the two innermost leaves were split along the midvein and sterilized by stirring in a 2.6% sodium hypochlorite solution which contained approximately 0.1% of the surfactant triton X-100 for 1.5 min. The leaf bases were then rinsed three times in sterile water. Each leaf half was transversly cut into six sections (3 to 5 mm each) from the base towards the apex and plated onto modified SH medium (59) that contained 30 μM dicamba (SH-30) as described by Conger et al. (14). Zeatin was added at

concentrations of 0.001, 0.01 or 0.1 μM to observe cytokinin effects on embryogenesis. The sections from one half of each leaf were plated on a control plate (SH-30 medium) and sections from the other half were plated on SH-30 medium containing various levels of cytokinins. Leaf sections were incubated in the dark at 22^{o}C for 4 to 5 weeks and then were transferred to hormone free (SH-0) medium. Plantlet formation was assessed after 3 weeks of culture with a 16 hour day/8 hour night and 22^{o}C/18^{o}C repectively. A germination percent was calculated by removing 10 to15 embryos with distinct coleoptile notches from leaf sections of each plate and placing them on SH-0 medium and incubating as above. The number of embryos was estimated as the number of plants formed on the leaf sections divided by the germination rate (71). This procedure of estimating embryo number was performed because it was not feasible to count the actual number of embryos on each leaf section from each plate. Differences in embryogenesis between control and cytokinin treatments were determined by a paired t-test.

Quantitation of PGRs

The two innermost leaves from tillers of the same plants described above were harvested. The lower 3 cm of the leaves (used in tissue culture) of both genotypes were frozen in liquid nitrogen, lyophylized and stored refrigerated in a vacuum desiccator until used.

For embryogenic subclones, the next 3 cm of the leaves were also cut and treated as above. Whole tillers of the embryogenic subclone, II-10-16, and nonembryogenic genotype, I-39, were also harvested and treated as described above.

Approximately 0.5 g of dried tissue was ground with a polytron in 80% MEOH to which 0.2 g l^{-1} of butylated hydroxytoluene (BHT) was added. The extraction procedure is outlined in Figure 1. After overnight extraction, chlorophyll and other nonpolar compounds were removed by partioning the extract with hexane. The extract was separated into IAA and cytokinin fractions by increasing the pH to 8.6 and partitioning with ETOAC (60). The cytokinin fraction (ETOAC) was dried, resuspended in water and slurried with PVP to remove phenolics. After filtering, it was adjusted to pH 6.4 with HCl or KOH and partitioned against water saturated 1-butanol. The 1-butanol was dried and the residue was resuspended in 10 ml of water. The sample was passed through a C-18 Sep-pak cartridge and cytokinins were eluted with 10 ml of 35% MEOH (27).

The IAA fraction (aqueous) was treated as described in method A by Jensen and Juntilla (38). The fraction was slurried with PVP to remove phenolics and filtered. The filtrate was adjusted to pH 3.5 and passed through a C-18 Sep-pak cartridge. The cartridge was rinsed with 2 ml of 20% MEOH in 10 mM acetic acid and IAA was eluted with 6 ml of 60% MEOH. The MEOH was evaporated under vacuum, the left over water was adjusted to pH 3 and partitioned

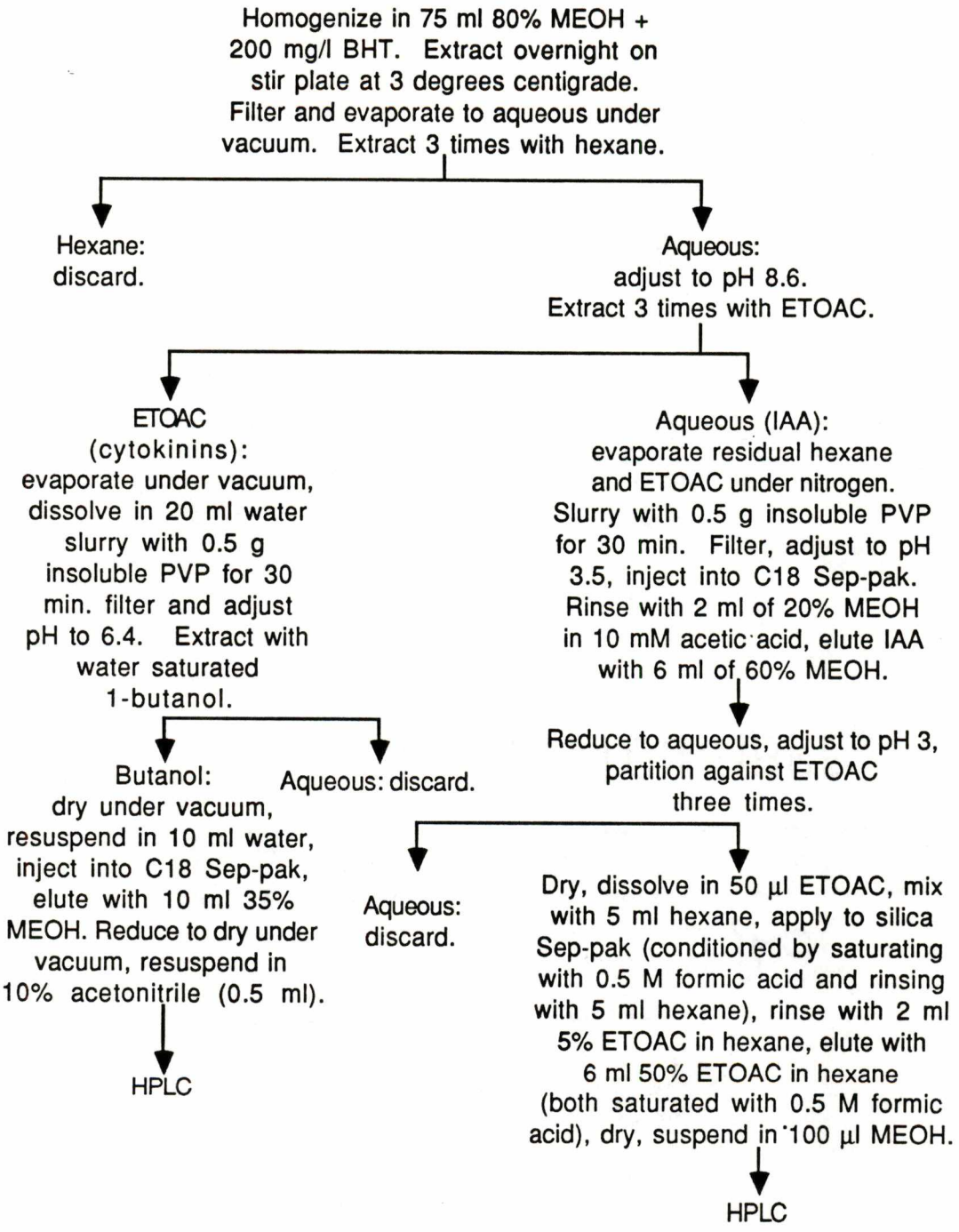

Figure 1: Extraction scheme.

against ETOAC. The ETOAC was dried under nitrogen, the residue was dissolved in 50 µl of ETOAC and mixed with 5 ml of hexane. The mixture was passed through a Silica Sep-pak cartridge (conditioned as shown in Figure 1). The cartridge was then rinsed with 2 ml of 5% ETOAC in hexane and IAA was eluted with 6 ml of 50% ETOAC in hexane.

Both fractions were dried, resuspended in the solvents given in Figure 1, and subjected to HPLC analyses under the appropriate conditions (given below). Only 25 to 50 µl of the cytokinin extract (0.5 ml) was injected but all 100 µl of the IAA extract was injected for each analysis.

High performance liquid chromatography of cytokinins was performed using a 25 cm X 4.5 mm Partisil 5-ODS 3 column connected to a Waters HPLC system containing duel pumps, a gradient programmer, a variable UV detector (set at 270 nm) and a data integrator. The solvent was 10% acetonitrile with 90% 0.1 mM phosphate buffer (pH 7) run isocratically with a flow rate of 0.8 ml min^{-1}.

For IAA analysis, HPLC was performed with the above column using a single Waters pump and fluorescence detector (excitation 280 nm, emmission 360 nm) and a Hewlet Packard data integrator or the pumps and integrator mentioned above. The solvent was 40% MEOH in 0.1 mM Phosphate buffer (pH 6.6) to which the ion pairing reagent,

tetrabutylammonium phosphate was added at a concentration of 0.01 M. The flow rate was 0.8 ml min^{-1}.

For quantitation, standard cytokinins and IAA concentrations were analyzed. Regressional analysis of peak areas was used to produce the equations for calculating concentrations in plant samples. Total cytokinins were analyzed as the sum of ZR, DHZR, Z and DHZ. Individual cytokinin types were also analyzed as was the ratio of total cytokinins/IAA. Differences between embryogenic and nonembryogenic plants and between the basal 3 cm and the next 3 cm were determined using linear contrast.

Statistical Analyses

All statistical analyses were performed using SAS (Statistical Analysis System, Cary, N.C.). Numbers generated for IAA and cytokinins were corrected for losses incurred during extraction. These correction factors (recovery rate) were determined by running the extraction outlined in Figure 1 on samples containing no plant material but known amounts of IAA, ZR, DHZR, DHZ and Z and on subsamples of plant material spiked with known amounts of these compounds.

CHAPTER IV

RESULTS

The cytokinins ZR, DHZR, Z and DHZ were identified in orchardgrass leaves by similar retention times to standard cytokinins and by enhancement of the suspect peaks by the addition of standards. A chromatogram of standard cytokinins is shown in Figure 2 with a plant extract given in Figure 3. The respective cytokinins are labled for each chromatogram.

Indole-3-acetic acid was identified in extracts by a similar retention time to standard IAA and by enhancement of the suspect peak by standard IAA. Figure 4 shows chromatograms of an IAA standard and a sample extract.

Calculated mean values for total cytokinin content, ZR, DHZR, Z and DHZ are given in Table 1. These values are corrected for the recovery rates of 50% for ZR, 35% for DHZR, 38% for Z and 53% for DHZ. The recovery rates are lower compared to some methods of cytokinin analysis; however the technique given in Figure 1 is faster and easier to perform than many other procedures. Recovery rates for blank samples and for spiked plant samples were not different and were combined to give the recovery rate percentages for these cytokinins and IAA (see Table 6 in Appendix for these data. Also given are standard curves for cytokinins and IAA used for calculating values in samples [Figures 6 to 10 in Appendix]). Table 1 does not

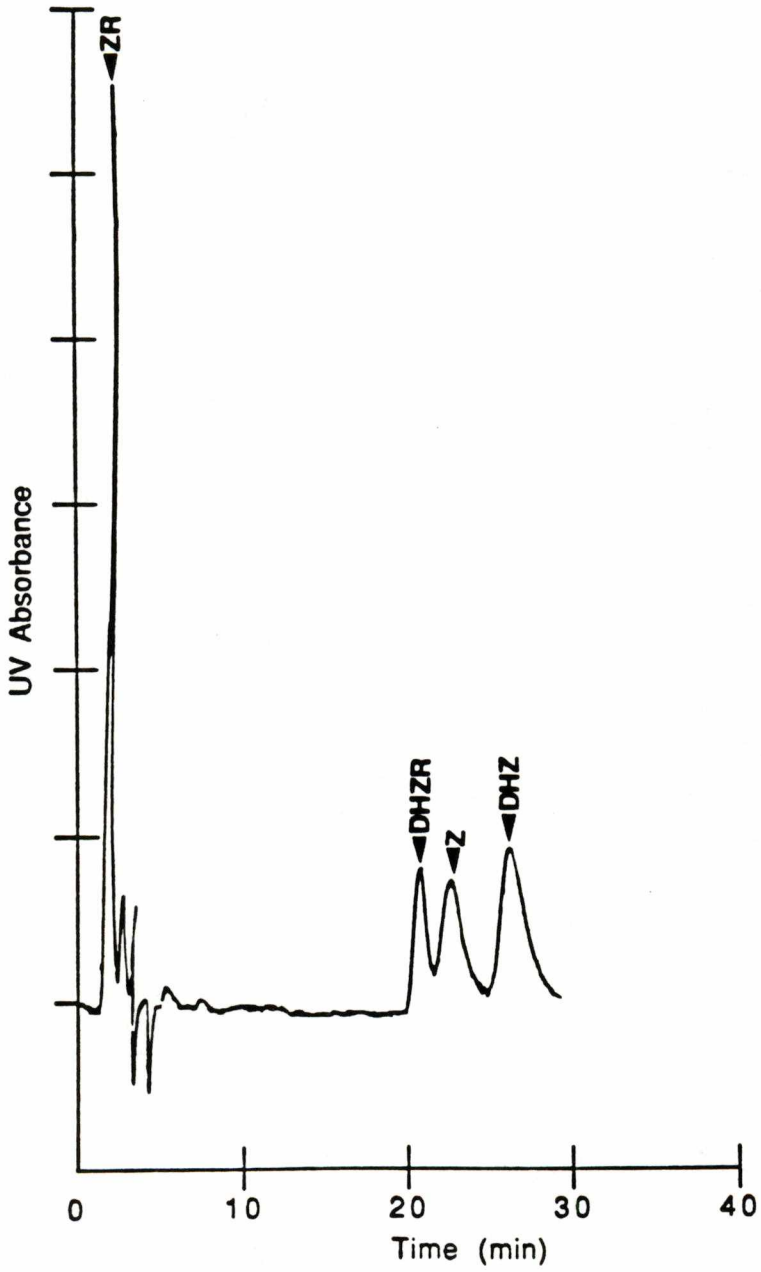

Figure 2: Chromatogram of standard cytokinins. Peaks contained 90 ng of zeatin riboside (ZR), 36 ng of dihydrozeatin riboside (DHZR), 36 ng of zeatin (Z) and 36 ng of dihydrozeatin (DHZ) chromatographed on a Partisil ODS-3 column with 10% acetonitrile in 0.1 mM phosphate buffer at 0.8 ml min^{-1} and ultraviolet detection at 270 nm.

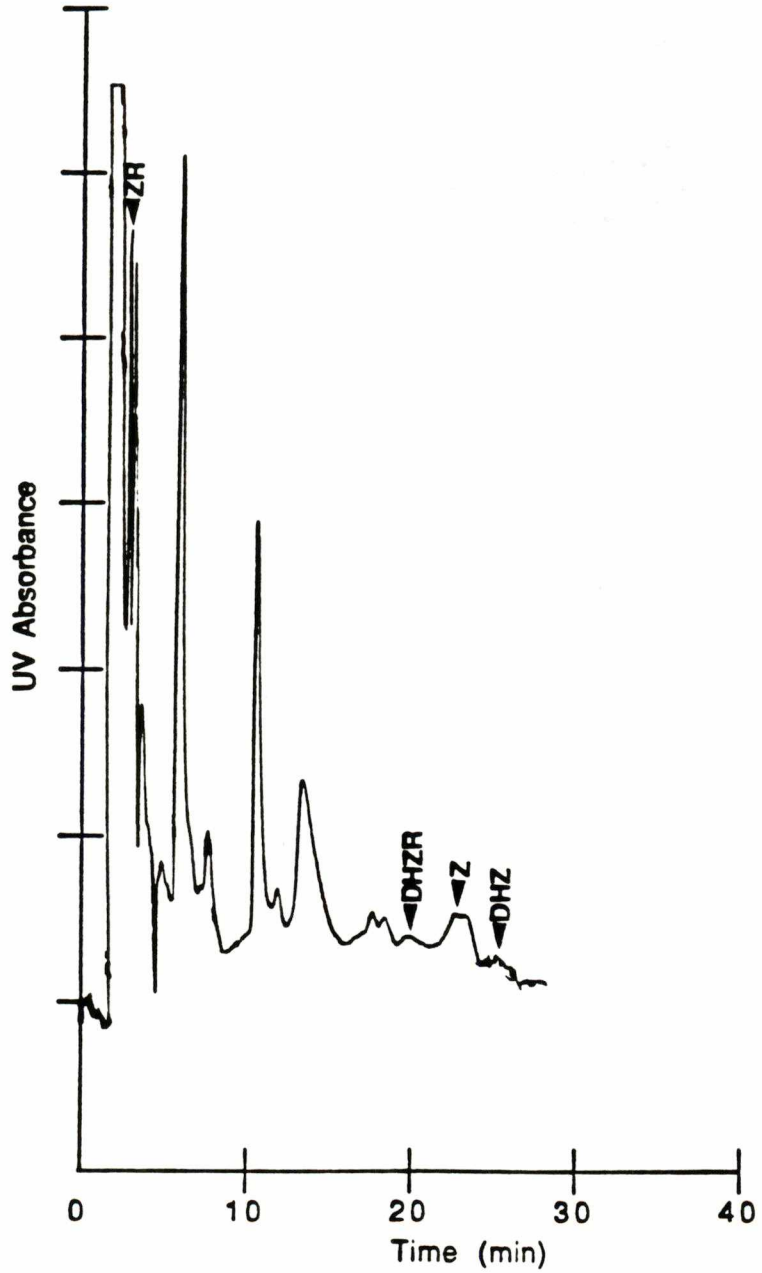

Figure 3: Chromatogram of a leaf extract. Peaks contained 103 ng of zeatin riboside (ZR), 6 ng of dihydrozeatin riboside (DHZR), 3 ng of zeatin (Z) and 1 ng of dihydrozeatin (DHZ) chromatographed on a Partisil ODS-3 column with 10% acetonitrile in 0.1 mM phosphate buffer at 0.8 ml min^{-1} and ultraviolet detection at 270 nm.

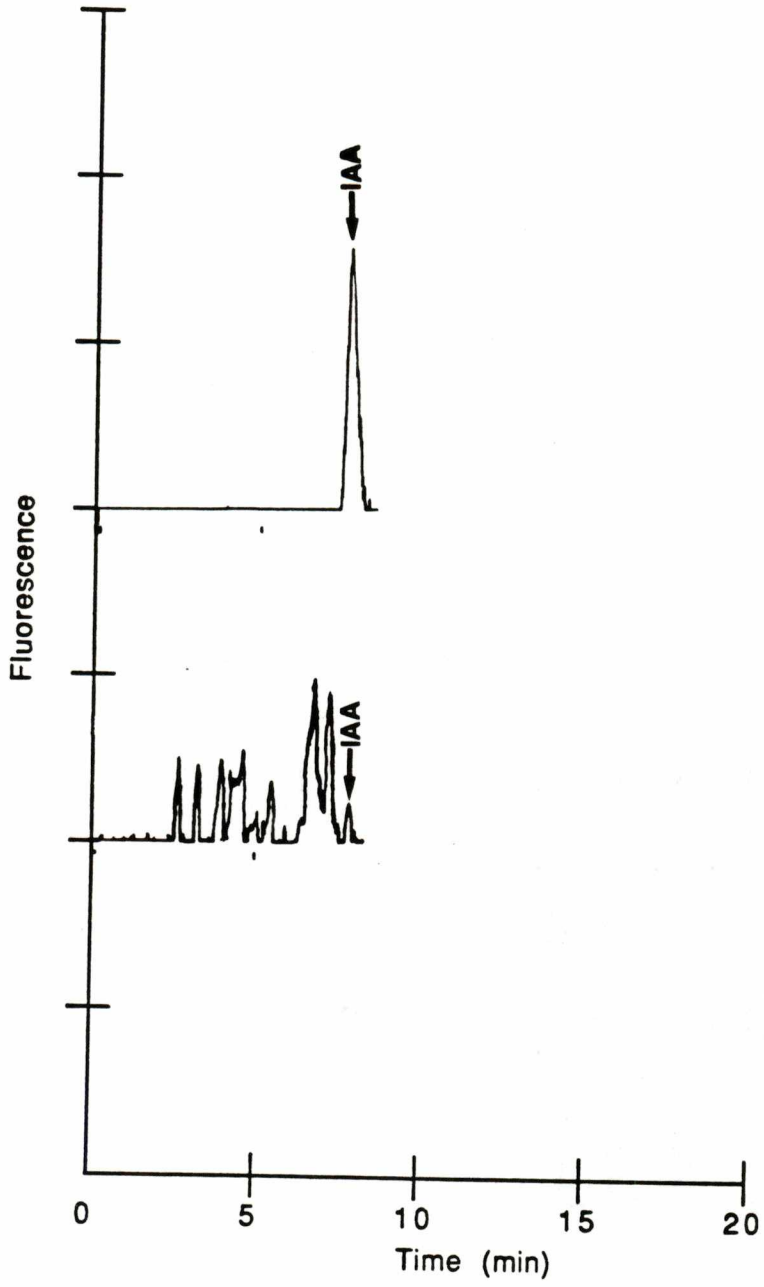

Figure 4: Chromatograms of indole-3-acetic acid (IAA) and a plant extract. Peaks contained 280 ng of IAA for the standard and 8 ng of IAA for the plant extract (top and bottom chromatograms repectively) chromatographed on a Partisil ODS-3 column with 40% methanol in 0.1 mM phosphate buffer with 0.01 M tetrabutylammonium phosphate at 0.8 ml min^{-1} and fluorescence detection (excitation 280 nm, emmission 360 nm).

Table 1: Mean cytokinin concentrations. Means for zeatin riboside (ZR), dihydrozeatin riboside (DHZR), zeatin (Z), dihydrozeatin (DHZ) and total cytokinin concentrations ($\mu g\ g^{-1}$) in the basal 3 cm of embryogenic (designated with a B) and nonembryogenic leaves and in the next distal 3 cm of embryogenic leaves (designated with a D) are shown.

Genotype	Number of Replications	Mean ZR	Mean DHZR	Mean Z	Mean DHZ	Mean Total
I-39B	5	7.13	0.89	1.81	0.92	10.74
I-34B	6	4.96	0.88	1.28	0.86	8.06
II-10-10B	6	1.05	0.33	0.51	0.19	2.08
II-10-16B	6	1.74	0.81	0.36	0.08	3.05
II-10-10D	3	1.07	1.12	2.15	0.19	4.47
II-10-16D	3	1.68	0.17	0.17	0.03	2.04

contain the cytokinin value obtained during replication 3 in the genotype I-39 which was deleted from the data set because it was determined to be an outlier by the technique described by Dixon and Massey (15) for processing data for extreme values. All of the deleted points were rejected at the 0.05 level of significance. Rejection of the cytokinin value did not change the conclusions for cytokinin variables.

Mean values for IAA and the ratio of total cytokinin content/ IAA are given in Table 2. Indole-3-acetic acid means were corrected with the recovery rate of 52% and do not contain the IAA values determined in replication 2 for the genotypes I-34 and I-39 since these values were found to be outliers (15). Deletion of these points from the data did not change the conclusions for IAA. The conclusions for the ratio of cytokinins/IAA did change due to the corresponding decrease in the standard error. The recovery rate of 52% is typical for IAA analysis.

Linear contrasts generated from the data for the cytokinin variables are given in Table 3. Results of these contrasts showed that there were significant differences in total cytokinin content between embryogenic and nonembryogenic genotypes. The nonembryogenic genotypes contained greater amounts of cytokinins than the embryogenic subclones. Zeatin riboside and DHZ were found in significantly greater quantity in nonembryogenic genotypes than in

Table 2: Indole-3-acetic acid (IAA) concentrations (ng g^{-1}) and the ratios of cytokinins/IAA. Values given are for the basal 3 cm of embryogenic (designated with a B) and nonembryogenic leaves and in the next distal 3 cm of embryogenic leaves (designated with a D).

Genotype	Number of Replications	Mean IAA	Mean Ratio
I-39B	5	26.25	0.41
I-34B	4	22.91	0.35
II-10-10B	6	18.32	0.11
II-10-16B	6	20.93	0.15
II-10-10D	3	9.47	0.47
II-10-16D	3	2.66	0.77

Table 3: F-values generated from linear contrasts of the variables zeatin riboside (ZR), dihydrozeatin riboside (DHZR), zeatin (Z), dihydrozeatin (DHZ), and total cytokinins. Plants are designated as (E) and nonembryogenic (NE) plants.

Contrast	ZR	DHZR	Z	DHZ	Total Cytokinin
E vs NE	20.17*	0.55	2.09	7.26*	23.49*
I-39NE vs I-34NE	1.85	0.08	0.19	0.00	0.83
II-10-10E vs II-10-16E	0.24	1.81	0.02	0.08	0.21
basal 3 cmE vs next 3 cmE	2.41	0.32	0.87	3.82	0.43

*significant at the 0.05 level.

embryogenic subclones. There were no significant differences in cytokinin content between the two embryogenic subclones or between the two nonembryogenic genotypes. Cytokinin content was not significantly different between the basal sections of embryogenic leaves and more distal portions of the same leaves.

Indole-3-acetic acid levels between embryogenic and nonembryogenic genotypes were not significantly different; however, the ratio of cytokinins/IAA was significantly different (Table 4). Basal portions of embryogenic leaves contained higher levels of IAA than more distal portions. The ratio of cytokinins to IAA between these two portions were also significantly different. There were no significant differences found either between the two embryogenic subclones nor between the two nonembryogenic genotypes for these variables.

Total cytokinin concentration in the leaves was significantly higher in the nonembryogenic genotype. The nonembryogenic genotype used (I-39) contained higher levels of ZR and DHZ than the embryogenic subclone (II-10-16), but they were not significantly greater. The ratio of cytokinins/IAA also was not significantly different between genotypes (Table 5).

Leaves cultured on medium supplemented with 0.1, 0.01 or 0.001 μM Z produced significantly less embryos than leaves cultured on medium without this cytokinin (Figure 5).

Table 4: F-values generated from linear contrast of the variables indole-3-acetic acid (IAA) and the ratio of cytokinins/IAA. Plants are designated as embryogenic (E) or nonembryogenic (NE).

Contrast	IAA	Ratio
E vs NE	1.39	7.22*
I-39NE vs I-34NE	0.15	2.42
II-10-10E vs II-10-16E	0.30	1.60
basal 3 cmE vs next 3 cmE	7.60*	35.16*

*significant at the 0.05 level.

Table 5: Mean cytokinin and indole-3-acetic acid (IAA) concentrations for whole leaves. Concentrations given in $\mu g\ g^{-1}$ for zeatin riboside (ZR), dihydrozeatin riboside (DHZR), zeatin (Z), dihydrozeatin (DHZ), total cytokinin, $ng\ g^{-1}$ for IAA and $\mu g\ ng^{-1}$ for the ratio of cytokinins/IAA for whole leaves of the nonembryogenic genotype I-39 and the embryogenic subclone II-10-16.

Genotype	Mean ZR	Mean DHZR	Mean Z	Mean DHZ	Mean Total	Mean IAA	Mean Ratio
I-39	4.26	1.90	0.74	0.45	7.35*	12.11	0.61
II-10-16	2.83	0.45	0.24	0.01	3.53	16.39	0.22

*significantly different from II-10-16 at the 0.05 level by a t-test.

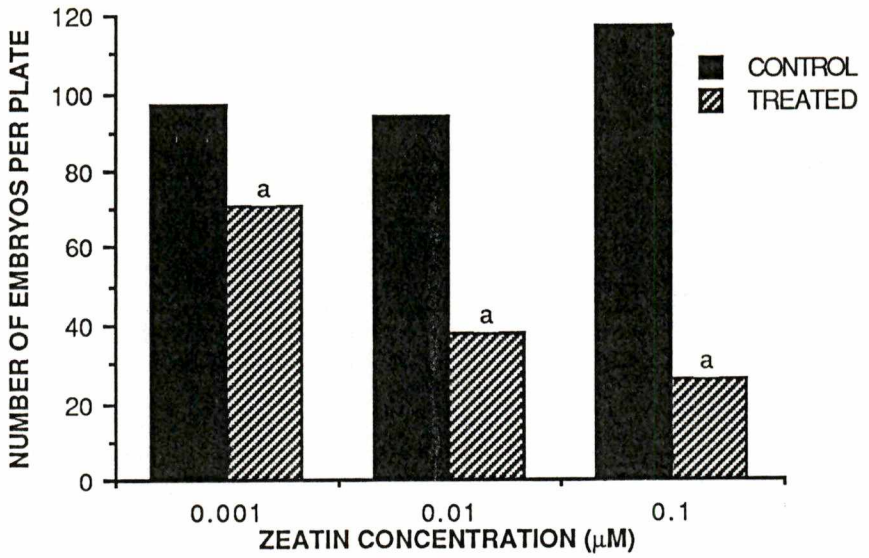

Figure 5: Effects of zeatin on somatic embryogenesis. Means followed with an "a" are significantly different from control means within each treatment at the 0.05 level by a paired t-test.

CHAPTER V

DISCUSSION

Cytokinins levels may be correlated with genotypic specific embryogenic responses since higher cytokinin concentrations were found in nonembryogenic genotypes than in two subclones of an embryogenic genotype. Addition of exogenous cytokinins also significantly inhibited embryogenesis form leaf sections in vitro. Cytokinins also had an inhibitory effect on embryogenesis in orchardgrass anther culture (62). Added cytokinins were also inhibitory to embryogenesis in soybean (42) and wheat (77). The differences in cytokinin levels are in agreement with the results of Rajasekaran et al. (54) who reported higher concentrations of cytokinins in nonembryogenic callus cultures than in embryogenic cultures of napiergrass. However, Tianran and Neuman (68) results showed that cytokinin levels were higher in embryogenic varieties of carrot than in nonembryogenic varieties. Likewise, higher cytokinin concentrations were found in regenerating tissues of soybean (*Glycine max*) cotyledons than in nonregenerating tissues (81).

Somatic embryogenesis was enhanced by 2IP at concentrations of $5X10^{-8}$ M to $5X10^{-7}$ M when added to anise suspension cultures in the first three days (logarithmic growth [16]). After this time,

addition of cytokinins did not affect embryogenesis. Apparently, cytokinins stimulated cell division which increased cell number and in turn produced more somatic embryos. Cytokinins were not thought to affect the actual regulation of embryogenesis. They did find, however, that 2IP levels of $5X10^{-6}$ M inhibited embryogenesis. Similar results were reported for carrot (19, 20), bean (9) and celery (3) cultures. Orchardgrass may be similar to other species in that optimum cytokinin concentrations are required for embryogenesis. Embryogenic subclones of orchardgrass may possess endogenous cytokinin concentrations that are optimum for embryogenesis; nonembryogenic genotypes may have inhibitory (excessive) amounts of these same cytokinins.

The genotypic response in orchardgrass did not seem to be influenced by endogenous IAA concentration. No significant differences in IAA concentrations were found between embryogenic and nonembryogenic genotypes. This finding conflicts with the results of Tianran and Neuman (68) who reported that embryogenic varieties of carrot contained higher levels of IAA than nonembryogenic varieties. Likewise, Rajasekaran et al. (54) found higher IAA concentrations in embryogenic suspension cultures than in nonembryogenic suspension cultures of napiergrass.

There were higher IAA concentrations in basal sections of leaves than in distal sections. This result is consistent with that of Rajasekaran et al. (54) for napiergrass. Indole-3-acetic acid

production is localized to meristematic regions which are the basal portions of orchardgrass and napiergrass leaves. Indole-3-acetic acid also has been shown to have a basipetal transport from the site of production. Therefore, it would not be expected for distal regions of grass leaves to contain greater amounts of IAA than basal regions. Furthermore, IAA may be metabolized or conjugated after synthesis which would account for a loss of free IAA in distal regions. Endogenous IAA may not be directly responsible for the gradient response in vitro since addition of IAA to cultures of distal portions of napiergrass leaves did not result in embryogenesis in these nonembryogenic regions (53). Traditionally only meristematic regions (nondifferentiated) of grasses have been capable of somatic embryogenesis or organogenesis in tissue culture; differentiated regions have consistently been nonresponsive (72, 73, 77, 78, 79). Hesemann and Schröder (30) reported that there was a loss of nuclear DNA in older (differentiated) leaves of rye (*Secale cereale* L.) which may account for the inability of differentiated regions to respond to tissue culture. However, Taylor and Vasil (67) did not find any loss in nuclear DNA content in differentiated portions of napiergrass leaves.

The ratio of total cytokinins/IAA was significantly different between genotypes and between basal and distal sections. Skoog and Miller (61) reported the importance of this ratio in manipulating the response of tobacco in tissue culture. How endogenous levels of IAA

or cytokinins are influenced by the introduction of exogenously applied PGRs is not known. Furthermore, auxin and cytokinin regulations may involve other PGRs not analyzed in this study. In some manner, alone or in conjunction, IAA and cytokinins work to cause cells to grow and develop (23).

CHAPTER VI

SUMMARY AND CONCLUSIONS

Endogenous cytokinin concentrations appear to be important in genotypic specific responses of orchardgrass leaves in vitro. Higher endogenous cytokinin concentrations in nonembryogenic genotypes compared to embryogenic subclones correlated with response of the tissue. The inhibitory effect of added cytokinins on somatic embryogenesis of cultured leaf sections further strengthen this hypothesis. Indole-3-acetic acid concentrations may have a role in the gradient effect since there were significant differences in IAA concentrations between basal and distal sections of leaves. The ratio of cytokinins/IAA may influence both gradient and genotypic responses since this ratio was significantly different on a genotypic and basal/distal basis.

Further studies with orchardgrass are required for elucidation of other embryogenic factors and for stronger confirmation of those found in this report. Experiments testing the effects of different ratios of cytokinins/auxin might help to further the understanding of hormone interactions. Experiments analyzing the effects of cytokinin inhibitors would help to prove/disprove the importance of endogenous cytokinin levels. Time course studies of PGR levels during tissue culture may be performed such as those done with carrot (68) and

napiergrass (54). Endogenous concentrations of both levels and types of cytokinins and IAA have been shown to change during culture. Other PGR's such as ABA or GA may also be analyzed for possible relationships to genotypic and gradient responses.

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APPENDIX

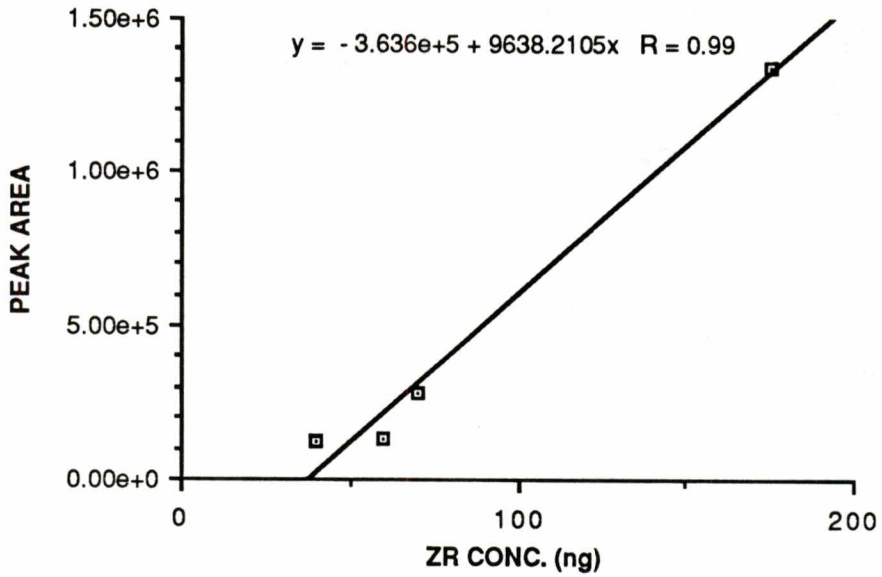

Figure 6: Standard curve for zeatin riboside (ZR). Given concentrations were chromatographed on a Partisil ODS-3 column with 10% acetonitrile 90% 0.1 mM phosphate buffer (pH 7) at 0.8 ml min^{-1} and ultraviolet detection at 270 nm.

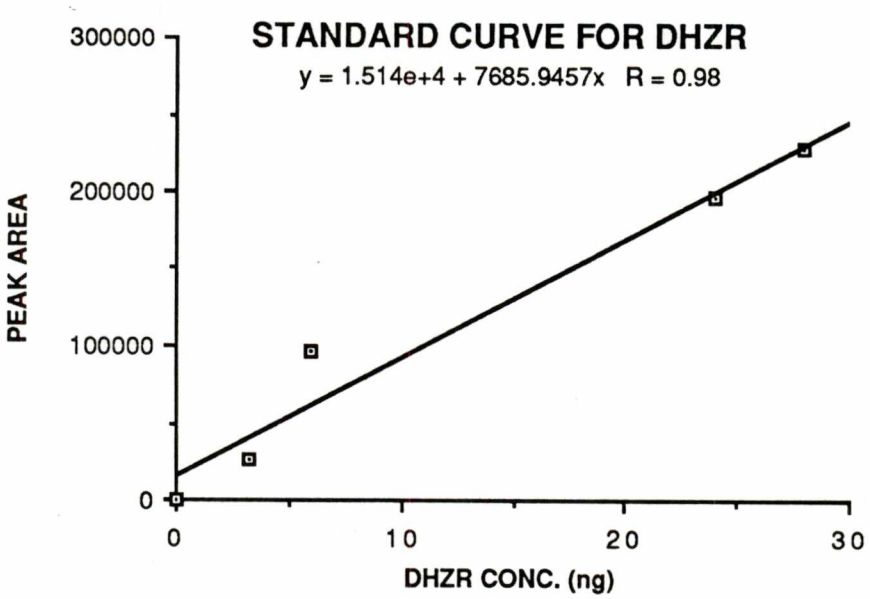

Figure 7: Standard curve for dihydrozeatin riboside (DHZR). Given concentrations were chromatographed on a Partisil ODS-3 column with 10% acetonitrile 90% 0.1 mM phosphate buffer (pH 7) at 0.8 ml min^{-1} and ultraviolet detection at 270 nm.

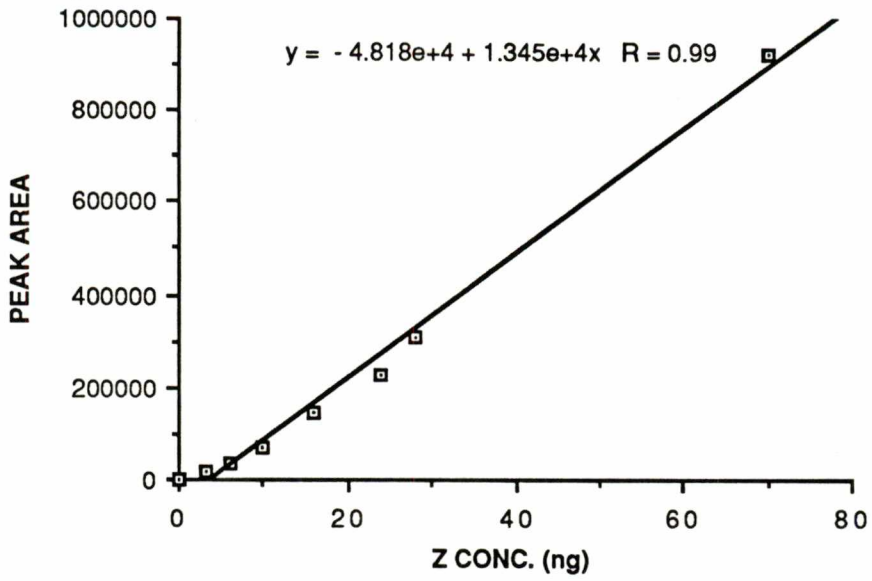

Figure 8: Standard curve for zeatin (Z). Given concentrations were chromatographed on a Partisil ODS-3 column with 10% acetonitrile 90% 0.1 mM phosphate buffer (pH 7) at 0.8 ml min^{-1} and ultraviolet detection at 270 nm.

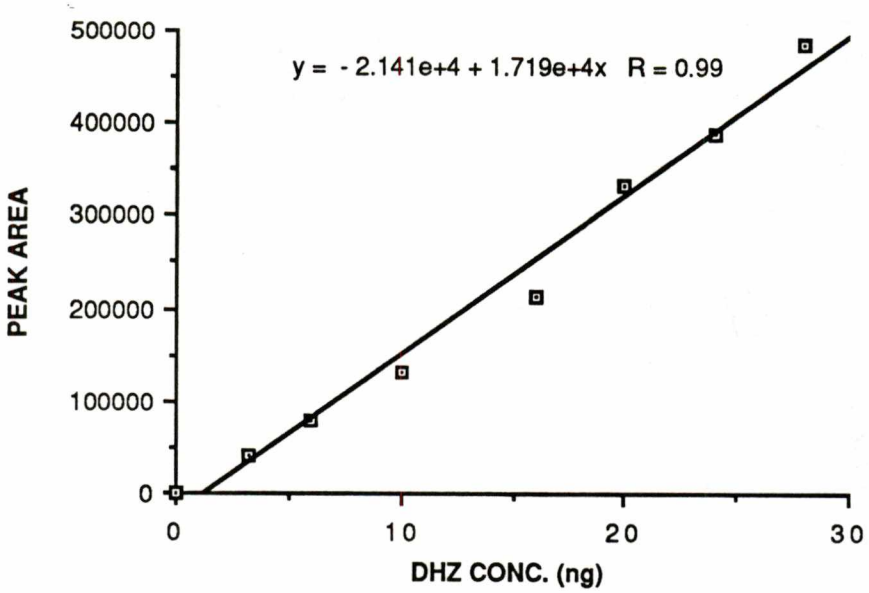

Figure 9: Standard curve for dihydrozeatin (DHZ). Given concentrations were chromatographed on a Partisil ODS-3 column with 10% acetonitrile 90% 0.1 mM phosphate buffer (pH 7) at 0.8 ml min^{-1} and ultraviolet detection at 270 nm.

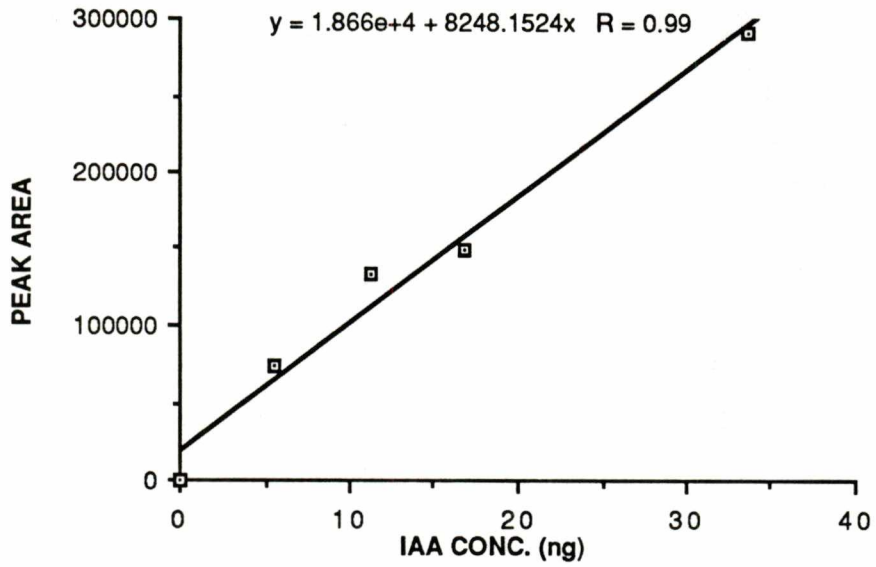

Figure 10: Standard curve for indole-3-acetic acid (IAA). Given concentrations were chromatographed on a Partisil ODS-3 column with 40% methanol in 0.1mM phosphate buffer (pH 6.6) with 0.01 M tetrabutylamonium phosphate at 0.8 ml min^{-1} and fluorescence detection (excitation 280 nm, emmission 360 nm).

Table 6: Recovery rates (%) for the plant growth regulators (PGRs). Zeatin riboside (ZR), dihydrozeatin riboside (DHZR), zeatin (Z), dihydrozeatin (DHZ) and indole-3-acetic acid (IAA) were analyzed in blank samples and spiked tissue samples (two replications each).

PGR	Plant Tissue	Blank	Mean Recovery
ZR	51.45 52.23	47.08 50.56	50
DHZR	21.00 43.02	32.00 42.00	35
Z	31.62 29.89	45.59 45.56	38
DHZ	44.86 67.30	60.78 40.02	53
IAA	63.96 36.97	50.59 55.59	52

VITA

Allan Richard Wenck was born on July 27,1963 in New Jersey to David and Patricia Wenck. He graduated from West Morris Central High School in 1981. He then went to Warren Wilson College in Swannanoa, North Carolina where he graduated with a B.A. in Biology and Environmental Studies on May 18, 1985. He was married to Mary Anne Duncan on May 19, 1985. Until March 1988 he was working for his Master of Science in Life Sciences at the University of Tennessee, Knoxville.