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PHYTOTOXINS FROM SHOOT EXTRACTS AND ROOT EXUDATES OF *AGROPYRON REPENS* SEEDLINGS

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Key Word Index—*Agropyron repens*; Poacea; quackgrass; seedlings; allelochemical; hydroxamic acids; ferulic acid; vanillic acid.

Abstract—Allelopathic constituents of ethylacetate extracts from shoots and root exudates of 10-day old *Agropyron repens* seedlings were investigated. The allelochemicals were identified by GC-mass spectrometry and comparison of retention times and mass spectra to data of respective reference compounds. In shoot extracts the cyclic hydroxamic acids 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (DIMBOA) and 2,4-dihydroxy-2*H*-1,4-benzoxazin-3-one (DIBOA), as well as the corresponding lactam derivative 2-hydroxy-1,4-benzoxazin-3-one (HBOA), were found. The concentration of major component DIBOA was 0.5 mg g^{-1} fr. wt, the concentration of DIMBOA was 0.02 mg g^{-1} fr. wt. Futhermore maleic, *t*-aconitic and citric acid were found. In order to estimate the allelopathic potential of living plants an investigation of root exudates was performed. The cyclic hydroxamic acids were identified as important constituents. Their concentrations were 0.4 μmol l^{-1} DIMBOA and 0.2 μmol l^{-1} DIBOA. Additionally 2,4-dihydroxy-7,8-dimethoxy-2*H*-1,4-benzoxazin-3-one (DIM_2BOA) was detected. Vanillic, ferulic and β-hydroxybutyric acid are also phytotoxins released by intact, living quackgrass seedlings.

INTRODUCTION

Quackgrass (*Agropyron repens*) is a highly aggressive Poaceae and its allelopathic interferences on higher plants are well known [1–4]. Since it is one of the widely spread weeds of the Northern Hemisphere the investigation of its allelopathic effects is of special importance. Numerous publications deal with the isolation and identification of inhibitory substances present in extracts of quackgrass shoots or rhizomes. Allelopathic effects of extracts from quackgrass rhizomes have been characterized by Le Fevre and Clagett [5], and Gabor and Veatch [6]. However, a chemical identification of the phytotoxins was not performed in these studies. Hagin [7] isolated well known auxin derivatives 5-hydroxyindol-3-acetic acid and 5-hydroxy-tryptophan. Tricin and a related flavonoid compound have been identified as allelochemicals from quackgrass shoots by Weston *et al.* [8].

Regarding the allelopathic potential of quackgrass, the investigation of phytotoxins released by intact, living plants is of special interest. In this study the identification of constituents from shoot extracts and root exudates of *Agropyron repens* is presented. Since young plants are known to show a comparably high allelopathic potential, the experimental identification of phytotoxins were performed with quackgrass seedlings in their early state of development by GLC–mass spectrometry.

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RESULTS AND DISCUSSION

In order to identify phytotoxins of *Agropyron repens*, water soluble constituents were extracted from shoots of young quackgrass seedlings. The TMSi derivatives of the related compounds were separated and identified by GC-mass spectrometry by comparison of retention times and mass spectra to data of respective reference compounds. The following substances were found to be constituents of the shoot extracts: malic acid (R_t 17.5 min, MS 335 [M − Me]$^+$ (21), 245 (21), 233 (35), 217 (10), 191 (15), 190 (15), 189 (16), 175 (14), 147 (100), 133 (25)), aconitic acid (R_t 25.5 min, MS 375 [M − Me]$^+$ (55), 346 (12), 300 (6), 285 (23), 229 (46), 215 (16), 211 (29), 147 (100), 133 (14)), citric acid (R_t 27.5 min, MS: 465 [M − Me]$^+$ (21), 437 (21), 375 (43), 363 (48), 347 (39), 305 (31), 273 (100), 217 (53), 211 (16), 147 (43)) and 4-hydroxycinnamic acid (R_t 30.6 min, MS 308 [M]$^+$ (61), 293 (62), 249 (35), 219 (100), 203 (18), 191 (8), 192 (8), 179 (29), 175 (12), 147 (8)).

Shoot extracts of *Agropyron repens* contained considerable amounts of di- and tricarboxylic acids. Malic, *t*-aconitic and citric acid have already been identified as constituents of quackgrass shoots by Nikolai [9]. A significant allelopathic effect of these compounds was not found.

GLC-peaks of derivatized shoot extract showed the same retention times as TMSi-derivatives of reference cyclic hydroxamic acids. These compounds were identified as 2,4-dihydroxy-2*H*-1,4-benzoxazin-3-one (DIBOA)

(R_t 26.0 min, MS 325 $[M]^+$ (37), 310 (100), 297 (13), 282 (6), 236 (7), 208 (55), 192 (44), 179 (29), 164 (55), 147 (60)), 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (DIMBOA) (R_t 33.0 min, MS 355 $[M]^+$ (35), 340 (39), 238 (75), 210 (13), 194 (100), 191 (48), 165 (9), 147 (35)) and the lactam of DIBOA, 2-hydroxy-1,4-benzoxazin-3-one (HBOA), R_t 21.5 min, MS 309 $[M]^+$ (100), 294 (23), 280 (8), 266 (21), 220 (7), 208 (14), 192 (25), 191 (13), 147 (52)). The spectra are in good correspondence with spectra given in the literature [10, 11]. The hydroxamic acids were also detected by the formation of blue-coloured $FeCl_3$-complexes in the preliminary thin layer chromatography. An R_f value of 0.55 was determined for DIBOA in chloroform–methanol (4:1). DIBOA was the major component of cyclic hydroxamic acids in shoots of germinated quackgrass. A concentration of 0.5 mg g^{-1} fr. wt DIBOA in shoots of germinated quackgrass was determined by analysing peak areas using a calibration straight line of the reference compound, whereas 0.02 mg g^{-1} fr. wt DIMBOA was found.

The decomposition of the 2,4-dihydroxy-1,4-benzoxazin-3-one in solution to the corresponding benzoxazolinone derivatives is often described [10, 11]. However, in our extracts these substances were not detectable. Cyclic hydroxamic acids are known to be very important bioactive compounds of Poaceae [12]. DIBOA and DIMBOA have already been detected in other species of the genus *Agropyron* (*A. cristatum*, *A. c. puberulum*, *A. desertorum*, *A. fragile*) by Copaja *et al.* [13]. Our study presented here substantiates the existence of these compounds in *Agropyron repens* as well. It is assumed that the functions of hydroxamic acids in quackgrass may be similar to those described from rye, corn and wheat [14–18], e.g. 2,4-dihydroxy-benzoxazin-3-ones have been related to allelopathic effects, the resistance of plants to insects and fungi, to the mineral nutrition and the detoxification of herbicides [12, 19].

Regarding the allelopathic potential of living quackgrass, phytotoxins released by young seedlings were studied. According to the definition of allelopathy the main feature of these interactions between the plants is that phytotoxins or precursors must be released into the soil and absorbed by an acceptor plant [20]. In this context the investigation of root exudates is of special importance. The identification of allelochemicals from quackgrass root exudates was performed in our study for the first time. The following phytotoxins were found to be constituents of the root exudate: β-hydroxybutyric acid (R_t 7.5 min, MS 233 $[M - Me]^+$ (14), 191 (36), 147 (100), 133 (11), 130 (7), 117 (47), 101 (6)), vanillic acid (R_t 25.5 min, MS 312 $[M]^+$ (79), 297 (100), 282 (32), 267 (55), 253 (36), 223 (61), 193 (28)) and ferulic acid (R_t 35.8 min, MS 338 $[M]^+$ (100), 323 (63), 308 (62), 293 (36), 279 (8), 249 (33), 219 (32)).

The concentrations of the phenolic acids were in the range of 10 nmol l^{-1}. Ferulic and vanillic acid have already been described as constituents of quackgrass shoot extracts by Hagin [7]. Further phenolic acids found in the study mentioned above such as *p*-hydroxybenzoic, sinapic and caffeic acid were not detected in our root exudates. β-Hydroxybutyric acid was described as an allelochemical existing in rye [20].

Furthermore, a number of cyclic hydroxamic acids were identified in the root exudates. DIMBOA is the major component of this group of substances. Concentrations up to 0.4 μmol l^{-1} DIMBOA and 0.2 μmol l^{-1} DIBOA are exuded. In contrast to the shoot extracts, the additional hydroxamic acid DIM_2BOA (R_t 36.5 min, MS 385 $[M]^+$ (42), 370 (30), 357 (4), 268 (40), 237 (100), 224 (17), 191 (48)) was found in the root exudate. This compound is released in lower concentrations compared with the other benzoxazinones. Traces of the corresponding lactam of DIMBOA (HMBOA: R_t 28.0 min, MS 339 $[M]^+$ (50), 324 (12), 310 (8), 296 (11), 250 (24), 238 (15), 222 (100), 206 (19), 147 (100)) were also detected.

In this paper, it is shown that phytotoxins of different substance classes are released by living, intact quackgrass seedlings. The high allelopathic potential of *Agropyron repens* is suggested to be a result of synergetic interactions of different allelochemicals. In this context cyclic hydroxamic acids may be of special importance because of their considerable effects on a variety of enzymes and auxin receptors [12].

EXPERIMENTAL

Extraction of shoots. Seeds of *Agropyron repens* were germinated on filter paper watered with tap water and grown in the dark at 20°. The shoots were extracted by a modified procedure described in ref. [21]. Shoots of 10-day-old seedlings (2.6 g fr. wt) were homogenized in 30 ml of distilled water. The homogenate was allowed to stand at room temp. for 30 min before being filtered through cheesecloth. The filtrate was heated to 70° and cooled rapidly to 15°. After filtration, pH was adjusted to 3 with 0.1 M HCl. The filtrate was partitioned ×4 against 50 ml freshly distilled EtOAc. The organic extract was evapd at 40° and dried *in vacuo*. A pale yellow extract was obtained (6.4 mg).

Collection and extraction of root exudates. Seeds of *Agropyron repens* were germinated in Petri dishes (6 cm i.d.) containing filter paper and 2 ml H_2O. The H_2O was changed every 2 days. Ten-day-old seedlings (10.7 g fr. wt) were transferred to 80 ml fresh H_2O. The plants were grown for 3 days at 20° with an 11 hr dark 13 hr light photoregime. After 3 days the H_2O was removed from the plant material (12.1 g fr. wt) and used for the following extraction procedure.

The H_2O phase was adjusted to pH 3 with 0.1 M HCl. After filtration the soln was partitioned ×4 against 125 ml freshly distilled EtOAc. The collected organic phase was dried over $MgSO_4$. The organic extract was evapd to dryness *in vacuo* at 40°. A yellow oily residue was obtained (2.2 mg).

Analytical methods. For TLC methanolic solns of crude extracts were applied to Merck silica gel plates. The plates were developed in a $CHCl_3$–MeOH mixture (4:1). An

$FeCl_3$-spray reagent was used for the detection of cyclic hydroxamic acids (5 g $FeCl_3 \cdot 6\,H_2O$ in 50 mol 95% EtOH, 0.5 ml of 1.5 M HCl) [22]. The hydroxamic acids were detected by blue spots. GC analyses were performed with a Hewlett–Packard capillary column Ultra 2 (5% phenyl-methyl polysiloxane, length 25 m and 0.2 mm diameter). GC-MS analyses were performed with an Automass 100 from Delsi Nermag (Unicam). Helium (1 bar) was used as carrier gas. The chromatograms were taken by split modus. The injection port was maintained at 280°. Oven temp. was 100° for 2 min followed by increases of 4° min^{-1} to 200° (4 min isotherm) and 4° min^{-1} to 280° (10 min isotherm). MS were obtained with electron impact of 70 eV.

Sample preparation. Methanolic solns of appropriate amounts of the extracts or reference compound were posited in micro reaction vials. The MeOH was evapd, 50 μl of BSTFA [*N,O*-bis(trimethylsilyl)-trifluoroacetamide] was added, and the reaction mixt. was kept at 70° for 1 hr. Samples of 2 μl were injected.

Reference compounds. 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3-one (DIMBOA): DIMBOA was isolated from a Et_2O extract of shoots of 7-day-old corn plants (*Zea mays* L., Marshall FAO 240) by a procedure described in ref. [21]. After purification by recrystallization from Me_2CO–hexane crystals were obtained. Mp 162–165° (lit. mp 163–164.5° [10]), GC-MS (TMSi-derivative): 355 $[M]^+$ (35), 340 (39), 238 (75), 210 (13), 194 (100), 191 (48), 165 (9), 147 (35).

2, 4-Dihydroxy-7, 8-dimethoxy-2*H*-1, 4-benzoxazin-3-one (DIM_2BOA): DIM_2BOA was isolated in mixture with DIMBOA from Et_2O extract of shoots of 7-day-old maize plants (*Zea mays* L., Marshall FAO 240). GC-MS (TMSi-derivative): 385 $[M]^+$ (42), 370 (30), 357 (4), 268 (40), 237 (100), 224 (17), 191 (48).

2,4-Dihydroxy-2*H*-1,4-benzoxazin-3-one (DIBOA): DIBOA was isolated from Et_2O extract of shoots of rye (*Secale cereale* L., Marder cultivar) by an analogous procedure to DIMBOA. After purification by recrystallization from Me_2CO–hexane crystals were obtained. Mp 152–155° (lit. mp 155–157° [10]), GC-MS (TMSi-derivative): 325 $[M]^+$ (37), 310 (100), 297 (13), 282 (6), 236 (7), 208 (55), 192 (44), 179 (29), 164 (55), 147 (60).

The reference compounds β-hydroxybutyric acid, 4-hydroxycinnamic acid, 4-hydroxy-3-methoxy-cinnamic acid, 4-hydroxy-2-methoxy-benzoic acid were commercially available from Sigma. 2-Benzoxazolinone was received from Aldrich.

REFERENCES

1. Osvald, H. (1948) *J. Ecol.* **38**, 192.
2. Penn. D. J. and Lynch, J. M. (1981) *J. Appl. Ecol.* **18**, 669.
3. Lovett, J. V. and Jokinen, K. (1984) *J. Agric. Sci. Finland* **56**, 1.
4. Weston, L. A. and Putnam, A. R. (1985) *Crop Sci.* **25**, 561.
5. Le Fevre, C. W. and Clagett, C. O. (1960) *Proc. North Cent. Weed Contr. Conf.* **14**, 353.
6. Gabor, W. E. and Veatch, C. (1981) *Weed Sci.* **29**, 155.
7. Hagin, R. D. (1989) *J. Agric. Food Chem.* **37**, 1143.
8. Weston, L. A., Burke, B. A. and Putnam, A. R. (1987) *J. Chem. Ecol.* **13**, 403.
9. Nikolai, H. (1982) Dissertation. Universität Heidelberg.
10. Atkinson, J., Morand, P., Arnason, J. T., Niemeyer, H. M. and Bravo, H. R. (1991) *J. Org. Chem.* **56**, 1789.
11. Woodward, M. D., Corcuera, L. J., Schnoes, H. K., Helgeson, J. P. and Upper, C. D. (1979) *Plant Physiol.* **63**, 9.
12. Niemeyer, H. M. (1988) *Phytochemistry* **27**, 3349.
13. Copaja, S. V., Barria, B. N. and Niemeyer, H. M. (1991) *Phytochemistry* **30**, 1531.
14. Barnes, P. and Putnam, A. R. (1987) *J. Chem. Ecol.* **13**, 889.
15. Perez, F. J. (1990) *Phytochemistry* **29**, 773.
16. Queirolo, C. B., Andreo, C. S., Vallejos, R. H., Niemeyer, H. M. and Corcuera, L. J. (1981) *Plant Physiol.* **68**, 941.
17. Queirolo, C. B., Andreo, C. S., Niemeyer, H. M. and Corcuera, L. J. (1983) *Phytochemistry* **22**, 2455.
18. Pheto, M. (1992) *Acta Agron. Hung.* **41**, 167.
19. Pheto, M. (1992) *Acta Agron. Hung.* **41**, 57.
20. Shilling, D. G., Jones, L. A., Worsham, A. D., Parker, C. E. and Wilson, R. F. (1986) *J. Agric. Food Chem.* **34**, 633.
21. Woodward, M. D., Corcuera, L. J., Helgeson, J. P., Kelman, A. and Upper, C. D. (1978) *Plant Physiol.* **61**, 796.
22. Nair, M. G., Whitenack, C. J. and Putnam, A. R. (1990) *J. Chem. Ecol.* **16**, 353.