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Short communication

Gas chromatographic method for the analysis of allelopathic natural products in rye (*Secale cereale* L.)

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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.) using gas chromatography (GC). Sample preparation consists of extraction of freeze-dried rye vegetative tissue with aqueous ethanol followed by partitioning of the allelochemicals into ethyl acetate, evaporation, and derivatization using the trimethylsilylating reagent *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. GC analysis of the silylated mixture was performed using flame ionization detection. This method permits analysis of all known rye allelopathic agents including 2,4-dihydroxy-1,4-benzoxazin-3-one, its corresponding glucoside, 2-benzoxazolinone, β-hydroxybutyric acid, and β-phenyllactic acid. 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*

1. 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 [1–4]. 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 benzoxazolin-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 [5]. 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-phenyllactic acid (β-PLA) have been identified as allelopathic agents [6] (Fig. 1). No method for the quantitation of these additional allelopathic metabolites has been reported.

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. Environmental, cultural, and genetic factors are known

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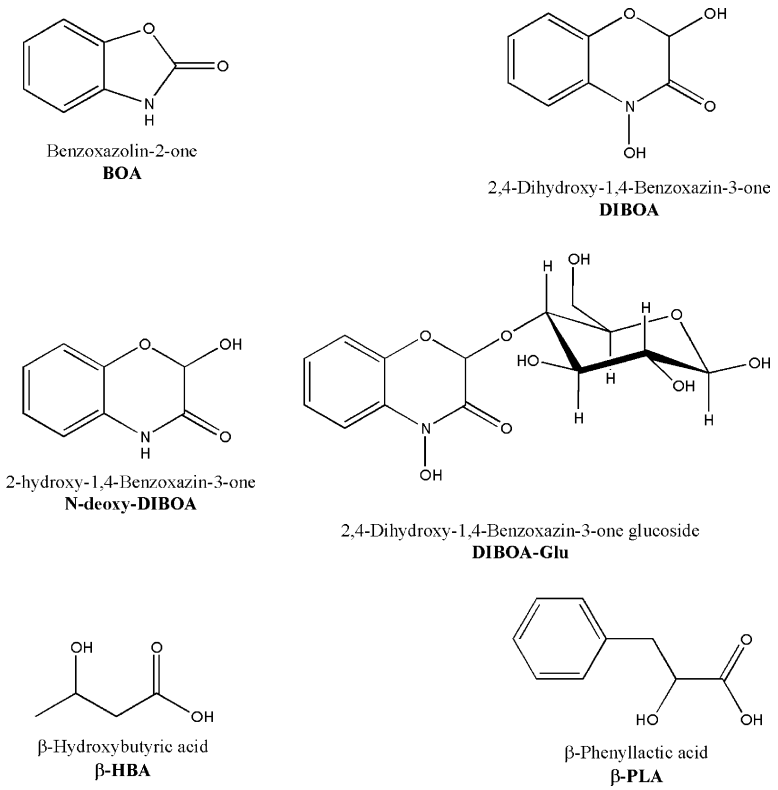

Fig. 1. Known allelopathic agents present in rye.

to affect levels of BOA metabolites in rye. 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 [7–9,11–15]. When extracted in aqueous solution, DIBOA can continue to degrade to BOA. Wu et al. [14] describes a gas chromatography (GC)/MS/MS method for the quantitation of allelopathic phenolic compounds as well as DIMBOA in wheat (*Triticum aestivum* L.). However, this method is focused primarily on the phenolic compounds and does not analyze the benzoxazinones in the glucosidic form. The analyses detailed in Lyons et al. [16] and in Yenish et al. [17] describe HPLC methods for quantitation of benzoxazinone content including DIBOA-Glu but, 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 with the intent of developing a high throughput screening system for analysis of genetic lines in a rye breeding program.

2. Experimental

2.1. Chemicals

Ethyl acetate (EtOAc) and *N*,*N*-dimethylformamide (DMF) (HPLC grade) were obtained from Fisher Scientific, Pittsburgh, PA, USA. Absolute ethanol (EtOH) was obtained from AAPER (Shelbyville, KY, USA). Standards of β-HBA, β-PLA, BOA, and octadecan-1-ol (Internal Standard) were purchased from Sigma–Aldrich, St. Louis, MO, USA. The BOA standard was further purified by re-crystallization from HPLC-grade methylene chloride (Fisher Scientific). Synthetic DIBOA standard was prepared 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 *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Pierce and mixed 1:1 with dry DMF.

2.2. DIBOA-glucoside isolation

Fourteen-day old rye seedlings (var. Bonel) were harvested then frozen with liquid N_2. The frozen biomass was stored at −80 °C until extraction. Extraction of DIBOA-Glu from the frozen tissue was accomplished using 500 mL of chilled acetone (–20 °C) per 500 g frozen shoot tissue. The tissue and acetone were homogenized in a blender for ap-

proximately 2 min. After the mixture warmed to 4 °C the tissue was briefly mixed again before filtering. The mixture was filtered through four layers of paper towels, and then filtered through a Whatman No. 4 filter paper. The filtrate was 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 −20 °C overnight. After freezing the sample was thawed and filtered through Whatman No. 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 20 μm nylon membrane filter and injected onto a 20 cm × 3 cm LiChroprep RP-8 column (E. Merck, Darmstadt, Germany). A mobile phase of water–MeOH (85:15) was pumped through the column at a rate of 2.0 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 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).

2.3. Analytical sample preparation

Fresh plant tissues were harvested from field or growth chambers, placed into labelled paper bags, and immediately frozen at −68.0 °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 0.50 g dried rye shoot tissue was weighed and transferred into empty 75.0 mL SPE (Alltech) reservoirs fitted with bottom frits (20 μm porosity). After adding 10.0 mL of 50% ethanol solution the reservoir was mixed with a vortexer for 1 min, the extract was then filtered through the reservoir by attaching it to vacuum manifold (VWR Scientific). An additional 5.0 mL of deionized (DI) water was filtered through the extracted tissue and the washed tissue was allowed to air dry under vacuum. The filtrate was transferred to a 25.0 mL volumetric flask, and brought to volume with DI water. 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 750 μL of 200 μg/mL octadecanol internal standard dissolved in toluene were reduced to dryness under a N_2 stream at 40 °C. 400 μL of MSTFA–DMF (1:1) were added to the sample/internal standard residue, the samples were capped under N_2, and then heated at 75 °C for 30 min. Samples were then transferred to autosampler vials for GC analysis.

2.4. 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 °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/s. The initial oven temperature was 100 °C, followed by a 5°/min temperature increase to 300 °C final temperature. The oven temperature increase began upon injection and the final temperature was maintained for 30 min. Data were 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, and DIBOA-Glu.

2.5. GC/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 70 eV. The mass range was set from 50 to 600 and data were collected at 1.1 scans per second. Component peaks were separated on a 30 m, 0.25 mm i.d., and 0.25 μm film thickness DB-XLB capillary column (J&W Scientific) with helium carrier gas flowing at a linear gas velocity of 37.1 cm/s. Initial oven temperature was 100 °C immediately increasing at a rate of 5 °C/min to 300 °C. The final temperature was held for 15 min.

3. Results and discussion

Limits of detection were determined to be 16.13 pmol/injection for DIBOA, 72.38 pmol/injection for DIBOA-Glu, and 173.8 pmol/injection for BOA. Equations for the standard curves were BOA- $f(x) = 1{,}146{,}000x - 379{,}900$, $R^2 = 0.985$, DIBOA- $f(x) = 1{,}560{,}000x - 44{,}020$, $R^2 = 0.999$, DIBOA-Glu- $f(x) = 2{,}255{,}000x - 319200$, $R^2 = 0.999$. Every curve consisted of four concentrations and each concentration was replicated three times. 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. Four

sequential extractions of the tissue showed that the initial extraction 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 procedure was developed using rye shoot tissue, however vegetative tissue at any stage of development could be analyzed, including crop residues. The ability to simultaneously examine DIBOA-Glu and the corresponding aglycone allows study of the breakdown and transformation of the benzoxazinones within the plant, thereby providing a useful tool for the examination of developmental and environmental effects on the hydrolysis of DIBOA-Glu. 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.

Table 1
Enzymatic hydrolysis of DIBOA-Glu

Compound	mmol/g dry wt	
	Pre-hydrolysis	Post-hydrolysis
BOA	1.034E−03	1.466E−03
DIBOA	1.161E−03	5.108E−03
DIBOA-Glu	7.744E−03	6.220E−04

Peak identities were confirmed by GC/MS analysis of the trimethylsilyl (TMS) derivatives. Mass fragments of components were compared to the mass fragmentation of known standards, published MS data, and through comparison with fragmentation data contained in the National Bureau of Standards 75 K and Wiley 275 K 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.

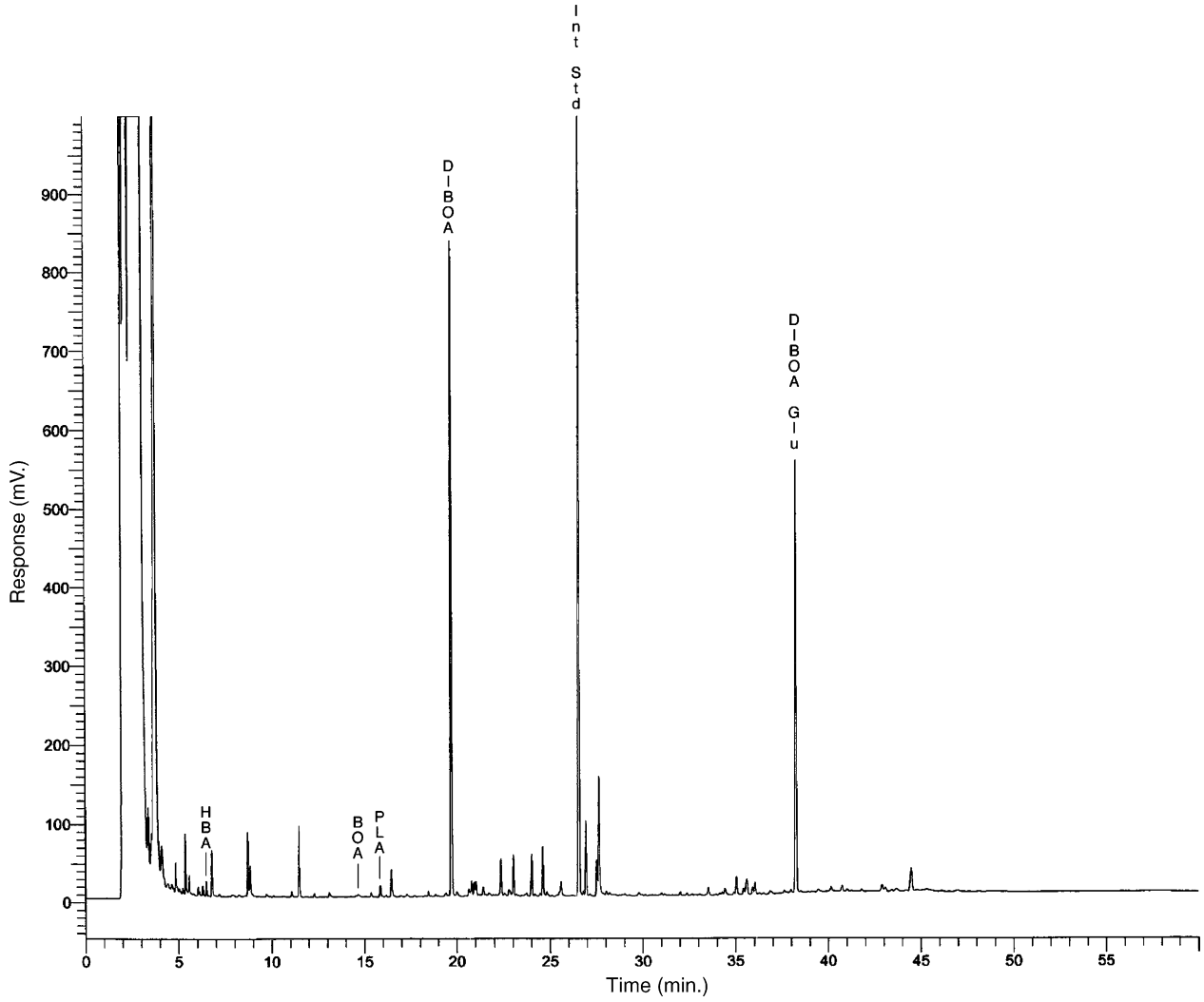

Fig. 2. Rye sample chromatogram.

β-HBA (t_R 6.32 min): *m/z* 233 (22%), 191 (60%), 147 (209%), 133 (27%), 117 (100%), 88 (29%), 73 (176%).

BOA (t_R 14.64 min): *m/z* 257 (37%), 247 (33%), 207 (67%), 192 (100%), 164 (85%), 147 (96%), 100 (33%), 73 (348%).

β-PLA (t_R 15.51 min): *m/z* 295 (12%), 267 (15%), 220 (24%), 193 (100%), 147 (85%), 131 (5%), 91 (22%), 73 (166%).

N-Deoxy-DIBOA (t_R 16.64 min): *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 (t_R 19.70 min): *m/z* 325 (31%), 311 (25%), 310 (100%), 208 (21%), 192 (21%), 164 (25%), 147 (31%), 73 (142%).

DIBOA-Glu (t_R 38.23 min): *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 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 benzoxazinone metabolism. Both β-HBA and β-PLA were detected at low levels confirming their presence in rye shoot tissue as possible allelochemicals [3]. Other major components identified by comparison with fragmentation patterns of known compounds found in the NBS 75 K and Wiley 275 K GC–MS libraries included palmitic acid (t_R 24.6 min), 4-methoxycinnamic acid (t_R 25.6 min), terpene alcohol (t_R 26.9 min), linoleic acid (t_R 27.7 min), and β-sitosterol (t_R 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 h before adding absolute EtOH to stop the reaction. The sample was worked up using the standard extraction and analysis procedure. After 24 h the amount of DIBOA-Glu declined to 8% of its original amount while the amount of DIBOA and BOA detected in the sample increased to 440% and 141% of the original amount (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.

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