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ISOPEROXIDASE PATTERN AND INTERNODE LENGTH GENOTYPE IN *PISUM*

PETER K. MACNICOL
Division of Plant Industry, CSIRO, P.O. Box 1600, Canberra, Australia

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Abstract—Inbred *Pisum sativum* lines of known constitution for the internode length genes *Le*, *La* and *Cry*, and representing four height phenotypes, were grown to the 7-internode stage in the light. Six cationic isoperoxidases, making up *ca.* 90% of the activity of stem extracts, were resolved by concave gradient elution from Dowex 50 columns and shown to run as single peroxidase bands on starch gel electrophoresis. They were all able to oxidise IAA in the presence of 2,4-dichlorophenol, but fell into two groups with widely differing IAA oxidase/peroxidase ratios. The isoperoxidase patterns were independent of both genotype and phenotype for internode length, thus making it unlikely that these loci exert their effect on internode extension via control of synthesis of a particular isoperoxidase. Amongst the lines screened polymorphism was detected involving two of the isoperoxidases, and limited F_2 data suggest that these two variants are determined by alleles of a single gene. Isoperoxidase patterns of stem extracts of 6 other *Pisum* species did not differ significantly from the two found in *P. sativum*.

INTRODUCTION

THERE IS considerable evidence which suggests that enzymic auxin destruction may be a major factor in the control of stem elongation. Van Overbeek[1] found positive correlations between dwarfing and IAA inactivation by mesocotyl and coleoptile sections of maize seedlings. Dwarf forms in fact usually[1–4] but not always[5,6] contain more total peroxidase than genetically related tall ones. Furthermore, peroxidase content is increased in plants whose growth is inhibited by CCC and AMO-1618,[7] and conversely decreased in certain dwarfs induced to elongate by gibberellin treatment.[8] Nevertheless, the partial nature of these correlations and the reservations expressed by some workers[9,10] about the physiological significance of IAA oxidation make it desirable to seek further experimental evidence.

Chromatographic[11] and electrophoretic[12,13] fractionation has shown that peroxidase in

[1] VAN OVERBEEK, J. (1935) *Proc. Nat. Acad. Sci.* **21**, 292.
[2] ROSS, H. (1948) *Z. Ind. Abst. Vererbungslehre* **82**, 187.
[3] KAMERBEEK, G. A. (1956) *Acta Bot. Neerl.* **5**, 257.
[4] EVANS, J J. and ALLDRIDGE, N. A. (1964) *Phytochemistry* **4**, 499.
[5] VON ABRAMS, G. J. (1953) *Plant Physiol.* **28**, 443.
[6] MULLER, H. P. (1969) *Phytochemistry* **8**, 1867.
[7] GASPAR, T. and LACOPPE, J. (1968) *Physiol. Plant.* **21**, 1104.
[8] MCCUNE, D. C. and GALSTON, A. W. (1959) *Plant Physiol.* **34**, 416.
[9] BRIGGS, W. R., STEEVES, T. A., SUSSEX, I. M. and WETMORE, R. H. (1955) *Plant Physiol.* **30**, 148.
[10] CLELAND, R. E. (1969) in *Physiology of Plant Growth and Development* (WILKINS, M. B., ed.), p. 49, McGraw-Hill, London.
[11] MACNICOL, P. K (1966) *Arch. Biochem. Biophys.* **117**, 347.
[12] SIEGEL, B. Z. and GALSTON, A. W. (1967) *Plant Physiol.* **42**, 221.
[13] MILLS, A. K. and CROWDEN, R. K. (1968) *Australian J. Biol. Sci.* **21**, 1131.

crude extracts of *Pisum*, as in other plants, can be resolved into organ-specific patterns which change during ontogeny. Not only do these 'isoperoxidases' exhibit different oxidase to peroxidase activity ratios,[11] but they also respond differently to extraction.[14] The idea that they are isolation artefacts[15] is rendered unlikely by the recent finding[16] that the isoperoxidases of horseradish root differ markedly in primary structure. It is therefore an interesting possibility that one of these isoperoxidases, as a result of physiological specialization, is the functional IAA oxidase *in vivo*, the other forms having other roles. This could explain the incompleteness of the abovementioned correlations between total peroxidase activity and internode length. If genetic dwarfing in *Pisum* is due to IAA destruction by such an isoperoxidase, then the presence or absence of this enzyme should depend on the genotype for stem elongation.

The length of internodes in *Pisum* is controlled at three main loci,[17,18] namely *Le*, *La* and *Cry*.* Plants carrying *Le* are tall irrespective of the other genes, while in a *le le* background either of the dominant genes *La* or *Cry* produces a dwarf phenotype. However, plants recessive for all three genes may be either 'cryptodwarf' (*le la cry*^{c}) or 'slender' (*le la cry*^{s}), according to which of the two recessive *cry*-alleles is carried. These grow respectively to about two-thirds or twice the height of the normal varieties. *Pisum* lines of known genotype with respect to these genes, and representing these four phenotypes, are available. The above hypothesis can therefore be tested by comparison of their isoperoxidase patterns.

Apart from their relevance to the physiological role of peroxidase, the results described below contribute to the chemotaxonomy of *Pisum*, genetically one of the best-known genera of higher plants.

RESULTS

The patterns resulting from starch gel electrophoresis of the isoperoxidases separated on Dowex 50 columns, and of crude 'Alaska' stem extract, are presented in Fig. 1, which is a composite diagram of several gels. This type of representation was necessitated by the instability of the C_1 and C_2 isoenzymes following chromatographic isolation. The isoperoxidases are numbered in order of decreasing mobility towards the cathode. The weak band between C_2 and C_3 has not been numbered because its appearance is spasmodic. The presence of this component in the unadsorbed Dowex peak may be due to its frontal elution by other components of the extract.

Chromatographic resolution of the additional isoperoxidases C_5 and C_6 (see Fig. 2) is the result of extending the salt gradient to higher concentration than used previously.[11] The relative size of the unadsorbed peak depends upon the efficiency of dialysis of the crude extract: insufficient dialysis enlarges it at the expense of the C_2–C_1 peak, as has probably occurred in the case of Lamm Line 8a in Fig. 2.

* Other dwarfing genes to have been described are 'micro' (*Lm*)[19] whose recessive allele reduces the internode length of all phenotypes mentioned above, and the dominant *Cry* allele series of Von Rosen;[20] material of the latter is no longer available (Von Rosen, personal communication).

[14] MACNICOL, P. K. (1973) *Phytochemistry* **12,** 1269
[15] KEILIN, D. and HARTREE, E. F. (1951) *Biochem. J.* **49,** 88.
[16] SHIH, J. H. C., SHANNON, L. M., KAY, E. and LEW, J. Y. (1971) *J. Biol. Chem.* **246,** 4546.
[17] LAMM, R. (1937) *Hereditas* **23,** 38.
[18] LAMPRECHT, H. (1962) *Agric Hort. Genet* **20,** 23.
[19] LINDQUIST, K. (1951) *Hereditas* **37,** 389.
[20] VON ROSEN, G. (1958) *Hereditas* **44,** 123.

In the present study the limited data on IAA oxidase/peroxidase activity ratios given previously[11] were extended to cover the whole range of enzymes, giving the following values (ΔA_{261}/min/peroxidase unit at 25°): unadsorbed material, 0·026; C_1, 0·032; C_2, 0·019; C_3, 0·24; C_5, 0·24; C_6, 0·17.

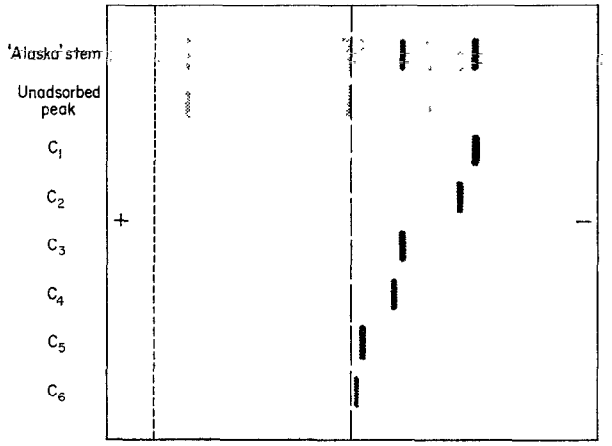

FIG. 1. DIAGRAM OF STARCH GEL ELECTROPHORESIS OF 'ALASKA' STEM EXTRACT AND OF ISOPEROXIDASE PEAKS FROM DOWEX 50 COLUMNS, USING THE ASHTON–BRADEN[28] SYSTEM AND STAINING WITH GUAIACOL–H_2O_2.

The thin lines in the centre indicate the position of sample application; the dotted line indicates the position of the borate front.

Table 1 gives details of the pea lines investigated and the isoperoxidase patterns of their stem extracts determined as chromatographic elution profiles. Under the growth conditions employed the heights of the four phenotypes with 7 expanded internodes were: tall, 21–28

TABLE 1. GENOTYPES, PHENOTYPES AND ISOPEROXIDASE PATTERNS OF *Pisum sativum* LINES

Line	Genotype*	Reference	Phenotype	Isoperoxidase pattern†					
				A + N‡	C_1	C_2	C_3	C_4	C_5	C_6
Bismarck	*Le La Cry*	Lindquist[19]	Tall	+	++	+	++	–	+	+
De Winton	*Le La Cry*	Marx (pers comm.)		+	++	+	–	+	+	Trace
Alaska-7	*Le La Cry*			+	–+	+	++	Trace	+	+
Lamm Line 30	*le La Cry*		Dwarf	+	++	+	++	Trace	+	+
Witham Wonder	*le La cry*^{c}	Lamm[17]		+	++	+	++	Trace	+	+
Acacia	*le La cry*^{s}	Lamm[17]		+	++	+	++	Trace	+	+
De Haan 204 1	*le La cry*^{s}	Lamm[17]		+	++	+	Trace	++	+	+
Frühe Niedrige	*le la Cry*	Lamm[17]		+	++	+	–	++	+	Trace
De Haan 201 1	*le la Cry*	Lamm[17]		+	++	+	++	Trace	++	+
Lamm Line 8a	*le la cry*^{c}	Marx (pers comm)	Cryptodwarf	++	++	+	–	++	+	Trace
Lamprecht Line 1329	*le la cry*^{c}	Lamprecht (pers. comm)		+	++	+	Trace	++	+	+
Lamme Line 64	*lel a cry*^{s}	Marx (pers comm)	Slender	+	++	+	+	–	Trace	Trace
Lamprecht Line 1118	*le la cry*^{s}	Lamprecht (pers. comm)		+	++	+	Trace	++	+	Trace

* Homozygous for the genes listed.
† Visual assessment of peaks in elution profile
‡ Anionic + neutral components not adsorbed at pH 6·2.

cm; dwarf, 8–12 cm; cryptodwarf, 18–22 cm; slender, 35–45 cm. All of the profiles are qualitatively invariant for isoperoxidases C_1, C_2, C_5 and C_6 and contain either C_3 with a

trace of C_4 ('C_3-type') or vice versa ('C_4-type'). There were no marked differences between profiles in relative height of a given peak.

Figure 2 gives actual elution profiles for 5 selected genotypes representing all 4 phenotypes. These genotypes allow comparison, for each gene, of the effect of dominant and recessive alleles; clearly none of the genes *Le*, *La* or *Cry* controls the isoperoxidase pattern. Table 1 shows in addition that three of the phenotypes may have either a C_3- or C_4-type of pattern.

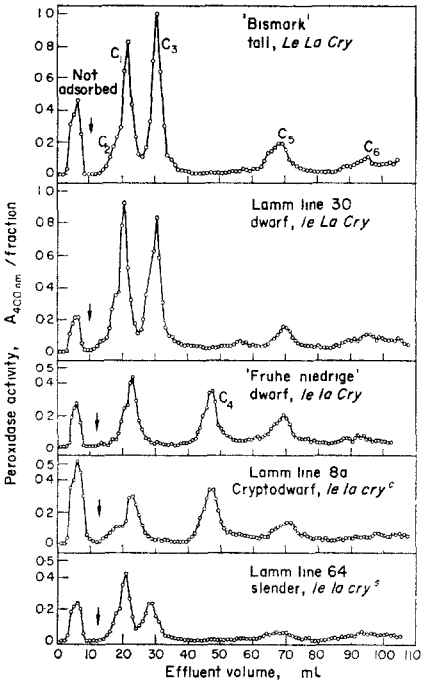

FIG. 2. ISOPEROXIDASE PROFILES RESULTING FROM CHROMATOGRAPHY ON DOWEX 50 COLUMNS OF STEM EXTRACTS OF INBRED *Pisum* LINES OF FOUR PHENOTYPES.

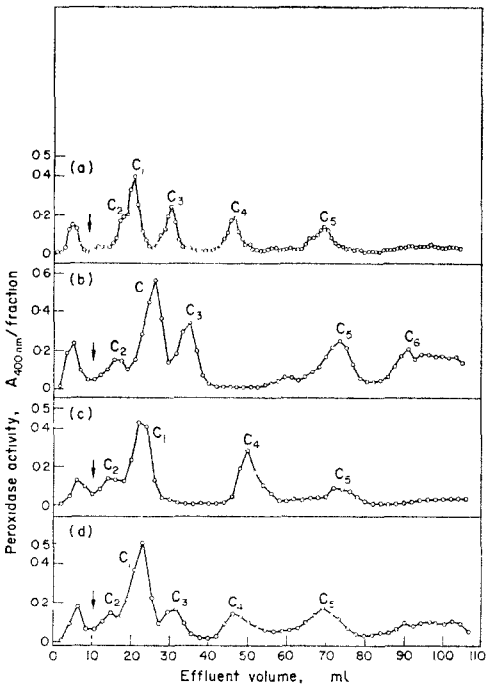

FIG. 3. ISOPEROXIDASE PROFILES OF STEM EXTRACTS OF (A) THE F_1 HYBRID BETWEEN 'FRUHE NIEDRIGE' AND LAMM LINE 30, AND (B), (C), (D) THE F_2 FROM THIS CROSS.

Arrow indicates beginning of salt gradient.

As far as total peroxidase content is concerned, it can be seen from Fig. 2 that tall and dwarf phenotypes do not necessarily differ, nor do dwarf and cryptodwarf. The lower content in Lamm Line 64 (slender) is not correlated with phenotype, as the content in Lamprecht Line 1118 (slender) is similar to that in dwarfs such as 'Frühe Niedrige'.

Figure 3 shows the profiles that result from crossing a C_3-type (Lamm Line 30) with a C_4-type line ('Frühe Niedrige'), and from selfing the hybrid. Both enzymes are present in the hybrid in about equal amount. The F_2 are of 3 types, containing either C_3 with a trace of C_4, or vice versa, or both peaks in diminished amount. Of 20 F_2 plants extracted, these three types occurred in the ratio 3:3:4, which is a reasonable approximation to a ratio of 1:1:2. The other such F_1 hybrid examined was between 'Witham Wonder' and 'Frühe Niedrige'); it also contained C_3 and C_4 in roughly equal amount. In this case the F_2 were not grown.

A limited survey of other *Pisum* species showed isoperoxidase patterns similar to the two found in *Pisum sativum*. Thus *P. humile* (1 sample), *P. abyssinicum* (3 samples), *P. elatius* (3 samples) and *P. jomardii* (2 samples) all had C_4-type patterns, whereas *P. fulvum* (1 sample) and *P. nepalensis* (1 sample) had C_3-type patterns.

DISCUSSION

In a study of this type the question arises whether the isoenzymic composition of the extract is representative of the tissue from which it came. Although the presence of covalently bound peroxidase in cell walls[21] makes a definitive answer difficult, results presented elsewhere[14] suggest that all the isoperoxidases accessible to mild extraction procedures are being removed.

The chromatographic system employed here has important advantages over the conventional method of gel electrophoresis.[12,13] These are firstly the clear resolution of isoperoxidases C_3 and C_4, and secondly the quantitative relationship between elution profile peak height and peroxidase content.[11] This relationship does not normally hold in zone electrophoretic scans, where staining of the bands typically proceeds until limited by hydrogen donor concentration or enzyme inactivation.

All of the isoperoxidases were able to oxidize IAA in an assay system containing 2,4-dichlorophenol as cofactor. It was noticeable that the isoperoxidases eluted at low ionic strength were more unstable and had much lower relative IAA oxidase activity than those eluted at high ionic strength. However, their high k_1/k_4 ratios[11] make it unlikely that these forms are degraded. The molecular basis of these differences, and whether they reflect physiological specialization towards particular oxidation or hydroxylative functions, are interesting topics for future research.

From the chromatographic elution profiles it may be concluded that the complement of cationic isoperoxidases in stem extracts is independent of both genotype and phenotype for internode length. This conclusion is strengthened by the electrophoretic experiments: the isoperoxidase peaks each contain a single peroxidase component with mobility corresponding to that of a component of the crude extract; furthermore all the bands from the crude extract are accounted for as peaks.

Anionic and neutral isoperoxidases comprise at most 10% of the peroxidase activity of well-dialysed stem extracts, and were not investigated in the present study. Not only is their combined IAA oxidase/peroxidase activity ratio low, but their simple electrophoretic, composition does not appear to leave much scope for variation. Also, although this work has concentrated on the qualitative aspect of the isoperoxidase pattern, the data indicate that neither total peroxidase content nor relative content of particular isoperoxidases is obviously correlated with genotype or phenotype.

The conclusion, that internode length genotype at the three loci investigated does not control formation of a specific IAA-destroying isoperoxidase, suffers from a limitation. If such an enzyme were produced transitorily during the differentiation of an internode, then its presence might be 'swamped' by the peroxidase complement of already differentiated internodes which make up the bulk of the extracted material. Apart from direct control of peroxidase type or amount, genetic control of IAA oxidase activity could also be exerted

[21] BARNETT, N. and CURTIS, C. R. (1970) *Plant Physiol.* Suppl. 46, 14.

indirectly via phenolic cofactors and inhibitors.[22] In view of the probable location of such compounds in the cell vacuole,[23] this possibility appears unlikely.

Endogenous gibberellin level is thought to play an important role in stem elongation, but whether this is exerted via promotion of IAA destruction, retardation of its synthesis, or some other mechanism, is controversial.[24,10] However, convincing evidence has been produced[25] that regulation of gibberellin level is not the basis of action of the *Le* locus in *Pisum*, nor of the greater elongation of slender and cryptodwarf relative to dwarf phenotypes.

The present study has detected polymorphism of the *Pisum* isoperoxidases involving the components C_3 and C_4, which on the basis of the limited amount of F_2 data obtained appear to be determined by alleles of a single gene. A similar situation with maize pollen isoperoxidases was described by Scandalios,[26] who concluded that these are monomeric proteins. Recent work[16] shows indeed that the isoperoxidases of horseradish root are each composed of a single polypeptide chain. However, assuming that this is also true of *Pisum* isoperoxidases, the presence in C_3-types of a trace of C_4, and vice versa, is still not explained. The close relationship between the *Pisum* isoperoxidases C_3 and C_4 is confirmed by their similar IAA oxidase/peroxidase ratio, electrophoretic mobility and behaviour towards differential extraction with EDTA.[14] The similarity of the isoperoxidase patterns of other *Pisum* species to those of *P. sativum* and the presence in these species of the C_3 and C_4 components (although C_4 predominates) appears to indicate either that the species are closely related or that the pattern is determined by physiological functions of the isoperoxidases.

EXPERIMENTAL

Pisum seed was sterilized with 1% sodium hypochlorite solution and sown in sterile vermiculite in a glasshouse controlled to 21° (day) and 16° (night). After germination the plants were watered with half-strength Hoagland solution. Shoots of plants with 7 fully expanded internodes were excised just above the cotyledons, freed from leaves and stipules, cut into short lengths and ground in a chilled mortar with sand and 50 mM Na phosphate buffer-10 mM EDTA-200 mM NaCl, pH 7·2 (1 ml per stem). Subsequent operations were performed at 5°. The brei was centrifuged at 20000 *g* for 10 min and the residue re-extracted twice. Glycerol to a concentration of 10% was added to the combined extract, which was stored at −20° for up to 1 month with no change in elution profile.

Chromatography was performed at 5° on 10 × 1 cm columns of Dowex 50W–X2, 200–400 mesh resin in the Na^+ form, pretreated by the method of Moore and Stein[27] and equilibrated with 10 mM Na phosphate buffer, pH 6·2. The tissue extract was thawed, centrifuged and the supernatant dialysed for 3 hr against the same buffer before application to the column, followed by a 1 ml buffer rinse. Elution was with 100 ml linear or concave gradients of NaCl in buffer as specified below; concave gradients were generated in a cylindrical mixing vessel having three times the cross-sectional area of the cylindrical reservoir vessel. The flow rate was 30 ml/hr, and 1 ml fractions were collected. The peroxidase activity of these fractions was measured with the rapid assay previously described[11] the whole fraction being reacted with 20 mM guaiacol–2 mM H_2O_2 at 22° for 1 min, the reaction terminated with 4 M NaOH, and the absorbance at 400 nm measured after 1 hr.

In order to obtain sufficiently concentrated samples of the isoperoxidases for electrophoresis, preparative chromatography using heavy loading (extract equivalent to several seedlings) was employed. 'Alaska' leaf extract was used as the source of C_1 and C_2 (linear gradient to 0·1 M NaCl), 'Alaska' stem extract as the source of unadsorbed peroxidase, C_3, C_5 and C_6 (concave gradient to 0·6 M NaCl) and 'Graue Niedrige' stem extract as the source of C_4 (linear gradient to 0·4 M NaCl). The peaks were located by rapid assay of 0·1 ml aliquots of the resulting fractions. The peak fractions containing C_2, C_5 and C_6 were concentrated about 5-fold in an Amicon ultrafiltration cell over a UM-2 membrane.

[22] BOTTOMLEY, W., SMITH, H. and GALSTON, A. W. (1966) *Phytochemistry* **5**, 117
[23] PRISTUPA, N. A., PETROVA, R. K. and SHALAMBERIDZE, T. KH. (1970) *Tsitologiya* **12**, 403
[24] SACHS, R. M. (1965) *Ann. Rev. Plant Physiol.* **16**, 73.
[25] MCCOMB, A. J. and MCCOMB, J. A. (1970) *Planta* **91**, 235.
[26] SCANDALIOS, J. G. (1969) *Biochem. Genetics* **3**, 37.
[27] MOORE, S. and STEIN, W. H. (1951) *J. Biol. Chem.* **192**, 663

For analytical chromatography of stem extracts the loading was reduced to an aliquot equivalent to a single seedling. A concave gradient to 0·6 M NaCl was used.

Horizontal starch gel electrophoresis was performed in 12% gels (Connaught starch), using the pH 8 lithium borate/TRIS citrate system of Ashton and Braden,[28] at 28 V/cm and 50 mA for 4 hr. The samples were applied in small rectangular inserts of Whatman 3MM filter paper. During the runs both sides of the gels were cooled by metal blocks through which water at 10° was circulated. The sliced gels were stained in 30 mM guaiacol–3 mM H_2O_2 to reveal the peroxidase bands.

IAA oxidase activity of the isolated isoperoxidases was measured at pH 5·0 and 25° in the assay system previously described,[11] using a Cary 14 spectrophotometer. Rates were expressed as ΔA_{261}/min.

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[28] Ashton, G. C. and Braden, A. W. H. (1951) *Australian J. Biol. Sci.* **14**, 248.