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A Possible Role of Divalent Manganese Ions in the Photoinduction of Phenylalanine Ammonia-Lyase

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ABSTRACT

Divalent Mn ions cause an increase in the level of phenylalanine ammonia-lyase in gherkin hypocotyls. With the exception of Mg ions, which had a small effect, no other metal ion has so far been found which could replace the Mn ion in this respect. Invertase and peroxidase were not significantly affected by the Mn treatment. The increase in phenylalanine ammonia-lyase activity is explained by the removal, under the influence of Mn ions, of hydroxycinnamic acids, which cause repression of phenylalanine ammonia-lyase synthesis and/or inactivation of phenylalanine ammonia-lyase. Arguments are advanced for the hypothesis that photochemical transformations of Mn complexes are involved in the photoinduction of phenylalanine ammonia-lyase in dark-grown gherkin seedlings.

Exposure of dark-grown gherkin seedlings to the light causes an increase in the level of PAL[1] (EC 4.1.3.5) (4). In this respect blue light is the most effective; the photoinduction is probably not mediated by phytochrome (4, 5). The nature of the events leading to the changes in the PAL level is still obscure. In this paper evidence in favor of a role of Mn will be presented. Such a function of Mn is not entirely unexpected, since it has been found that irradiation of dark-grown maize seedlings brings about the mobilization of Mn in the plant (13).

MATERIALS AND METHODS

The experiments were performed with 3-day-old dark-grown gherkin seedlings *Cucumis sativus* L., cv. "Venlose niet plekkers," strain Tercken VI (9). Unless otherwise stated, the seedlings were cut immediately above the roots and positioned side by side in Petri dishes, with the cut surface in the solution and the cotyledons hanging over the rim. The irradiations were carried out with blue light of 150 $\mu w/cm^2$ (19). Determination of hydroxycinnamic acids (*p*-coumaric acid plus ferulic acid) and extraction and assay of PAL were performed as described before (4, 9). Invertase was assayed by polarimetry (1) and peroxidase was determined with the guaiacol method. Protein was measured by the Lowry method (17). Manganese and other metal ions were determined with a flame spectrophotometer.

[1] Abbreviation: PAL: phenylalanine ammonia-lyase.

RESULTS

Effect of Manganese on the Development of PAL. In preliminary experiments, seedlings were grown in darkness on filter paper. After 3 days, the water of half the seedlings was replaced by a solution of 30 mM $MnCl_2$. After 24 hr on this solution in darkness, the PAL level in the hypocotyl was 240% of that in the control plants kept on water. The possibility that this was due to activation of PAL by Mn ions which had been carried into the *in vitro* test system could be ruled out. Mn concentrations of up to 50 μM appeared not to affect the PAL activity, whereas higher concentrations were slightly inhibitory.

In order to achieve a more rapid uptake of Mn ions, the investigations were continued with seedlings which had been deprived of their roots and which had then been placed with the cut surface of the hypocotyl into the salt solution. A disadvantage of this method was that it introduced a wound effect: an increase in PAL activity in the tissue adjacent to the cut surface (6). Therefore, throughout this paper, data on PAL activity are reported separately for the upper and the lower half of the hypocotyl, of which the former was not affected by the cutting (Fig. 1). In this figure the changes in Mn concentration and PAL activity are presented in respect of seedlings which were either incubated in the Mn solution for 4 hr and then transferred to water or remained in the solution for 24 hr. Throughout the whole hypocotyl there was a much larger increase in the PAL level than in the preceding experiment. This increase lagged behind the accumulation of Mn and was maintained as long as more Mn was taken up. If cycloheximide was applied simultaneously with Mn, the increase in PAL activity was much lower, indicating that *de novo* protein synthesis was involved (see Table IV).

The Mn treatment led to brownish purple staining of the gherkin hypocotyl, a phenomenon observed by other workers (14–16, 21) in a number of plants. They ascribed this to the formation of tannins or tannin-like substances. The incubation during 24 hr in a 30 mM $MnCl_2$ solution in darkness temporarily inhibits the elongation growth of the gherkin seedlings. The Mn treatment does not impair the further development of the plants.

Specificity of Manganese Ions in Inducing PAL Activity. Table I contains a list of various metal salts which have been tested for their capability of changing the PAL level in gherkin hypocotyls. In addition to the Mn salts, only $MgCl_2$ had a stimulatory effect. The effect of the 3 mM solution was rather small. Further investigation of the effect of Mg was hampered by the fact that concentrations higher than 3 mM were lethal to the plants. A number of other metal ions, such as Ca^{2+}, Cu^{2+}, and Co^{3+}, had a lowering effect on the PAL level. The table shows further that the anion produced little effect.

Another question was that of specificity with respect to the enzymes affected by the Mn treatment. Irradiation caused a

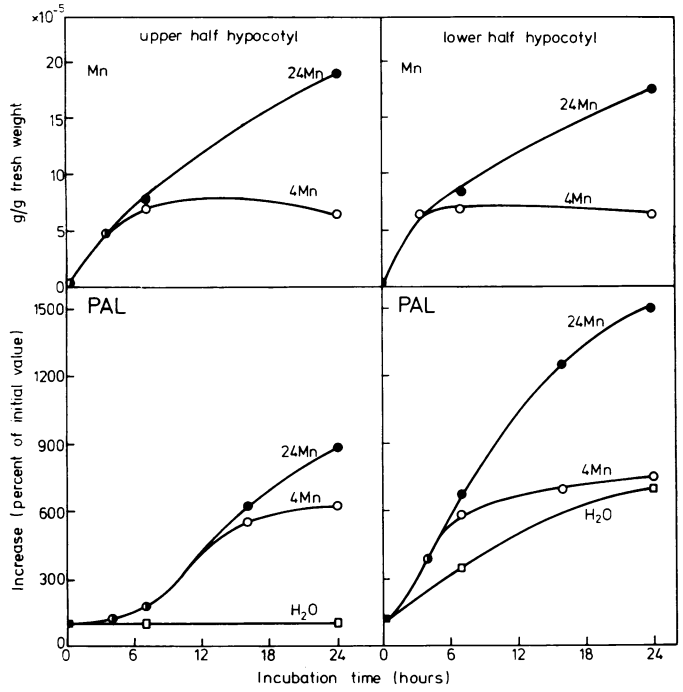

FIG. 1. Accumulation of Mn and the development of PAL in the upper and lower half of the hypocotyl, of gherkin seedlings that were incubated in a 30 mM $MnSO_4$ solution for 24 hr or in a 30 mM $MnSO_4$ solution for 4 hr and then transferred to water. All experiments were at 25 C in darkness.

Table I. *Effect of Various Metal Salts on the PAL Activity in Gherkin Hypocotyls*

The seedlings were incubated in the solution at 25 C in darkness during 24 hr. Values are in per cent of the controls incubated in distilled water. The PAL activity in the control plants was 5.7 and 21 nmole cinnamic acid/g fresh wt·min in the upper and lower half of the hypocotyl, respectively.

Salt	Concn	PAL Actvity in Hypocotyl	
		Upper half	Lower half
	mM		
LiCl	3	90	65
LiCl	30	80	60
NaCl	3	80	105
NaCl	30	90	109
KCl	30	115	100
NH_4Cl	30	100	105
$MgCl_2$	3	158	126
$MnCl_2$	30	940	182
$MnNO_3$	30	880	190
$MnSO_4$	0.03	95	117
$MnSO_4$	0.3	110	128
$MnSO_4$	3	288	170
$MnSO_4$	30	960	208
$CaCl_2$	30	80	52
$CuCl_2$	1	105	53
$CuCl_2$	30	90	50
$FeCl_3$	1	95	56

change in the level of PAL but did not affect the levels of peroxidase and invertase in the gherkin hypocotyl. The same applies to plants incubated in a 30 mM $MnCl_2$ solution. After 24 hr on this solution in darkness, the peroxidase and invertase activities were respectively 105 and 97% of that in the control plants maintained on water. The total amount of protein was about 30% lower as compared to that of the control plants.

Mechanism of the Manganese Effect. In gherkin hypocotyl tissue the PAL level is enhanced by irradiation and by wounding. Arguments have been advanced that different mechanisms are involved (6). Thus, sucrose and IAA enhance the PAL level in wounded tissue but inhibit the photoinduction of PAL. Table II shows that these two compounds lessen the effect of Mn with respect to the induction of PAL, indicating that the effect of Mn is related to that of irradiation rather than to that of wounding.

It was further found that in the hypocotyl of seedlings which were simultaneously irradiated during 24 hr and incubated in a $MnSO_4$ solution the PAL level was higher than in seedlings irradiated alone or incubated in a $MnSO_4$ solution in darkness (Table III). The amount of *p*-coumaric acid, one of the end products of the pathway in which PAL is a key enzyme, was much higher in the seedlings which are only irradiated than in those simultaneously incubated in a manganese solution. Moreover, ferulic acid, another end product, was completely absent in the seedlings treated with Mn in the light. The same holds for the seedlings treated with Mn in the dark. Although a high PAL level was reached in these seedlings, the amount of *p*-coumaric acid present at the end of the 24-hr period was low.

Figure 2 shows that when seedlings are treated with Mn in

Table II. *Effects of IAA and Sucrose on the Changes in the PAL Level Caused by Incubation in a Mn Solution*

The seedlings were incubated in the various solutions at 25 C in darkness during 24 hr.

Solution	PAL Activity in Hypocotyl	
	Upper half	Lower half
	%	
H_2O	100[1]	100[2]
$MnSO_4$, 30 mM	850	210
IAA, 1 mM	68	138
Sucrose, 0.15 M	71	163
$MnSO_4$, 30 mM + IAA, 1 mM	139	141
$MnSO_4$, 30 mM + sucrose, 0.15 M	102	193

[1] 6.2 nmoles cinnamic acid/g fresh wt·min.
[2] 24 nmoles cinnamic acid/g fresh wt·min.

Table III. *Interaction between Irradiation and Mn Treatment with Respect to the PAL Activity and the Amounts of* p-*Coumaric and Ferulic Acid in Gherkin Hypocotyls*

The seedlings were incubated at 25 C for 24 hr. The $MnSO_4$ solution was 30 mM.

Treatment	PAL Activity in Hypocotyl		*p*-Coumaric Acid in Hypocotyl		Ferulic Acid in Hypocotyl	
	Upper half	Lower half	Upper half	Lower half	Upper half	Lower half
	nmole cinnamic acid/g fresh wt·min		*nmole/g fresh wt*			
H_2O, 24 hr dark	6	22	70	30	10	10
H_2O, 24 hr blue light	9	30	380	230	110	100
Mn, 24 hr dark	51	46	90	40	0	0
Mn, 24 hr blue light	62	66	280	200	0	0

the dark the amount of hydroxycinnamic acids first increases and then declines. The decline in hydroxycinnamic acids is much less if after 16 hr in the dark the plants are exposed to the light. In these conditions irradiation has a lowering effect on the development of PAL activity. These results are in line with previous observations (6–8) that a high level of hydroxycinnamic acids goes with a low PAL level. Another way to enhance the level of hydroxycinnamic acids is by feeding the seedlings with the precursors phenylalanine or cinnamic acid (3). The data presented in Table IV demonstrate that simultaneous application of these compounds and Mn results in lower PAL levels than when Mn is given alone.

DISCUSSION

Incubation of dark-grown gherkin seedlings in a Mn solution results in the conversion of hydroxycinnamic acids into other products, probably tannins, and in an increase in the PAL level. It is likely that both phenomena are causally related. The hydroxycinnamic acids, which are end-products of the pathway in which PAL is a key enzyme, repress PAL synthesis and/or induce a system which inactivates PAL (4, 18, 22, 23). The removal of these compounds would thus lead to an increase in the rate of synthesis of PAL and/or a decrease in the rate of inactivation of PAL. Thereafter the increase in PAL activity may cause the rate of synthesis of hydroxycinnamic acids to increase. In the interpretation of the data the possibility has to be taken into account that the hydroxycinnamic acids are present in different cell compartments and that only a certain proportion will affect the PAL level. The action of Mn might be due to a specific catalytic function in a peroxidase-oxidase reaction (12). The conversion of hydroxycinnamic acids in such a reaction has been demonstrated *in vitro* (2). The above mechanism would explain why PAL is specifically affected by the Mn treatment.

An observation probably related to the phenomena described in this paper has been made by Feruya and Galston (11), who found that *in vitro* preincubation of pea homogenates with Mn caused the destruction of certain phenolic compounds. The occurrence of a similar process *in vivo* can be implied from the effect of Mn on the IAA oxidase system of cotton (20).

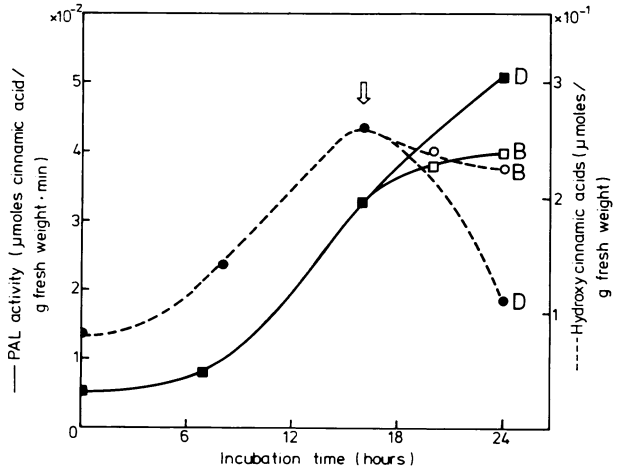

FIG. 2. Time course of the changes in the PAL level and in the amount of hydroxycinnamic acids in the upper half of the hypocotyl of gherkin seedlings that were incubated at 25 C in a 30 mM $MnSO_4$ solution. Closed symbols: seedlings which were kept in darkness; open symbols: seedlings which after 16 hr in darkness were transferred to blue light (indicated by the arrow).

Table IV. *Influence of Phenylalanine, Cinnamic Acid, and Cycloheximide on the Changes in the PAL Level and the Amount of Hydroxycinnamic Acids Induced by Manganese*

The seedlings were incubated in the various solutions at 25 C in darkness for 24 hr.

Incubation Medium	PAL Activity in Hypocotyls		Hydroxycinnamic Acids in Hypocotyls	
	Upper half	Lower half	Upper half	Lower half
	nmole cinnamic acid/ g fresh wt·min		*nmole/g fresh wt*	
H_2O	7	26	80	55
$MnSO_4$, 30 mM	48	46	105	70
$MnSO_4$, 30 mM + cinnamic acid, 1 mM	23	22	190	400
$MnSO_4$, 30 mM + phenylalanine, 10 mM	16	14	140	80
$MnSO_4$, 30 mM + cycloheximide, 30 μM	10.5	6	360	140

Mn salts are the only naturally occurring compounds found so far which have the same effect as light on the PAL activity in gherkin seedlings. Sucrose and IAA appeared to inhibit the induction of PAL by Mn in a similar way as they inhibit its photoinduction. Joyard and Fourey (13) recently reported that maize seedlings in the period following the transfer from darkness to the light exhibit important changes in the status of Mn inside and outside the proplastids. Part of the Mn might by this process get into a condition in which it is temporarily able to catalyze the conversion of hydroxycinnamic acids in certain cell compartments and thus to give rise to an increase in PAL activity. In addition to this, the possibility should be considered that Mn may be recycled in the light. After leaving the oxidative process in the trivalent state, the Mn could be reduced to the divalent state, as has been demonstrated in *in vitro* systems (10). This might explain why in darkness a similar increase in PAL activity can be obtained only if high Mn concentrations are used.

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