ABSTRACT

FINNEY, MATTHEW MARTIN. Effect of Environment on, and Analysis of, Allelopathic Natural Products in Rye (*Secale cereale* L.). (Under the direction of David A. Danehower.)

This research examined the effects of environmental factors such as temperature, fertility, and water status, on the production of a class of allelopathic natural products in rye. These compounds, the benzoxazinones, were first identified and studied in the 1960's as fungal resistance factors in cereal crops. The primary phytotoxic compounds are: 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), its corresponding glucoside (DIBOA-glu), and 2-benzoxazolinone (BOA). Nutrient availability was found to have the greatest overall effect on the production of allelochemicals in rye. Plants grown under a higher fertility regime had significantly greater concentrations of allelopathic compounds in their tissue. Plants grown with low water availability also had elevated levels of allelopathic compounds in their tissue in comparison with those plants grown with adequate moisture. Subjecting rye to elevated growing temperatures also led to a significant increase in allelochemical content. All results were weighed against the established theories of plant defense, optimal defense theory and carbon / nutrient theory. Results did not conform to one particular theory, but were supported by portions of each. This work also describes the development of a new method for analysis of these compounds as well as other allelopathic agents found in rye tissue, β-hydroxybutyric acid (*β*-HBA), and β-phenyl lactic acid (β-PLA). Sample preparation consists of extraction of freeze-dried rye vegetative tissue with aqueous ethanol (50%) followed by partitioning of the allelochemicals into an ethyl acetate, evaporation, and derivatization using the trimethyl silylating reagent MSTFA. GC analysis of the silylated mixture was performed

using a 20M DB-5 megabore capillary column and a temperature program increasing from 100 to 300 C at 5^{O}/ min with detection by FID. Injector temperature was 250^{O} C and detector temperature was 325^{O} C. Identities of all compounds were confirmed by GC/MS analysis.

EFFECT OF ENVIRONMENT ON, AND ANALYSIS OF, ALLELOPATHIC NATURAL PRODUCTS IN RYE (*SECALE CEREALE* L.)

By
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Biography

Matthew Finney was born and raised in Evansville, IN. Working for the family business, a southern Indiana farm supply store, he developed a deep interest in agricultural science, which he pursued by studying at Purdue University in the Agronomy department. After obtaining his B.S., Matt chose to further engage his academic interests by enrolling in a Masters degree program in the Crop Science department at North Carolina State University.

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Introduction

Allelopathy is defined as the deleterious process by which one organism (almost exclusively limited to plants in the common usage of the word) influences others through the escape or release of toxic or inhibitory substances (Oxford English Dictionary Online. 2003, http://dictionary.oed.com/entrance.dtl). The scientific study of allelopathy as a specific phenomenon coincided with the coining of the term in the 1930's, but observations of allelopathic effects date back to at least 300 B.C. (Rice 1984). Even today, several definitions of allelopathy exist. Popularly defined, allelopathy encompasses all biochemical interactions between plants and microorganisms, both inhibitory and stimulatory (Rice 1984). Other definitions include plant-insect and plant higher animal interactions, or do not include stimulatory effects. Regardless of the definition, for allelopathy to occur, chemicals must be transferred from one organism to another. (Eihnling 1995, Rice 1984) This process of transfer distinguishes allelopathy from competition, with which allelopathy is often grouped (Rice 1984). Competitive effects involve the removal of a factor, such as water, light, or nutrients from a plant's environment by other plants. The combined effects of allelopathy and competition are referred to as interference (Rice 1984). Allelopathic effects extend beyond mere plant (weed) suppression, to include: vegetation patterns, plant succession, seed preservation, germination of fungal spores, the nitrogen cycle, mutualistic associations, crop

productivity, and plant defense (Einhellig 1995, and Rice 1984). Thus, allelopathy has implications in many aspects of agriculture.

It has long been recognized that some crops reduce the growth of weeds. Practices such as planting "smother crops" in a rotation or intercropping system have been implemented by farmers for hundreds of years (Worsham 1989). Many of these cropping systems utilize the allelopathic potential of plants. The planting of allelopathic cover crops and the use of phytotoxic mulches are the most commonly utilized methods for taking advantage of allelopathy in a weed management system. Allelopathic cover crop use in a no-till or reduced tillage system gives the added advantage of physical weed suppression as well as allelopathic and competition effects (Worsham 1989).

Rye (*Secale cereale* L.) is a commonly used cover crop. Its use is due to both high biomass production as well as its widely recognized allelopathic capabilities. In 1986, Barnes and Putnam clearly demonstrated the allelopathic potential of rye. Residues of rye suppressed the emergence of lettuce (*Lactuca sativa* L.) and proso millet (*Panicum miliaceum* L.) more effectively than a wood shaving mulch control treatment (Barnes and Putnam 1986). In addition to lettuce and millet, rye residue affected cress (*Lepidium sativum* L), and baryardgrass (*Echinochloa crus-galli* L.) to a lesser extent. The phytotoxicity of both rye residue and aqueous rye extract manifested itself in reduced radicle elongation of each species (Barnes and Putnam 1986).

The following year, Barnes et al. (1987) determined the identity of the principal phytotoxic compounds contained in rye residue. The two compounds were identified as 2,4-dihydroxy-1, 4(2*H*)-benzoxazin-3-one (DIBOA, CAS # 17359-54-5) and 2(3*H*)-benzoxazlinone (BOA, CAS#59-49-4). Each of these compounds inhibited cress radicle elongation. However, DIBOA alone inhibited the growth of barnyardgrass. Other phytotoxic compounds have also been isolated from rye. The compounds β-hydroxybutyric acid (β-HBA), and β-phenyl lactic acid (β-PLA) have been identified as allelopathic agents (Shilling 1986).

DIBOA and BOA are members of a class of compounds known as benzoxazinones (Bx). BOA is a less phytotoxic breakdown product of DIBOA (Niemeyer 1988). These compounds were first identified and studied in the 1960's as fungal resistance factors in cereal crops. Infection levels of Northern Corn Leaf Blight (*Helminthosporium turcicum*) are inversely correlated to benzoxazinone levels in maize (*Zea mays*), DIMBOA (2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one, CAS #115893-52-4), the principle benzoxazinone found in maize, [Fig1] decreased germination of the spores (Niemeyer 1988). Benzoxazinone derivatives have also been found to have biological activity against insect pests and bacterial pathogens (Niemeyer 1988, Niemeyer and Perez 1995). In maize, resistance to the European corn borer (*Ostrinia nubilalis*) correlates with increased benzoxazinone levels in the plant and as benzoxazinone concentration declines resistance to corn borer declines. Aphid (*Rhopalosiphum madis*) resistance in maize and wheat (*Titicum aestivum*) increases (Niemeyer 1988). Benzoxazinones added to insect diets increased larval mortality, slowed development, and reduced offspring.

Benzoxazinones have been found in most species in the Gramineae and vary in structure from species to species (Niemeyer 1988; Friebe 2001). DIBOA is principally found in rye while in maize and wheat the primary benzoxazinone is DIMBOA, a methoxylated form (Niemeyer 1988). Biosynthetic pathways for DIBOA and DIMBOA are identical across the Gramineae (Glawischnig et al. 1999). The hydroxamic biosynthetic pathway shares several early intermediates with the tryptophan biosynthetic pathway. Frey and Gierl (1999) demonstrated that the labeled precursors, indole and anthranilic acid, form labeled DIBOA, DIMBOA, and tryptophan in maize cells. Labeled tryptophan, however, did not form any of the benzoxazinones. Five genes located on the short arm of chromosome four in maize encode for the principal enzymes required for DIBOA synthesis [Fig. 2]. The first gene, (*Bx1*), encodes the key branch point enzyme between benzoxazinone and tryptophan biosynthetic pathways. This enzyme is very similar to the tryptophan synthase-a subunit and catalyzes the formation of free indole. The next genes in the series, *Bx2-Bx5* contain the genetic code for cytochrome P450-dependent monooxygenases that catalyze a series of four hydroxylations, which ultimately result in the formation of DIBOA (Frey and Gierl 1999; Sicker et al. 2000). The activity of these enzymes in rye and barley (a non-benzoxazinone producer) was tested to determine the similarity of the pathways between species (Glawischnig et al. 1999). All reactions identified in maize occurred identically in rye (Glawischnig et al. 1999). Specific activities were also similar in both species. Barley showed no activity for any of the enzymes tested (Glawischnig et al. 1999). Rye and maize are evolutionarily distant among Gramineae species. Therefore, the similarity of the biosynthetic pathways in each species suggests the evolution of these genes occurred early in grass species' development (Frey and Gierl 1999; Gierl and Frey 2001).

Benzoxazinones are present in all parts of Gramineae species, but are absent in the seed (Niemeyer 1988). Benzoxazinone levels increase after germination and typically reach maximum concentration a few days after germination in maize and wheat. In rye benzoxazinones reach maximum concentration later in growth and development (Niemeyer 1988). Benzoxazinones are stored in vacuole as an inactive glucoside. Glycosylation typically occurs at the 2-postion of the azinone ring system. Upon wounding or leaf senescence the glucoside is cleaved by β-glucosidases stored in the plastid and the active benzoxazinone aglycone released into the environment (Barnes et al. 1987; Sicker et al. 2000).

In 1995, Yenish et al. published a study on the release, breakdown and disappearance of benzoxazinones in rye residue. Rye cover crops were cut, the resulting crop residue was placed in a field environment in fiberglass mesh residue bags, and data was collected over two years. Concentrations of benzoxazinones in the rye residue declined to 50% of initial levels within 10 to 12 days after cutting. The levels reduced in a logarithmic pattern and theoretically decline to zero mol / Ha after 121 and 168 days in year 1 and 2 of the study, respectively (Yenish et al. 1995).

Analytical Methods

Varied methods have been developed for the quantitative analysis of benzoxazinones in rye tissue. Most of the published methods use high performance liquid chromatography (HPLC) as the major tool for analysis. The method described in Gianoli and Niemeyer (1997) has

been utilized in many other studies of benzoxazinone levels in rye. The method requires plant material to be macerated with 1 mL of H_2O and the extract left at room temperature for 15 min before adjusting the pH of the mixture to 3 using 0.1N H_3PO_4. The tissue is removed from the aqueous extract by centrifugation of the mixture at 13000g for 15 min and subsequent removal of the supernatant. An aliquot (50μL) of the supernatant is injected into the HPLC equipped with a RP-100 Lichrospher-C18 column using a dual solvent system with solvent A (Methanol) and B (0.05% aqueous H_3PO_4) run in a linear gradient of 0-7 min 30% solvent A, 7-9 min 100% solvent A, and 9-13 min 30% solvent A. The solvent flow rate is a constant 1.5 mL/min and detection of compounds is accomplished by measurement of UV absorption at 263nm.

Another HPLC method described in Yenish et al. (1995) requires 10 g of ground dried rye shoot tissue to be extracted with 300 mL methanol. The plant tissue is homogenized in a blender for a period of 5 minutes the tissue is allowed to settle for 1 min, followed by additional homogenization for 5 min. The mixture is filtered through cheesecloth; the filtrate re-filtered through Whatman #1 filter paper, and is then reduced to 20 mL in vacuo with a rotary evaporator. The concentrated mixture is subjected to additional filtration with Whatman #1 filter paper then passed through a 0.22mm nylon membrane filter, before analysis. For analysis 25 μL of the concentrated filtrate are injected into an HPLC with a μBondapack C18 pre-column and an 8 mm x 10 cm Nova-Pak C18 column. An isocratic solvent system of 75:25 Methanol: H2O is pumped at a 1.5 mL/ min flow rate. UV detection is at 255nm.

The analysis used by Burgos, et al. (1999) adapted the Yenish method using the same HPLC conditions, but altering the sample preparation. In their adaptation, 15 g of rye tissue is extracted with 150 mL of H_2O. The mixture is filtered through cheesecloth then, 150 mL of acetone is added to precipitate the mixture. It is then centrifuged at 2200 g for 10 min at 2° C. The supernatant is removed and partitioned twice with 150 mL hexane in a separatory funnel. The aqueous fraction is reserved then extracted twice with diethyl ether, (200 mL followed by 150 mL). The ether fractions are then combined and dried over anhydrous $MgSO_4$. The dried ether is evaporated *in vacuo*, and the residue dissolved in 10 mL of methanol. A 15 μL sample is analyzed for benzoxazinone content using the same method as Yenish.

Mwaja et al. (1995), describe another method of sample preparation and analysis. In this method 40 g of ground dried rye shoot tissue are added to 720 mL H_2O and shaken at 200 rpm and 4° C. The extract is then filtered through cheesecloth and centrifuged at 9500 g for 15 min. The supernatant is then subjected to a series of filtrations using filter papers, Whatman #s 4, 1, and 42. After filtration the extracts are partitioned six times each with 150 mL of hexane and 150 mL diethyl ether. The ether extract is then partially purified using Baker bond C18 sep-pack eluted with 2 mL methanol and then filtered twice through a 0.2 μm nylon membrane filter. The filtered samples are reduced to dryness, weighed and re-dissolved in chloroform to form a 1 mg/ mL solution. Using HPLC analysis, 20 μl of the chloroform solution is injected on a Waters Nova Pak C18 column of 3.9mm x 150mm. An isocratic system with a mobile phase of 75:25, pH 3 (acetic acid) H_2O: acetonitrile is pumped at a flow rate of 1.0 mL/ min. Detection is by UV absorbance at 254 nm.

Zúñiga et al. (1983) describes two methods for determining benzoxazinone content, a $FeCl_3$ method and a TLC-UV method. The plant extracts are prepared the same in both methods. The youngest leaves of a plant were macerated in H_2O at room temperature followed by filtration through cheesecloth. The pH of the extract was reduced to 3 by addition of HCl followed by 15 minutes of centrifugation at 6000 *g*. After centrifugation the supernatant was removed and extracted with two volumes of Et_2O three times. The combined organic layers were reduced to dryness and used for the quantitation procedures. For the $FeCl_3$ procedure the residue was dissolved in the $FeCl_3$ reagent (50 g $FeCl_3 \cdot 6H_2O$, 500 mL 95% EtOH, and 5 mL 1.5M HCl) and the absorbance measured at 590 nm. The absorbance reading are compared to a standard curve of know concentration. For the TLC-UV method the organic residue was dissolved in 0.2 mL EtOH and spotted on a GF 254 silica gel TLC plate (5 x 20 cm). The plate was developed in Benzene: Et2O (1:4) and those bands with the same R_f as standards of the benzoxazinones were extracted with EtOH. The absorbance of the extracted fraction was measured at 263 nm, and the concentration values calculated using a standard curve.

Barria et al. (1992) developed a gas chromatography method for benzoxazinone analysis. Fresh shoot tissue, 20 – 50 mg, was macerated three times with 0.33 mL H_2O. The extract was then centrifuged at 10000 *g* for 15 min, the supernatant removed, and filtered (0.45 mm). The filtered sample was then lyophilized, and the dried samples dissolved in 50 mL of N,O-bis(trimethylsilyl)acetamide (BSTA). The samples were then heated for 15 minutes at 52^{O} C. 5µL were injected into a CBPS capillary column. Injector and detector temperature were

both set at 300^{O} C. Initial oven temperature is held at 170^{O} C for 3 minutes, followed by an increase of 3^{O} C / min for 12 minutes.

Environmental Effects on Allelopathy

Researchers have struggled to understand the influence of the environment on allelopathy. Environmental factors alter allelopathic interactions through effects on the plant, the target species, and the transfer of the agents between the plants. Several established theories address the impact of the environment on allelochemical production. Studies have shown allelopathic plant species increase production of phytotoxic secondary metabolites when subjected to environmental stresses such as mineral deficiency, extreme temperatures, moisture stress, or extreme light levels (Einhellig 1989).

More elaborate theories of plant defense have been proposed, including the theories of optimal defense and the carbon-nutrient theory. Optimal defense theory relates to plant-herbivore defenses, while the carbon–nutrient theory concerns defense compounds in general.

Optimal defense theory results from the synthesis of several different studies (Rhoades 1979). Two hypotheses and four corollary hypotheses define optimal defense theory. The primary hypotheses are that:

1) Evolution and allocation of defenses is done to maximize individual fitness,

2) Costly defense is effective; less well defended individuals have higher fitness costs when enemies are present.

Corollary hypotheses in the optimal defense theory state that

a) defenses evolve in proportion to the amount of risk and inverse proportion to cost of defense,

b) defense allocation matches risk of attack and value of specific tissues,

c) defense allocation increases or decreases in direct proportion to presence or level of predatory attack, and

d) environmental stresses, or a reduction in resources, reduces allocation to defense.

Results of individual studies should be evaluated according to optimal defense theory hypotheses, but the theory does not universally explain the impact of environmental stress or predator attack on defense metabolism. Results from many studies do not hold to the tenets of the optimal defense theory because defense response to the environment differs according to the class of compound or compounds produced by a given species (Bazzaz 1987).

The carbon-nutrient theory also attempts to explain plant defense responses as they are influenced by environmental factors. This theory explains changes in defense allocation according to the available resources and growth habits of a given plant species. Principally, this theory regards carbon and nitrogen as primary resources in the plant. Each element has priorities with regards to allocation and use within the plant (Mooney 1983). The relative availability of these resources affects the adaptation and evolution of a plant's defenses. Nutrient availability affects the evolution of both the level and type of defense used by a given species. Concerning the general level of defense, adaptation and response among species indicates increased defense allocation when resources are limited. However, within a

species, defense allocation may become elevated when available growth resources reach levels above growth requirements (Bazzaz 1987). The type of defense is affected in that species adapted to a nutrient-limiting environment utilize carbon-based secondary metabolites, while those species in a carbon-limited environment develop nitrogen-based defense compounds (Bryant 1983). Plants in a nutrient-deficient environment have growth rates reduced more than photosynthate production rates, leading to an abundance of available carbon. This carbon accumulates in the plant as carbohydrates and carbon-based defense compounds (Bryant 1983). Likewise, when plant nutrient concentrations reach levels above those required for growth, synthesis of nitrogen-based defense compounds increases. This includes situations where carbon, and therefore growth, is limited as well as fertilization treatments or other sudden nutrient releases (Bryant 1983). [Fig. 3] Nitrogen is highly mobile and recycled within the plant; therefore, it is advantageous for the plant to store any excess abundance of nitrogen within the plant in forms that contribute to plant defense (Mooney et al. 1983).

Previous studies examining the affect of environment on allelopathy in rye provided information on the production of allelopathic compounds, principally DIBOA, its corresponding glucoside (DIBOA-glucoside), and their breakdown product, BOA. Better understanding of the production and regulation of allelochemicals in rye under varying environmental conditions is necessary to improve the efficacy of cover crops in weed control.

When rye (Var. Wheeler) was grown under low, medium, and high fertility regimes, the middle range fertility treatment performed the best in a rye shoot tissue allelopathic bioassay

on cress (*Lepidium sativum* L.*)* and barnyardgrass (*Echinochloa crus-galli*) radicle growth (Mwaja et al. 1995). Dried rye shoot tissue was placed in soil, the soil was covered with filter paper, wetted, and cress and barnyardgrass seeds were added to the filter paper. Radicle growth was measured after 72 hours. Comparisons of allelopathic activity were also made between rye grown under monoculture and polyculture with hairy vetch (*Vicia villosa* Roth). Both systems were grown under low, medium, and high fertility regimes, and rye tissues from each of these were evaluated by bioassay. Under monoculture, the medium fertility regime was significantly more toxic than the low and high treatments in the cress bioassay. Under polyculture, the medium and high fertility tissue was significantly more toxic to cress than rye tissue in the low fertility treatment. In the barnyardgrass bioassay the medium fertility monocultured rye tissue was significantly more toxic than other monoculture tissues. There were no significant differences between the three fertility regimes grown under polyculture in the barnyardgrass assay. The authors closely examined activity and quantity of phytotoxic compounds in a rye extract bioassay. Dried shoot tissue was extracted with H_2O and partitioned with hexane and diethyl ether. The hexane and ether extracts were combined, reduced to dryness, and re-dissolved in chloroform to form solutions of total extractable metabolites (not necessarily phytotoxic) of decreasing concentrations (666, 333, 160, 83, 42, and 21 μg/ml). Within both the monoculture and polyculture treatments, toxicity of extracts measured in I_{50} values (concentration of total extract required to produce 50% inhibition of cress radicle growth) decreased with higher fertility. Monoculture /low fertility gave an I_{50} value of 177.2 μg/mL, while the high fertility/ monoculture treatment resulted in an I_{50} value of 275 μg/mL. Patterns within the polyculture treatment were similar, while comparing monoculture with polyculture reveals higher toxicity in the polyculture treatments for each

fertility regime. The concentration of BOA and DIBOA in the total extractable compounds decreased as fertility levels increased for both mono- and poly culture, correlating with decreased toxicity of the extracts. Total extract recovery was greatest for the medium fertility regimes for both monoculture rye and rye /vetch polyculture, with the monoculture treatment giving the highest total recovery. These results would explain the high performance of the medium fertility treatments in the rye shoot tissue bioassay. In the extract bioassay the concentration of extracts was equalized across treatments, and thus was a measurement of effects of fertility and cropping system on total extract composition (percentage of phytotoxic metabolites) not production. Therefore, this study suggests nutrient deficiencies lead to increases of benzoxazinone concentration in the total extractable metabolites present in rye. The increase in concentration could be due to a greater decrease in production of other extractable compounds rather than an increase in benzoxazinone production under nutrient stress. In fact, it is possible to conclude that adequate fertility may lead to an actual increase in benzoxazinone production along with an increase in other metabolites. If however, nutrient deficiency conditions were increasing the production benzoxazinones, this result would support the optimal defense theory.

DIBOA concentrations in grain rye decrease as the plant proceeds through its normal growth cycle. It is not fully understood if the decrease in DIBOA concentration is closely linked to the growth and development of the plant or is regulated by other signal pathways. In an effort to examine the relationship between DIBOA concentration and biomass production, Gianoli et al. (2000) looked at the effect of reduced pot volume on shoot biomass and

DIBOA. The authors grew plants under restrictive pot volumes and compared them to control treatments with adequate pot volume. They evaluated the effects of reduced pot volume at two stages of growth. (Two scales, Feekes and Zadoks, exist for determining the growth and development of small grains. The Feekes scale is commonly used in North America, while Zadoks is prevalent in the rest of the world; for a complete description of these scales see Zadoks et al. 1974 and the Southern Small Grains Resource Management Handbook found at http://www.ces.uga.edu/pubcd/B1190.htm.) Decreasing pot volume decreased shoot biomass as the plant proceeded through the growth stages. In the study, the effect of reduced pot volume on biomass accumulation was highly significant at Growth Stage (GS) 30 (Zadoks scale- leaf sheath erection), but not significant at the earlier GS 14 (four leaves unfolded). Those volume-restricted plants at GS 14 displayed elevated DIBOA concentration and total DIBOA content in comparison to the control treatment. Thus, rye responds to a limitation of resources at this early stage with an elevation in level of defense compounds. Such a response adheres to the principles of optimal defense theory. Rye plants at GS 30 had no significant difference in DIBOA concentration between volume-restricted and control treatments, and total DIBOA content was much greater in the control treatment, due to the higher biomass. These results match the predictions of optimum defense theory as well. Plants at later developmental stages are more robust and therefore in less need of chemical defense, whereas plants in juvenile stages of growth are highly vulnerable and in greater need of defense.

Defoliation is also known to affect the concentration of allelochemicals in rye. Collantes et al. (1997) found significant effects of defoliation and age on shoot DIBOA concentration.

The concentration of DIBOA increased under defoliation treatment and decreased as the plants matured. Optimal defense theory indicates that elevated stress levels will trigger an increase in chemical defense, and these results would confirm that prediction. These researchers also examined the contribution of the primary leaf to total biomass and DIBOA concentration in the plant. Defoliation did not have any significant effect on DIBOA concentration in primary leaves of rye. This result suggests that DIBOA production is primarily achieved through new tissue growth. The decreasing contribution of the primary leaf over time as a percentage of total DIBOA content supports the conclusion that the elevation of DIBOA content is achieved through new growth.

The same research group followed up on the defoliation study in 1999 (Collantes et al. 1999). Plants underwent a primary defoliation treatment (1X) and some were followed with a second defoliation treatment (2X). The biomass distribution differed significantly as a function of defoliation treatment. Shoot biomass was significantly lower in the multiple defoliation treatment than in the single defoliation treatment or in the non-defoliated control. Root biomass was the same for all treatments. Benzoxazinone concentrations in the shoot tissue of the treatments paralleled the results for shoot biomass. The consecutive defoliation treatments had a significantly lower benzoxazinone concentration in the shoots than either the non-defoliated or single defoliation treatment. However, the concentration of benzoxazinone derivatives in the root exudates of the consecutive defoliation treatment was significantly higher than the non-defoliated treatment. The allocation of benzoxazinones throughout the plant was also affected by the consecutive defoliation treatment. Less of the total benzoxazinone content was found in shoots of the multiple defoliation treatment, while

a greater percentage was found in both roots and root exudates in comparison to the other treatments. Plants under more severe attack respond with greater intensity than plants under more moderate stress. Optimal defense theory would dictate that the plants defoliated for the second time have limited above ground biomass and must defend themselves more vigorously than plants under control or single defoliation treatments. Both defoliation treatments increased the ratio of aglycone to glucoside. As mentioned previously, leaf injury or senescence brings the glucoside in contact with b-glucosidase through the disruption of cell membranes (Barnes et al. 1987, Yenish et al. 1995). Plants subjected to defoliation treatments would be expected to convert DIBOA-glucoside to the aglycone.

Burgos et al. (1999) examined the effects of cultivar and age differences on allelochemical content in rye. A wide range of differences in both biomass production and benzoxazinone content existed in the array of rye cultivars tested. The variety "Bonel" was found to have the highest DIBOA and BOA concentration at boot stage (Feekes 9). Bonel was also found to be a low biomass producer (seventh out of eight varieties tested). In bioassays, Bonel also had the highest activity on the species *Eleusine indica* (goose grass), however there was a weak linear relationship (R^2=0.50 p=0.04) between GR_{50} (concentration where growth rate is inhibited by 50%) and benzoxazinone concentration. This could be due to the presence of other, non-benzoxazinone, phytotoxic compounds. In addition, the concentration of benzoxazinone in the variety Bates, was found to peak at sixty days after planting. Peak concentration of benzoxazinones in relation to maturity was not examined for the other varieties. Differences in benzoxazinone production among varieties is not addressed by any

of the defense theories, but does suggest genetic differences lead to differences in benzoxazinone production.

Age, maturation, cultivar, and fertility effects were examined by Reberg-Horton et al. (2002). Ten, fall planted, cultivars treated with four nitrogen rates were harvested over the growing season at three separate dates, 105, 140, and 158 days after planting. The growth stages of the varieties at the time of the harvests ranged from 5 to 7 (Feekes) for the first harvest, 6 to 8 for the second harvest, and 9 to 10 for the final harvest. Both allelopathic activity bioassays and quantitative DIBOA analyses were performed on the sample tissues. The bioassay activity, as measured by root growth inhibition, decreased over time for both goose grass (*Eleusine indica*) and pigweed (*Amaranthus retroflexus*) indicator species. DIBOA concentrations decreased in parallel with the decline in bioassay activity. The rate of decline in phytotoxic activity differed by variety. The variety 'Wheeler' had a significantly less steep decline in activity over time. The other cultivars also differed in the rate of reduction of DIBOA concentration. Winter types of rye had a slower rate of decline between the first two sampling dates compared with the facultative and spring types. Wheeler, a winter type, had a slower rate of phonological development therefore at all sampling dates this cultivar was at a more juvenile growth stage than other varieties. As expected, biomass for all cultivars and treatments increased over time, as DIBOA concentration decreased. However, if results were expressed as the total quantity of DIBOA per unit area in a field, probably the best measure of the allelopathic potential of rye, the DIBOA/ area was found to increase for facultative varieties between the first two harvests. This suggests that there may be a peak time for chemical weed suppression by the rye when used as a cover crop. All fertility treatments

responded similarly, most likely due to high residual nitrogen present at the study site. One significant difference was detected however. The varieties Bonel, Wrens Abruzzi, and an unnamed NC variety had a significantly higher DIBOA concentration at the first sampling date than all other treatments. In the bioassay, no nitrogen effect was detected.

Reberg-Horton et al. (2002) also undertook a screening of rye varieties and accessions, where all varieties were harvested as they reached one specific growth stage, Feekes 9 (boot stage), over a period of 30 days. In this study, the highly toxic and slower developing Wheeler was not found to be the most toxic variety, suggesting that its principal advantage as an allelochemical producer lies in its slower maturation. Differences between cultivars do exist, indicating a difference in genetic allelopathic potential, and/ or effects of the environment at the time of harvest. These differences suggest that efforts to breed rye varieties with enhanced DIBOA content and allelopathic potential could lead to development of even better allelopathic rye cover crops.

Studies on environmental effects on benzoxazinone production in related species (wheat, maize, etc.) can also provide insight into the regulation of allelochemical production in rye. Although the patterns of allocation differ over the life cycle of the plant, and corn and wheat contain different forms of benzoxazinones, all benzoxazinone producing Gramineae species share common early steps in their respective biosynthetic pathways.

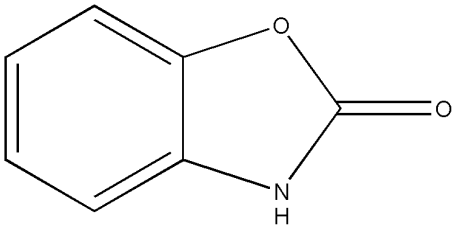
In 1997, Gianoli and Niemeyer examined the effects of aphid infestation and nitrogen fertilization on benzoxazinone production in wheat. Wheat plants at growth stages 10, 11,

and 12 (Zadoks) were infested with the bird cherry oat aphid (*Rhopalosiphum padi* L.). Treatments at each growth stage included two infestation periods, of 16 and 48 hours, and two nutrient regimes, high and low. Both aphid infestation and leaf age had significant effects on benzoxazinone levels. The 48-hour aphid treatment resulted in an increased level of benzoxazinones, and the GS 11 growth stage resulted in the highest levels of benzoxazinone metabolite concentrations. Once again, plants under elevated stress (herbivory) responded with an increased defense allocation adhering to the predictions of optimal defense theory. Benzoxazinone levels were elevated in the high nutrient level treatments, but the effect was marginally non-significant with a p-value of 0.061. No interactions of treatments were significant.

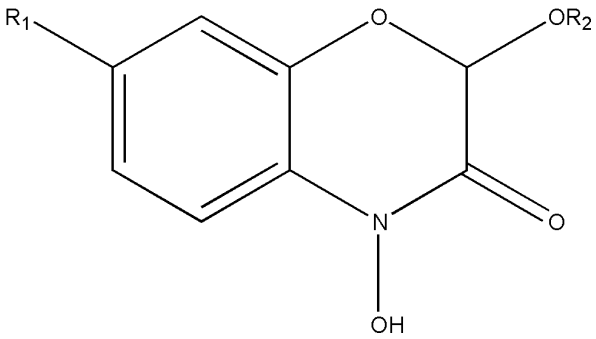
The same group also published a study examining the effects of temperature and photoperiod on benzoxazinone levels in wheat seedlings (Gianoli and Niemeyer 1997). Wheat plants were grown under three different temperatures (15^{O}, 20^{O}, and 25^{O} C) and three different photoperiods (8, 12, and 16 hours of light) in a factorial array. All shoot biomass was removed when a treatment reached growth stage 12 (Zadoks), and not at a set time across treatments. Total benzoxazinones accumulated directly with increasing temperature. This effect was highly significant ($p < 0.001$). The photoperiod treatments demonstrated no significant effects or trends, however the interaction of photoperiod and temperature effects was marginally significant ($p< 0.05$). Although initially the data seems to imply that increased temperatures or temperature stress increase benzoxazinone production, additional analysis using plant growth rate as a covariate demonstrated that the temperature effect was related to enhanced growth rate as opposed to temperature stress phenomenon. Therefore,

those plants in the elevated temperature environment had more rapid growth rates than those in the lower temperature environments, and those plants with the rapid growth rate had greater total benzoxazinone content. The authors suggest that the differences in benzoxazinone content are directly linked to growth rate and are not effects of the environment on the benzoxazinone metabolic pathway.

Information generated through the study of environmental and cultural impacts on allelopathy is useful for developing improved weed management strategies, especially strategies designed to reduce the use of conventional crop inputs. Unfortunately, findings regarding the production and regulation of allelopathic compounds and their activities in the plant environment are complex and do not lead to obvious and immediate improvements in weed management strategies. Additional work is needed to improve the understanding of both the production and regulation of allelochemicals within the plant. The release, persistence, and actions of these compounds within the plant environment also need further study. The findings of such studies should also be integrated into new weed management research strategies. The ability to harness these interactions will lead to improved production systems with reduced inputs. These improved methods will be a boon to sustainable agricultural practices.



BOA



DIBOA	R1=H	R2=H
DIMBOA	R1=OCH_3	R2=H
DIBOA-Glu	R1=H	R2=Glu

Figure 1
Benzoxazinones Present in Rye

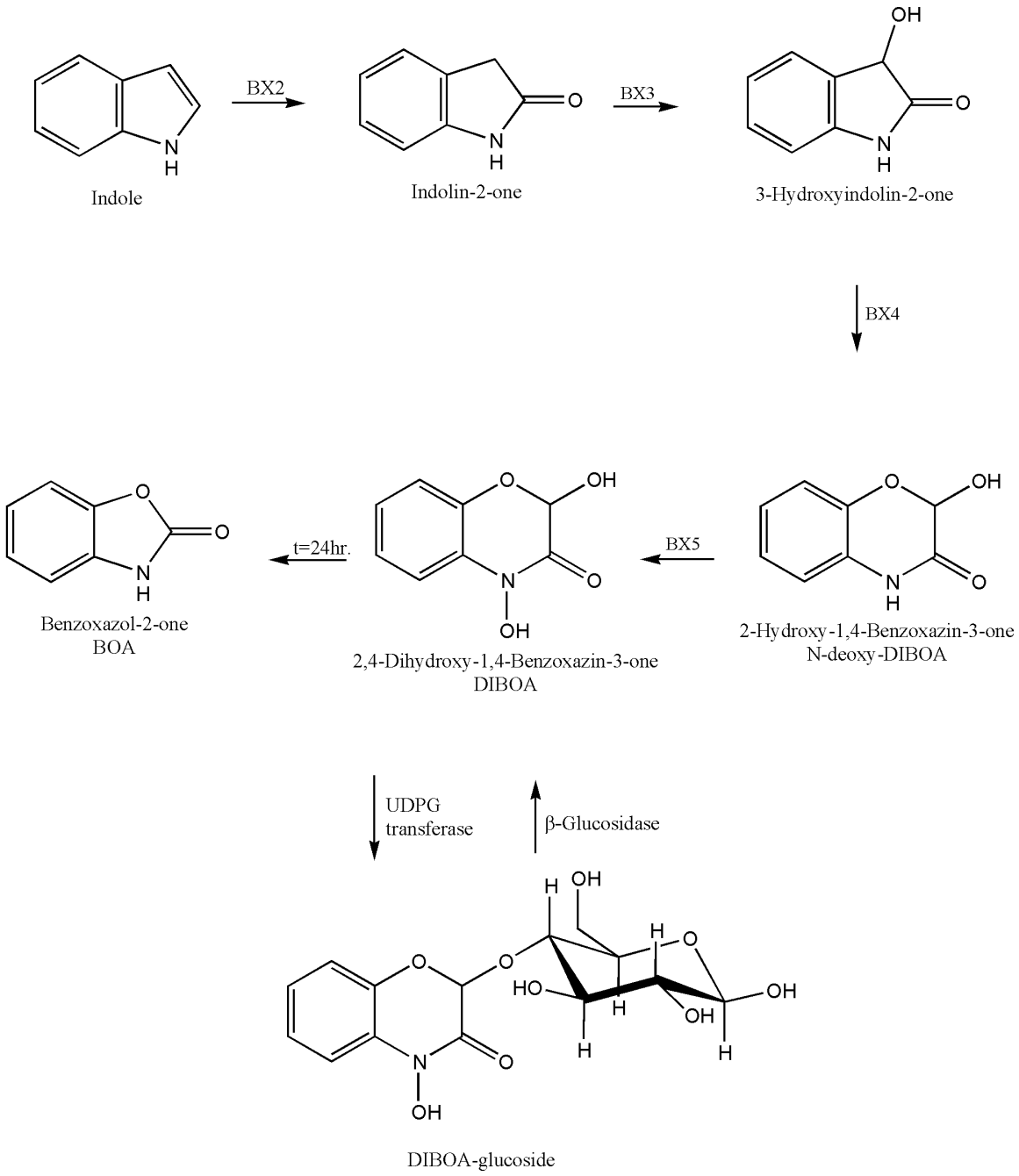


Figure 2
DIBOA Biosynthetic Pathway
Adapted from Masters-Moore 1999

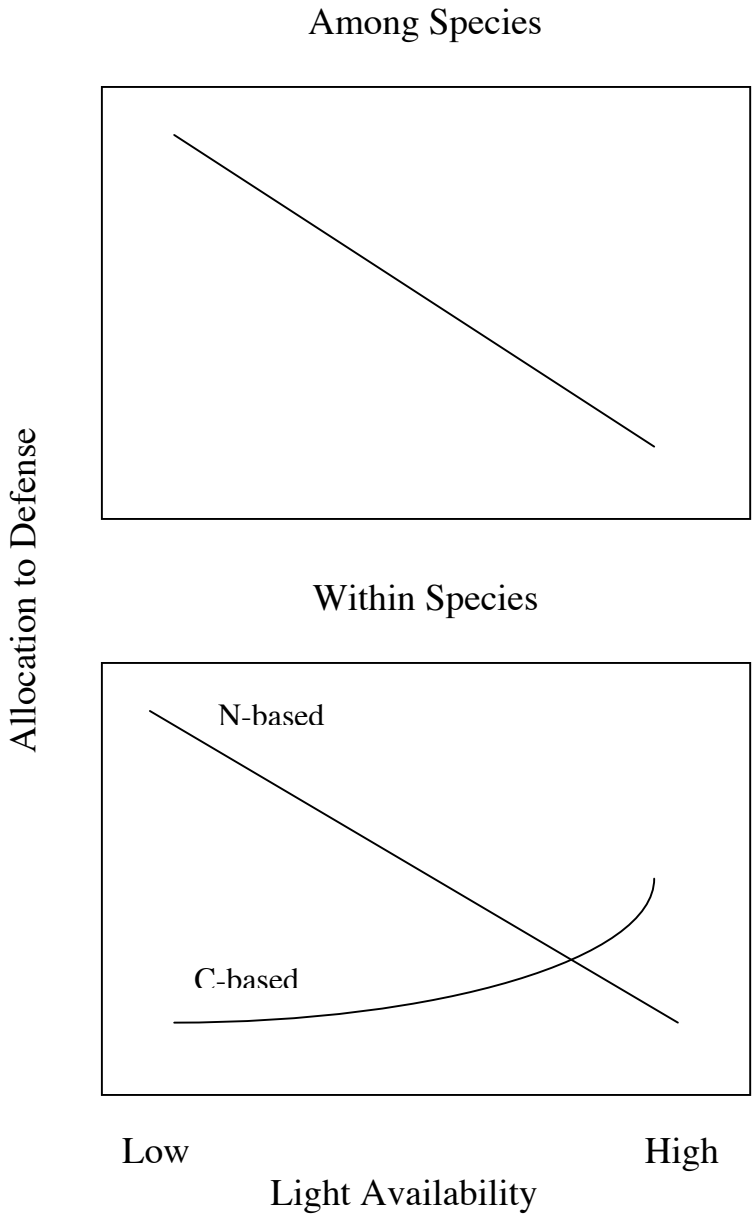


Figure 3
General Trends in Defense Allocation in Carbon / Nutrient Theory
Adapted from Bazzaz et al. 1987

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Chapter II

A Gas Chromatographic Method for the Analysis of Allelopathic Natural Products in Rye (Secale cereale L.)

For submission to: Journal of Chromatography A

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Abstract

Accurate and reproducible methods for the analysis of plant allelochemicals are a requirement for the study of chemical interactions between plants. This paper describes a method for sample preparation and quantitative analysis of the allelopathic chemical content of rye (*Secale cereale* L.). Sample preparation consists of extraction of freeze-dried rye vegetative tissue with aqueous ethanol followed by partitioning of the allelochemicals into an ethyl acetate, evaporation, and derivatization using the trimethyl silylating reagent MSTFA. GC analysis of the silylated mixture was performed using a 20M DB-5 megabore capillary column and a temperature program increasing from 100 to 300 C at 5^{O}/ min with detection by FID. Injector temperature was 250^{O} C and detector temperature was 325^{O} C. This method permits analysis of all known rye allelopathic agents including 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), its corresponding glucoside (DIBOA-glc), 2-benzoxazolinone (BOA), β-hydroxybutyric acid (*b*-HBA), and β-phenyl lactic acid (β-PLA). Identities of all compounds were confirmed by GC/MS analysis.

Keywords: Benzoxazinones, DIBOA, DIBOA-Glucoside, BOA, Phenyllactic Acid, Hydroxybutyric Acid, GC, Allelopathy, Hydroxamic Acids, Allelochemicals, Rye, *Secale cereale*

Introduction

Analysis of natural products responsible for weed suppression is essential for the study of allelopathy. Grain rye (*Secale cereale* L.) is often touted for its allelopathic qualities, and therefore is frequently used and studied as a weed-suppressive cover crop. A series of naturally-occurring benzoxazinones are thought to be primarily responsible for allelopathic weed suppression in cereal rye [1]. These compounds include: 2,4-dihydroxy-1,4-benzoxazin-3 (4*H*)-one (DIBOA CAS # 17359-54-5); its corresponding glucoside, 2-(2, 4-dihydroxy-1,4 (2*H*)-benzoxazin-3 (4*H*)-one)-β-D-glucopyranoside (DIBOA-glu), and benzoxaxolin-2-one (BOA CAS#59-49-4) (Fig 1). Within rye tissue, DIBOA is present in the vacuole, primarily in the stable, non-toxic, glucoside form [2]. Upon wounding or leaf senescence, cellular membranes break down releasing endogenous plant β-glucosidases that cleave DIBOA-glu forming the toxic aglycone, DIBOA [1]. In addition to the allelopathic benzoxazinones, other phytotoxic compounds have also been isolated from rye. The compounds β-hydroxybutyric acid (β–HBA), and β-phenyl lactic acid (β–PLA) have been identified as allelopathic agents [3] (Fig 1). No method for the quantitation of these additional allelopathic metabolites has been reported.

The benzoxazinones (DIBOA-Glu, DIBOA, and BOA) were first identified and studied in the 1960's as fungal resistance factors in cereal crops. Infection levels of Northern Corn Leaf Blight (*Helminthosporium turcicum*) are inversely correlated to benzoxazinone levels in maize (*Zea mays)*, and DIMBOA (2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one, CAS #115893-52-4), a benzoxazinone found in maize) extracts was shown to decrease germination of the spores [4]. Benzoxazinone derivatives have also been found to be

defensive agents against insect pests and bacterial pathogens [4,5]. In maize, resistance to the European Corn Borer (*Ostrinia nubilalis*) correlates with increased benzoxazinone levels in the plant. Aphid (*Rhopalosiphum madis*) resistance in maize increases when benzoxazinone content is elevated, and aphid resistance in wheat (*Titicum aestivum*) shows a similar correlation [4]. Effects of this class of compounds when added to insect diets include increased larval mortality, slowed development, and reduced offspring. Additionally, resistance to the corn borer decreased as the age of the maize increased and the benzoxazinone concentration declined [4].

In 1986, Barnes and Putnam first demonstrated the allelopathic potential of rye [6]. Residues of rye suppressed the emergence of lettuce (*Lactuca sativa* L.) and proso millet (*Panicum miliaceum* L.) more effectively than a wood shaving mulch control treatment. In addition to lettuce and millet, rye residue affected cress (*Lepidium sativum* L), and baryardgrass (*Echinochloa crus-galli* L.) to a lesser extent. The phytotoxicity of both rye residue and aqueous rye extracts manifested itself in reduced radicle elongation of each species.

Allelopathic weed suppression is a complex phenomenon that is only partially understood, and highly variable in a field environment. Previous studies that examined allelochemical production in annual rye under specific environmental or cultural conditions have provided some information on the role that environment plays in variability of allelopathic compounds and the resulting control of weeds in field settings, Levels of BOA metabolites in rye have been found to be affected by numerous environmental, cultural, and genetic factors. Previous work demonstrated differences in rye allelochemical production as a function of fertility [7],

growth stage [8, 9], growth rate[10], defoliation [11] and cultivar [8]. However, these studies, while valuable, do not provide a complete understanding of the variability in rye allelochemical production.

Reproducible quantitative analytical methods are an important tool in the study of allelopathic activity. Current methods of evaluating allelochemical content in rye focus on the benzoxazinones, and usually only DIBOA [7,9]. Most methods utilize aqueous tissue extraction that results in hydrolysis of DIBOA-glu to its corresponding aglycone, DIBOA [7-9, 11-13]. When done in aqueous solution, DIBOA can continue to degrade to BOA. The analyses detailed in Lyons et al. [14] and in Yenish et al. [15] describe HPLC methods for quantitation of benzoxazinone content including DIBOA-glu however, these methods of analysis do not include analysis of the β-HBA and β-PLA contained in the rye tissues. The following paper details a method for the quantitative analysis of all DIBOA metabolites as well as both β-HBA and β-PLA.

Experimental

Chemicals

Ethyl Acetate (EtoAc) and *N, N*-Dimethylformamide (DMF) (HPLC grade) were obtained from Fisher Scientific, Pittsburgh, PA. Absolute ethanol (EtOH) was obtained from AAPER (Shelbyville, KY). Standards of β−HBA, β−PLA, BOA, and octadecan-1-ol (Internal Standard) were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. The BOA standard was further purified by re-crystallization from HPLC-grade methylene chloride (Fisher Scientific). Synthetic DIBOA standard was prepared synthetically in the laboratory

of W. S. Chilton, NCSU Botany Department. DIBOA glucoside was isolated from 14 day-old rye seedlings using the method described below. For silylation of the samples, the derivatization reagent MSTFA was purchased from Pierce Chemical and mixed 1:1 with dry DMF.

DIBOA-Glucoside Isolation

Fourteen-day old rye seedlings (var. Bonel) were harvested then frozen with liquid N_2. The frozen biomass was stored at –80º C until extraction. Extraction of DIBOA-Glu from the frozen tissue was accomplished using 500 mL of chilled acetone (-20º C) per 500 grams frozen shoot tissue. The tissue and acetone were homogenized in a blender for approximately 2 min. After the mixture warmed to 4º C the tissue was briefly mixed again before filtering. The mixture was then filtered through 4 layers of paper towels, and then filtered through a Whatman #4 filter paper. The filtrate was then reduced *in vacuo* in order to remove the acetone. Waxes and chlorophylls remained adhered to the rotary evaporation sample flask while the DIBOA-Glu remained in the aqueous solution. The sample was then frozen at –20º C overnight. After freezing the sample was thawed and filtered through Whatman #4 filter paper to remove any remaining chlorophyll and waxes. After this filtration step the sample had light red coloration. This solution was then re-filtered through a 20um nylon membrane filter and injected onto a 20cm X 3cm LiChroprep RP-8 column (E. Merck, Darmstadt Germany). A mobile phase of 85% H2O/15% MeOH was pumped through the column at a rate of 2 mL/ min. Detection was set at 262 nm and all fractions were collected and saved. Fractions corresponding to the DIBOA-Glu peaks were collected and pooled. The pooled fractions (24 mg/ mL) were then reduced to dryness under vacuum,

and dissolved in MeOH and stored at -20° C. For re-crystallization, the MeOH solution was then evaporated under N_2 and the residue dissolved in a minimum volume of hot MeOH. Hot toluene was then added drop-wise until the mixture remained cloudy. The MeOH/Toluene solution was then filtered through a nylon membrane (0.45 μm) and the sample evaporated under N_2 stream at 40° C leaving a light brown powder. The authenticity of the sample was verified by GC retention time and GC-MS analysis of the silylated derivative (see below).

Analytical Sample Preparation

Fresh plant tissues were harvested from field or growth chambers, placed into labeled paper bags, and immediately frozen at –68° C. Frozen tissues were freeze-dried using a modified Virtis Freeze Drying apparatus. The freeze-dried tissue was ground using a Wiley mill (20-mesh sieve) and the ground tissue was then stored in plastic bags at -20° C. Approximately 0.5000g dried rye shoot tissue was weighed and transferred into empty 75 mL SPE (Alltech) reservoirs fitted with bottom frits (20 μm porosity). After adding 10mL of 50% ethanol solution the reservoir was mixed with a vortexer for one minute, the extract was then filtered through the reservoir by attaching it to vacuum manifold (VWR Scientific). An additional 5.0 mL of DI H_2O, was then allowed to filter through the extracted tissue and the washed tissue was allowed to air dry under vacuum The filtrate was then transferred to a 25 mL volumetric flask, and brought to volume with DI H_2O. A 10.0 mL aliquot of the filtrate was partitioned three times with equal volumes (5.0 mL) of EtOAc. The ethyl acetate layers were combined and dried overnight with sodium sulfate.

A 10.0 mL aliquot of dried EtoAc and 750μL of 200μg/mL octadecanol internal standard dissolved in Toluene were reduced to dryness under a N_2 stream at 40^O C. 400 μL of 1:1 MSTFA:DMF were added to the sample/internal standard residue, the samples were capped under N_2, and then heated at 75^O C for 30 min. Samples were then transferred to autosampler vials for GC analysis.

Gas Chromatographic Analysis

GC analysis was used to resolve and quantify compounds in the plant extract. An Agilent 6890N gas chromatograph equipped with a 20 M DB-5 megabore column (0.53 mm diameter, 1.5 μm film thickness, J&W Scientific was used for analysis. 0.5 μL of the derivatized sample was analyzed using splitless injection and an injector temperature of 250^O C. Flame ionization detection was utilized with a detector temperature of 325^O C. Column flow of the Helium (UHP) carrier gas was set to a linear gas velocity of 43.0 cm/second. The initial oven temperature was 100^O C, followed by a 5°/minute temperature increase to 300^O C final temperature. The oven temperature increase began upon injection and the final temperature was maintained for 30 minutes. Data was collected using the Perkin-Elmer TotalChrom 6.2 system. Quantitation of peaks was done using the internal standard method. Appropriate multi-level calibration curves were run for BOA, DIBOA, DIBOA-glu, β-PLA, and β-HBA.

CG/MS

GC/MS analysis was carried out on a mixed standard of compounds of interest and representative plant samples to confirm the identity of component peaks. Sample preparation

and derivatization was as described above. Analyses were performed on a HP 5890 GC equipped with a HP 5970 Mass Selective Detector operating in the EI mode at 70eV. The mass range was set from 50 to 600 and data was collected at 1.1 scans per second. Component peaks were separated on a 30M DB-XLB 0.25mm ID, and 0.25μm film thickness (J&W Scientific) capillary column with Helium carrier gas flowing at a linear gas velocity of 37.1 cm/ second. Initial oven temperature was 100°C immediately increasing at a rate of 5°/minute to 300°C. The final temperature was held for 15 minutes.

Results and Discussion

Limits of detection were determined to be 12.9 nmol for DIBOA, 57.9 nmol for DIBOA-Glu, and 139 nmol for BOA. Equations for the standard curves were BOA- $f(x)=1146000x - 379900$, $R^2=0.984$, DIBOA- $f(x)=1560000x - 44020$, $R^2=0.999$, DIBOA-Glu- $f(x)=2255000x - 319200$, $R^2=0.999$. Retention time matching of standards and GC/MS analysis were used to confirm peak identity. The sample preparation method provided a consistent and rapid method for extraction of rye shoot tissue for all known allelopathic compounds. The initial extraction of the sample preparation procedure removed 96% of total DIBOA in the tissue. This procedure was developed with the intent of using it in a high throughput system for analyzing large numbers of samples including the analysis of accessions and crosses in a breeding program to develop rye varieties optimized for allelopathic potential and cover crop use. This method is consistent and reproducible and emphasizes extraction of benzoxazinone compounds, while also providing an analysis of other known allelochemicals should they be more abundant in other varieties or crosses. The ability to simultaneously examine DIBOA-Glu and the corresponding aglycone allows

study of the breakdown and transformation of the benzoxazinones within the plant providing a useful tool for the examination of developmental and environmental effects on the hydrolysis of DIBOA glucoside. Such studies will provide a better understanding of allelopathy in cereal rye and will hopefully lead to improvements in its use as an effective and consistent weed control tool for use in sustainable agronomic systems.

Peak identities were confirmed by GC/MS analysis of the TMS derivatives. Mass fragments of components were compared to the mass fragmentation of known standards,-published MS data (Table 2), and through comparison with fragmentation data contained in the NBS 75K and Wiley 275 libraries. The common TMS fragments of m/z 73 and 147, which are typically the largest fragment ions in most of the mass spectra examined were not considered for the base fragment ion.

β–HBA: m/z 233 (22%), 191 (60%), 147 (209%), 133 (27%), 117 (100%), 88 (29%), 73 (176%).

BOA: m/z 257 (37%), 247 (33%), 207 (67%), 192 (100%), 164 (85%), 147 (96%), 100 (33%), 73 (348%).

β–PLA: m/z 295 (125), 267 (15%), 220 (24%), 193 (100%), 147 (85%), 131 (5%), 91 (22%), 73 (166%).

N-deoxy-DIBOA: m/z 310 (28%), 309 (100%), 294 (26%), 280 (13%), 266 (30%), 208 (15%), 192 (26%), 191 (13%), 165 (19%), 147 (55%), 73 (125%).

DIBOA: m/z 325 (31%), 311 (25%), 310 (100%), 208 (21%), 192 (21%), 164 (25%), 147 (31%), 73 (142%).

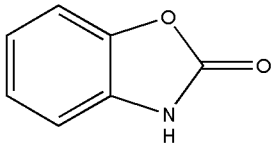
DIBOA-Glucoside: m/z 362 (34%), 361 (100%), 331 (20%), 309 (17%), 271 (14%), 236 (42%), 217 (31%), 169 (17%), 147 (31%), 129 (17%), 103 (20%), 73 (120%)

Notably, N-deoxy-DIBOA (R.T. 16.64) was detected at low levels in samples analyzed using this method. This compound is the immediate precursor to DIBOA and demonstrates the potential utility of the method described herein in studying benzoxazolinone metabolism. Both β-HBA and β-PLA were detected at low levels confirming their presence in rye shoot tissue as a possible allelochemicals [3]. Other major components identified by comparison with fragmentation patterns of known compounds found in the NBS 75K and Wiley 275 GC-MS libraries included palmitic acid (R.T. 24.6 min), 4-methoxy cinnamic acid (R.T. 25.6 min), terpene alcohol (R.T. 26.9 min), linoleic acid (R.T. 27.7 min), and β-sitosterol (R.T. 44.5 min).

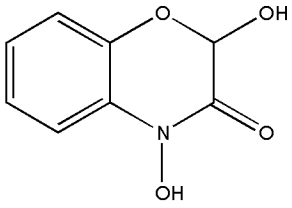
The identity of the DIBOA-glu peak was confirmed by hydrolysis of the glucoside-containing fraction into the aglycone and glucose. Plant material high in DIBOA-glu content and relatively low in DIBOA and BOA content was subjected to enzymatic hydrolysis and analysis. A rye tissue extract was treated with an aqueous β-glucosidase solution for 24 hours before adding absolute EtOH to stop the reaction. The sample was worked up using the standard extraction and analysis procedure. After 24 hrs the DIBOA-glu peak had decreased to 4% of its original area while the DIBOA and BOA peaks had increased by 957% and 1133% respectively (Table 1).

GC analysis gives good resolution of both the early and late eluting compounds. This is important due to the wide range of retention times of the compounds of interest (Fig. 2).

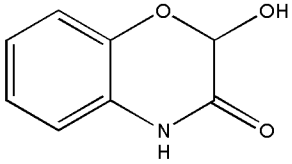
Since equal peak areas of the glucoside and aglycone peaks do not represent equivalent allelopathic activity, it is suggested that the total benzoxazinone concentration should be presented in millimoles per gram of dried plant tissue.



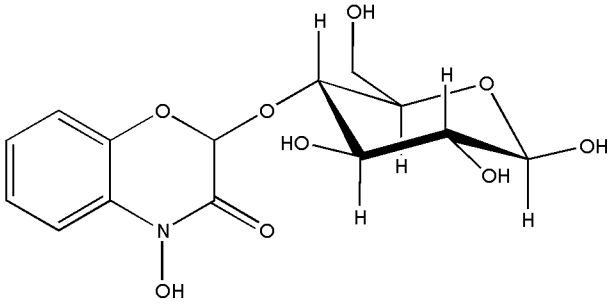
Benzoxazol-2-one



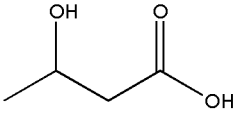
2,4-Dihydroxy-1,4-Benzoxazin-3-one



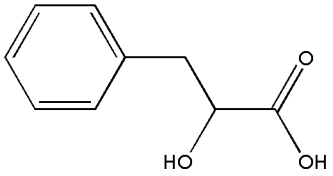
2-hydroxy-1,4-Benzoxazin-3-one



2,4-Dihydroxy-1,4-Benzoxazin-3-one glucoside



β-Hydroxybutyric acid



β-Phenyllactic acid

Figure 1
Known Allelopathic Agents Present in Rye

Table 1
Enzymatic Hydrolysis of DIBOA-Glu

	BOA Peak Area	DIBOA Peak Area	DIBOA-Glu Peak Area
Pre-hydrolysis	14916.05 μv*s	559578.77 μv*s	5501559.06 μv*s
Post-hydrolysis	180123.47 μv*s	2612122.21 μv*s	148367.76 μv*s

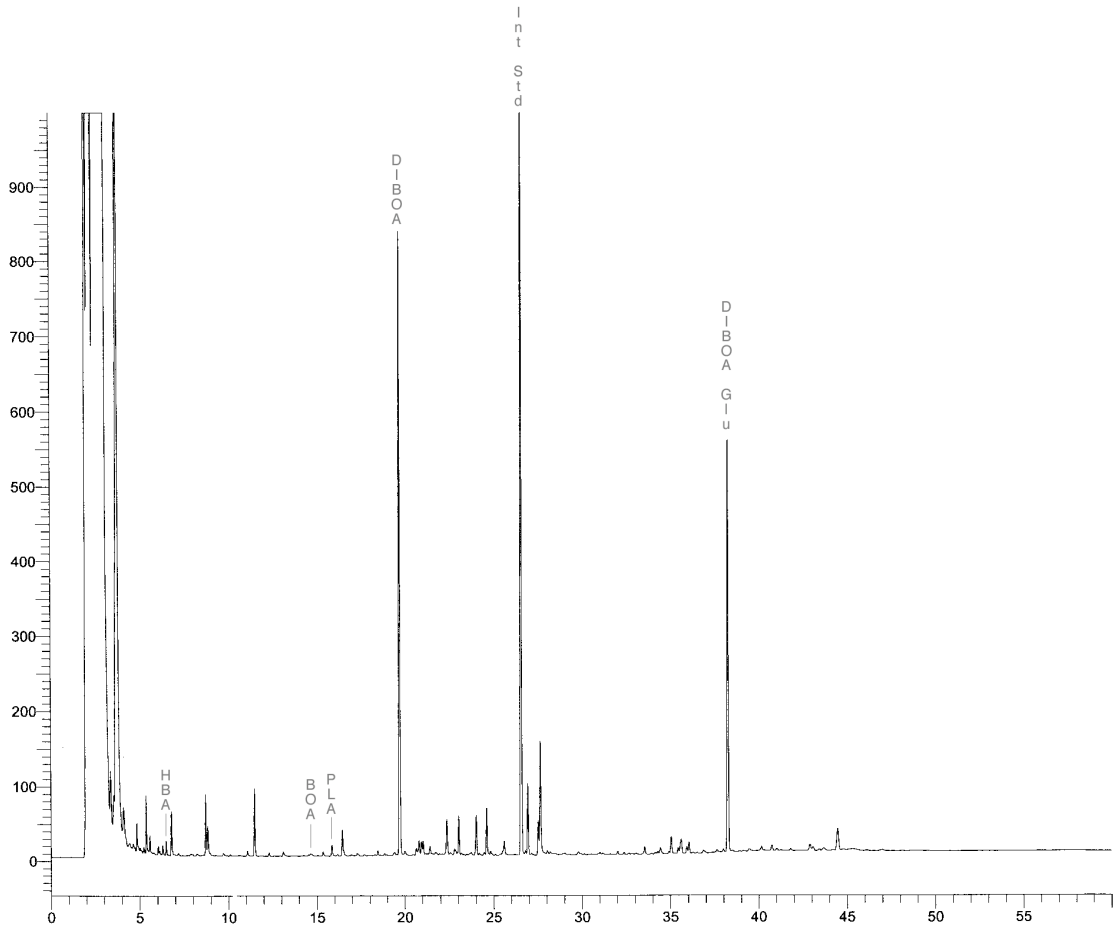


Figure 2
Rye Sample Chromatogram

Table 2
Previously Published Mass Fragments of Rye Allelochemicals

Compound	Retention Time	MW	MW of TMS der	Major Mass Fragments Derivatized- TMS (E. I.)	Major Mass Fragments Non-Derivatized (E.I.)
β-HBA	6.32 min	104	248	45, 73, 88, 101, 117, 147, 191, 204, 217, 233 [3]	
BOA	14.64 min	135.12			
β-PLA	15.51 min	166	310	45, 73, 91, 103, 133, 147, 161, 177, 193, 205, 220, 235, 253, 267, 295 [3]	135, 52, 51 [14]
DIBOA	19.70 min	181.15	325.39	325, 310, 208, 192, 179, 164, 147, 136, 73 [8]	181, 165, 152, 149, 136, 135, 109, 108 [14] 181, 165, 163, 135 [1]
DIBOA-glu	38.23 min	356.27	716.87		343, 327, 165, 163 149, 136, 135, 120, 109 [14]

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Chapter III

The Effect of Temperature, Fertility, and Water Status on Production of Alleleopathic Natural Products in Rye (*Secale cereale* L.)

Introduction

Rye (*Secale cereale* L.) is a commonly used cover crop. Rye's utility as a cover crop is due to both high biomass production, which increases soil organic matter as well as impeding weed seed germination and growth as well as its widely recognized allelopathic capabilities. In 1986, Barnes and Putnam clearly demonstrated the allelopathic potential of rye. In a laboratory study, residues of rye suppressed the emergence of lettuce (*Lactuca sativa* L.) and proso millet (*Panicum miliaceum* L.) more effectively than a wood shaving mulch control treatment (Barnes and Putnam 1986). The following year, Barnes et al. (1987) identified the principal phytotoxic compounds contained in rye residue. The two compounds were identified as 2,4-dihydroxy-1, 4(2*H*)-benzoxazin-3-one (DIBOA, CAS # 17359-54-5) and 2(3*H*)-benzoxazlinone (BOA, CAS#59-49-4) [Fig. 1]. Each of these compounds inhibited radicle elongation in cress (*Lepidium sativum* L). However, DIBOA alone inhibited the growth of barnyardgrass (*Echinochloa crusgalli* L.) (Barnes et al 1987).

DIBOA and BOA are members of a class of compounds known as benzoxazinones. BOA is a less phytotoxic breakdown product of DIBOA (Niemeyer 1988). These compounds were first identified and studied in the 1960's as fungal resistance factors in cereal crops. Benzoxazinone derivatives have also been found to have biological activity against insect pests and bacterial pathogens (Neimeyer 1988, Niemeyer and Perez 1995).

Benzoxazinones are present in all parts of the plant, but the seed (Niemeyer 1988). Benzoxazinone levels increase after germination and typically reach maximum concentration a few days after germination in maize and wheat; rye reaches maximum concentration later in its growth and development (Niemeyer 1988). Benzoxazinones are usually stored in plant tissue in an inactive glucoside form. Glycosylation typically occurs at the 2-postion of the azinone ring system. Upon wounding or leaf senescence glucosidases are released, the glucoside is cleaved, and the active benzoxazinone aglycone is released into the environment (Barnes et al 1987).

More recently, researchers have begun to study the influence of environmental factors on allelopathy. Environmental factors alter allelopathic interactions through effects on the allelopathic plant, the target species, and the transfer of the agents between the plants. Several established theories address the influence of environment on allelochemical production. Many studies have shown allelopathic plant species increase production of phytotoxic secondary metabolites when subjected to environmental stresses such as mineral deficiency, extreme temperatures, moisture stress, or extreme light levels (Einhellig 1989).

Previous studies examining the effect of environment on allelopathy in rye provided information on the production of allelopathic compounds, principally dihydroxybenzoxazilinone (DIBOA), its corresponding glucoside (DIBOA-glucoside), and their breakdown product benzoxazilinone (BOA). Studies have demonstrated differences in rye allelochemical production according to fertility (Mwaja et al. 1995), growth stage

(Burgos et al. 1999; Gianoli et al. 2000), growth rate (Gianoli and Niemeyer 1997), defoliation (Collantes et al. 1999) and cultivar (Burgos et al. 1999). However, a complete understanding of environmental factors on rye allelochemical production has yet to emerge. For example, Reberg-Horton et al. (2002) found no effect of nitrogen rates on the toxicity or production of benzoxazinones, but Mwaja et al. (1995) found increased toxicity of rye extracts grown with low added nitrogen. Burgos et al. (1999) demonstrated cultivar differences in benzoxazinone production, but it is unknown if varieties may respond differently according to the environment. No data exists on the impact of moisture stress in rye, but corn (*Zea mays* L.) seedlings grown under moisture stress had increased levels of benzoxazinones (Richardson and Bacon 1993). Better understanding of the production and regulation of allelochemicals in rye under varying environmental conditions is necessary to improve the efficacy of cover crops in weed control. The purpose of this study is to examine rye allelochemical production in two cultivars under a variety of environmental conditions, specifically fertility, water status, and temperature.

Materials and Methods

Two rye cultivars, Wheeler and Wrens Abruzzi, were grown under three temperature regimes (low, mid, and high). Seeds of each variety were planted in plastic pots (1650 mL) and given nutrient solution [Table 1] once per day for 14 days in a controlled environment at 17^{O} C to allow for germination and early growth. After14 days, plants were placed into three controlled environment chambers each at day/ night temperatures of $12^{O}C/5^{O}C$ (A), $17^{O}C/6^{O}C$ (B), and $22^{O}C/10^{O}C$ (C), respectively. Plants in each chamber were arranged according to a modified split-split plot design. The main plot factor was harvest, sub-plot

factor was variety, and sub-sub-plot factor was watering/ nutrient regime replicated within harvest and variety combinations. In each temperature treatment, the plants were grown under treatment from four different watering/ nutrient regimes (high water/ high nutrient levels, high water / low nutrient level, low water / high nutrient level, and low water / low nutrient level) in order to induce moisture and/ or nutrient deficiency stresses. Administration of water/ nutrient treatments was determined by visual examination of plant and soil conditions by the investigator. Harvests of shoot biomass were taken at three growth stages throughout the growing season to determine the season long effects of the environmental conditions on allelochemical content. The designated growth stages were 3- tillering, 7- jointing, 9-boot (Feekes scale). After 59, days temperatures in all three chambers were increased to: $22^{O}C/10^{O}C$ (A), $27^{O}C/15^{O}C$ (B), and $32^{O}C/19^{O}C$ (C). The increase in temperature was modeled after average (1984-1996) spring temperature increases at the, National Weather Service weather station data in Goldsboro, NC (3520N 07758W). The initial day/ night temperatures were based on the average March high and low temp and the increase in temperatures based on the May high and low temperatures. The middle temperature chamber was set at the March and May day/ night means the low temperature chamber was the mean temperatures minus five degrees and the high temperature chamber the means plus five degrees.

Analysis

Analysis of plant chemical constituents was done by the method described in Chapter II. Briefly, fresh plant tissues were harvested from the growth chambers, placed into paper bags, and stored at -68^{O} C. Frozen tissues were freeze-dried and ground using a Wiley mill

equipped with a 20-mesh screen. 0.5 grams of freeze-dried rye shoot tissue were weighed using an analytical balance, transferred to a capped test tube and extracted with 10mL of 50% ethanol solution. The extracted tissue was filtered using a 75 mL SPE reservoir (Alltech) equipped with a 20 μm frit. An additional 5.0 mL of DI H_2O, was used to wash the tissue and the filtrate collected. The extract was diluted to 25 mL with DI H_2O. A 10.0 mL aliquot of the filtrate was partitioned 3 times with equal volumes (5 mL) of ethyl acetate. The ethyl acetate layers were combined and any residual H_2O removed overnight over anhydrous sodium sulfate.

A10 mL aliquot of dried EtOAc solution and 750μL of 200μg/mL (toluene) octadecanol internal standard were reduced to dryness under a N_2 stream at 40° C.
The dried residue was derivatized with 400 μL of 1:1 MSTFA:Dimethylformamide.
The samples were capped under N_2 and heated at 75° C for 30 min. Silyated samples were then transferred to autosampler vials for analysis.

GC analysis was used to resolve and quantify compounds in the plant extract. An Agilent 6890N gas chromatograph equipped with a 20 M DB-5 column (0.53 mm diameter, 1.5 μm film thickness, J&W Scientific) was used for analysis. 0.5 μL of the derivatized sample was analyzed using split-less injection and an injector temperature of 250° C. Flame ionization detection was utilized with a detector temperature of 325° C. Column flow of the Helium (UHP) carrier gas was set to a linear gas velocity of 43.0 cm/second. The initial oven temperature was 100°C, followed by a 5°/minute temperature increase to 300° C final temperature. The oven temperature increase began upon injection and the final temperature

was maintained for 30.0 minutes. Data was collected using the Perkin-Elmer TotalChrom 6.2 system. Quantitation of peaks was done using the internal standard method. Standards of DIBOA, DIBOA glucoside, and BOA were either purchased or prepared as described in Chapter II. Individual benzoxazinone contributions (mM of DIBOA, DIBOA-glucoside, and BOA) were summed and the effect of treatments was expressed as the change in total benzoxazinone content (mmol) per gram dry weight of tissue. Analysis of variance (Proc GLM SAS 6.2, Cary, NC) was carried out on concentration and log transformed ratios, with Harvest tested against Rep within Harvest, Variety and Variety by Harvest tested against Rep by Variety within Harvest, and all other effects tested against Error.

Results

Two subsets of the data were analyzed; this is due to the large amount of lost treatments resulting from the elevation of temperatures mid-study. The "high" and "middle" (temp. levels '2' and '3') temperature treatments were well beyond the optimum growth temperatures for rye. Even with frequent watering, plants in these chambers grew extremely slowly or died. The first three harvests in the low temperature chamber are treated as one subset of data. The first harvest across all three temperatures chambers is also treated as a subset.

In the low temperature dataset the addition of nutrient solution had the greatest effect ($F_{1, 51}$= 181.64, $p < 0.0001$) on DIBOA production. Rye grown under high fertility had the greatest concentration of total benzoxazinones at all three harvests [Table 2]. Water status was also important to allelochemical production ($F_{1, 51}$= 81.10, $p < 0.0001$). Those plants under

moisture stress (- H_2O) had higher total benzoxazinone concentration than those plant grown under optimum moisture availablitiy. Growth stage and maturity of the plant was also important to benzoxazinone production ($F_{2, 51}$= 25.41, $p < 0.0002$). Peak levels of benzoxazinone concentration occurred at the second harvest (Feekes stage 7). The harvest by nutrient interaction ($F_{2, 51}$= 17.03, $p < 0.0001$) was significant. The change in concentration as the rye matures is greater in the high fertility treatment than in the low fertility treatment [Fig 2]. Other two- way interactions of interest are the harvest by moisture level ($F_{2, 51}$= 9.30, $p <0.0004$), and the harvest by variety ($F_{2, 51}$=4.92, $p <0.0359$) interactions. The harvest by moisture level interaction appeared similar to the harvest by nutrient level interaction with the moisture stressed (-H_2O) treatment increasing in concentration more rapidly as the plant matured [Fig 3]. The harvest by variety interaction was significant due to the steeper decline in concentration at GS 9 in the variety 'Abruzzi' [Fig 4].

The first harvest dataset also demonstrated that fertility had the greatest overall effect on benzoxazinone production ($F_{1, 53}$= 71.28, $p < 0.0001$) with the high fertility treatment having the largest benzoxazinone concentration [Table 3]. Temperature was also important ($F_{2, 53}$= 26.39, $p < 0.0002$) as higher temperatures led to elevated benzoxazinone concentrations. Moisture status was also important ($F_{1, 53}$= 14.40, $p < 0.0004$). Those plants grown under moisture stress had higher benzoxazinone concentrations. The variety by temperature interaction ($F_{2, 53}$= 8.45, $p < 0.0086$) was of interest due to the response of the variety Abruzzi to an increase in temperature. In 'Abruzzi', the mean concentration of benzoxazinones in response to increased temperature dropped from temperature level 1 (low) to temperature 2 (mid.). However, 'Wheeler' followed a linear progression of benzoxazinone concentration

increasing with temperature [Fig 5]. There were two additional two-way interactions of significance, nutrient level by moisture level ($F_{1, 53}$= 8.29, p< 0.0058) and temperature by nutrient level ($F_{2, 53}$= 7.27, p< 0.0016). The significance of fertility by moisture status interaction occurs because there was no difference in the benzoxazinone concentration under the high fertility treatment, while under low fertility, moisture stressed plants had higher concentrations of benzoxazinones [Fig 6]. The temperature by fertility interaction was significant due to the larger slope from the 'mid' to 'high' temperature under the high fertility treatment than in the low fertility treatment [Fig 7].

To determine the form of benzoxazinone present in the plant as a function of the treatments a ratio of mmol BOA + mmol DIBOA / mmol DIBOA-Glu was calculated for all treatments. In both sets of data, fertility level was very important (First Harv.- $F_{1, 53}$= 133.43, p< 0.0001, Low Temp. $F_{1, 51}$= 77.7, p < 0.0001) to the ratio. The high fertility treatment had increased ratio values, signaling a shift to the DIBOA and BOA forms of benzoxazinones. Moisture status also had an effect on the ratio ($F_{1,53}$= 23.7, p< 0.0001) in the first harvest dataset [Table 3]. The plants grown with abundant water had an increased ratio. In the low temperature dataset the moisture status was not important [Table 2]. Growth stage of the plant also had a significant effect ($F_{1, 51}$= 34.50, p< 0.0001) on the form of benzoxazinone. As the plants developed, an increasing amount of benzoxazinones were present in the BOA and DIBOA form.

Discussion

In both sets of data, the addition of nutrient solution had the greatest effect on benzoxazinone concentration. Since the benzoxazinones are nitrogen-containing molecules, the carbon/nutrient theory of defense allocation would predict that any abundance of nitrogen beyond that which is needed for growth could be utilized in benzoxazinone production. Results of this study lend support for this theory of defense allocation. Mwaja et al. (1995) found increased toxicity of rye extracts when grown under low-level fertility treatments. However, in rye tissue bioassays shoot biomass from the medium and high fertility treatments was more toxic. Quantitative analysis of the tissue also indicated rye grown under the medium fertility treatment had the highest concentration of DIBOA (Mwaja et al 1995).

The higher concentration of benzoxazinones in moisture stressed plants indicated a response consistent with the predictions of optimal defense theory. However, the carbon/ nutrient theory could also apply in that moisture stress lead to a reduction in growth rate; therefore any nitrogen that may normally be utilized for growth was directed to defense. Support for the carbon / nutrient theory also comes from Richardson and Bacon (1993). They showed that benzoxazinone concentrations increased as growth rate decreased in moisture stressed corn seedlings.

In the first set of harvests across all three temperatures, elevated temperatures led to increased benzoxazinone concentration, thus adhering to the principles of optimal defense theory. If the elevated temperatures caused a reduction of plant growth rate, the increase in benzoxazinone concentration could be due to nitrogen normally utilized in growth and

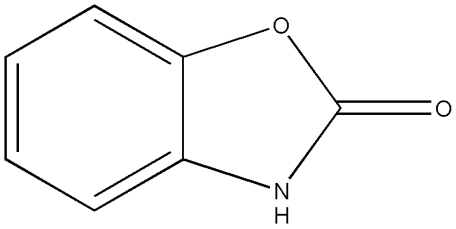
development being directed toward defense. However, a previous study of temperature effects on hydroxamic accumulation in wheat showed that significance of the temperature effect disappeared when plant growth rate was used as a covariate (Gianoli and Niemeyer, 1997). Since plant growth rate was not measured in this study it is not possible to determine if growth rate interacts with temperature with regard to benzoxazinone production.

The change in benzoxazinone concentration as the plant grows and develops is to be expected. Previous studies have shown the rye cultivar 'Bates' reaching peak benzoxazinone concentration at 60 days after planting (Burgos et al. 1999). No indication of growth stage at day 60 is given. Both varieties used in this study reached the peak concentration at harvest 2 (Feekes 7). The ability to continue to accumulate benzoxazinones fairly late into development is one of the principle benefits of using rye as a winter cover crop. Benzoxazinone concentration was evaluated over the life cycle of the plant to test for interactions of growth stage with other factors, and no interactions with harvest were significant.

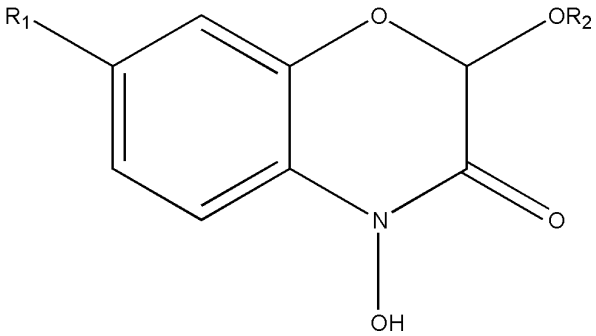
Increased leaf scenesence as the plant ages likely accounts for the shift in forms to the aglycone. The reason for the nutrient addition effect is less clear. Treatment effects on BOA levels were insignificant and levels of BOA in the tissue were very low across all treatments. The breakdown of DIBOA to BOA occurs over time and is not mediated by an enzyme. Therefore, it is unlikely the shift in form was due to an effect of the extraction or analysis procedure. A β-glucosidase enzyme catalyzes the shift from DIBOA-Glu to the aglycone. No data exists on the effects of elevated nutrient levels on the activity of the

enzyme within the plant. However, Fauci and Dick (1994) found β-glucosidase activity in soil was elevated in proportion to the addition of inorganic nitrogen. Increased β-glucosidase activity under elevated nitrogen levels would explain the shift from the glucoside to the aglycone. In DIBOA-Glu biosynthesis UDPG transferase mediates the reaction to form the glucoside from the aglycone. No data exists on the impact of elevated nutrient levels on the enzyme activity. Reduced activity of this enzyme could be another reason for the change in ratio.

These results can be used as a basis for additional study of the impact of environmental factors on allelochemical production in rye. Ultimately better understanding of allelochemical production can lead to improvements in weed suppression in cover crop systems. In particular, additional experiments in timing and level of nitrogen applications would be of great benefit to growers using cover crops.



BOA



DIBOA	R1=H	R2=H
DIMBOA	R1=OCH_3	R2=H
DIBOA-Glu	R1=H	R2=Glu

Figure 1
Benzoxazinones Present in Rye

Table 1
Nutrient Solution Analysis

Element	Conc (ppm)
N	106.23
P	10.41
K	111.03
Ca	54.40
Mg	12.40
Fe	5.00
S	13.19
Mn	0.113
B	0.24
Zn	0.013
Cu	0.005
Co	0.0003
Mo	0.005
Na	11.04

Table 2
Main Effect Means of Low Temperature Data

	GS 3	GS 7	GS 9	GS lsd (.05)	High Fert.	Low Fert.	Fert. lsd (.05)	High H2O	Low H2O	H2O lsd (.05)
[Bx]mmol/Kg	6.685	9.653	8.025	0.729	10.188	6.097	0.595	6.769	9.503	0.595
Ratio	0.377	0.554	0.59	0.057	0.607	0.408	0.047	0.529	0.484	0.047

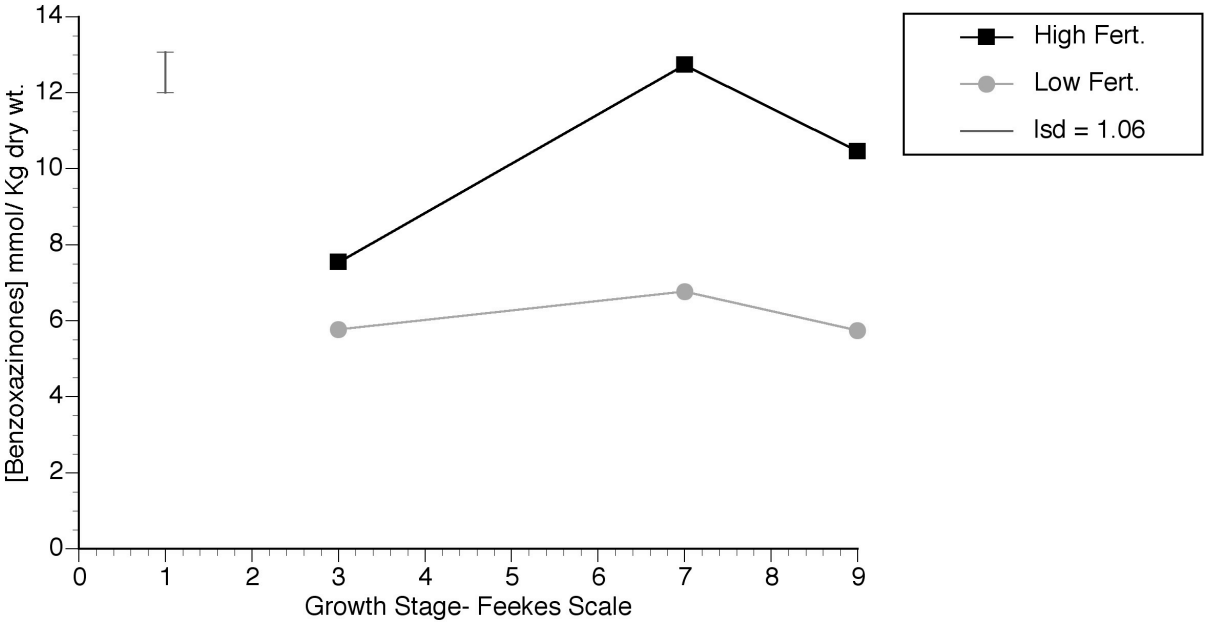


Figure 2
Growth Stage by Fertility Interaction, Low Temperature Dataset

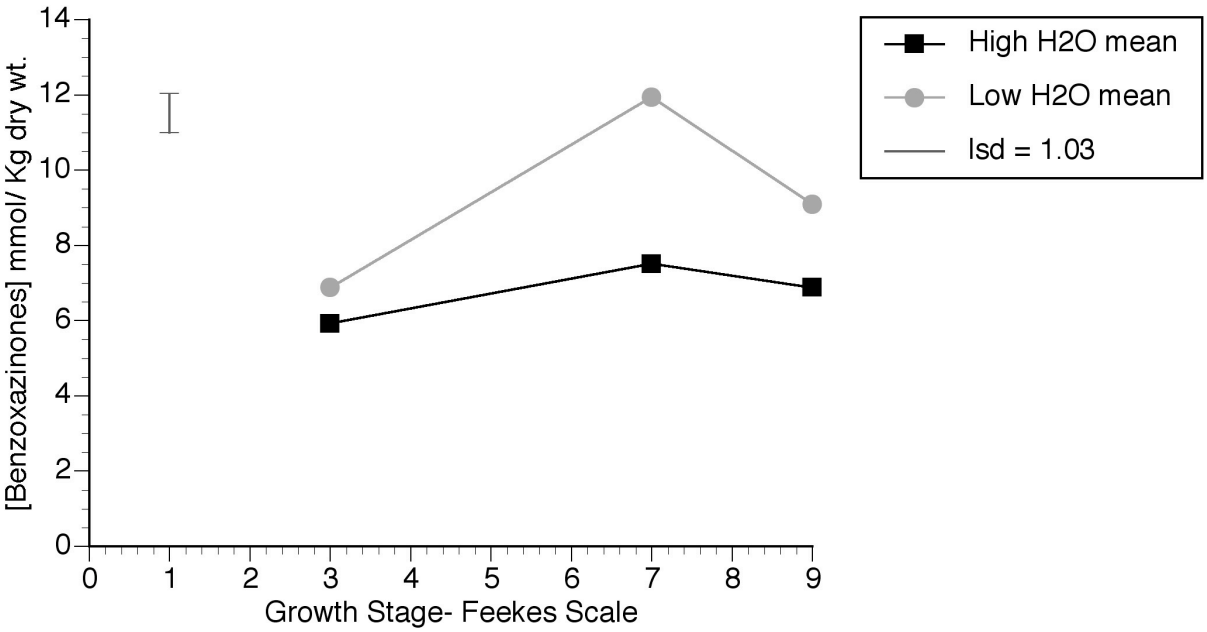


Figure 3
Growth Stage by Moisture Level Interaction, Low Temperature Dataset

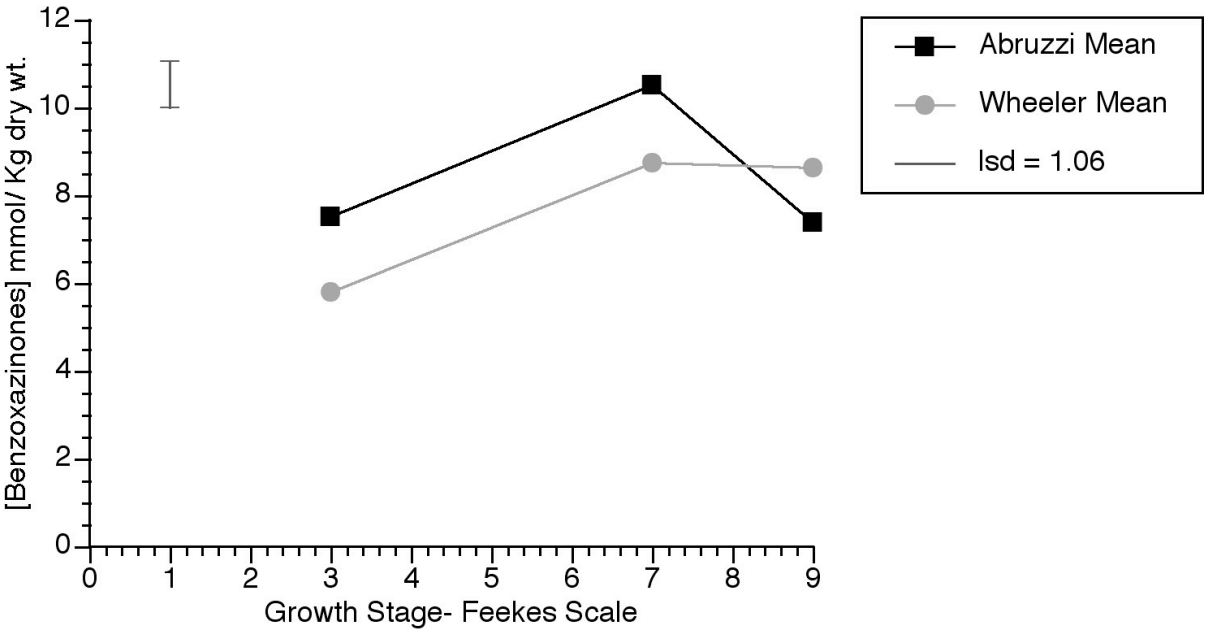


Figure 4
Growth Stage by Variety Interaction, Low temperature Dataset

Table 3
Main Effect Means of First Harvest Data

	Low Temp.	Med. Temp.	High Temp	Temp. lsd (.05)	High Fert.	Low Fert.	Fert. lsd (.05)	High H2O	Low H2O	H2O lsd (.05)
[Bx]mmol/Kg	6.685	7.016	9.496	0.76	9.045	6.414	0.621	7.135	8.365	0.621
Ratio	0.379	0.319	0.317	0.046	0.443	0.228	0.037	0.381	0.293	0.037

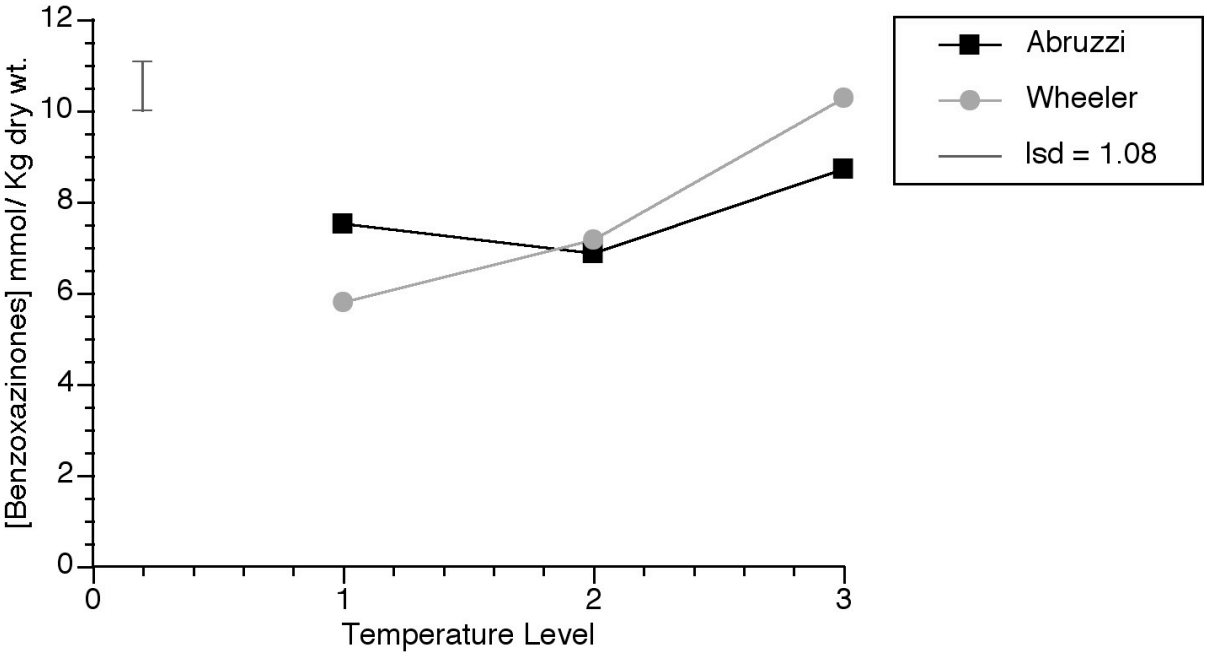


Figure 5
Variety by Temperature Interaction, First Harvest Dataset
Temp 1= Low, Temp 2= Med, Temp 3=High

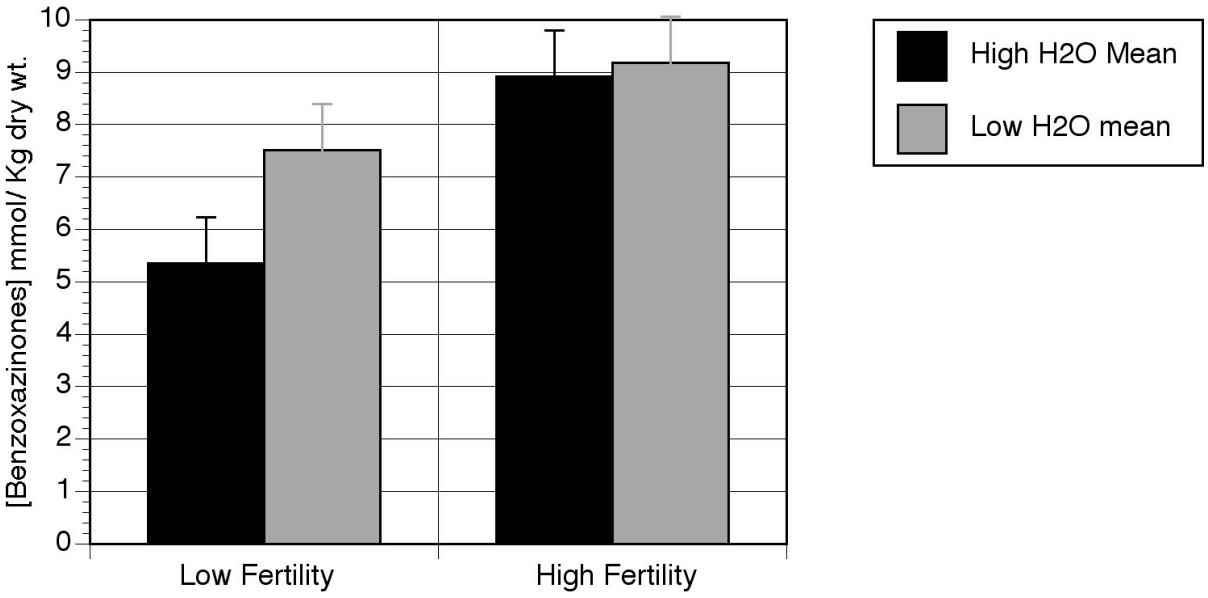


Figure 6
Fertility by Moisture Level Interaction, First Harvest Dataset

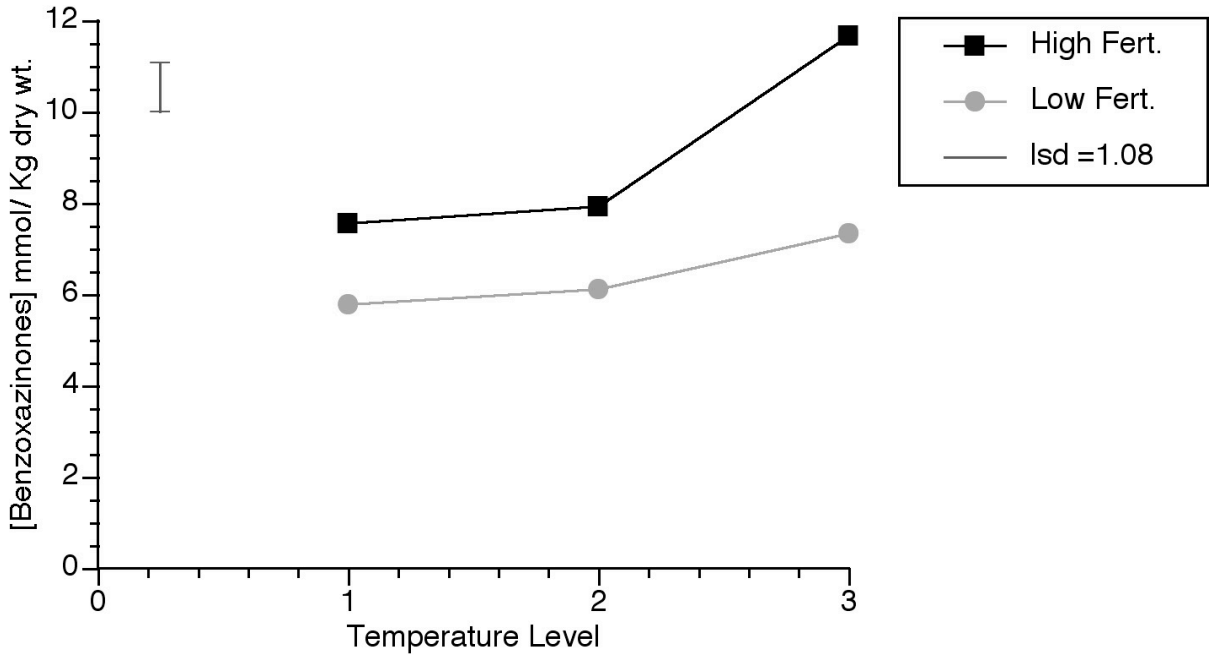


Figure 7
Temperature by Fertility Interaction, First Harvest Dataset
Temp 1= Low, Temp 2= Med, Temp 3=High

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APPENDICES

Appendix A

Low Temperature Dataset- Concentrations ANOVA Table

All numbers were analyzed as mmol/ g dry wt concentrations

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	41	0.00097396	0.00002376	11.25	0.0001
Error	51	0.00010771	0.00000211		
Corrected Total	92	0.00108167			

R-Square	C.V.	Root MSE	HXTOTAL Mean
0.900426	17.89514	0.00145	0.00812

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HARV	2	0.00014814	0.00007407	35.07	0.0001
REP(HARV)	9	0.00002623	0.00000291	1.38	0.2218
VARIETY	1	0.00000910	0.00000910	4.31	0.0430
VARIETY*HARV	2	0.00003979	0.00001990	9.42	0.0003
VARIETY*REP(HARV)	9	0.00003637	0.00000404	1.91	0.0708
H2O	1	0.00017127	0.00017127	81.10	0.0001
N	1	0.00038361	0.00038361	181.64	0.0001
N*H2O	1	0.00000086	0.00000086	0.41	0.5252
HARV*H2O	2	0.00003929	0.00001964	9.30	0.0004
VARIETY*H2O	1	0.00000435	0.00000435	2.06	0.1573
VARIETY*HARV*H2O	2	0.00000215	0.00000108	0.51	0.6034
HARV*N	2	0.00007193	0.00003596	17.03	0.0001
VARIETY*N	1	0.00000132	0.00000132	0.63	0.4323
VARIETY*HARV*N	2	0.00000032	0.00000016	0.08	0.9277
HARV*N*H2O	2	0.00001935	0.00000967	4.58	0.0148
VARIETY*N*H2O	1	0.00000122	0.00000122	0.58	0.4509
VARIETY*HARV*N*H2O	2	0.00000276	0.00000138	0.65	0.5247

Tests of Hypotheses using the Type III MS for REP(HARV) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HARV	2	0.00014814	0.00007407	25.41	0.0002

Tests of Hypotheses using the Type III MS for
VARIETY*REP(HARV) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VARIETY	1	0.00000910	0.00000910	2.25	0.1677
VARIETY*HARV	2	0.00003979	0.00001990	4.92	0.0359

Appendix B

First Harvest Dataset- Concentrations ANOVA Table:

All numbers were analyzed as mmol/ g dry wt concentrations

Dependent Variable: HXTOTAL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	41	0.00055797	0.00001361	5.93	0.0001
Error	53	0.00012169	0.00000230		
Corrected Total	94	0.00067966			

R-Square	C.V.	Root MSE	HXTOTAL Mean
0.820952	19.56876	0.00152	0.00774

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TEMP	2	0.00015013	0.00007507	32.69	0.0001
REP(TEMP)	9	0.00002560	0.00000284	1.24	0.2920
VARIETY	1	0.00000006	0.00000006	0.02	0.8756
VARIETY*TEMP	2	0.00004128	0.00002064	8.99	0.0004
VARIETY*REP(TEMP)	9	0.00002198	0.00000244	1.06	0.4043
H2O	1	0.00003306	0.00003306	14.40	0.0004
N	1	0.00016366	0.00016366	71.28	0.0001
N*H2O	1	0.00001974	0.00001974	8.60	0.0050
TEMP*H2O	2	0.00001013	0.00000506	2.20	0.1203
VARIETY*H2O	1	0.00000157	0.00000157	0.68	0.4120
VARIETY*TEMP*H2O	2	0.00001198	0.00000599	2.61	0.0830
TEMP*N	2	0.00003399	0.00001700	7.40	0.0015
VARIETY*N	1	0.00000121	0.00000121	0.53	0.4718
VARIETY*TEMP*N	2	0.00002122	0.00001061	4.62	0.0141
TEMP*N*H2O	2	0.00000930	0.00000465	2.02	0.1422
VARIETY*N*H2O	1	0.00000638	0.00000638	2.78	0.1014
VARIETY*TEMP*N*H2O	2	0.00000079	0.00000039	0.17	0.8432

Tests of Hypotheses using the Type III MS for REP(TEMP) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TEMP	2	0.00015013	0.00007507	26.39	0.0002

Tests of Hypotheses using the Type III MS for
VARIETY*REP(TEMP) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VARIETY	1	0.00000006	0.00000006	0.02	0.8821
VARIETY*TEMP	2	0.00004128	0.00002064	8.45	0.0086

Appendix C

Low Temperature Dataset- Ratio ANOVA Table:
Ratios were log transformed

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	41	2.72713848	0.06651557	5.07	0.0001
Error	51	0.66966476	0.01313068		
Corrected Total	92	3.39680324			

R-Square	C.V.	Root MSE	R2 Mean
0.802854	22.61655	0.11459	0.50666

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HARV	2	0.90999476	0.45499738	34.65	0.0001
REP(HARV)	9	0.11870808	0.01318979	1.00	0.4487
VARIETY	1	0.05442142	0.05442142	4.14	0.0470
VARIETY*HARV	2	0.02563016	0.01281508	0.98	0.3838
VARIETY*REP(HARV)	9	0.15454442	0.01717160	1.31	0.2562
H2O	1	0.07228881	0.07228881	5.51	0.0229
N	1	1.02025809	1.02025809	77.70	0.0001
N*H2O	1	0.19739375	0.19739375	15.03	0.0003
HARV*H2O	2	0.12029881	0.06014941	4.58	0.0148
VARIETY*H2O	1	0.01423945	0.01423945	1.08	0.3026
VARIETY*HARV*H2O	2	0.00603212	0.00301606	0.23	0.7956
HARV*N	2	0.05187214	0.02593607	1.98	0.1492
VARIETY*N	1	0.02939635	0.02939635	2.24	0.1408
VARIETY*HARV*N	2	0.01254192	0.00627096	0.48	0.6230
HARV*N*H2O	2	0.02854796	0.01427398	1.09	0.3449
VARIETY*N*H2O	1	0.00263783	0.00263783	0.20	0.6559
VARIETY*HARV*N*H2O	2	0.06472124	0.03236062	2.46	0.0951

Dependent Variable: R2

Tests of Hypotheses using the Type III MS for REP(HARV) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
HARV	2	0.90999476	0.45499738	34.50	0.0001

Tests of Hypotheses using the Type III MS for
VARIETY*REP(HARV) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VARIETY	1	0.05442142	0.05442142	3.17	0.1087
VARIETY*HARV	2	0.02563016	0.01281508	0.75	0.5013

Appendix D

First Harvest Dataset- Ratio ANOVA Table:

Ratios were log transformed

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	41	1.94546919	0.04745047	5.70	0.0001
Error	53	0.44152705	0.00833070		
Corrected Total	94	2.38699624			

R-Square	C.V.	Root MSE	R2 Mean
0.815028	27.07987	0.09127	0.33705

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TEMP	2	0.05396778	0.02698389	3.24	0.0471
REP(TEMP)	9	0.05337303	0.00593034	0.71	0.6955
VARIETY	1	0.05314710	0.05314710	6.38	0.0146
VARIETY*TEMP	2	0.02632224	0.01316112	1.58	0.2156
VARIETY*REP(TEMP)	9	0.11283161	0.01253685	1.50	0.1705
H2O	1	0.19745585	0.19745585	23.70	0.0001
N	1	1.11155398	1.11155398	133.43	0.0001
N*H2O	1	0.18283231	0.18283231	21.95	0.0001
TEMP*H2O	2	0.03502893	0.01751447	2.10	0.1322
VARIETY*H2O	1	0.00106909	0.00106909	0.13	0.7216
VARIETY*TEMP*H2O	2	0.00700620	0.00350310	0.42	0.6589
TEMP*N	2	0.01327875	0.00663937	0.80	0.4560
VARIETY*N	1	0.00737085	0.00737085	0.88	0.3512
VARIETY*TEMP*N	2	0.06490703	0.03245351	3.90	0.0264
TEMP*N*H2O	2	0.01503366	0.00751683	0.90	0.4118
VARIETY*N*H2O	1	0.00808486	0.00808486	0.97	0.3290
VARIETY*TEMP*N*H2O	2	0.00459890	0.00229945	0.28	0.7599

Tests of Hypotheses using the Type III MS for REP(TEMP) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TEMP	2	0.05396778	0.02698389	4.55	0.0431

Tests of Hypotheses using the Type III MS for
VARIETY*REP(TEMP) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
VARIETY	1	0.05314710	0.05314710	4.24	0.0696
VARIETY*TEMP	2	0.02632224	0.01316112	1.05	0.3892