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CORRELATION BETWEEN PHYTOTOXICITY ON ANNUAL RYEGRASS (*Lolium rigidum*) AND PRODUCTION DYNAMICS OF ALLELOCHEMICALS WITHIN ROOT EXUDATES OF AN ALLELOPATHIC WHEAT

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Abstract—An improved allelopathic correlation between phytotoxicity measured in root growth bioassay upon annual ryegrass (*Lolium rigidum* Gaud.) and the concentrations of a selection of dynamically produced allelochemicals quantified in the root exudates of cv. Khapli wheat (*Triticum turgidum* ssp. *durum* (Desf.) Husn.) monitored during the first 15 days of wheat seedling growth in a sterile, agar–water medium, has been established. Changes over the 15-day growth period in the quantities of five exuded benzoxazinones and seven phenolic acids were measured simultaneously using GC/MS/MS. Substantiating pure compound dose–response measurements were conducted over a range of concentrations for the putative allelochemicals within the wheat exudates. One synergism-based proposal using the monitored compounds to explain the observed low-exudate-concentration phytotoxicity was explored, but was found to be experimentally inadequate.

Key Words—Allelopathic correlation, wheat root exudate, production dynamics, benzoxazinones, phenolic acids, allelochemicals, synergism, GC/MS/MS.

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INTRODUCTION

Crop plant root exudation has been chemically investigated from as early as 1921 when Lyon and Wilson (1921) studied the liberation of organic substances through the roots of growing plants. Rovira (1969) has written a review covering plant root exudation. Allelopathic secondary metabolites such as α-methoxyphenylacetic acid have been observed to move from one plant to another through their root systems (Preston et al., 1954), and as one of the important world crops, the root exudates from wheat have become a focus of allelopathic study.

The literature on wheat (*Triticum* spp.) contains a number of studies on its allelochemicals, singling out such compound types as the benzoxazinoids, the phenolic acids, and scopoletin (Baghestani et al., 1999) as the main active constituents. Some studies deal with the phenolics alone (e.g., Guenzi et al., 1966; Lodhi et al., 1987, Baghestani et al., 1999; Wu et al., 2001a,b), some with benzoxazinoids alone (e.g., Copaja et al., 1991; Perez and Ormeno-Nunez, 1991; Petho 1992; Wu et al., 2001c), while some deal with both phenolics and benzoxazinoids (e.g., Wu et al., 1999, 2000a). A small number of wheat studies include the production dynamics of allelochemicals (e.g., Argandona et al., 1981; Zuniga et al., 1990; Nicol et al., 1992; Nakagawa et al., 1995, Copaja et al., 1999), while a smaller number focus upon wheat root exudates (e.g., Perez and Ormeno-Nunez, 1991; Petho, 1992; Kobayashi, et al., 1996; Baghestani et al., 1999; Wu et al., 2000a,c). To our knowledge, no wheat study has been published that attempted to deal simultaneously with the dynamics of both phenolic acids and benzoxazinoids production within root exudate.

The significance of root exudates to plant–plant allelopathy lies in the fact that soil-borne exudates are the main source of contact between donor and receiver plants. Any serious examination of crop plants with allelopathic potential for weed control must necessarily focus upon exudate content. A simplistic quantification of allelochemical levels inside donor plant roots alone is not adequate (Perez and Ormeno-Nunez, 1991; Niemeyer and Perez, 1995; Wu et al., 2000a) because exudation appears to be an active metabolic process (Wu et al., 2001c) dependent upon genetic factors. Thus, of a global selection of 58 wheat cultivar exudates analyzed (Wu et al., 2001c), only 11 were found to exude detectable amounts of the hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), one of the main allelochemicals of wheat, despite the fact that 57 of those cultivars had measurable quantities of DIMBOA within their roots. Of the 58 cultivars, one in particular exuded relatively high concentrations of both phenolic acids and DIMBOA, viz., cv. Khapli (*Triticum turgidum ssp. durum* (Desf.) Husn.), an Indian wheat. Khapli, therefore, presented a favorable donor target for indepth allelochemical studies toward receiver annual ryegrass (*Lolium rigidum* Gaud.), and as a source of germplasm possibly suitable for breeding new wheats of high quality, high yield, and weed inhibiting ability.

Whether phenolic acids actually play a role in wheat allelopathy remains a vexing question. Presently, two schools of thought exist. One is based upon a large body of published material containing circumstantial evidence suggesting the assumption to be correct, while the second withholds belief until the definitive experiments are performed and proof of cause–effect convincingly demonstrated. That *Triticum aestivum* L. is allelopathic toward *L. rigidum* Gaud. (Wu et al., 2000b) and exudes phenolic acids from the roots is not contended (Kobayashi et al., 1996; Baghestani et al., 1999, Wu et al., 2000a, 2001b, 2002), and neither is the individual acids' phytotoxicities, as measured against a range of test plants (e.g., Rasmussen and Einhellig, 1979; Liebl and Worsham, 1983; Lodhi et al., 1987; Blum et al., 1992; Schulz et al., 1994). However, a point of contention surrounds the perception that previous dose–response studies performed on pure individual phenolic acids, such as ferulic or coumaric, require concentrations at 1.0 mM or above to cause significant toxicity, whereas the actual concentrations associated with real plant sources measured experimentally are less than this by a factor of 10–1000. A possible answer to this apparent concentration requirement is the idea of synergism (or multiple additivities), whereby in the usual plant-produced mixture, the constituents combine their biological activities to produce a concerted effect equivalent to a much higher concentration of the most active compound. A limited amount of support exists for this idea (e.g., Asplund, 1969, Rasmussen and Einhellig, 1979). Certainly, the concept of synergistic enhancement of biological activity has been long accepted in agriculture and applied to herbicidal mixtures (Sharma et al., 1982; Simpson and Stoller, 1996). However, the occurrence is rare, and determining the applicability of synergism to plant exudates containing a hundred or more components is a daunting task not yet known to have been undertaken; insufficient evidence presently exists to accept this explanation for any low-concentration phytotoxicity of phenolics.

Although the literature contains no reports (Eljarrat and Barcelo, 2001) on the simultaneous determination of the two distinct groups of allelochemicals in wheat (benzoxazinoids and phenolic acids) apart from our own limited results incorporating DIMBOA using a GC/MS/MS method (Wu et al., 1999, 2000a), we recognized the ability of this technique to handle simultaneously a range of compounds from both groups (Schulz et al., 1994) and to open new ground for exploring any causal relationship that might exist at the molecular level between observed phytotoxic effect in bioassay and the putative allelopathic agents.

The work of Petho (1992) and our own earlier unpublished findings on the benzoxazinones present in exudates from *Triticum aestivum* pointed to the presence of such constituents as DIMBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2-hydroxy-1,4-benzoxazin-3-one (HBOA), 2-hydroxy-7-methoxy,1,4-benzoxazin-3-one (HMBOA), and 2,7-dihydroxy-1,4-benzoxazin-3-one (DHBOA). Of these benzoxazinones, only DIBOA and DIMBOA are hydroxamic acids (hydroxyl group on the nitrogen atom at position 4). According to Hashimoto and Shudo

(1996), it is only such acids that possess the necessary level of allelochemical activity. However, the other three benzoxazinones have weak activity (Friebe, 2001) and for caution's sake deserved not to be automatically discounted in any causative exploration, particularly as Macias et al. (2002) found the lactam 2-deoxy-HMBOA to show hydroxamic acid-like activity against *L. rigidum*. All five benzoxazinones therefore, were, chosen, together with the seven phenolic acids of our earlier work (Wu et al., 2000a), for determination by GC/MS/MS on the exudate from Khapli wheat grown in a neutral, sterile, agar–water medium.

Our aims were to: (1) monitor changes in the quantities of identified phenolic acids and benzoxazinones in the exudates of seedlings of an allelopathic wheat during the course of the first 15 days growth in sterile agar–water; (2) record the accompanying changes in levels of phytotoxicity by bioassay against root growth of annual ryegrass; (3) investigate any correlation between wheat exudate allelochemical concentrations and the corresponding observed phytotoxicity on ryegrass; and (4) substantiate the phytotoxicities of the selected individual phytotoxins toward annual ryegrass using pure compound dose–response measurements.

METHODS AND MATERIALS

Plant Material. Seeds of wheat (*T. turgidum* ssp. *durum* cv. Khapli) [AUS #378] were obtained from the Australian Winter Cereals Collection, Tamworth. Seeds of annual ryegrass (*Lolium rigidum* Gaud.) were obtained commercially. Agar technical was purchased from Amyl Media Pty Ltd.

Sterilization and Pregermination. The pretreatment of wheat and ryegrass seeds has been previously described (Wu et al., 2000c). Wheat seeds were surface-sterilized by soaking them in 70% ethanol for 2.5 min, followed by four rinses with sterilized distilled water. Seeds were soaked in 2.5% sodium hypochlorite solution for 15 min, followed by five rinses with sterilized distilled water. The surface-sterilized seeds of wheat and ryegrass were each soaked in sterilized water for imbibition in light at 25°C for 24 hr and then rinsed with fresh sterilized water. Finally, wheat seeds were incubated in light at 25°C for another 24 hr, and ryegrass seeds incubated in light at 25°C for 48 hr.

Bioassay of Wheat Exudates. The method described previously (Wu et al., 2000a) was slightly modified to assess the allelopathic activity of wheat seedling exudates. Glass beakers (500 ml) containing 30 ml of 0.3% agar–water (nonutrients) were autoclaved. Twenty-four pregerminated wheat seeds were uniformly selected and aseptically sown on the agar surface in each beaker. Beakers were wrapped with parafilm to prevent contamination and evaporation from the agar surface and placed in a controlled environment growth cabinet with an L/D cycle of 13 hr/11 hr and a temperature cycle at 25/13°C. The fluorescent light intensity in the cabinet was 42.72 ± 1.92 μEm^{-2} s^{-1} (PAR) (Quantum/Radiometer/Photometer, model LI-185B, LI-COR, Inc.). Wheat seedlings were allowed to grow for 2, 4,

6, 8, 11, and 15 days. Seedlings were carefully removed from the agar, and 10 pregerminated seeds of ryegrass (*L. rigidum*) were aseptically sown onto each of the respective agar surfaces. Ryegrass sown on agar–water with no wheat beforehand was used as a control. Experiments were arranged in a randomized complete block design with three replicates. Ryegrass-containing beakers were wrapped with parafilm and placed back into the growth cabinet, and the seeds were allowed to grow for 7 days. At the end of this period, the lengths of ryegrass roots and shoots were determined as the means of the three replicates.

Collection of Root Exudates from the Agar Medium. Methods of collection and extraction of allelochemicals for wheat root exudates were identical to those reported previously (Wu et al., 1999). After the growth of 24 wheat seedlings in 0.3% agar–water medium (nutrient-free) for 2, 4, 6, 8, 11, and 15 days, the wheat seedlings were uprooted from the soft growth medium and their roots rinsed twice with 5-ml distilled water to remove any residual agar. Each of the respective washings was returned to its corresponding agar–water medium. The agar–water was collected, adjusted to pH 3.0 by dropwise addition of 0.01 M HCl, stirred thoroughly, and sonicated at 5°C for 15 min. The agar medium was extracted three times with 60-ml portions of diethyl ether. Ether layers were combined and evaporated on a rotary evaporator under reduced pressure at 35°C until the volume of residual solution was approximately 2 ml. The ether residue was transferred into a 2-ml minivial, and the solvent was removed by nitrogen gas blow-down.

Preparation of Silyl Derivatives and GC/MS/MS Analysis. Minivials containing agar extract samples were further dried in an air oven at 60°C for 30 min. After each vial had been cooled to ambient temperature, 1.0 ml of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was added to each, and then the vial sealed with its septum cap. Vials were shaken well, heated at 60°C for 30 min to complete derivatization, and allowed to cool to ambient temperature in a desiccator over P_2O_5. Silylated samples were analyzed for selected phenolic acids and benzoxazinones under the same set of GC/MS/MS instrument parameters as earlier reported (Wu et al., 2000a). The MS/MS parameters chosen for determination of the five selected benzoxazinones (under identical GC conditions as for phenolics) are as stated in Table 1. Allelopathic compounds found in the wheat agar extracts were identified by comparing their retention times and daughter mass spectra with those in the user-library created earlier from authentic reference compounds, kindly supplied by Prof. D. Sicker, University of Leipzig. Quantifications were carried out in triplicate using multipoint external standard calibration curves based upon the same reference compounds.

Pure Compound Dose–Response Data. Defined concentrations of four benzoxazionones and five phenolic acids were tested for biological activity against annual ryegrass. Bioassays were performed in 9-cm petri dishes lined with one layer of filter paper, where 12 pregerminated ryegrass seeds were uniformly

TABLE 1. ION-TRAP MS/MS METHOD PARAMETERS FOR BENZOXAZINONES[a]

Benzoxazinone[b]	Retention time (min)	Precursor ion (m/z)	Excitation: Nonresonant amplitude (V)	Excitation: Storage level (m/z)	Quantifying product ion (m/z)
HBOA	13.04	309	50	75	266
DIBOA	15.73	310	50	75	164
HMBOA	17.28	339	46.5	75	222
DHBOA[c]	18.65	397	49	75	280
DIMBOA	20.18	340	46.5	75	194

[a] Mass isolation window (m/z) was set at 3 and excitation time at 20 ms.
[b] Identified as trimethylsilyl derivatives (TMS).
[c] Identified as *tris*-TMS derivative ($M = 397$) by mass spectral comparison with Woodward et al. (1979) and estimation based upon calibration for HMBOA.

distributed after complete evaporation of the solvents. Controls received only pure solvent. After 7 days of incubation in 4.0 ml water at 25°C; 15 hr light/9 hr dark cycle, measurements were taken of the ryegrass root lengths. Assays were repeated four times.

RESULTS

Allelopathic Effect. The effect of wheat root exudate upon the growth of annual ryegrass roots (means of three replicates) across a 15-day period of juvenile wheat growth is shown in Figures 1–3. While ryegrass root growth was inhibited

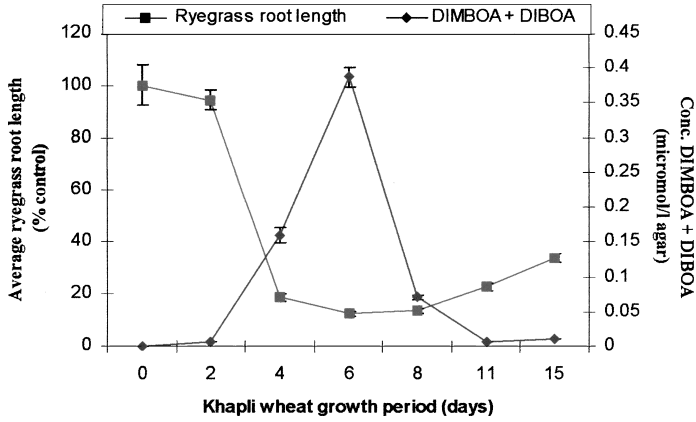

FIG. 1. Relation between phytotoxic effect on ryegrass root length and concentration of active hydroxamic acids in cv. Khapli wheat root exudate (means ± standard errors). Absence of error bars indicates that error bars are smaller than the symbol representing the mean.

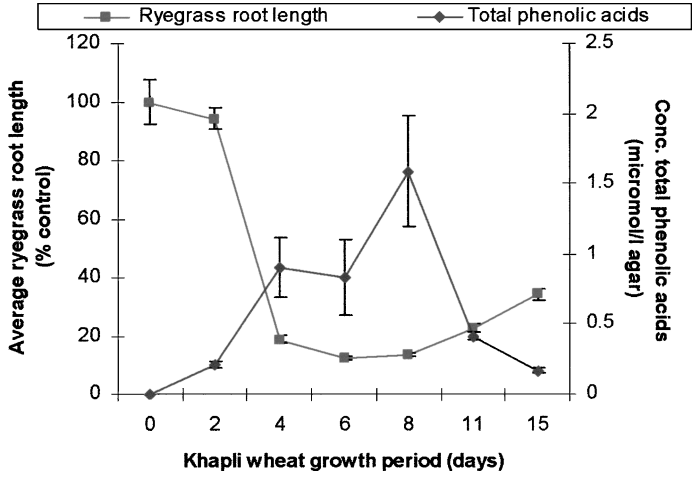

FIG. 2. Relation between phytotoxic effect on ryegrass root length and concentration of total phenolic acids in cv. Khapli wheat root exudate (means ± standard errors). Absence of error bars indicates that error bars are smaller than the symbol representing the mean.

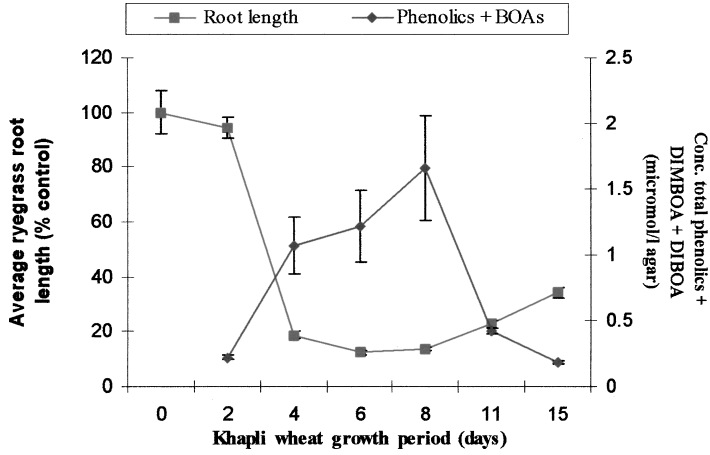

FIG. 3. Relation between phytotoxic effect on ryegrass root length and concentration of total measured phenolics + DIMBOA + DIBOA in cv. Khapli wheat root exudate (means ± standard errors). Absence of error bars indicates that error bars are smaller than the symbol representing the mean.

to a greater extent than shoot, both root and shoot inhibition was greatest in the day 6 to day 8 zone. This maximum coincides with the highest levels of measured allelochemicals (Table 2).

The parallels that exist between measured allelochemical concentrations in the wheat growth medium and the concurrent growth inhibition in the root length of test plant annual ryegrass are shown in Figures 1 and 2. Figure 1 shows an exact coincidence between the maxima of hydroxamic acids concentration and ryegrass root inhibition on day 6, while Figure 2 shows that the total measured phenolic acids maximize on day 8, a little later than maximum inhibition. The relation between ryegrass inhibition and total hydroxamic acids plus phenolic acids appears in Figure 3, where it is evident that as the combined allelochemical concentration drops in the latter days, so the allelopathic effect upon ryegrass roots also declines. These results suggested an association of the 12 measured allelochemicals with the phytotoxicity observed.

Quantitation of Allelochemicals. The concentrations of benzoxazinones and phenolic acids in Khapli wheat exudate at 6 different time periods across the first 15 days of seedling growth are given in Table 2. Both DIBOA and DIMBOA reached their highest concentrations on day 6 (0.146 ± 0.018 μmol/l and 0.241 ± 0.015 μmol/l agar–water, respectively. The highest level found for any of the benzoxazinones was 0.424 ± 0.023 μmol/l on day 8 for HBOA. All benzoxazinones reached maximal concentrations in the day 6 to day 8 zone before declining. Individual phenolic acids had more variability across the 15-day observation period, however the total concentration of phenolic acids showed a similar (though broader) maximum between day 4 and day 8. The highest combined concentration of measured allelochemicals from both classes occurred at day 8 with 2.312 ± 0.682 μmol/l agar.

Correlation between Allelochemical Concentration and Phytotoxicity. Prompted by the above parallels, a wide range of both single and multivariate regressions were attempted between the dependent variable (Y) "relative phytotoxicity" (defined as ryegrass root control length divided by ryegrass root test length under bioassay), and a range of independent variables (Xn) all involving some measure of allelochemical concentration within the agar wheat growth medium, and arranged in (nonchronological) order of ascending magnitude. The resulting plot represented a natural dose–response curve. After statistical testing, the best regression took the form of a "relative phytotoxicity" versus Ln (total phenolic plus hydroxamic acids) plot (one variate, nine compounds), which for a linear model gave $r^2 = 0.914$ ($P < .005$) (see Figure 4). Taken together, the concentrations of the seven phenolic acids plus DIBOA plus DIMBOA explain 91% of the variation in growth inhibition of annual ryegrass.

Response of Ryegrass to Pure Allelochemicals. Table 3 presents the results of the pure compound dose–response treatments for five phenolic acids (*cis* and *trans* isomers combined as one) ranging from 10^{-8} to 10^{-2} molar, based upon ryegrass root length measurements (4 replicates). Similar results appear for the

TABLE 2. QUANTIFICATION OF BENZOXAZINONES AND PHENOLIC ACIDS IN WHEAT SEEDLING EXUDATES[a]

Compound	2 Days[b]	4 Days	6 Days	8 Days	11 Days	15 Days
HBOA[c]	0.042 ± 0.002	0.189 ± 0.027	0.168 ± 0.022	0.424 ± 0.023	0.082 ± 0.016	0.129 ± 0.009
DIBOA	0.006 ± 0.001	0.083 ± 0.017	0.146 ± 0.018	0.070 ± 0.004	0.006 ± 0.001	0.010 ± 0.000
HMBOA	0.020 ± 0.001	0.092 ± 0.011	0.154 ± 0.020	0.209 ± 0.004	0.115 ± 0.013	0.103 ± 0.012
DHBOA[d]	ND	0.023 ± 0.002	0.026 ± 0.000	0.019 ± 0.000	0.016 ± 0.000	0.016 ± 0.000
DIMBOA	ND	0.076 ± 0.010	0.241 ± 0.015	ND	ND	ND
Total benzoxazinones	0.068 ± 0.002	0.463 ± 0.035	0.735 ± 0.38	0.722 ± 0.024	0.219 ± 0.021	0.258 ± 0.015
p-Hydroxybenzoic acid	0.031 ± 0.010	0.430 ± 0.353	0.418 ± 0.453	1.158 ± 0.680	0.051 ± 0.003	0.029 ± 0.011
Vanillic acid	0.012 ± 0.005	0.046 ± 0.032	0.030 ± 0.033	0.016 ± 0.004	0.048 ± 0.007	0.020 ± 0.006
cis-*p*-Coumaric acid	0.008 ± 0.008	0.037 ± 0.031	0.091 ± 0.083	0.081 ± 0.030	0.022 ± 0.012	0.004 ± 0.003
Syringic acid	0.025 ± 0.009	0.101 ± 0.049	0.077 ± 0.057	0.071 ± 0.020	0.047 ± 0.017	0.017 ± 0.006
cis-Ferulic acid	0.014 ± 0.002	0.027 ± 0.023	0.029 ± 0.026	0.014 ± 0.006	0.027 ± 0.017	0.014 ± 0.002
trans-*p*-Coumaric acid	0.075 ± 0.030	0.136 ± 0.056	0.098 ± 0.055	0.167 ± 0.028	0.098 ± 0.018	0.039 ± 0.011
trans-Ferulic acid	0.050 ± 0.016	0.130 ± 0.064	0.089 ± 0.059	0.083 ± 0.024	0.122 ± 0.033	0.046 ± 0.014
Total phenolic acids	0.215 ± 0.038	0.907 ± 0.370	0.832 ± 0.473	1.590 ± 0.682	0.415 ± 0.047	0.169 ± 0.023

Note: ND = not detected.
[a] Data expressed as mean concentration of compound ± SD in μmol/l agar-water ($N = 3$).
[b] Days growth of cv. Khapli wheat seedlings in sterile agar-water medium, 24 seeds per beaker.
[c] Determined as their trimethylsilyl ethers/esters (TMS).
[d] Estimated from the calibration curve for pure HMBOA.

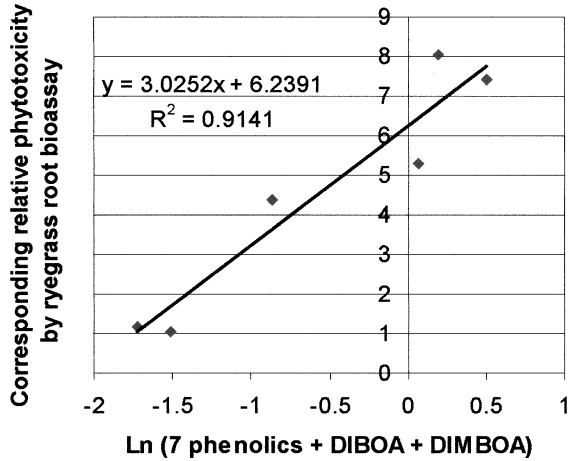

FIG. 4. Correlation between allelochemical concentrations (μmol/l) in Khapli wheat seedlings growth medium and phytotoxicity on annual ryegrass root growth ($P < 0.005$). Plot represents a natural dose–response to measured allelochemicals at points within an observed 15-day wheat growth period.

benzoxazinones whose concentrations ranged from 10^{-8} to 10^{-3} molar (DHBOA unavailable). The phenolic acids affected *Lolium rigidum* in a characteristic manner, exhibiting little phytotoxicity until their individual concentrations reached a value close to 10^{-3} molar (Schulz et al., 1994). In this respect, they are about as active as the natural lactam benzoxazinones HBOA and HMBOA that do not possess the seemingly important OH group on position 4 of the oxazinone ring. As expected, the two hydroxamic acids DIBOA and DIMBOA displayed higher levels of activity, quite noticeable at 10^{-4} molar. However, during the 15-day growth monitoring period, the sum of total phenolics plus total benzoxazinones never exceeded 3.02 μmol/l. This is a concentration 100 times less than what appears to be necessary for equivalent activity with pure allelochemicals applied to ryegrass in isolation. As synergism among these allelochemicals has been offered in the literature as one explanation for high bioactivity from a low-dose mixture, an artificial mixture with the day 6 exudate composition (minus DHBOA which was unavailable) was prepared at 1.55 μmol/l and tested against ryegrass root growth in our usual bioassay. Instead of root inhibition it resulted in a slight stimulation (Table 4). If bioactivity at low concentration on day 6 was due to synergism, we were not able to duplicate the correct mixture composition with the above design. Of course there may be a number of reasons for our not testing the "true synergistic mix," one of which may simply be the fact we chose only nine allelochemicals to combine. There may well be other unknown contributors present in the real exudate that have not been considered and play an essential role.

TABLE 3. DOSE–RESPONSE RESULTS FROM PURE PHYTOTOXIN TREATMENTS OF RYEGRASS SEEDLINGS

	Average root length of ryegrass (mm) (4 replicates)									
Concentration (μmol/l)	0.0 (control)[a]	0.01 (10^{-8} M)	0.1 (10^{-7} M)	1 (10^{-6} M)	10 (10^{-5} M)	100 (10^{-4} M)	1000 (10^{-3} M)	5000 (5×10^{-3} M)	10000 (10^{-2} M)	l.s.d. at 0.05 level
Phytotoxin										
p-Hydroxybenzoic acid	62.7	58.4	61.8	63.8	58.8	58.5	63.8	6.4	0.0	7.0
Vanillic acid	62.7	65.9	67.4	66.9	64.7	67.2	48.7	2.1	0.0	6.9
Coumaric acids	62.7	65.0	72.9	66.8	64.5	65.2	54.9	0.4	0.1	10.2
Syringic acid	62.7	74.4	71.6	68.5	72.6	65.8	63.0	0.2	0.0	4.5
Ferulic acids	62.7	61.5	57.8	62.4	64.4	61.1	59.7	24.6	0.1	4.4
Concentration (μmol/l)[b]	(control)	0.01 (10^{-8} M)	0.05 (5×10^{-8} M)	0.1 (10^{-7} M)	1 (10^{-6} M)	5 (5×10^{-6} M)	10 (10^{-5} M)	100 (10^{-4} M)	1000 (10^{-3} M)	
HBOA	62.7	62.8	65.8	75.4	73.1	69.7	66.2	66.2	49.3	6.2
DIBOA	62.7	69.7	65.2	70.2	68.8	67.3	69.1	53.2	1.7	5.7
HMBOA	62.7	68.8	70.3	69.8	64.3	65.2	70.6	70.2	47.5	5.8
DIMBOA	62.7	61.5	69.2	66.7	73.2	65.2	71.6	45.3	7.4	5.9

[a] Control was distilled water.
[b] DHBOA unavailable.

TABLE 4. ATTEMPTED COPY OF MAXIMUM PHYTOXICITY MIXTURE EFFECT

Phytotoxic compounds	Concentration phytotoxins on day 6[b] (μmol/l agar)	Day 6, % of control ryegrass root length
		15
p-Hydroxybenzoic acid	0.418	
Vanillic acid	0.03	
Coumaric acids	0.189	
Syringic acid	0.077	
Ferulic acids	0.118	
HBOA[a]	0.168	
DIBOA	0.146	
HMBOA	0.154	
DIMBOA	0.241	
Artificial Chemical Mix	Total conc. mix = 1.55	
Artificial Mix Result	Root length ryegrass = 68.5 mm, H_2O control = 62.7 mm (4 replicates)	

[a] DHBOA unavailable.
[b] Maximum phytotoxicity occurred at 6–8 days of Khapli wheat growth.

DISCUSSION

Under sterile agar conditions of the bioassay, the dynamics of exuded benzoxazinones displayed a rapid increase in concentration up to a maximum at 6–8 days of wheat seedling growth followed by a decline to low levels by 15 days (cf. Nicol et al., 1992; Copaja et al., 1999). The reason for the decline was not experimentally pursued, and is not well understood. One explanation could be associated with the limited half-life these compounds possess in near-neutral solution. For example, DIMBOA has a half-life of 5.3 hr at 28°C at pH 6.75 (Woodward et al., 1978) and decomposes to the more stable MBOA (a benzoxazolinone) that also has inhibitory activity, but to a lesser degree (Hashimoto et al., 1996; Burgos et al., 1999; Macias et al., 2002). While the phenolic acids as a group followed a similar overall change during the 15-day monitoring period, the concentration of individual compounds fluctuated. It is likely that their decline toward the end of the experimental period is due to reabsorption by the growing wheat plants—a process observed earlier in wheat Kobayashi et al., (1996). However, if absorption is a possibility, then even in sterile conditions a decarboxylase in wheat roots may be capable of converting *p*-hydroxycinnamic acid derivatives into their corresponding hydroxystyrenes (vinylphenols), which are more phytotoxic than their precursors (Liebl and Worsham, 1983). This could possibly help to sustain the observed effect even after the 15-day wheat growth period when phenolic acid (and benzoxazinone) concentration has become quite low. The present experiments were not designed to test this possibility. The observed decrease in ryegrass root inhibition after 8 days of wheat growth not only correlates with the

concentration of benzoxazinones and phenolic acids found herein, but is consistent with the findings of Schulz et al. (1994) and Macias et al. (2002).

Regarding the failure of the artificial phytotoxins mix to duplicate the response of ryegrass roots at wheat growth day 6 (Table 4), it is unlikely that the two bioassay systems used for comparison—agar/water/seedlings versus filter paper/water/pure phytotoxins—would behave differently, as each system is a simple polysaccharide/water matrix. The difference is more likely accounted for by the additional (mostly unknown) components within the agar assay that come from the wheat exudate. Other possible contributions to this difference may arise from the degree of spontaneous breakdown in the hydroxamic acids that can occur over a longer period of time in the agar system than in the filter paper chemoassay, thereby allowing accumulation of moderately toxic benzoxazolinones (BOA; MBOA) for the commencement of the 7-day ryegrass growth period.

While numerous statistical correlations have been reported in the plant allelopathy literature, only a few attempts have been made to study correlation between observed allelopathy effect and the actual concentrations of specific allelochemicals found in the donor exudate. This is understandable given the complexity of many allelopathic plant products. Such products often contain different primary and secondary metabolites (Vancura, 1964), many of which are unknown, and only a few of which have any recognized phytotoxicity against the test plant of interest. What allelopathic role is played by most of the individual constituents in these phytotoxic mixtures can only be guessed. Considering the large number of exuded compounds and the enormous number of possible combinations of these compounds that would need to be tested for phytotoxicity, the finding of proof for simple synergism (if that is the true picture) between root exudate components as a molecular explanation for plant allelopathy will prove to be a difficult task.

Nevertheless, causative explanations for allelopathic effects at a molecular level are considerably assisted by any significant correlations that can be found that link particular compounds to observed effects. Some limited progress in this area has been made (Ben-Hammouda et al., 1995, Burgos et al., 1999; Wu et al., 2002). The correlation of Burgos et al. (1999) showed a significant ($P = 0.04$), but weak ($r^2 = 0.50$) linear relationship between root growth of *Eleusine indica* (measured as dilutions of *Secale cereale* cultivar extracts causing 50% root growth inhibition), and Ln (DIBOA + BOA) in *Secale cereale* cultivar shoot tissue (μg/g tissue). The weak relationship prompted these workers to comment that "compounds other than hydroxamic acids may also be involved in growth suppression." However, in view of the usual transportation mechanism required in plant allelopathy, plant-parts studies are not as likely as exudate studies to yield strong correlations between observed phytotoxicity under bioassay, and the concentrations of active compounds. Better correlations are more likely found using root exudate concentrations because it is exudate components (or their soil metabolites) that are transported to the receiver plant.

The correlation between allelopathic wheat exudates (including cv. Khapli) and annual ryegrass toxicity that we achieved earlier (Wu et al., 2002) using DIMBOA plus seven phenolic acids in a linear model, 8-variate, multiple regression ($r^2 = 0.504$, $P < 0.01$) indicated a possible additive or synergistic effect among those (and other) compounds. The widening of the compound range in the present study to examine the effect of an additional four benzoxazinones has led to an improved univariate, linear correlation between toxicity on ryegrass and putative allelochemicals in wheat exudate where a significant ($P < 0.005$) and strong ($r^2 = 0.914$) association has been established, based upon the inclusion of one additional hydroxamic acid (DIBOA). Most correlations (uni- or bivariate) attempted between ryegrass toxicity and the two hydroxamic acids alone were unsatisfactory (not significant), with the best using as independent variable Ln (DIMBOA + DIBOA), which resulted in $r^2 = 0.678$ ($P < 0.05$), noticeably below the previous coefficient of determination (0.914) that also incorporates the phenolic acids. This suggests an interactive relationship between both classes of compound in accounting for the observed toxicity on ryegrass. A capacity to account for 91% of test-plant bioassay response in terms of defined molecular concentrations of natural allelopathic agents appears to be one of the best results of its type so far recorded in the allelopathy literature (Belz and Hurle, 2001). It is worth making the comparison between this natural dose–response result using a partially informed selection of exudate components and the artificial extreme obtained by observing the effects of a single pure allelochemical (DIBOA) dose–response treatment of the *Lolium* species *perenne* (Shulz et al., 1994) where a linear regression of "relative phytotoxicity" vs. DIBOA concentration was $r^2 = 0.965$ ($P < 0.001$) (our calculations). The comparison helps place a perspective upon the progress that is being made toward understanding the chemical basis of plant allelopathy.

Nevertheless, further improvement is likely to be gained in the case of Khapli wheat by the future inclusion of other possible bioactive exudate components such as the benzoxazolinones and bioactive coumarins like scopoletin. Scopoletin is a recognized phytotoxin toward Italian ryegrass (*Lolium multiflorum* Lam.) (Fay and Duke, 1977) and is an established constituent of *Triticum aestivum* exudate (Baghestani et al., 1999). Interestingly, Korableva et al. (1969) report that scopoletin is more effective as a growth retardant when used in combination with caffeic acid than when used alone. This fact raises speculation as to the joint roles of the potent phytotoxins (e.g., DIMBOA) and the less active phenolic acids within the overall allelopathic wheat mix. It appears that the primary effect of phenolic acids on plant roots is an alteration of water and nutrient uptake (Blum et al., 1999), perhaps by working through cell permeability, and that this effect has more to do with outer surface contact concentration than with the actual uptake of phenolics. If so, in exuded donor mixtures such as those we examined from wheat, the phenolic acids may be playing a cell permeability role on the root cells of the receiver plant,

and, thereby, opening the way for easier uptake of the more potent components like hydroxamic acids.

Another possiblity lies with the hydroxystyrene phytotoxins (Liebl and Worsham, 1983) that may be formed at the point of exudation from wheat by possible decarboxylase action on various cinnamic acids (Kobayashi et al., 1996) even under sterile conditions, and, therefore, deserves consideration in future study, as do any other newly discovered secondary metabolites in wheat exudate that are known to exhibit phytotoxicity. Further experimentation along these lines is presently being undertaken.

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