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SUBSTRATE PEROXIDATION PATTERNS IN VASCULAR PLANTS AND THEIR MODIFICATION BY EXPOSURE TO MERCURY VAPOUR

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Key Word Index—Angiosperms; peroxidase; stress; mercury vapour.

Abstract—Donor substrate peroxidation patterns appear to reflect both taxonomic affinities and certain physiological states of the plant. When peroxidase activities in *Mimosa*, *Dionaea*, *Tillandsia*, *Nephrolepis* and *Salvinia* cell-free extracts were compared with activity of crystalline horseradish enzyme using six phenolic donors, the horseradish, *Mimosa* and *Dionaea* patterns were most similar, *Tillandsia* less so, and the others more distant. The most remote was *Nephrolepis*, as one of the phenols was not a substrate. After exposure of intact plants to mercury vapour-saturated air for 10 days, peroxidase activity was increased in every species as reflected by at least one, and up to six, of the phenolic substrates (which include pyrogallol, gallic acid, guaiacol and *p*-methylaminophenol). The substrate peroxidation pattern for the increments of activity induced by mercury vapour was entirely different from the baseline patterns prior to mercury vapour exposure.

INTRODUCTION

In their survey of the plant peroxidase literature of the decade 1970–1980, Gaspar *et al.* [1] list approximately 150 papers, out of a total of 1500, concerned with abscission, ethylene, aging, stress and wounding. A similar proportion of the papers presented at the 1985 Geneva symposium on plant peroxidases [2] were addressed to these same areas.

Heme enzyme activity has long been associated with seed viability, but the specific association of peroxidase (E. C. 1.11.17) changes with injury and stress is more recent. Increases in total peroxidative activity have been reported in many cases of exposure to ozone, drought and chilling, salinity, gravity and radiation, hypoxia and other injurious agents [1, 2]. Changes in peroxidase are not limited to overall activity, but include alterations in isozyme patterns by salinity [3], hypoxia [4], etc.

Exposure of the plant to most of these factors commonly results in early senescence and is manifested by abscission presumably via enhanced ethylene production [5]. In 1976 mercury vapour (Hg°) was observed to induce ethylene formation initially, followed by abscission, in *Coleus* and *Citrus* explants [6]. Subsequently [7] it was shown that mature plants especially were heavily defoliated by exposure to as little as 0.05 μg Hg° per litre of air; much higher concentrations of $HgCl_2$, both as an aerosol and in aqueous solution, produced necrotic lesions but little or no abscission.

We have now found that although mercury vapour does promote an increase in peroxidase activity in a variety of vascular plants, this enhancement and its extent are highly species and donor-substrate dependent, to a wholly unexpected degree.

RESULTS AND DISCUSSION

The term 'activity' as used here, refers to the kinetic quantity, ΔO.D/min/mg, and not to the actual heme-protein concentration. Changes which might affect activity include the appearance of a new, more reactive redox co-factor, but we deem this improbable because all our test compounds are known to be direct donor substrates for peroxidase [8]. Another alternative is enhanced activity resulting from structural changes or variations induced at the molecular level, including synthesis of new isozymes.

We have chosen to use a highly simplified standard procedure for the assessment of the effects of mercury vapour on peroxidase activity (see Experimental). The application of fractionation and purification procedures to these extracts would entail the risk of creating isozymal artifacts with substrate specificities different from those with the extract (and the plant tissues). The best test of our methodology can be found in the comparison of purified horseradish peroxidase with all three extracts from closely related vascular plants [9], (Tables 1 and 2) as discussed below.

Whatever the mechanistic basis for the enhancement of peroxidase activity in plants exposed to toxic doses of mercury vapour, the consistency of the response is clearly in evidence (Table 3). As expected [8], the peroxidase in all species tested used pyrogallol, gallic acid, guaiacol and *p*-methylaminophenol as donor-substrates, but *Dionaea* preparations could not peroxidize dimethoxyphenol; *Nephrolepis* could not utilize dimethoxyphenol, and oxidized methyl gallate but slowly (Table 3). These few vascular plant specimens include a highly specialized angiosperm and very common sort of fern, the latter displaying several 'all-or-none' specificities. Of equal or greater importance is the existence among these plants of systematic similarities and differences in the relative rates of substrate peroxidation. We have already suggested [9] that the ratios of oxidation rates for an array of substrates normalized to the rate for one of these substrates provides an index of comparative value both phylogenetically and physiologically. The reference molecule of choice may vary but we have selected pyrogallol because it appears to be closest to a 'universal' substrate, and related to the rate

Table 1. The performance of horseradish peroxidase as a comparison standard

	pH	(mm)	Peroxidase 0.2	1.0 (OD/min)	(μM) 2.0	Substrate Rate ratio
Pyrogallol	7.5	425	0.022	0.110	0.201	1.00
Gallic acid	4.5	425	0.034	0.169	0.322	1.40 ± 0.12
Methyl gallate	4.5	425	0.063	0.303	0.612	2.88 ± 0.15
Guaiacol	6.1	470	0.036	0.180	0.348	1.67 ± 0.05
2,6-Dimethoxyphenol	6.1	580	0.021	0.102	0.200	0.95 ± 0.03
p-(*N*-Methylamino)phenol	5.2	580	0.357	1.65	3.46	15.82 ± 0.80

Hydrogen peroxide 20 mM.
All donor substrates 10 mM; temperature 24 ± 0.5°.
Values are based on the means four replicates; mean error ±9%.

Table 2. Substrate rate ratios for horseradish peroxidase and peroxidase containing preparations from five vascular species; all substrate rates are normalized to pyrogallol = 1.00

Substrate ratio	Horseradish peroxidase	Peroxidase in *Mimosa*	*Dionaea*	*Tillandsia*	*Nephrolepis*	*Salvinia*
Pyr/Pyr	1.00	1.00	1.00	1.00	1.00	1.00
Gall/Pyr	1.40	1.28	1.20	0.90	0.79	0.60
MeGal/Pyr	2.88	2.60	2.71	0.88	0.20	0.25
Gua/Pyr	1.67	1.65	1.43	1.50	0.79	0.79
DMP/Pyr	0.95	0.76	0.40	0.35	0.00	0.20
MAP/Pyr	15.82	16.82	10.36	8.95	0.76	3.79

Table 3. Substrate peroxidation patterns in cell-free extracts of five vascular species and their modification by prior exposure of the plants to mercury vapour

Species	Exposure to mercury vapour (days) 0	3	10
	(a) Pyrogallol (Pyr)		
Mimosa	0.151 ± 0.020 (100)	0.418 ± 0.061 (277)*	0.574 ± 0.087 (377)*
Dionaea	0.014 ± 0.003 (100)	0.022 ± 0.004 (154)	0.142 ± 0.021 (1014)*
Tillandsia	0.099 ± 0.010 (100)	0.142 ± 0.011 (142)*	0.270 ± 0.044 (271)*
Nephrolepis	0.042 ± 0.006 (100)	0.042 ± 0.005 (100)	0.046 ± 0.009 (109)
Salvinia	0.077 ± 0.009 (100)	0.096 ± 0.012 (125)	0.132 ± 0.008 (171)*
	(b) Gallic acid (Gall)		
Mimosa	0.194 ± 0.028 (100)	0.243 ± 0.033 (126)	0.547 ± 0.017 (282)*
Dionaea	0.016 ± 0.004 (100)	0.018 ± 0.002 (113)	0.058 ± 0.010 (63)
Tillandsia	0.090 ± 0.006 (100)	0.150 ± 0.009 (167)*	0.291 ± 0.022 (321)*
Nephrolepis	0.012 ± 0.004 (100)	0.007 (57)	0.003 (25)
Salvinia	0.046 ± 0.007 (100)	0.048 ± 0.004 (106)	0.105 ± 0.013 (233)*

Table 3. *continued*

Species	Exposure to mercury vapour (days) 0	3	10
	(c) Methyl gallate (MeGal)		
Mimosa	0.392 ± 0.060 (100)	0.531 ± 0.060 (136)	0.798 ± 0.095 (204)*
Dionaea	0.038 ± 0.004 (100)	0.064 ± 0.009 (168)*	.0133 ± 0.015 (350)*
Tillandsia	0.089 ± 0.012 (100)	0.138 ± 0.023 (155)	0.203 ± 0.029 (229)*
Nephrolepis	0.010 ± 0.001 (100)	0 —	0.002 —
Salvinia	0.019 ± 0.003 (100)	0.019 ± 0.003 (100)	0.020 ± 0.009 (105)
	(d) Guaiacol (Gua)		
Mimosa	0.250 ± 0.022 (100)	0.632 ± 0.058 (253)*	1.562 ± 0.150 (604)*
Dionaea	0.020 ± 0.003 (100)	0.033 ± 0.004 (165)	0.136 ± 0.010 (680)*
Tillandsia	0.160 ± 0.008 (100)	0.119 ± 0.007 (75)	0.211 ± 10.017 (132)*
Nephrolepis	0.033 ± 0.005 (100)	0.039 ± 0.006 (117)	0.036 ± 0. + 020 (109)
Salvinia	0.006 ± 0.002 (100)	0.074 ± 0.009 (122)	0.120 ± 0.008 (182)*
	(e) Dimethoxyphenol (DMP)		
Mimosa	0.115 ± 0.014 (100)	0.080 ± 0.011 (70)	0.070 ± 0.008 (60)
Dionaea	0.006 —	0.006 —	0.004 —
Tillandsia	0.035 ± 0.005 (100)	0.045 ± 0.006 (128)	0.077 ± 0.007 (220)*
Nephrolepis	0.020 ± 0.005 (—)	0.002 (—)	0 (—)
Salvinia	0.020 ± 0.003 (100)	0.086 ± 0.004 (180)	0.048 ± 0.013 (240)*
	(f) *p*-(*N*-Methylamino)phenol (MAP)		
Mimosa	2.45 ± 0.231 (100)	3.21 ± 0.30 (133)	5.87 ± 0.49 (240)*
Dionaea	0.145 ± 0.010 (100)	0.190 ± 0.033 (131)	0.297 ± 0.014 (205)*
Tillandsia	0.90 ± 0.11 (100)	1.15 ± 0.21 (120)	3.41 ± 0.51 (384)*
Nephrolepis	0.032 ± 0.005 (100)	0.119 ± 0.031 (372)*	0.245 ± 0.098 (765)*
Salvinia	0.253 ± 0.034 (100)	0.428 ± 0.051 (168)*	0.436 ± 0.071 (174)*

*Activity increase upon exposure to mercury vapour is significant at $p < 0.05$.
†Relative percent in parenthesis.

of peroxidation of the other five substrates when a purified horseradish (*Armoracia*) enzyme is used (Table 1). When the 'substrate-rate-ratio' from our experimental species is compared with the reference enzyme (Table 2), the pattern of similarities and differences is evident. The horseradish enzyme pattern is close to *Mimosa* and to the Venus Flytrap, *Dionaea*. *Tillandsia*, a monocotyledonous plant differs more, and further differences appear between fern species which separate them from the angiosperms and from one another.

A three-day exposure to mercury vapour evokes significant increases in peroxidase activity in *Mimosa*; in *Tillandsia*, as reflected in the oxidation of pyrogallol and gallic acid; in *Dionaea*, as shown by methyl gallate only; and in *Nephrolepis*, as shown by *p*-methylaminophenol only. After 10 days the effect of mercury vapour was more general as shown in *Mimosa* and *Tillandsia* where oxidation of five or six substrates was now affected; and by Dionaea where its oxidation of four substrates was now increased. Enhancement of peroxidase activity in

Nephrolepis was shown with *p*-methylaminophenol only, but by *Salvinia* with every substrate save methyl gallate. Hence of the 30 possible combinations of number of substrates × number of plant species, there are 21 instances of significant enhancement of peroxidase activity after a 10-day exposure to mercury vapour.

To examine further the relation of that increment to the control or baseline peroxidase, the difference (10-day rate − 0-day rate) has been normalized to pyrogallol as above in the substrate 'rate ratio' procedure (Table 4). Even the most superficial comparison of Tables 1 and 4 shows that the factors responsible for incremental peroxidative activity have completely unique substrate rate ratios, distinct from baseline peroxidases in the experimental plants and from the horseradish standard alike.

We have considered the possibility that mercury itself participates directly. Young plants grown from seed in mercury vapour-saturated air (14 μg l) will, after 1–2 weeks, accumulate 200–300 μg Hg per g dry wt, or about 1.0–1.5 μmol/g [10]. Elemental mercury, however, does not exhibit peroxidase-like catalytic activity. When added to peroxidase preparations, it does not modify the enzyme's activity toward common substrates. These negative observations do not preclude the possibility that specific proteins evoked by mercury-intoxication combine with the metal to form an oxidase.

It will be recalled that exposure to other stresses—salinity, drought, gravity, radiation, etc.—results in significant increases in peroxidase activity without any involvement of mercury at all. Thus the nature of the induced or evoked peroxidatic activity remains unclear, but the response itself is consistent with a growing body of literature linking peroxidases and stress. The two unusual features of our present work are the fact that non-ionic mercury vapour consistently shows physiological activity, and that there is a remarkable degree of substrate selective character in the peroxidatic response to mercury vapour exposure.

EXPERIMENTAL

Mimosa pudica L. ('sensitive plant') and *Dionaea muscipula* L. ('Venus Flytrap') were 11–12 weeks old when used and the bromeliad *Tillandsia fasciculata* SW. were all about 6 months old. Boston fern, *Nephrolepis exaltata* var. *bostoniensis* Davenport was about 18 weeks old and mature vegetative fronds of a *Salvinia* sp. were at least 16–18 weeks. Routinely, each replicate represents 3 or 4 shoots homogenized in M/100 phosphate buffer, centrifuged free of particulate matter and tested within a few min. In general, one ml of supernatant represented 10–100 mg. of fresh weight. For assay, 1.0 ml of suitably diluted supernatant was mixed with 5 ml of 10 mM donor substrate in M/10 phosphate of desired pH, any trace of colour or turbidity adjusted to zero, and H_2O_2 added with rapid mixing to give a final 20 mM concentration. The reactions at 25 ± 1° were run for 3 min, although rates were based on 30–90 sec A readings. Other specific details are summarized in Table 1. The reference standard in this study was prepared from Worthington crystalline horseradish peroxidase, R.Z. = 3.1, PZ = 3000, batch MPODD 8F. The overall error of mean values was 9%.

The substrates used were pyrogallol, Kodak ACS reagent grade 1,2,3-trihydroxybenzene; gallic acid, Baker ACS reagent grade of 3,4,5-trihydroxybenzoic acid; methyl gallate, Aldrich purified (98%) gallic acid methyl ester; guaiacol, Baker Analysed *o*-methoxyphenol; 2,6-dimethoxyphenol, Aldrich purified (99%); and methylaminophenol, Kodak purified (99%+) *p*-(*N*-methylamino)phenol sulphate. All the substrates are white crystalline or colourless liquid materials.

Exposure of the plants to mercury was accomplished by sealing them in clear 400 l plexiglass chambers with Hg° (liq.)-filled syracuse dishes at each of the corners. No more than a few hundred g (fr. wt) of plant tissue were exposed at any time, assuring adequate environmental support for gas exchange. Exposure took place on a natural diurnal cycle with an 11–12 hr day and 25–30° temp. range.

Means of replicated experiments, each involving 3–4 plants, and their standard deviations are given. Significance (*) by *t*-test is given for $p \leqslant 0.05$.

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Table 4. Substrate rate ratio for the increment of peroxidase activity induced by 10 days. exposure to mercury vapour

Substrate ratio	Peroxidase in				
	Mimosa	*Dionaea*	*Tillandsia*	*Nephrolepis*	*Salvinia*
Pyr/Pyr	1.00	1.00	1.00	N	1.00
Gall/Pyr	0.87	0.32	1.44	N	1.08
MeGal/Pyr	0.93	0.73	0.67	O	N
Gua/Pyr	3.10	0.91	0.76	N	1.08
DMP/Pyr	N	N	2.45	O	0.49
MAP/Pyr	8.09	1.19	14.8	+	3.33

N, No increment.
O, Not a substrate.
+, Significant increment.

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