Phytochemistry 1974, Vol 13, pp 2695 to 2702 Pergamon Press Printed in England

INDUCTION OF POLYPHENOL OXIDASE IN GERMINATING WHEAT SEEDS

SHANT R TANEJA and R C. SACHAR

Division of Biochemistry, Indian Agricultural Research Institute, New Delhi-110012, India

(*Revised Received* 25 *January* 1974)

Key Word Index—*Triticum aestivum*, Gramineae, wheat, germinating seeds, induction of polyphenol oxidase, gel electrophoresis

Abstract—A 50- and 100-fold increase in the *o*-diphenolase activity was observed respectively in excised coleoptiles and roots of wheat seedlings after germination for 4–5 days This increased activity was associated with the appearance of several new multiple forms of *o*-diphenolase on acrylamide gels The embryo-less half-seeds dissected from seedlings, however, revealed only a three-fold increase in *o*-diphenolase activity, without any alteration in the pattern of multiple forms Cycloheximide substantially inhibited the activity and appearance of multiple forms of *o*-diphenolase, whereas actinomycin D failed to bring about a similar response Protein synthesis was probably necessary for the formation of new multiple forms Unlike *o*-diphenolase activity which was present in all parts of the seedling, the monophenolase activity was confined to the embryo-less endosperm A 5–7-fold increase in monophenolase activity was observed in the embryo-less half-seed dissected from the seedling A single broad band of monophenolase developed on acrylamide gels This persisted during the early period of seed germination without addition of new multiple forms No inhibition of monophenolase activity was observed in seeds treated with cycloheximide or actinomycin D

INTRODUCTION

IN RECENT years, the regulation of enzyme induction in germinating and developing seeds of higher plants has received considerable attention.[1–4] Experimental findings by many workers support the view that the modulation of enzyme activity may be controlled by a variety of growth substances.[5] Further, it has been observed that the increased enzyme activity could be either due to *de novo* synthesis[1] or to activation of pre-existing enzyme.[6,7]

At present, little information is available on the regulation of polyphenol oxidase in higher plants However, a few reports indicate that the activity of this enzyme is stimulated by the application of ethylene[8] and indoleacetic acid.[9,10] Tissue injury in sweet potato can also trigger the activity of polyphenol oxidase.[11,12] The *o*-diphenolase activity in root

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slices of sweet potato is inhibited by the addition of actinomycin D, puromycin and blasticidin S, indicating that the rise in activity results from the *de novo* synthesis of the enzyme [13]

We have observed that the monophenolase and *o*-diphenolase activities of polyphenol oxidase represent two distinct enzymes in wheat grain [14] The activity of these enzymes, particularly that of monophenolase, is low in mature seeds. Preliminary observations indicated that the activity of *o*-diphenolase is considerably increased during seed germination Such enhancement could operate at transcriptional or translational level, or represent a case of activation of the dormant enzyme Since polyphenol oxidase is known to exist in multiple forms,[15] stimulation of this enzyme may be associated with the appearance of new multimeric forms We have now studied the induction of monophenolase and *o*-diphenolase in germinating seeds of wheat. Inhibitors of RNA and protein synthesis were employed for determining the level of control during induction of these enzymes

RESULTS

Activity and multiple forms of o-*diphenolase in wheat seedling*

The *o*-diphenolase activity increased from 0·2 A units/mg protein/min by nearly 2·5-fold during the first 24 hr of seed germination Thereafter, activity declined on the 3rd day to a value 20% greater than that found in ungerminated seed Subsequently, activity showed a slight increase to the 6th day of seed germination when it was 0·3 A units/mg protein/min

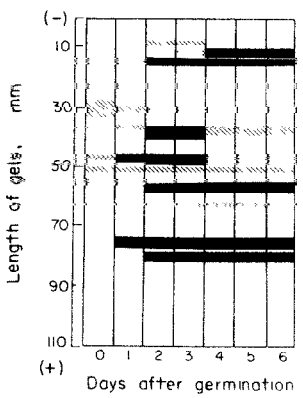

FIG 1 DIAGRAM OF *o*-DIPHENOLASE OF GERMINATING WHEAT SEEDS SEPARATED BY ELECTROPHORESIS
The acrylamide gels were stained with DL-DOPA (1·5 mg/ml in 80% EtOH) and destained in 30% EtOH ■, high activity, ▩ medium activity, □ low activity

The crude extract prepared from mature grains revealed six multiple forms of *o*-diphenolase activity on incubating the gels with DL-DOPA (Fig 1) Two new bands appeared after 24 hr germination and two more multiple forms appeared in 2-day-old seedlings In addition to these, some of the pre-existing bands showed higher activity and one of the slow-moving bands present in the mature grain could no longer be detected on the

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gels. Thus, in a 2-day-old seedling, there were 9 multiple forms showing *o*-diphenolase activity and 11 bands were observed on the 3rd day of germination. Thereafter, the pattern of multiple forms remained essentially unaltered, except that some of the bands gave a feeble reaction with DL-DOPA. Ten multiple forms of *o*-diphenolase were found in a 6-day-old seedling. Significantly, the two prominent fast-moving multiple forms found 2 days after germination persist with the same intensity during subsequent stages of seed germination.

TABLE 1 EFFECT OF CHLORAMPHENICOL AND CYCLOHEXIMIDE ON THE *o*-DIPHENOLASE ACTIVITY

No	Additions	*o*-Diphenolase activity (A units of 430 nm/mg protein/min)	% Of control
1	None	0 24	100
2	Chloramphenicol (200 μg/ml)	0 24	100
3	Cycloheximide		
	(a) 1 μg/ml	0 29	120
	(b) 2 μg/ml	0 33	137
	(c) 5 μg/ml	0 38	157

The *crude extract* was obtained from seeds germinated in presence of antibiotics for 48 hr and the activity of the enzyme assayed with catechol (10 mg/ml)

The appearance of multiple forms of *o*-diphenolase is known to occur by the phenomenon of association of monomeric subunits [15] The fast-moving multiple forms which appear during germination of wheat seeds possibly represent monomers. This was revealed by experiments with sodium dodecyl sulphate (SDS). Treatment of crude extract (from embryos dissected from 2-day-old seedlings) with SDS (0·1%) and mercaptoethanol (0·1%) led to the dissociation of multiple forms into a fast-moving band of very high intensity which coincided with the two fast moving bands of the untreated extracts.

TABLE 2 EFFECT OF CYCLOHEXIMIDE AND ACTINOMYCIN D ON THE ACTIVITY OF *o*-DIPHENOLASE OF EMBRYOS AND EMBRYO-LESS HALF-SEEDS DISSECTED FROM 2-DAY-OLD SEEDLINGS

No	Additions to seeds	Excised embryos *o*-Diphenolase activity A units at 430 nm/mg protein/min	Inhibition (%)	Excised embryo-less half-seeds *o*-diphenolase activity A units at 430 nm/mg protein/min	Inhibition (%)
1	None	15 0	—	0 57	—
2	Cycloheximide (5 μg/ml)	6 9	54	0 47	18
3	EtOH (1%)	7 0	53	0 37	35
4	EtOH (1%) + actinomycin D (50 μg/ml)	6 7	54	0 40	30

The seeds were germinated in the presence of antibiotic for 48 hr Since actinomycin D in its final concentration contained 1% EtOH, seeds grown in the presence of 1% EtOH served as control for the above treatment

The possibility of *de novo* synthesis of *o*-diphenolase was tested by making use of inhibitors of protein synthesis. When wheat seeds were grown for 48 hr in the presence of cycloheximide (1–5 μg/ml), there was a gradual decline in the number and intensity of the newly

appearing multiple forms. Out of the nine multiple forms observed in the control, the antibiotic (5 μg/ml) completely eliminated five forms and the remaining bands gave only a feeble activity. Although one would expect low activity of this enzyme in cycloheximide-treated germinating seeds, there was in fact a stimulation in the activity of *o*-diphenolase (Table 1) This discrepancy was resolved when we examined the activity of this enzyme in different morphological parts of the seedling. Cycloheximide inhibited the activity of *o*-diphenolase in excised embryo but not in the embryo-less grain tissue (see Table 2). Unlike cycloheximide, chloramphenicol (200 μg/ml) showed no inhibition of multiple forms and activity of *o*-diphenolase (Table 1)

No inhibition of *o*-diphenolase activity was observed in seeds germinated in the presence of actinomycin D (12·5, 25 and 50 μg/ml) and also the number of multiple bands remained unaltered on acrylamide gels

Activity and multiple forms of o-diphenolase in excised root, coleoptile and embryo-less half-seed

A preliminary observation indicated that the activity of *o*-diphenolase varies in different morphological parts of the seedling We therefore determined the relative activity of *o*-diphenolase in root, coleoptile and embryo-less grain excised from seedling at various stages of seed germination Roots possessed the greatest activity, followed by coleoptile, while the embryo-less half-seed (endosperm) showed only a low activity (Fig 2) Since it was not feasible to separate coleoptile from root in 1-day-old seedling, the study at this stage was confined to the excised whole embryos There was a 15-fold increase in the *o*-diphenolase activity in one-day-old embryonic tissue

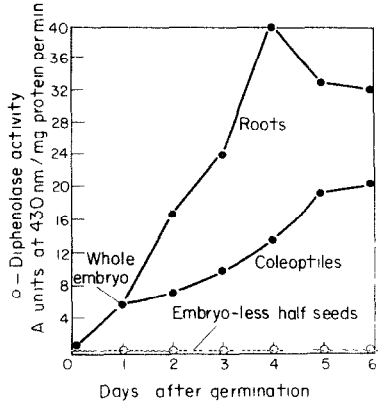

FIG. 2. LEVELS OF *o*-DIPHENOLASE ACTIVITY IN ROOTS, COLEOPTILES AND EMBRYO-LESS HALF-SEEDS EXCISED FROM GERMINATING SEEDS OF WHEAT

Since the roots and coleoptiles could not be conveniently separated during the early stages, whole embryos were dissected from ungerminated seeds and one-day-old seedlings for measuring the activity of *o*-diphenolase Catechol (10 mg/ml) served as a substrate for the assay of enzyme

Two multiple forms of *o*-diphenolase were observed in embryo dissected from ungerminated seed. The embryos excised from 24-hr-old seedlings showed several new multiple forms. In addition, one of the bands in ungerminated embryo showed high activity on acrylamide gels (Fig. 3a).

The *o*-diphenolase activity in roots continued to increase for 4 days and then showed a slight decline (Fig. 2). The roots exhibited a 108-fold increase in *o*-diphenolase activity over the embryos dissected from ungerminated seeds. Roots excised from 2-day-old seedlings showed 11 multiple forms on acrylamide gels. Thereafter, the pattern remained unchanged except for the addition of one band in 3-day-old and another slow-moving band in 5-day-old roots respectively (Fig. 3b).

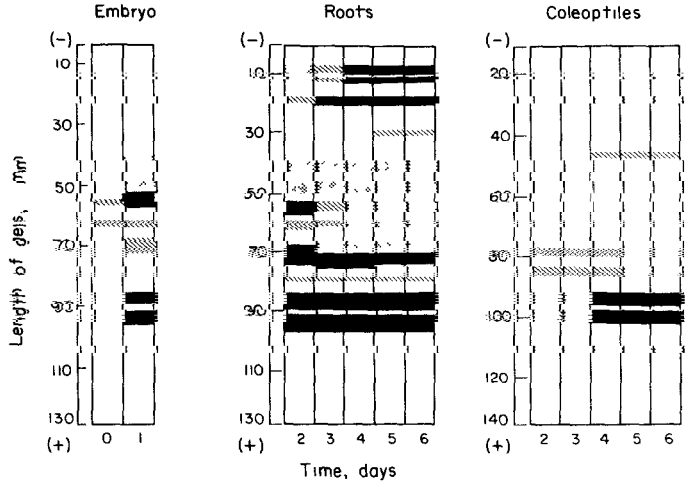

FIG 3 (a) SCHEMATIC REPRESENTATION OF ACRYLAMIDE GEL ELECTROPHORESIS OF *o*-DIPHENOLASE MULTIPLE FORMS IN *crude extracts* OF EMBRYO EXCISED FROM UNGERMINATED SEED (0) AND 24-hr-OLD SEEDLING (1)
(b) *o*-Diphenolase multiple forms on acrylamide gels in roots excised from 2, 3, 4, 5 and 6 day-old seedling (c) *o*-Diphenolase multiple forms on acrylamide gels in coleoptiles excised from 2, 3, 4, 5 and 6 day-old seedlings The gels were stained with DI-DOPA (1 5 mg/ml) ■, High activity, ▩, medium activity, ▨, low activity

The *o*-diphenolase activity in coleoptiles was approximately 50% less than that in roots, but was substantial in comparison with the embryo-less half-seed (Fig. 3c). There was a 52-fold increase in *o*-diphenolase activity in extracts prepared from coleoptile tissue (Fig. 2). Five fast-moving multiple forms o-diphenolase were observed in 2-day-old seedlings, of which two became intense at subsequent stages of coleoptile development. Also, two slow-moving bands appeared in 4-day-old tissue, followed by the addition of a single band 6 days after germination. Thus in all, there were 8 multiple forms of *o*-diphenolase activity in coleoptiles (Fig. 3c).

The *o*-diphenolase activity in the embryo-less half-seed increased until the 4th day of germination and then it remained more or less constant. A 3-fold increase in *o*-diphenolase activity was observed in the embryo-less half-seeds dissected from 4-day-old seedlings. Four slow-moving multiple forms were present in the embryo-less half-seed dissected from mature grain and these persisted without any alteration in germinating seeds.

Effect of cycloheximide and actinomycin D on o-diphenolase activity of excised embryo and embryo-less half-seed

Seeds germinated in the presence of cycloheximide showed a retardation of embryo growth. Cycloheximide caused a 54% inhibition of enzyme activity in isolated embryos (Table 2) with simultaneous disappearance of several multiple forms of *o*-diphenolase. No significant change in the activity (Table 2) and gel pattern of *o*-diphenolase was observed in embryo-less half-seeds dissected from cycloheximide-treated seeds.

The effect of actinomycin D on *o*-diphenolase activity was also studied in excised embryos and embryo-less half-seeds (Table 2). As actinomycin D solution (50 μg/ml) contained 1% EtOH, the control seeds were also soaked in the same concentration of EtOH. EtOH (1%)* reduced the activity of *o*-diphenolase in dissected embryos to 53% and the newly-formed bands gave a feeble reaction on acrylamide gels. Actinomycin D neither decreased the activity nor affected the pattern of multiple forms over that of controls. Similarly, actinomycin D caused no detectable change in the activity and multiple forms of *o*-diphenolase in embryo-less half-seeds.

Monophenolase activity

The dry seeds of wheat showed low activity of monophenolase which increased several fold following germination. The enzyme extracts prepared from isolated roots, coleoptiles and embryo-less half-seeds were tested with L-tyrosine for studying the monophenol oxidase function. The monophenolase activity appears to be restricted to the grain as no such activity was detectable in coleoptile and root tissues. Further studies on monophenolase were therefore confined to the embryo-less half-seed dissected from the seedling. There was a five-fold increase in monophenolase activity in the first 24 hr of germination. This level of activity was maintained in a 3-day-old seedling. A second rise in activity was observed on 4th day of seed germination. Finally in a 6-day-old seedling, there was a seven-fold increase in monophenolase activity over the embryo-less half-seed dissected from ungerminated grain. Cycloheximide and actinomycin D failed to inhibit the activity of monophenolase in embryo-less half-seeds.

Enzyme extracts prepared from the grain tissue were fractionated on acrylamide gels and tested with L-tyrosine. The fraction obtained from ungerminated seeds showed a single fast-moving band of monophenolase activity. The same band gave intense activity without any alteration in its position on gels in the germinating seeds. There was no further addition of multiple forms in 1–6-day-old seedlings.

DISCUSSION

Previous workers have reported a two- to five-fold increase in polyphenol oxidase activity in developing tissues of a variety of higher plants.[8,10,13,16] In the present investigation, we have measured the activity of monophenolase and *o*-diphenolase in germinating seeds of wheat. The total rise in *o*-diphenolase activity was only two- to three-fold. However, an altogether different picture emerged when activity was measured in the isolated parts of the seedling. Compared with the whole seedlings, the excised coleoptiles and roots

* When the concentration of EtOH was increased from 1 to 5% during seed germination, there was further inhibition in the activity and appearance of new multiple forms. However addition of EtOH to the crude extract had no inhibitory effect on *o*-diphenolase activity.

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revealed a considerable stimulation of *o*-diphenolase activity. A 50- and 100-fold increase in the enzyme activity was observed in coleoptile and root tissues respectively, whereas the embryo-less half-seed showed only a three-fold increase. Clearly, therefore, the bulk of the enhanced activity was located in the embryonic region of the seedling. This, however, was masked to a considerable extent if the activity was measured in the whole seedlings.

We have shown that the multiple forms developed on acrylamide gels with L-tyrosine and DL-DOPA truly represent polyphenol oxidase activity in our system.[14] The phenolics could serve as substrates for peroxidase activity bands, provided peroxides are available in the gels. Sheen[17] has reported that unwashed gels are contaminated with peroxides produced by the polymerizing catalyst, ammonium persulphate. Crude extract fractionated on exhaustively washed gels and subsequently stained with L-tyrosine and DL-DOPA showed the same number of multiple forms as observed on unwashed gels, thereby excluding the possibility of an artifact caused by peroxidase activity bands. It appears that exhaustive washing of gels was unnecessary in our system and that peroxides were possibly eliminated during the period of 60 min, when concentrating the protein sample on gel columns.

The inducibility of *o*-diphenolase during seed germination may be correlated with the appearance of several new multiple forms The quiescent embryo possessed two bands of *o*-diphenolase activity. The number of multiple forms increased in the embryonic region of the seedling, there being 8 bands in coleoptile and 13 in root tissue. The electrophoretic mobility of multiple forms of *o*-diphenolase also varied in different regions of the seedling. The embryo-less half-seed showed only four slow-moving bands, while the roots and coleoptiles possessed both slow and fast-moving multiple forms, some of which were common to both tissues.

Actinomycin D had no effect on the activity and multiple forms of *o*-diphenolase. The occurrence of stable *m*RNA capable of supporting protein synthesis is already reported in germinating wheat embryos.[18] The lack of inhibition of *o*-diphenolase activity by actinomycin D in our system could be due to the existence of a stable form of *m*RNA for coding polyphenol oxidase. Cycloheximide inhibited the activity of *o*-diphenolase in excised embryonic tissue, while its effect was not so severe in the embryo-less half-seeds. Also, the newly appearing multiple forms of *o*-diphenolase were inhibited to a considerable extent in seeds germinated in presence of cycloheximide. These experiments favour the view that the increase in *o*-diphenolase activity in excised coleoptile and root tissues could be due to the *de novo* synthesis of the enzyme. The situation, however, is somewhat different in root slices of sweet potato, where the activity of *o*-diphenolase is reported to be inhibited both by actinomycin D and puromycin.[13]

Whereas the *o*-diphenolase activity was prevalent in all the morphological parts of the wheat seedlings, the monophenolase activity could be detected only in the grain tissue. Further analysis of the grain revealed that this enzyme is confined to the aleurone layers and is not found in the endosperm tissue.[19] A seven-fold increase in the activity of monophenolase was observed in embryo-less half-seeds excised from 6-day-old seedlings. However, no new multiple bands of monophenolase were formed during seed germination. In-

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stead, a single broad band, initially present in the ungerminated seeds persisted in 1–6-day-old seedlings. No inhibition of monophenolase activity was observed in seeds germinated in presence of cycloheximide or actinomycin D. The enhanced activity of monophenolase during seed germination can be taken as an indication of enzyme activation and not due to *de novo* synthesis.

EXPERIMENTAL

Seed germination. Freshly harvested wheat seeds (cv. *Sharbati Sonora*) were surface sterilized in a soln of $HgCl_2$ (0·1%) for 10 min, rinsed thoroughly with sterile H_2O and spread over a layer of white sand in Petri dishes containing 20 μg/ml of chloramphenicol. The seeds were grown in the dark at 25°.

Treatment of seeds with chloramphenicol, cycloheximide and actinomycin D. The sterilized seeds were grown in the presence of different concentrations of cycloheximide (1, 2, 4 and 5 μg/ml), chloramphenicol (100 and 200 μg/ml) and actinomycin D (12·5, 25 and 50 μg/ml prepared in 0·25, 0·5 and 1·0% EtOH respectively) for 48 hr and used for the preparation of *crude extracts* for the assay of polyphenol oxidase.

Enzyme extraction. The enzymes were extracted from germinating seeds with 0·05 M phosphate buffer (pH 6·6). The homogenate was centrifuged at 20000 *g* for 15 min. The supernatant (*crude extract*) was used for the assay of monophenolase and *o*-diphenolase activities. The above procedure was also followed for the preparation of *crude extracts* of whole embryo, root, coleoptile and embryo-less half-seed excised from seedling.

Assay and acrylamide gel electrophoresis of polyphenol oxidase. The monophenolase and *o*-diphenolase activities of *crude extracts* were measured at 430 nm using catechol (100 mg%) and L-tyrosine (1 mg%) as substrates. The incubation mixture contained 2 ml of substrate, 0·1–0·5 ml of *crude extract* and 0·05 M phosphate buffer (pH 6·6) added to make the total vol. 4 ml. Omission of substrate from the incubation mixture served as control. Prior to mixing, all the ingredients were maintained at 37°. In the case of monophenolase activity, the mixture containing L-tyrosine and buffer was oxygenated for 5 min before the addition of *crude extract*. The activity of monophenolase is expressed as absorbance (A) units at 430 nm/5 mg protein/3 hr, while that of *o*-diphenolase as A units at 430 nm/mg protein/min.

Crude extracts were subjected to acrylamide gel electrophoresis using Tris glycine buffer (pH 8·3).[20] About 1·5 mg of protein sample was layered on each gel column. The penetration of the sample required 1 hr after which the gels were flushed and the run completed in 1 hr. Exhaustive electrophoretic washing of gels for 6 hr was unnecessary in our system since unwashed gels gave no indication of any contamination of peroxides. The gels were incubated with DL-DOPA (1·5 mg/ml) and L-tyrosine (0·5 mg/ml) solns at 37° for developing *o*-diphenolase and monophenolase multiple forms respectively. Other monophenols, such as phenol (1 mg/ml), *p*-cresol (1 mg/ml) and vanillic acid (1 mg/ml) were also tested, but these proved ineffective in detecting monophenolase multiple forms. The substrates were prepared in 80% EtOH. The gels were destained and stored in 30% EtOH.[21] The *crude extract* prepared from embryos, excised for 2-day-old seedlings, was mixed with SDS (0·1%) and mercaptoethanol (0·1%) and fractionated by acrylamide gel electrophoresis in the presence of additional SDS (0·1%) and mercaptoethanol (0·1%) added to Tris–glycine buffer. The gels were stained with DL-DOPA (1·5 mg/ml). We have tested both DL-DOPA and catechol for the colorimetric assay of *o*-diphenolase activity. It has been reported that catechol gives higher activity of *o*-diphenolase than DL-DOPA.[22] However, in the present study when DL-DOPA and catechol were tested for developing the multiple forms of *o*-diphenolase, catechol gave very few bands as compared to DL-DOPA. We therefore used DL-DOPA for staining gels and catechol for colorimetric assay.

Protein estimation. Protein was determined according to the procedure of Lowry *et al.*[23]

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