Pergamon

PII: S0031-9422(96)00677-2

Phytochemistry, Vol. 44, No. 6, pp. 979–983, 1997
Copyright © 1997 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0031–9422/97 $17.00+0.00

EFFECTS OF 2,4-DIHYDROXY-1,4-BENZOXAZIN-3-ONES ON THE ACTIVITY OF PLASMA MEMBRANE H^+-ATPASE

ANNETTE FRIEBE, UDO ROTH, PETER KÜCK, HEIDE SCHNABL and MARGOT SCHULZ*

Institut für Landwirtschaftliche Botanik, Universität Bonn, Meckenheimer Allee 176, 53115 Bonn, Germany

(*Received in revised form* 17 *August* 1996)

Key Word Index—*Avena sativa*; *Vicia faba*; allelopathy; plasma-membrane; H^+–ATPase; 2,4-dihydroxy-1,4-benzoxazin-3-one; DIBOA; DIMBOA; 2(3*H*)-benzoxazolinone; BOA.

Abstract—The action of 2,4-dihydroxy-1,4-benzoxazin-3-ones and their corresponding benzoxazolinone (BOA) derivatives on the activity of plasma membrane H^+-ATPase from roots of *Avena sativa* and *Vicia faba* was investigated. Significant inhibitory effects were found with 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one in concentrations of 0.25 mM or higher. Benzoxazolinone compounds caused a weaker inhibition of the enzyme. At low effector concentrations stimulatory effects were observed. The H^+-ATPase activity of *A. sativa* and *V. faba* was reduced by DIBOA to the same extent. The allelopathic effects of DIBOA and BOA on radicle elongation of *A. sativa* are correlated with the H^+-ATPase activity. Copyright © 1997 Elsevier Science Ltd

---

INTRODUCTION

2,4-Dihydroxy-1,4-benzoxazin-3-ones are important secondary metabolites of the *Poaceae* involved in plant resistance to pests and diseases [1]. In addition, these substances and their decomposition products are associated with allelopathic effects. Phytotoxic influences on radicle growth of various monocotyledenous and dicotyledenous plants were found for 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) [2–4]. Inhibitory effects on the energy metabolism of chloroplasts and mitochondria have been described, e.g. the chloroplast ATPase coupling factor is inhibited by cyclic hydroxamic acids [5–8]. In addition, various enzymes such as the proteases papain [9] and α-chymotrypsin [10] are deactivated.

This paper reports on the sensitivity of the plasma membrane (PM) H^+-ATPase to DIBOA and DIMBOA. This enzyme acts as the main electrogenic pump of the PM. The maintenance of the proton gradient is a basic requirement for the transport of ions and molecules through the PM. Because of its central role in plant cell physiology, the activity of PM H^+-ATPase is closely related to radicle growth [11, 12]. In order to assess the significance of the molecular effects of DIBOA and DIMBOA on plant development, bioassays were carried out in parallel. The respective decomposition products of the cyclic hydroxamic acids benzoxazolinone (BOA) and 6-methoxy-benzoxazolinone (MBOA) were investigated as well.

RESULTS AND DISCUSSION

The preparation of PM vesicles of high purity is an essential requirement for the experimental determination of allelopathic effects on the PM H^+-ATPase. In order to ensure optimal conditions the ATPase activity of the microsomal fraction and the PM fraction prepared by two-phase partitioning were measured. The activities of PM, tonoplast and mitochondrial ATPases were determined by the use of specific inhibitors and are shown in Table 1.

DIMBOA in concentrations of 0.5 and 1 mM inhibited the vanadate sensitive H^+-ATPase by 6.6 and 12.5% in the microsomal fraction. In contrast, the same inhibitor concentrations led to a 40.6 and 44.9% inhibition of the PM H^+-ATPase.

The effects of the cyclic hydroxamic acids DIBOA and DIMBOA on PM H^+-ATPase activity of *Avena sativa* and *Vicia faba* are given in Table 2. The activity of the controls were 4.88 ± 0.42 (SE) nkat mg^{-1} prot. for *A. sativa* and 5.35 ± 0.92 nkat mg^{-1} prot. for *V. faba*. Inhibition of the enzyme by DIBOA and DIMBOA was observed for inhibitor concentrations from 0.25 mM up to 2 mM. Compared with DIBOA the methoxy derivative was found to be a stronger inhibitor. In contrast to DIBOA, a significant decrease in the activity was also observed in the presence of this substance at a concentration of 0.1 mM.

---

*Author to whom correspondence should be addressed.

Table 1. Characteristics of ATPase activity in the microsomal fraction and in the plasma membrane fraction prepared by two-phase partitioning (values are means ± S.E.; four replicates)

	Sp. activity (nkat mg^{-1} protein)	
	Microsomal fraction	Fraction of plasma membrane vesicles
Plasma membrane ATPase	2.96 ± 0.58	5.60 ± 1.34
Tonoplast ATPase	0.51 ± 0.16	0.11 ± 0.10
Mitochondrial ATPase	0.42 ± 0.19	0.12 ± 0.12

Table 2. Influence of cyclic hydroxamic acids on the plasma membrane H^+-ATPase activity of *A. sativa* and *V. faba*

	Activity of membrane H^+-ATPase (% control)		
Concentration (mM)	*A. sativa* (DIMBOA)	*A. sativa* (DIBOA)	*V. faba* (DIBOA)
0.05	108.6±0.4	—	—
0.10	88.2±2.9	100.2±0.9	98.7±12.1
0.25	71.4±1.7	87.1±1.7	90.1±7.0
0.50	59.4±6.8	79.9±2.0	86.1±0.5
1.00	55.1±2.6	74.6±2.1	71.4±2.6

The reactivity of cyclic hydroxamic acids towards sulphydryl groups is supposed to be one reason for their inhibitory effects on various enzymes [5, 13]. This reaction may also be responsible for the inhibition of the PM H^+-ATPase, since at least one exposed cysteine residue at the active centre is of special importance for the maintenance of the enzyme's conformation [14]. The stronger inhibition of the ATPase by DIMBOA can be explained by the presence of the strong electron-donating methoxy group, greatly enhancing the reactivity of the hydroxamic acid. No significant differences in the extent of inhibition was found on comparing the effects of DIBOA on *A. sativa* and *V. faba* PM ATPases. It may be supposed that conserved regions of the enzyme are involved in the inhibition.

Cyclic hydroxamic acids decompose in solution, yielding BOAs. The effects of the decomposition products MBOA and BOA on the PM H^+-ATPase activity from *A. sativa* was investigated. Inhibitory effects were found with concentrations from 0.5 to 2.0 mM. At lower concentrations, stimulatory effects were observed. Compared with the effects of cyclic hydroxamates the inhibition of H^+-ATPase activity by the corresponding BOAs was less pronounced (Fig. 1).

According to our studies, both hydroxamic acids and their corresponding decomposition products cause a rapid inhibition of the PM H^+-ATPase. DIMBOA has been described as an inhibitor for a wide variety of enzymes including proteases and chloroplast ATPase [5, 9, 10]. Nevertheless, the inhibitory influence of cyclic hydroxamic acids on the PM H^+-ATPase activity may be of special importance with regard to the allelopathic potential of the compounds. The location of the enzyme in the plasma membrane implies early interactions with absorbed allelochemicals. Furthermore, the enzyme has an important role in plant cell physiology. Therefore, these molecular effects can account for the allelopathic influences of benzoxazinones and related compounds.

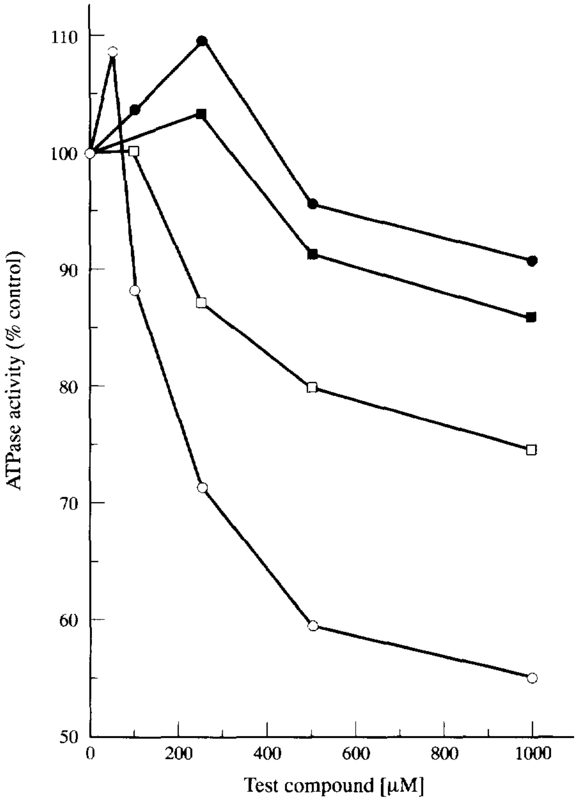

Fig. 1. Effects of BOA (■), MBOA (●), DIBOA (□) and DIMBOA (○) on the activity of H^+-ATPase after a 15-min preincubation.

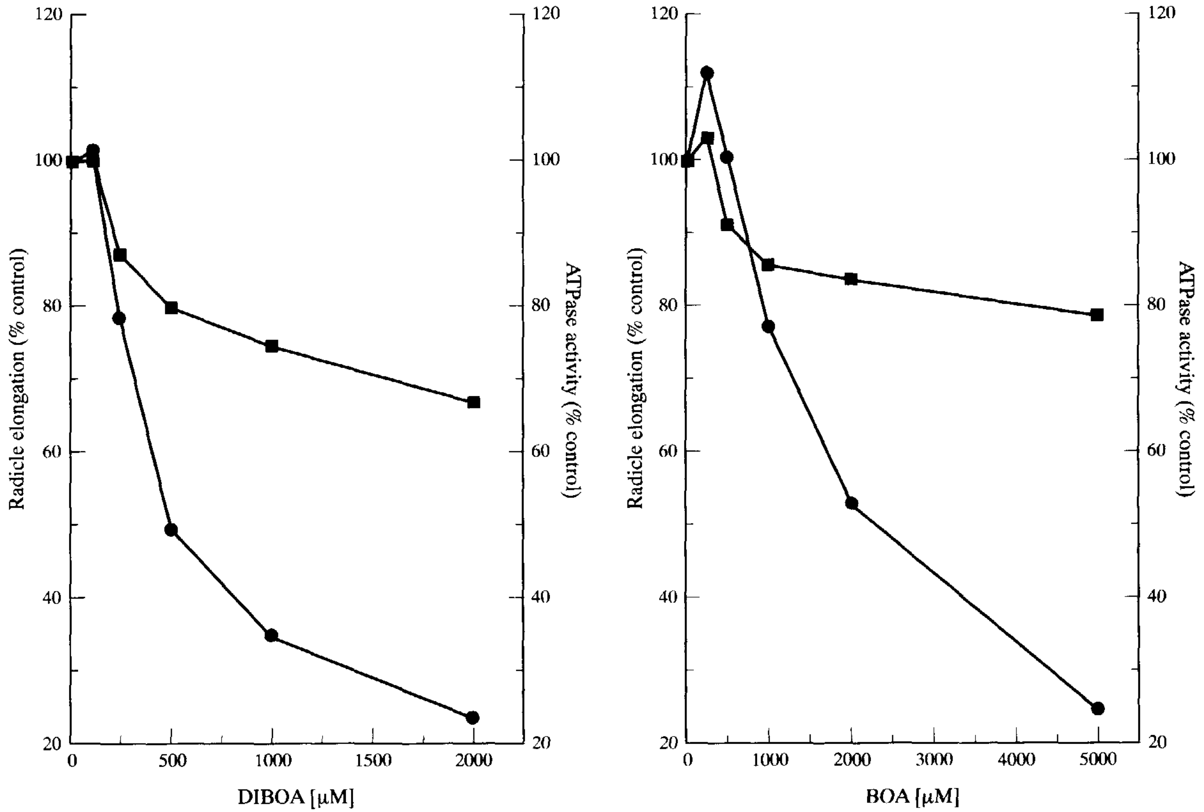

Fig. 2. Response of H$^+$-ATPase (■) and radicle elongation of oat (●) to BOA and DIBOA. ATPase was incubated in assay buffer containing BOA and DIBOA, respectively.

In order to prove the physiological importance of the inhibition of PM H^+-ATPase for plant growth, radicle elongation tests were performed. The effects of DIBOA and BOA on root elongation of *A. sativa* and ATPase activity are shown in Fig. 2.

The allelopathic action of both DIBOA and BOA on root elongation is closely related to their effects on PM ATPase. The concentration dependent inhibition of this enzyme is correlated with a strong reduction of root elongation. A decrease in PM ATPase activity to *ca* 80% leads to an inhibition of radicle elongation of *ca* 50%. However, it is well known that BOAs interfere with auxin-induced growth [15, 16], e.g. 6-methoxy-2-benzoxazolinone inhibits shoot and root elongation. Hasegawa *et al.* [17] assumed an allelochemical potential for these compound by acting as antiauxins. A multiple interaction of benzoxazinones and their decomposition products with numerous physiological and biochemical events has, therefore, to be taken into consideration, although the observed correlation between growth reduction and ATPase activity is striking.

Stimulatory effects of allelochemicals in low concentration on acceptor plants are often described in the literature, e.g. [2, 18, 19]. Nevertheless, there are only few explanations for this phenomenon at the molecular level. For BOA and MBOA an increased PM ATPase activity was found at effector concentrations of 0.1 and 0.25 mM. As can be seen from the radicle elongation test this molecular effect is correlated with a stimulation of root development by low concentrations of BOA. Thus, the stimulation of membrane-bound H^+-ATPase may account as well for the stimulatory effects of the investigated allelochemicals.

EXPERIMENTAL

Isolation of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA). DIMBOA was isolated from 7-day-old corn seedlings (*Zea mays* L., Marshall FAO 240) by a procedure described in ref. [20]. After purification by recrystallization from Me_2CO–hexane, crystals were obtained; mp 161.5–163.5° (lit. 163–164.5° [21]).

Isolation of 2,4-dihydroxy-2H-1,4-benzoxazin-3-one (DIBOA). DIBOA was isolated from an extract of shoots of rye (*Secale cereale* cv. Marder) by an analogous procedure to that described for DIMBOA. After purification by recrystallization from Me_2CO–hexane, crystals were obtained; mp 152–155° (lit. 155–157 [21]).

Preparation of PM vesicles. PMs were prepd from 7-day-old seedlings of *A. sativa* cv. Jumbo and *V. faba* cv. Alfred. All steps were carried out at 4°. PM vesicles of *A. sativa* were prepd by a modification of the method described in ref. [14]. Roots (30 g) were homogenized in 30 ml buffer (30% sucrose, 250 mM Tris–HCl, pH 7.5, 25 mM EDTA, 5 mM DTE, 0.05 mM Pefabloc, 2% insoluble PVPP, 10 mM ascorbic acid). According to the method of ref. [22] for the prep of the PM of *V. faba*, 40 g of roots were homogenized in 40 ml buffer (50 mM HEPES–KOH, pH 7.2, 3 mM EDTA, 3 mM DTE, 400 mM sucrose, 0.01% PMSF, 20% insoluble PVPP for inhibition of high phenoloxidase activity in broadbean tissue). Homogenates were filtered through 3 layers of cheese-cloth and the filtrates centrifuged for 20 min at 10 000 *g*. The resulting pellets were discarded and the supernatants were centrifuged for 30 min at 80 000 *g*. The pellets, i.e. the microsomal frs, were resuspended in 1.7 ml buffer (5 mM K-Pi buffer, pH 7.8, 4 mM KCl, 330 mM sucrose). PM vesicles were purified from the microsomal membrane fr. by 2-phase partitioning according to the method of ref. [23]. The microsomal fr. (1.5 g) was used for a 6-g phase mixt. [6.3% (w/w) Dextran (T 500), 6.3% (w/w) PEG 3350, 330 mM sucrose, 5 mM K-Pi buffer, pH 7.8]. The final phase was washed (× 2) with 3 vols buffer (330 mM sucrose, 4 mM KCl, 5 mM K-Pi buffer, pH 7.8) and centrifuged for 35 min at 100 000 *g*. The pellet was resuspended in 400–600 μl buffer. Protein was assayed by the method of ref. [24] using BSA as standard.

ATPase activity. ATP hydrolysis was determined at pH 6.5 (125 mM sucrose, 50 mM KCl, 0.015% Triton X-100) with 5 mM MgATP in the presence of 1 mM NaN_3 (inhibitor of mitochondrial ATPase), 0.2 mM $(NH_4)_6Mo_7O_{24}$ (inhibitor of acid phosphatase) and 50 mM $NaNO_3$ (inhibitor of vacuolar ATPase). Protein (10–15 μg) were used per assay. The final assay vol. was 1 ml. Allelochemicals were added from 100-fold concd stock solns in EtOH. Controls included 1% EtOH. After starting the reaction by addition of ATP, samples were incubated for 20 min at 37°. Released phosphate was measured according to ref. [25]. Each assay was performed in triplicate and all measurements were repeated.

Bioassays. The phytotoxicity of DIBOA and BOA was tested by radicle elongations assays with *A. sativa* cv. Jumbo. Stock solns of 15, 30, 60, 120 and 300 mM BOA and 7.5, 15, 30, 60 and 120 mM DIBOA were prepd in MeOH. Stock soln (50 μl) was added to glass pots (11 cm high, 5.5 cm diameter) with 26 g autoclaved glass pearls; 50 μl MeOH was also added to the controls. Pots were shaken thoroughly to ensure homogeneous distribution of the soln. The MeOH was allowed to evaporate completely for at least 6 hr. Six seeds of *A. sativa* were placed in each pot and watered with 3 ml tap water. After incubation for 6 days at 20° in the dark the radicle length was measured and expressed as a percentage of the control.

REFERENCES

1. Niemeyer, H., *Phytochemistry*, 1988, **27**, 3349.
2. Perez, F. J., *Phytochemistry*, 1990, **29**, 773.
3. Barnes, J. P. and Putnam, A. R., *Journal of Chemical Ecology*, 1987, **13**, 889.
4. Perez, F. J. and Ormeno-Nunez, J., *Journal of Chemical Ecology*, 1991, **17**, 1037.

5. Queirolo, C. B., Andreo, C. S., Niemeyer, H. M. and Corcuera, L. J. *Phytochemistry*, 1983, **22**, 2455.
6. Massardo, F., Zuniga, G. E., Perez, L. M. and Corcuera, L. J., *Phytochemistry*, 1994, **35**, 873.
7. Niemeyer, H. M., Calcaterra, N. B. and Roveri, O. A., *Biochemical Pharmacology*, 1986, **35**, 3909.
8. Queirolo, C. B., Andreo, C. S., Vallejos, R. H., Niemeyer, H. M. and Corcuera, L. J., *Plant Physiology*, 1981, **68**, 941.
9. Perez, F. J. and Niemeyer, H. M., *Phytochemistry*, 1988, **28**, 1579.
10. Cuevas, L., Niemeyer, H. M. and Perez, F. J., *Phytochemistry*, 1989, **29**, 1429.
11. Hager, A., Debus, G., Edel, H.-G., Stransky, H. and Serrano, R., *Planta*, 1991, **185**, 527.
12. Rayle, D. L. and Cleland, R., *Plant Physiology*, 1992, **99**, 1271.
13. Niemeyer, H. M., Corcuera, L. J. and Perez, F. J., *Phytochemistry*, 1982, **21**, 2287.
14. Serrano, R., *Methods in Enzymology*, 1988, **157**, 533.
15. Hoshi-Sakoda, M., Usui, K., Iskizuha, K., Kosemura, S., Yamamura, S. and Hasegawa, K., *Phytochemistry*, 1994, **37**, 297.
16. Hayashi, T., Gotok, K., Oknishi, K., Okamura, K., Asamizu, T., *Phytochemistry*, 1994, **37**, 1611.
17. Hasegawa, K., Togo, S., Urashima, M., Mizutani, J., Kosemura, S. and Yamamura, S. *Phytochemistry*, 1992, **31**, 3673.
18. Chase, W., Nair, M. G. and Putnam, A. R., *Journal of Chemical Ecology*, 1991, **17**, 9.
19. Schulz, M., Friebe, A., Kück, P., Seipel, M. and Schnabl, H., *Angewandte Botanik*, 1994, **68**, 195.
20. Woodward, M. D., Corcuera, L. J., Helgeson, J. P., Kelman, A. and Upper, C. D., *Plant Physiology*, 1978, **61**, 796.
21. Atkinson, J., Morand, P., Arnason, J. T., Niemeyer, H. M. and Bravo, H. R., *Journal of Organic Chemistry*, 1991, **56**, 1789.
22. Blum, W., Key, G. and Weiler, E. W., *Physiology of Plants*, 1988, **72**, 279.
23. Larsson, C., in *Isolation of Membranes and Organelles from Plant Cells*, eds J. L. Hall and A. L. Moore. Academic Press, London, 1983, pp. 277–309.
24. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.
25. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. and Candia, O. A., *Analytical Biochemistry*, 1979, **100**, 95.