Physiological Activity of Some Aminophosphonates

Krzysztof Bielecki[a,*], Anna Dziamska[b] and Janusz Sarapuk[b]

[a] Department of Botany and Plant Physiology, Agricultural University, Cybulskiego 32, 50-205 Wrocław, Poland. E-mail: bielecki@ozi.ar.wroc.pl
[b] Department of Physics and Biophysics, Agricultural University, Norwida 25, 50–375 Wrocław

* Author for correspondence and reprint requests

Z. Naturforsch. **56 c,** 995–998 (2001); received June 29/August 28, 2001

Aminophosphonates, Peroxidation, Conductance

Influence of some new aminophosphonates on electrolyte leakage from cucumber (*Cucumis sativus* cv "Wisconsin") cotyledons as well as on the content of chlorophyll and activity of guaiacol and pyrogallol peroxidase were studied. Concentration of malondialdehyde (MDA), one of the end-products of lipid peroxidation, was also measured.

It was found that aminophosphates influenced the parameters observed to various extents, depending on their structural features and the concentration used. Most active modifiers were those possessing sufficiently long hydrocarbon substituents at the nitrogen atom and/or iso-propyl chain at the phosphorus atom.

Introduction

Xenobiotic exposure may induce cellular injury due to membrane damage, which would seriously affect cellular structures and function (Crowley, 1980). The toxicity is usually related to the redox cycling phenomenon which promotes the formation of free radicals, identified as responsible for oxidative stress. Frequent and long-termed duration of oxidative stress leads to various pathological changes in organisms, dangerous maladies including (Braughler and Hall, 1989; Piotrowski *et al.*, 1990; Adams and Odunze, 1991; Gey, 1993; Smith *et al.*, 1994). The free radical-mediated effects include DNA damage, enzyme inactivation, and cellular or subcellular membrane peroxidation resulting in the generation of lipid hydroperoxides and complexes of carbonyl compounds including malondialdehyde (Pryor, 1984; Slater, 1984). Cellular damage is usually preceded by the impairment of antioxidant biochemical mechanisms that quench radicals before they initiate molecular effects. Among oxidative defenses, the antioxidant enzymes catalase, peroxidase (POX, EC 1.11.1.7) and superoxide dismutase are involved in the scavenging of reactive oxygen species (Castillo, 1992; Chance and Maehly, 1955; Foyer *et al.*, 1994; Kenyon and Duke, 1985, Knörzer *et al.*, 1996).

In peroxisomes, mitochondria and cytosol catalase play a key role in controlling the level of hydrogen peroxide (Foyer, 1994). Guaiacol and pyrogallol peroxidases, modulated by xenobiotics, are among the various peroxidases found in plant cells (Castillo, 1992). In addition to being implicated in the reaction of polymerisation of lignin precursors, their activity is also induced during many stress events (Knörzer *et al.*, 1996).

This work contains observations on activity of guaiacol and pyrogallol peroxidases activities in cucumber cotyledons treated by some aminophosphonates synthesized for potential use as herbicides. Generally, they constitute two groups of compounds, acyclic and cyclic, with different substituents at the carbon, phosphorus and nitrogen atoms in individual compounds. The aim of these studies was to select the best potential biologically active aminophosphonates. Studies on peroxidases activity were accompanied by measurements of conductance of aminophosphonates-treated tissue, lipid peroxidation and chlorophyll content in cotyledons.

Materials and Methods

Cucumber (*Cucumis sativus* cv 'Wisconsin') was grown under constant fluence of 150 μmol m^{-2} s^{-1}. Cotyledons from 7-d-old seedlings were used for experiments. Discs of 7 mm diameter were cut avoiding the midrib with a brass cork borer. The discs were rinsed in water and floated 24 h under constant light in 0.25 mM and 1 mM aminophos-

0939–5075/2001/1100–0995 $ 06.00

phonate solutions. Conductivity of the treatment solution was assayed with the OK-102/1 conducometer (Radelkis, Hungary). Enzymes were extracted by grinding discs in 100 mM K-phosphate buffer (pH 7.0) at 4 °C. After centrifugation at 15,000×*g* for 10 min the supernatant was used for all assays.

Formation of purpurogallin catalysed by pyrogallol peroxidase was followed at 430 nm and used for P-POD activity (Knörzer *et al.*, 1996). An absorbance coefficient of 2.47 mM^{-1} cm^{-1} was used. The reaction mixture contained potassium phosphate buffer (50 mM, pH 7.0), pyrogallol (20 mM), H_2O_2 (1 mM) and enzyme extract (0.28 μg protein) in final volume 1 cm^3. Reaction was started by adding H_2O_2.

For guaiacol peroxidase the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 5 mM H_2O_2, 0,25% guaiacol and enzyme extract (0.14 μg protein). The enzyme activity was measured by monitoring the increase in absorbance at 470 nm (absorbance coefficient of 26.6 mM^{-1} cm^{-1}) during polymerization of guaiacol into tetraguaiacol (Chance and Maehly, 1955).

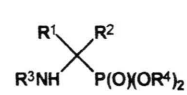
The malondialdehyde (MDA) in cotyledon discs and bathing solution was assayed. Ten discs were homogenized with 8 cm^3 5% TCA [Moran *et al.*, 1994]. 1.5 cm^3 of 0.65% in 20% TCA to 1.5 cm^3 of the extract from plant tissues or bathing medium was added. The mixture was heated in boiling water bath for 20 min, cooled quickly and centrifuged at 5000×*g* for 15 min. Absorbance of the supernatant was measured at 440, 532 and 600 nm. A_{532} represents the maximum absorbance of TBA-MDA complex, A_{600} the correction for nonspecific turbidity and A_{440} interference generated by TBA-sugar complex. MDA equivalents were calculated using the absorbance coefficient 0.156 M^{-1} cm^{-1} (Hodges *et al.*, 1999).

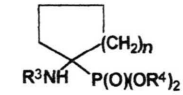
Chlorophylls were extracted in 80% acetone (Lichtenthaler, 1987).

All biochemicals reagents were of the highest quality available.

Aminophosphonates studied were synthesized at the Department of Organic Chemistry, Biochemistry and Biotechnology, Technical University of Wrocław. Their general structure and particular substituents are given in Table I. Synthesis details as well as spectral data are given elsewhere (Wieczorek *et al.*, 2000; 2001).

Table I. The structure and substituent groups of the aminophosphonates.





Comp. No.	R^1	R^2	R^3	R^4	*n*
1			n-C_4H_9	i-C_3H_7	2
2			n-C_8H_{17}	i-C_3H_7	1
3			n-C_8H_{17}	C_2H_5	2
4			n-C_8H_{17}	n-C_4H_9	2
5			C_2H_4OH	n-C_4H_9	2
6			$C(CH_2OH)_2CH_3$	n-C_4H_9	1
7	CH_3	n-C_3H_7	n-C_4H_9	n-C_4H_9	
8	CH_3	CH_3	n-$C_{14}H_{29}$	n-C_4H_9	
9	CH_3	n-C_4H_9	n-C_5H_{11}	n-C_4H_9	

n = 1 is for compounds with a pentane ring, *n* = 2 for these with a hexane ring.

Results and Discussion

The results of aminophosphonate-induced changes in conductivity of the efflux are shown in Table II. Some differences between the values of conductance are small, which permit to divide the compounds into three groups. The sequence of the activity of those groups is like follows:

(2, 3) > (7, 8, 9) > (1, 4, 5, 6).

It can be easily seen that the changes in conductivity are almost exactly followed by aminophosphonates-induced lipid peroxidation (Table II). On the other hand, the chlorophyll content (Table II) roughly follows the reverse sequence. The obvious conclusion is that the more damaged the membrane tissue is less chlorophyll content is found. This conclusion is confirmed by the observation that chlorophyll content decreased with increase in concentration of a particular aminophosphonate while both the conductance and the malondialdehyde (MDA) concentration increased. Compounds containing hydroxyl groups in their substituent at the nitrogen atom (compounds **5** and **6**) exhibited a weak influence on both conductance and chlorophyll content, especially when used in the lowest of concentrations used. On the other hand, the strongest modifiers were found to be compounds **2**, **3** and **8**. All of them have a hydrocarbon chain containing at least 8 carbon atoms. It seems that such a substituent should be

Table II. Effect of 0.25 mM and 1 mM of aminophosphonates on efflux of electrolyte from cucumber (*Cucumis sativus* cv "Wisconsin") cotyledons, malondialdehyde (MDA) and chlorophyll content in cotyledons and on pyrogallol and guaiacol peroxidases activities in cotyledons.

Parameters	Conc. [mM]	Compounds 1	2	3	4	5	6	7	8	9
Conductance	0.25	282	475	380	100	82	70	180	350	145
[$\mu S\ cm^{-1}$]	1.00	420	500	570	210	310	185	550	510	600
MDA	0.25	105	452	307	182	121	132	196	275	141
[% of control]	1.00	175	405	428	232	232	215	399	319	373
Chlorophyll	0.25	62	29	53	83	99	94	78	55	88
[% of control]	1.00	37	12	17	78	72	59	16	41	34
Guaiacol perox. activity	0.25	7	6	120	160	27	100	44	349	117
[% of control]	1.00	48	14	6	200	135	128	12	124	4
Pyrogallol perox. activity	0.25	70	26	120	216	121	109	59	330	99
[% of control]	1.00	24	20	55	185	47	148	22	116	34

Specific activities of the controls were [mmol min^{-1} mg^{-1} protein]: guaiacol peroxidase –0.47; pyrogallol peroxidase –1.02. Standard deviations did not exceed 14% for MDA determination and 9% for determination of other parameters.

combined with a short one at the phosphorus atom. Another conclusion is that acyclic compounds (**7** and **9**) are more effectively influencing the parameters studied than cyclic ones (**1** and **4**), probably due to the slightly greater lipophilicity produced by the substituents R^3 and R^4 of the cyclic aminophosphonates.

It is worth noting that the majority of the aminophosphonates studied were tested for potential antioxidative activity. It was found that this activity, although not very intensive, depended in similar way on their structural features. Especially good correlation between conductance and antioxidative activity experiments was found for compounds containing hydroxyl groups at the nitrogen atom substituents. Their antioxidative activity was significantly lower than that of other compounds (Kleszczyńska and Sarapuk, 2001).

It was shown previously that membrane destruction by organophosphorous compounds is closely related to lipid peroxidation (Linsel *et al.*, 1988). Our studies evidence that the protective enzymes pyrogallol and guaiacol peroxidases follow a pattern which indicates general cellular disruption. Pyrogallol peroxidase seems to be more sensitive to aminophosphonates. This is not surprising, because peroxidase is localized in the vacuole and near the plasmalemma and both tonoplast and plasmalemma have been shown to be sites of damage caused by aminophosphonates. The results obtained indicate that the sequences of increase in activity of both peroxidases mimic quite well that obtained for change in chlorophyll content of cellular extracts (Table II). A slight dependence of their activity on aminophosphonate concentration was also observed. These results may be interpreted as a response of cotyledons to aminophosphonate-induced oxidative stress, especially in view of extraplastidic H_2O_2 quenching by peroxidase.

Acknowledgments

This work was supported by the Polish Research Committee (KBN), grant no. 6 P04G 050 17. We also thank K. Kulma for her technical assistance.

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