Phytochemistry, Vol. 21, No. 8, pp. 1875–1880, 1982.
Printed in Great Britain.

0031-9422/82/081875-06$03.00/0
Pergamon Press Ltd.

INDOLEACETIC ACID OXIDASE ACTIVITY IN MAIN STEM HOMOGENATES OF FLAX GENOTROPHS AND GENOTYPES

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(*Revised received* 6 *July* 1981)

Key Word Index—*Linum usitatissimum*; Linaceae; flax; IAA-oxidase, peroxidase; genotypes and genotrophs; stem gradients.

Abstract—The IAA-oxidase and peroxidase capabilities along the length of the main stem tissues of two flax genotrophs L and S and two flax genotypes R and M were examined *in vitro*. Stem gradients for peroxidase activity increased basipetally in all plant types, as did IAA-oxidase activity gradients at non-rate-limiting concentrations of Mn^{2+}. Correlations between peroxidase activity and non-rate-limited IAA-oxidase activity supported the contention of dual activities on the same molecule. At rate-limiting concentrations of Mn^{2+}, IAA-oxidase activity did not correlate with peroxidase activity. Plant type differences were detected in rate-limited IAA-oxidase activity. This activity was higher in the stem region immediately above the cotyledons (axillary buds) of the more branched types, L and R, than in the sparsely branched types, *S* and M.

INTRODUCTION

Environmentally induced heritable changes have been demonstrated in flax (*Linum usitatissimum* L.)[1] and tobacco (*Nicotiana rustica*)[2], which both are, or can be, completely inbred. Contrasting large (L) and small (S) flax genotrophs resulted from one generation of treatment of a completely inbred flax genotype with high levels of soil-supplied inorganic nutrients, in particular NPK and NK. NPK treatment yielded progeny which were markedly larger than those from NK-treated parental plants. NPK progeny were also much more branched than NK. The differences in plant size and weight between the two progeny groups have persisted virtually undiminished for at least 17 generations grown in uniform environmental conditions. The similarities and essential features of such environmentally induced changes in flax have been summarized[1].

Biochemical studies with L and S have involved the phytohormone systems, due to their possible role in mediating plant size and branching differences. Indole derivatives such as indole acetic acid (IAA) form one facet of these sytems; one may look at IAA synthesis and/or degradation. Quantitative assay of IAA in plant tissues still poses formidable technical problems, in spite of recent advances[3], whereas assay of enzymes concerned with IAA degradation, such as peroxidase (donor: H_2O oxidoreductase EC 1.11.1.7), is relatively straightforward. Not only can IAA oxidase (IAA-ox) and peroxidase activities be readily measured, but individual peroxidase isozymes can be separated, assayed and purified through gel electrophoresis and CC [4]. The availability of these techniques provided the technical rationale for looking at IAA degradation in flax genotrophs.

The genetics of control over genotroph differences (L–S) in peroxidase isozymes showed that the relative mobility (R_m) differences between two corresponding isozymes in L and S resulted from two alleles at one locus, with complete dominance of the allele for the faster moving (L) variant[5]. Higher peroxidase activity of S isozymes was due to greater production of S isozyme protein compared to L[4]. Peroxidase activity gradients increasing basipetally were found in flax genotrophs[6] and genotypes[7]. Purified peroxidase isozymes possessed both perocidase and IAA-ox activity[4], corroborating other suggestions[8] that both functions resided on the same molecule. Basipetal gradients of IAA-ox activity have been reported also in young shoots, e.g. of Japanese morning glory[9].

The studies reported here concerned *in vitro* measurement of peroxidase and IAA-ox capabilities at points along the main stem tissue of flax genotrophs L and S. A genotype with sparse basal branching, that is, a 'flax' type similar to the S genotroph in this respect, was included, as well as a 'linseed' genotype resembling L. In terms of degree of basal branching, therefore, the highly branched and bushy L genotroph was paralleled by the Royal (R) linseed genotype, and S was paralleled by the flax Mandarin (M). IAA-ox activity measurement reflected tissue extract capability in degrading exogenously supplied IAA.

Degradation (decarboxylation) of IAA can be enhanced or inhibited by a number of naturally occurring phenolic acids[10, 11]. Anion exchange resin used during extract preparation removed virtually all phenolic peroxidase inhibitors present[12]. *In vitro* degradation of IAA is also affected by the presence of Mn^{2+}[13]. IAA-ox activity of extracts was, therefore, measured in the absence of exogenous Mn^{2+} to obtain the 'basal activity', and also over an Mn^{2+} range to allow 'potential activity' (i.e. IAA-ox activity when Mn^{2+} was not rate limiting) to be obtained.

RESULTS

IAA-ox activity response to Mn^{2+} concentration

All extracts showed a curvilinear increase in activity with increasing Mn^{2+} concentration. Table 1 shows examples of this response and gives for comparison the IAA-ox activity response of purified horseradish peroxidase (HRP) in a range of IAA concentrations. The activity vs Mn^{2+} concentration response curves mirrored the reaction velocity vs substrate concentration curve of HRP except that most extracts displayed basal activity, i.e. in the absence of Mn^{2+}. For each extract, subtracting basal activity from the IAA-ox activities at each Mn^{2+} concentration adjusted the activity response curves to pass through the origin. For the majority of extracts the Michaelis–Menten equation suitably described these adjusted response curves. Using the weighted regression analysis, the regression coefficients K_m/V_{max} and $1/V_{max}$ were significant for 25 of the 32 extracts. $1/V_{max}$ and V_{max} derived from it were significant for 28 of the 32. V_{max} estimated the increase in activity from basal activity to the activity when Mn^{2+} was not rate limiting. The maximal potential IAA-ox activity ('potential activity') of an extract was, therefore, the sum of its basal activity and its V_{max} value.

The mean values for the basal and potential activities of the plant type–stem section combinations are given in Table 2. Table 3 summarizes the analyses of variance for these two parameters.

Basal IAA-ox activity and activity at low Mn^{2+} concentrations

Activity was significantly higher in section 2 than in the mean of the three other sections (except in L). The basal activities and their analysis indicated interactions between the genotrophs and the stem sections. Examinations of the raw data showed that plant type–stem section interactions occurred below 0.20 mM Mn^{2+}. The activity data at M^{2+} below 0.20 mM Mn^{2+} for each stem section of the four plant types are given in Table 4 together with F-values from analyses conducted on each stem section separately. Mean IAA-ox activity at low Mn^{2+} concentrations was higher for L and R than for S and M respectively in section 1. It was lower for L and S and showed no difference between R and M in sections 2 and 3. No differences between the four plant types were detected in section 4.

Potential IAA-ox activity

Potential activity decreases from the cotyledons to the apex. Mean potential activity was significantly higher for S than L, all four plant types displayed linear decreases up the stem and the slope of the linear decrease in S was significantly steeper than the mean of L, R and M.

Peroxidase activity

Table 2 gives the mean peroxidase activities and Table 3 summarizes the analyses of variance of the peroxidase activity data. Peroxidase activity was significantly higher in S and R than in L and M, respectively, and decreases from the cotyledons to the apex in all plant types. The activity decrease up the stem was curvilinear and there were significant plant type–stem section interactions. The stem section gradients were examined separately for each plant type. The activity gradient for S was significantly different from those of L, R and M.

Comparison of peroxidase activity and IAA-ox activity parameters

Table 2 shows the correlation coefficients derived from covariance analyses comparing peroxidase activity with basal and potential IAA-ox activity. The between plant type–stem section combination correlation coefficients were positive and significant for potential activity and not significant for basal activity. Within plant type–stem section correlation coefficients (error line correlations) indicated that the

Table 1. Typical IAA-oxidase response to $MnCl_2$ concentration (stem section 1) in plant types L, S, R and M: purified horseradish IAA-oxidase activity response to substrate concentration

$MnCl_2$ concentration (mM)	IAA-oxidase activity (A/30 min/ml)				Purified horseradish peroxidase			
					Purified* isoenzyme no. 4		IAA concentration (g/ml)	IAA-oxidase activity (A/30 min/ml)
	L	S	R	M	L	S		
0.00	0.11	0.00	0.11	0.00	2.8	2.8	5	0.22
0.05	0.14	0.01	0.11	0.01	—	—	10	0.43
0.10	0.20	0.08	0.14	0.05	2.7	2.7	15	0.61
0.20	0.23	0.12	0.16	0.08	2.7	2.9	20	0.80
0.30	—	—	—	—	2.7	2.9		
0.40	0.29	0.52	0.30	0.13	2.9	2.9	25	0.96
0.60	0.33	0.64	0.31	0.16	—	—	30	1.08
1.00	0.35	0.76	0.37	0.22	—	—	35	1.08
1.40	0.36	1.12	0.41	0.23	—	—	40	1.11
1.80	0.37	1.08	0.42	0.23	—	—	45	1.01

*A/0.3 min/ml purified isozyme concentrate.

Table 2. Mean peroxidase activity and IAA-oxidase parameter values and correlation coefficients in plant types L, S, R and M

Enzyme activity	Section	L	S	R	M	Mean
Peroxidase activities	1	9.4	20.7	14.4	7.1	12.9
(ΔA/min/ml)	2	4.3	17.3	6.7	3.8	8.0
	3	1.9	6.4	2.5	1.4	3.0
	4	0.9	1.8	0.7	0.8	1.0
	Mean	4.1	11.5	6.1	3.3	—
Basal IAA-O ox activity	1	0.11	0.00	0.11	0.00	0.05
(ΔA/30 min/ml)	2	0.07	0.40	0.19	0.25	0.22
	3	0.01	0.14	0.00	0.04	0.05
	4	0.04	0.03	0.04	0.01	0.03
	Mean	0.06	0.14	0.08	0.07	—
Potential IAA-ox activity	1	0.43	3.64	0.55	0.35	1.24
(ΔA/30 min/ml)	2	0.39	2.40	0.48	0.53	0.95
	3	0.33	0.65	0.13	0.23	0.33
	4	0.14	0.39	0.13	0.15	0.20
	Mean	0.32	1.77	0.32	0.32	—

Correlation coefficients for comparisons of peroxidase activity with IAA-oxidase activity parameters.

	Basal IAA-ox	Potential IAA-ox
Between combinations	$r_{14} = +0.408$	$r_{14} = +0.855$**
Within combinations	$r_{15} = -0.791$**	$r_{15} = +0.584$*

*Significant at $P = 0.05$.
**Significant at $P = 0.01$.

underlying relationship between peroxidase activity and potential activity was also positive and significant but was negative and significant between peroxidase activity and basal activity.

DISCUSSION

There is controversy whether peroxidase and IAA-ox are the same enzyme (e.g. ref. [14]). Studies in this laboratory with flax peroxidases all indicate their capacity to function as IAA-ox, that is, although flax peroxidases may have additional roles within the plant tissues and, futhermore, may not be the only source of IAA-ox activity, they have the potential of playing a major role in the degradation of IAA. The dilemma lies not so much in whether peroxidase can function as IAA-ox but rather in whether it does function as such *in vivo*. Despite the problems of extrapolating from *in vitro* studies to the *in vivo* situation, results obtained *in vitro* for comparative studies are of value in that differences demonstrated under uniform *in vitro* conditions indicate the possibility of differences also occurring *in vivo*.

Under 'optimal' *in vitro* reaction conditions, an enzyme activity difference between two plant extracts would indicate either the presence of more enzyme, in one extract compared to the other, or a kinetic difference between the enzyme present in the two extracts. Ideally, to clearly distinguish between these two possibilities requires isolation and purification of the enzymes involved. The peroxidase isozymes of the total main stem tissue of the genotrophs and genotypes have been isolated and purified [4] and the following three points are relevant here: (1) Higher peroxidase activity in S and R compared to L and M was due to quantitative differences; (2) There was a basic correlation between the peroxidase and IAA-ox activities of the purified isozymes; (3) Despite this basic correlation, there was a difference between the genotrophs and between the genotypes in the balance between the two activities; IAA-ox activity per unit peroxidase activity was higher in S and M than in L and R respectively. As all purified flax isozymes possessed both peroxidase and IAA-ox activity, the correlation between peroxidase activity and potential IAA-ox activity for the plant extracts here suggests that potential activity for the most part measures the potential of peroxidase present in the extracts to function as IAA-ox. Further, genotroph or genotype differences in peroxidase or potential IAA-ox activity may be presumed to stem from quantitative differences and it seems realistic to assume that the extreme differences between different regions of the stem for both these activities also basically result from quantitative differences (i.e. less enzyme synthesized per unit fr. wt in progressing from the cotyledons to the apex). There were also some indications of differences in the balance of the two activities between the plant types and stem sections. For example, R and M differed significantly in peroxidase activity, but did not differ in potential

Table 3. *F*-values from analyses of variance of peroxidase activity and IAA oxidase parameter values

Comparison	Peroxidase activity	Basal IAA-ox	Potential IAA-ox
Types			
L − S	238.3**	1.50(N.S.)	69.52**
R − M	33.6**	< 1	< 1
(L + S) − (R + M)	85.3**	< 1	35.13**
Sections			
Linear	707.4**	1.40(N.S.)	46.36**
Quadratic	17.9**	3.64(N.S.)	< 1
Cubic	4.2(N.S.)	5.50*	2.13(N.S.)
Interactions			
(L − S) × (Lin)	83.7**	< 1	46.60**
× (Quad)	7.4*	4.58*	2.67(N.S.)
× (Cub)	12.6**	1.21(N.S.)	1.87(N.S.)
(R − M) × (Lin)	31.2**	< 1	< 1
× (Quad)	2.7(N.S.)	< 1	< 1
× (Cub)	< 1	< 1	< 1
(L + S) − (R + M)			
× (Lin)	21.5**	< 1	20.48**
× (Quad)	4.6*	< 1	1.07(N.S.)
× (Cub)	4.5*	< 1	< 1
Degrees of freedom	*F*1, 16	*F*1, 16	*F*1, 16

N.S. Not significant at $P = 0.05$.
*Significant at $P = 0.05$.
**Significant at $P = 0.01$.

IAA-ox activity. Furthermore, peroxidase activity decreased curvilinearly up the stem while the decrease in potential IAA-ox activity was linear.

Dichlorophenol and Mn^{2+} are considered to be cofactors in the IAA-ox mediated degradation of IAA. The experimentation conducted here placed emphasis on the role of Mn^{2+} while attempting to standardize as far as possible the reaction conditions with respect to phenolics. Endogenous phenolics were removed by anion exchange resin during plant extract preparation and a constant level of exogenous dichlorophenol was incorporated in all reaction mixtures. While naturally occurring phenolics may enhance or inhibit IAA-ox activity[10, 11], removal of phenolics during extract preparation was necessary because, firstly, *in vivo* phenolics may be spatially separated from the soluble enzyme fraction, possibly in the vacuoles[15], and secondly, during extraction of the soluble enzyme fraction phenolics may be oxidized to products which inactivate enzymes and may cause precipitation of soluble proteins[15]. The IAA-ox activity of the plant extracts was dependent on the Mn^{2+} concentration. Potential activity expressed the IAA-ox activity of an extract at optimal Mn^{2+} concentrations. Assaying each extract at a range of Mn^{2+} concentrations and applying the Michaelis–Menten equation eliminated the necessity of determining by trial and error the optimal Mn^{2+} concentration for each specific extract. Two aspects of the dependence of IAA-ox activity on Mn^{2+} were of interest. Firstly, purified flax peroxidase isozymes did not require Mn^{2+} as a cofactor, and secondly, the IAA-ox activity vs Mn^{2+} concentration curves simulated substrate concentration curves. Although both these aspects would seem to corroborate the suggestion that the role of Mn^{2+} in the degradation of IAA by IAA-ox is to free IAA from 'protection' by 'protection substances'[16] it is not clear at this stage whether such protection substances occur in flax plant extracts. Some preliminary experimentation with extracts of L and S has indicated that on gel filtration chromatography there is an elution peak, in the relatively high MW range, which suppresses the expression of the IAA-ox activity of commercial HRP. One point, however, is clear in that the *in vivo* level of Mn^{2+} may be crucial to the extent to which the potential IAAox activity is expressed, as can be seen by comparing the potential and basal IAA-ox activities of the plant types and stem sections.

Basal activity was detected in most extracts. However, no basal activity was detected for any of the extracts if they were dialysed against the homogenizing buffer before assay; dialysis removed from the extracts some component (possibly Mn^{2+}) which was responsible for the level of IAA-ox activity detected in the absence of exogenous Mn^{2+}. Extrapolation from IAA-ox activities to IAA levels and thence morphological differences would be a

Table 4. Mean IAA-oxidase activity at low $MnCl_2$ concentrations and F-values from analyses of variance in plant types L, S, R and M

Type	Section 1				Section 2			
	L	S	R	M	L	S	R	M
Mn^{2+} concn (mM)								
0.0	0.11	0.00	0.11	0.00	0.07	0.40	0.19	0.25
0.05	0.14	0.01	0.11	0.01	0.25	1.03	0.29	0.34
0.1	0.20	0.08	0.14	0.05	0.28	1.37	0.34	0.42
0.2	0.23	0.12	0.16	0.08	0.31	1.83	0.35	0.47
Mean	0.17	0.05	0.13	0.03	0.23	1.56	0.29	0.37
	Section 3				Section 4			
	L	S	R	M	L	S	R	M
Mn^{2+} concn (mM)								
0.0	0.01	0.14	0.00	0.04	0.04	0.03	0.04	0.01
0.05	0.17	0.65	0.10	0.16	0.05	0.01	0.07	0.05
0.10	0.23	0.66	0.10	0.17	0.08	0.03	0.08	0.06
0.20	0.29	0.66	0.10	0.20	0.11	0.09	0.10	0.10
Mean	0.17	0.53	0.08	0.14	0.07	0.04	0.07	0.06

	Section 1	Section 2	Section 3	Section 4
Comparison types†				
L − S	116.54**	36.61**	48.38**	3.72(N.S.)
R − M	72.98**	< 1	1.70(N.S.)	< 1
(L + S) − (R + M)	14.78**	11.18**	44.19**	< 1
Concentrations	30.94**	4.07*	11.74**	7.07*
Interactions	< 1	1.40(N.S.)	1.75(N.S.)	< 1

†Degrees of freedom $F1$, 16 for Types; $F3$, 16 for Concentrations; $F9$, 16 for Interactions.
N.S. Not significant at $P = 0.05$.
*Significant at $P = 0.05$.
**Significant at $P = 0.01$.

limited insight into the regulation of the growth differences between these plant types. However, the *in vitro* demonstration of genotroph and genotype differences in IAA-ox activity would indicate that further investigation of the role of IAA-oxidase and the possibility of IAA 'protector substances' in different regions of the stem is warranted.

EXPERIMENTAL

Plants were grown in controlled environment chambers on nutrient soln as detailed previously[6] and were harvested 30 days after germination. L, S and the genotypes R and M were grown in two replicates with *ca* 32 plants/type replicate. Each stem was subdivided into four equal length sections, corresponding sections of plants within types and replicates were pooled yielding 32 tissue samples. Tissue extracts were prepared by homogenizing in buffer (0.1 M NaPi) (25% w/v; tissue/buffer for each of the samples). Anion exchange resin was incorporated in the homogenization mixture (25% w/v Ambrite). Peroxidase activity was expressed as A_{470}/min/ml from the guaiacol–H_2O method. The basic procedure for assaying IAA-ox activity, involving reaction of exogenous IAA (in the presence of cofactors) with the enzyme sample [17] and assay of the unoxidized IAA after the reaction time [18], has been described previously [4]. Reaction time depended on the particular plant extract being examined. IAA-ox activity was expressed as ΔA_{530}/30 min/ml. 0.1 mM $MnCl_2$ was used in the basic procedure. However, as IAA-ox activity for the plant extract was dependent on the Mn^{2+} concn all extracts were assayed at eight concns between 0.05 and 1.8 mM. All extracts were also assayed without addition of $MnCl_2$; these assay results are referred to as basal activities. All IAA-ox activities were the means for duplicate assays; agreement between duplicates was extremely good, with typical coefficients of variation being between 3 and 5%.

Most extracts displayed basal activity. All extracts showed a curvilinear increase (of rectangular hyperbola form) in IAA-ox activity over the increasing $[Mn^{2+}]$ range. For each extract, the IAA-ox activities at each Mn^{2+} concn were first adjusted by subtracting the extract's basal IAA-ox activity. The linear transformation of the Michaelis–Menten equation:

$$\frac{S}{V} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} S,$$

(where $S = Mn^{2+}$ concn; V = adjusted IAA-ox activity; V_{max} = maximal adjusted IAA-ox activity and $K_m = Mn^{2+}$

conc at V_{max}) was then fitted using a weighted regression analysis with V^2/S^2 as weight[19, 20]. The regression coefficients K_m/V_{max} and $1/V_{max}$ were tested for significance. Standard errors were obtained for K_m and V_{max} for testing the significance of these parameters separately. Maximum potential IAA-ox activity (potential activity) for each extract was obtained by summing its basal IAA-ox activity and V_{max} value.

Standard analysis of variance and covariance techniques were applied. The analyses are summarized in the tables as F ratios and correlation coefficients. Data tables show mean values, averaged over replicates, for the plant type–stem section combinations. Stem sections were numbered 1–4 from the cotyledon increasing up the stem.

Acknowledgements—Thanks are extended to the National Research Council of Canada whose financial assistance made this work possible and to Dr. Alan Durrant, Aberystwyth, U.K. who very kindly provided seed of the L and S genotrophs.

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