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Changes in Enzyme Activity of Corn Seedlings After Foliar Application of Triacontanol*

Andrew P. Lesniak and Stanley K. Ries

Department of Horticulture, Pesticide Research Center, Michigan State University, East Lansing, Michigan 48824, USA

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Abstract. Changes in the activity of several enzymes in corn seedlings (*Zea mays* L.) after 1-triacontanol (TRIA) application have been analyzed. The specific activity of isocitrate dehydrogenase and 6-phosphogluconate dehydrogenase in corn seedlings treated with TRIA increased rapidly. Three days after treatment, the TRIA-treated seedlings showed 89% and 39% more ICDH and 6PGDH activity per mg protein, respectively, than the untreated plants. Malate dehydrogenase activity increased in treated plants at a rate approximately equivalent to the increase in soluble protein. Acid phosphatase, peroxidase, and alkaline phosphatase activity remained relatively constant on a per plant basis and decreased slightly on a per mg protein basis. No qualitative changes were observed in the isozyme patterns of the enzymes analyzed by starch gel electrophoresis, although quantitative changes consistent with the increases using spectrophotometric assays were observed.

Triacontanol (TRIA) is a naturally occurring straight-chain 30-carbon alcohol that stimulates plant growth (Ries et al. 1977, Zheng et al. 1981). TRIA has been shown to increase dry weight (Eriksen et al. 1981), reducing sugars (Ries et al. 1978), soluble protein (Ries and Wert 1977), and soluble nitrogen (Knowles and Ries 1981). The effects are specific for the 30-carbon alcohol and can be abrogated by trace amounts of C_{28} or C_{32} primary alcohol (Jones et al. 1979).

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Recently, an increase in starch phosphorylase activity was reported in an *in vitro* assay system (Houtz 1980). The effect, however, was dependent on application of TRIA to intact corn (*Zea mays*) tissues.

One of the most consistent responses to TRIA has been an increase in total soluble protein (Ries and Wert 1982). Alterations in enzyme activity could reflect this change more precisely than measurement of soluble protein. Analysis of changes in enzyme activity may also give insight on TRIA's mode of action and/or provide diagnostic indicators for the plant's response to TRIA. This study was therefore undertaken to determine the effect of TRIA on the activity of several enzymes. The enzymes chosen for analysis have been well characterized and are involved in both crucial catabolic pathways and in ancillary metabolism important to the normal growth of plants. In addition to spectrophotometric analysis, the multiple molecular forms of several of these enzymes can be analyzed by staining for their activity after starch gel electrophoresis. Both qualitative as well as quantitative changes can therefore be analyzed after treatment with TRIA.

Materials and Methods

Plant Material, Growth, and Treatment

Eight corn seeds (cv. Pioneer 3780) were planted per 18 cm clay pot containing a steam sterilized root medium consisting of two parts of soil mix (equal volumes peat, sand, and sandy loam soil) with three parts of Sunshine peat mix (Fisons Western Corp., Des Plaines, Illinois). After 5 to 7 days, the seedlings were thinned to the four most uniform plants per pot, prior to treatment with TRIA. Plants were blocked for size and treatments assigned to blocks using a random number table prior to treatment. Pots were fertilized with 250 ml of soluble 20-8.6-16.6 (N,P,K) fertilizer (1 g/L) 24 h before treatment. The pots were maintained in the greenhouse under 200–300 $\mu Em^{-2}s^{1}$ of supplemental light provided by high pressure metal halide lamps for 16 h daily. The night temperature was adjusted to 24 ± 3° C.

TRIA was synthesized by the method of Gibson (1982) and provided by Procter and Gamble Corporation, Cincinnati, Ohio, as a colloidal dispersion containing 0.1% (w/w) of the surfactant tallow alkyl sulfate (TAS, Laughlin et al. 1983). The TRIA used to make these dispersions was determined by repeated recrystallizations to be at least 99.7% pure. Seedlings were sprayed with TRIA (0.5 μg/L), using an adjustable plastic aerosol sprayer (Science Products Company Inc., Chicago, Illinois), to the drip point in the late afternoon. Controls were treated in an identical manner but were sprayed with a solution containing the surfactant only (0.005 μg/L). Previous results have shown that these extremely low rates of TAS do not have any effect on the growth of corn seedlings (Ries et al. 1977).

Extraction of Plant Tissues and Analytical Methods

Shoots were harvested and immersed in liquid nitrogen. The shoots were lyophilized, weighed, and then pulverized in liquid nitrogen in a mortar and

Table 1. Growth response, soluble protein, and soluble nitrogen content of 5-day-old corn shoots 72 h after TRIA treatment.

Treatment	Dry weight (mg/plant)	Soluble protein (Lowry) (mg/plant)	Soluble nitrogen (Kjeldahl) (mg/plant)
Control	166.0	12.0	1.40
+ TRIA	208.7**	14.3*	1.86*
% of control	125	119	133

*,** F value for the differences from the control significant at the .05 and .01 level, respectively. Each value is the average of six replicates.

pestle. Polyvinyl polypyrrolidone (PVPP) equivalent to the weight of the lyophilized plant tissue was added, and the mixture was suspended in 10 ml 0.05 M Tris/HCl, pH 7.0, containing 1 mM 2-mercaptoethanol. After further maceration, the slurry was centrifuged at 12,000 × *g* for 15 min. The supernatant solution was used in all assays.

Isocitrate dehydrogenase (ICDH) activity (E.C. 1.1.1.42) was assayed using the method of Curry and Ting (1976). Total peroxidase (Px) activity (E.C. 1.11.1.7) was analyzed by the oxidation of o-dianisidine at 460 nm as described by Gibson and Liu (1978). Malate dehydrogenase (MDH, E.C. 1.1.1.37) and 6-phosphogluconate dehydrogenase (6PGDH, E.C. 1.1.1.44) were analyzed as previously described (Ochoa 1955, Sako and Stahmann 1972). Acid phosphatase (AcP, E.C. 3.1.3.2) was analyzed using p-nitrophenyl phosphate as the substrate in 0.1 M Na acetate, pH 4.5, at 37° C for 15 min. Samples were made basic by the addition of 1.0 N NaOH prior to measuring the absorbance at 405 nm. Alkaline phosphatase (AlP, E.C. 3.1.3.1) was analyzed using p-nitrophenyl phosphate as the substrate in 0.1 M glycine/NaOH, pH 10.0, at 37° C for 15 min (Reid and Wilson 1971). An enzyme unit is defined as the amount of enzyme that converts 1 μmole of substrate to product in 1 min under the conditions described. Total protein was determined using the method of Lowry et al. (1951). Kjeldahl nitrogen was determined by the method of Ferrari (1979).

Starch gel electrophoresis was performed using the method of Scandalious (1969). Extracts were electrophoresed in 12% starch in 0.1 M Tris/citrate buffer, pH 8.3, using 0.1 M lithium/borate, pH 7.0, as the electrode buffer at 350–400 V with a current of 45 mA for 4–6 h at 4° C.

Results

Long-term Studies

A 19% increase in soluble protein and 25% increase in dry weight was observed in corn shoots 72 h after application of TRIA (Table 1). 6PGDH and ICDH activity on a per plant and per mg protein basis also increased significantly and paralleled the increase in soluble protein (Table 2). ICDH activity showed the greatest increase, with an apparent twofold increase in activity over the

Table 2. Enzyme activity in 5-day-old corn shoots 72 h after TRIA application.

Enzyme	Treatment	Units/plant	Units/mg protein	Units/g plant
Px	Control	24	2.0	0.15
	+ TRIA	26	1.8	0.12
	% of control	108	90	80
AcP	Control	3.7	0.31	0.02
	+ TRIA	3.7	0.26	0.02
	% of control	100	84	100
AlP	Control	0.33	0.03	0.02
	+ TRIA	0.33	0.02	0.02
	% of control	100	67	100
ICDH	Control	10.4	0.85	0.06
	+ TRIA	23.1*	1.6*	0.11*
	% of control	222	189	183
MDH	Control	164.7	13.8	0.99
	+ TRIA	206.7*	14.8	0.99
	% of control	126	107	100
6PGDH	Control	21.6	1.8	0.13
	+ TRIA	34.7*	2.5*	0.17*
	% of control	161	139	131

* F value for the differences from controls significant at the .05 level. Each value is the average of six replicates.

control. MDH activity, although significantly higher than the controls on a per plant basis, did not increase on a per mg protein basis.

Equally important was the lack of an apparent change in other enzymes analyzed in the treated plants. Px, AcP, and AlP activity after 72 h was not significantly different from the controls either on a per plant or on a per mg protein basis.

Short-term Studies

The initial long-term studies described above suggested an increase in 6PGDH, ICDH, and MDH at a rate equivalent to or greater than the increase in the dry weight or soluble protein in corn seedlings treated with TRIA. Short-term studies (75 and 1125 min) indicated that both 6PGDH and ICDH activity in corn seedlings treated with TRIA increased rapidly on a per plant basis. An increase of 19% and 26%, respectively, was apparent by 75 min (Table 3). If the data are expressed on a per mg protein basis, the increase in the activity of 6PGDH and ICDH occurred by 75 min, with 22% more ICDH and 16% more 6PGDH at 19 h. AcP, MDH, and Px activity, expressed either as activity per plant or per mg protein, varied little between control and treated plants.

A distinct problem in assessing units of enzyme activity per mg protein in the TRIA-treated samples is that the protein levels increased during the course of the experiment. This raises the magnitude of the change in 6PGDH and ICDH observed on a per plant basis. AcP activity, however, showed little change during the time course studied and is therefore a more accurate internal

Table 3. Enzyme activity in 5-day-old corn shoots of 75 min and 1125 min after TRIA application.

		Time after treatment (min)			
		75		1125	
Enzyme	Treatment	Units/plant	Units/mg protein	Units/plant	Units/mg protein
Px	Control	15.4	6.5	16.7	4.8
	+ TRIA	14.0	5.2	15.8	4.6
AcP	Control	1.8	1.0	2.1	0.8
	+ TRIA	1.8	0.8	2.0	0.7
ICDH	Control	8.0	2.2	11.1	2.3
	+ TRIA	10.1**	2.6*	14.1**	2.8**
MDH	Control	55.3	17.5	67.7	16.2
	+ TRIA	57.6	17.1	71.2	17.2
6PGDH	Control	51.4	7.8	78.5	9.8
	+ TRIA	61.2*	8.5*	98.2*	11.4*

*,** F value for the differences from the control significant at the .05 and .01 level, respectively. Each value is the average of three experiments with at least three replicates per experiment.

Table 4. Enzyme activity in 5-day-old corn shoots 75 min and 1125 min after TRIA application as a function of AcP activity.

		Time after treatment (min)	
		75	1125
Enzyme	Treatment	Units/unit AcP	Units/unit AcP
6PGDH	Control	22.3	32.4
	+ TRIA	27.9*	41.8*
	% of control	125%	129%
ICDH	Control	3.8	4.9
	+ TRIA	4.9**	6.5**
	% of control	129%	133%

*,** F value for the differences from the control significant at the .05 and .01 level, respectively. Each value is the average of three experiments with at least three replicates per experiment.

standard than mg protein in these studies. The change in ICDH and 6PGDH as a function of AcP activity is presented in Table 4.

Starch Gel Electrophoresis

To determine if the increase in enzyme activity was due to an increase in the activity of a particular isozyme after treatment, extracts were electrophoresed in starch gels and then stained for enzyme activity. A zymogram of Px activity is shown in Fig. 1. Peroxidase isozymes have been well characterized in both mutant and normal corn lines by Brewbaker and Hasegawa (1975). Since the isozymes of Px are under strict developmental control, they can serve as useful markers to indicate abnormal developmental patterns in corn. Both TRIA-

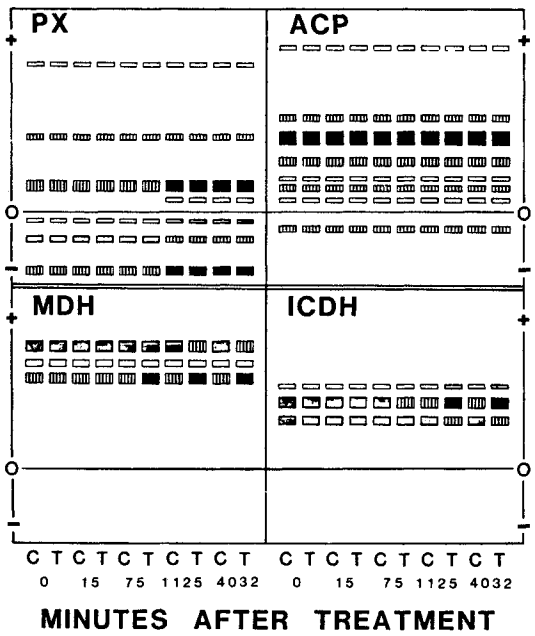

Fig. 1. Zymograms of enzyme activity after starch gel electrophoresis. Extracts were analyzed as described in *Materials and Methods*. C = control extract at each time interval. T = TRIA-treated extract at each time interval. Relative staining activity: (□) faint, (▨) weak, (▩) moderate intensity, (■) heavy intensity.

treated and control extracts showed the usual complement of leaf and shoot isozymes previously described (Brewbaker and Hasegawa 1975). Both the pattern of isozymes on the gels and the approximate staining intensity were essentially unchanged between the control and TRIA-treated samples during the time course.

The results of electrophoresis of ICDH and MDH were comparable to those obtained for Px isozymes. No discernible differences in the isozyme patterns between TRIA-treated and control extracts were observed. TRIA-treated samples consistently showed darker MDH and ICDH isozyme bands for equivalent volumes of extract electrophoresed at 1125 and 4032 min. Since all plant tissues were ground in equal volumes of buffer, this supports the spectrophotometric analysis showing an increase in total enzyme activity after application of TRIA.

Discussion

The results indicate that application of TRIA to corn seedlings results in increases in 6PGDH and ICDH activity. These increases occur within 75 min after treatment and persist for at least 3 days. MDH activity, which does increase on a unit per plant basis, increases at a rate equivalent to the increase in soluble protein in the plants. The activity of AcP, AlP, and Px remains unchanged from the control during the duration of these studies. Preliminary analysis indicates that similar patterns of response occur in rice after treatment with TRIA (not shown). The changes described above, however, do not appear to occur using the *in vitro* assay system, as has been noted for starch phosphorylase (Houtz 1980).

The results of starch gel electrophoresis support the increase in enzyme activity noted above. An increase in a specific isozyme, however, was not noted. These results suggest that there were no apparent developmental differences in TRIA-treated and control seedlings. Thus the action of TRIA, as assessed by Px isozyme patterns, does not appear to be due to precocious stimulation of developmental processes.

Large increases in both RNA and cellulase activity in pea epicotyls after IAA application have been previously described (Fan and Maclachlan 1967). Similarly, wounding also has been shown to result in the stimulation of several otherwise quiescent enzymes in potato slices (Uritani 1978). The specific means by which these increases occur, however, are unclear. There are several possible modes by which application of TRIA could result in stimulation of 6PGDH, ICDH, and MDH. The TRIA may stimulate dehydrogenase enzymes by binding to the enzymes and inducing a conformational change that would result in increased activity. In view of the fact that the increase in these enzymes did not occur until after 75 min and persisted for at least 3 days indicates that this mode of action is unlikely. Moreover, addition of TRIA to control extracts at the time of assay did not significantly alter the activity of these enzymes. Since the enzymes that showed the most significant increase in activity are all involved in crucial metabolic pathways, the use of specific transcriptional or translational inhibitors is precluded. Thus, whether the increase involves specific synthesis of new enzymes or perhaps the mobilization of a previously existing, quiescent pool remains to be determined. As judged by starch gel electrophoresis, however, any such mobilization appears to be broad and not restricted to a specific set of isozymes.

Regardless of the nature of the increase, 6PGDH, ICDH, and MDH may prove to be excellent diagnostic indicators of a response to TRIA. The activity of these enzymes reflects the increase in dry weight or soluble protein, but is considerably more specific. Further, AcP activity can now be used in other studies as an internal standard or reference to which other variables can be related. This should facilitate further studies on the mode of TRIA action.

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