Acta Bot. Neerl. 22(1), February 1973, p. 49–54

INDUCTION OF PHENYLALANINE AMMONIA-LYASE BY DICHLOBENIL IN GHERKIN SEEDLINGS

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SUMMARY

Treatment of dark-grown gherkin seedlings with dichlobenil (2,6-dichlorobenzonitrile) causes an increase in the phenylalanine ammonia-lyase (PAL) activity in the hypocotyl. This is explained by the following mechanism. Hydroxycinnamic acids, end products of the pathway in which PAL is a key enzyme, are normally mainly present in cell compartments separated from compartments containing phenol oxidizing enzymes such as peroxidases and phenol oxidases. Dichlobenil increases the permeability of membranes for hydroxycinnamic acids, making these acids available for the oxidizing enzymes. This results in the deposition of lignin-like material and in the decrease in the concentration of soluble hydroxycinnamic acids. In its turn this leads to derepression of PAL synthesis and/or diminished inactivation of PAL resulting in a higher PAL level.

1. INTRODUCTION

Irradiation of dark-grown gherkin seedlings causes an increase in the phenylalanine ammonia-lyase (PAL, EC 4.1.3.5) activity in the hypocotyl (ENGELSMA 1967a, b, 1968a). Recently it was found that incubation of the gherkin seedling in a Mn solution had the same effect on the PAL level as irradiation (ENGELSMA 1972). Furthermore the observation was made that the Mn treatment caused the conversion of hydroxycinnamic acids (derivatives of p-coumaric acid and ferulic acid) into other products, probably tannins. 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 (ENGELSMA 1967a, 1968b; MARCUS 1971; ZUCKER 1965, 1970). Therefore the decrease in the concentration of these compounds in certain cell compartments of Mn-treated plants provided a plausible explanation for the increase in the PAL level.

The PAL level in gherkin hypocotyl tissue can also be increased by cutting segments from the hypocotyl and floating them on water (ENGELSMA 1968b). In this case the effect was explained by the release of hydroxycinnamic acids into the incubation medium.

Other workers (DAAMS 1965, unpublished results; PRICE 1969) found that the treatment of young seedlings with dichlobenil (2,6-dichlorobenzonitrile) caused the accumulation of lignin-like substances in certain cells and in intercellular spaces. Hydroxycinnamic acids are precursors of the lignin-like material, and it might be expected that in this case, too, the disappearance of these compounds from certain cell compartments would cause the PAL level to increase. In this

paper it will be shown that treatment of gherkin seedlings with dichlobenil does indeed give rise to induction of PAL activity. The possible implication of this finding for the mechanism of the photo-induction of PAL is indicated.

2. MATERIAL AND METHODS

The experiments were performed with gherkin seedlings (*Cucumis sativus* L. 'Venlose niet plekkers', strain Tercken VI) grown in plastic boxes on moist filter paper. The treatment with dichlobenil started three days after sowing when the water in the box was replaced by a 10^{-4} M solution of this compound in distilled water. Dichlobenil is rather volatile in air. Therefore the boxes were closed with a plastic cover. The raising of the seedlings and the incubation with dichlobenil occurred in darkness at 25°.

Extraction and assay of PAL were performed as described before (ENGELSMA 1967a). Invertase was assayed by polarimetry (BACON 1955) and peroxidase was determined with the guaiacol method. The determination of hydroxycinnamic acids was carried out as described by ENGELSMA & MEIJER (1965). The lignin-like polyphenols were extracted from the dried material with 0.5 N NaOH at 70° and determined spectrophotometrically according to STAFFORD (1960).

The experiment in which the effect of dichlobenil on the leakage of hydroxycinnamic acid from hypocotyl tissue was studied, was carried out as follows. Three-day old dark-grown gherkin seedlings were irradiated during 8 hours with blue light (600 $\mu W/cm^2$, 25°) and then kept in darkness for another 16 hours (25°). Thus it was assured that the hypocotyl tissue contained a high concentration of hydroxycinnamic acids. From each of these seedlings 3 adjacent segments 8 mm in length were cut from the hypocotyl beginning immediately below the cotyledons. The segments were washed with distilled water during 30 min to remove the hydroxycinnamic acids from the disrupted cells. 300 segments were placed in a 50 ml Erlenmeyer flask in 20 ml of 10^{-4} M dichlobenil or distilled water and stirred gently. At different time intervals samples were removed from each flask for the determination of hydroxycinnamic acids. Immediately after removal of a sample the same amount of fresh solution was added to the flask.

3. RESULTS AND DISCUSSION

Fig. 1 shows that in the hypocotyl of gherkin seedlings treated at 25° with a 10^{-4} M dichlobenil solution the PAL level began to rise drastically after a time lag of about 8 hours. The increase continued for about 24 hours from the beginning of the treatment whereafter the PAL activity remained at a high and more or less constant level. If cycloheximide (3×10^{-5} M) was applied simultaneously with dichlobenil the increase in PAL activity was only 8% of that in plants incubated with dichlobenil alone. This indicates that de novo protein synthesis is involved. The levels of two other soluble enzymes, invertase and peroxidase, were only slightly higher after 24 hours incubation with dichlobenil (*table 1*), indicating that the effect of dichlobenil is rather specific with respect

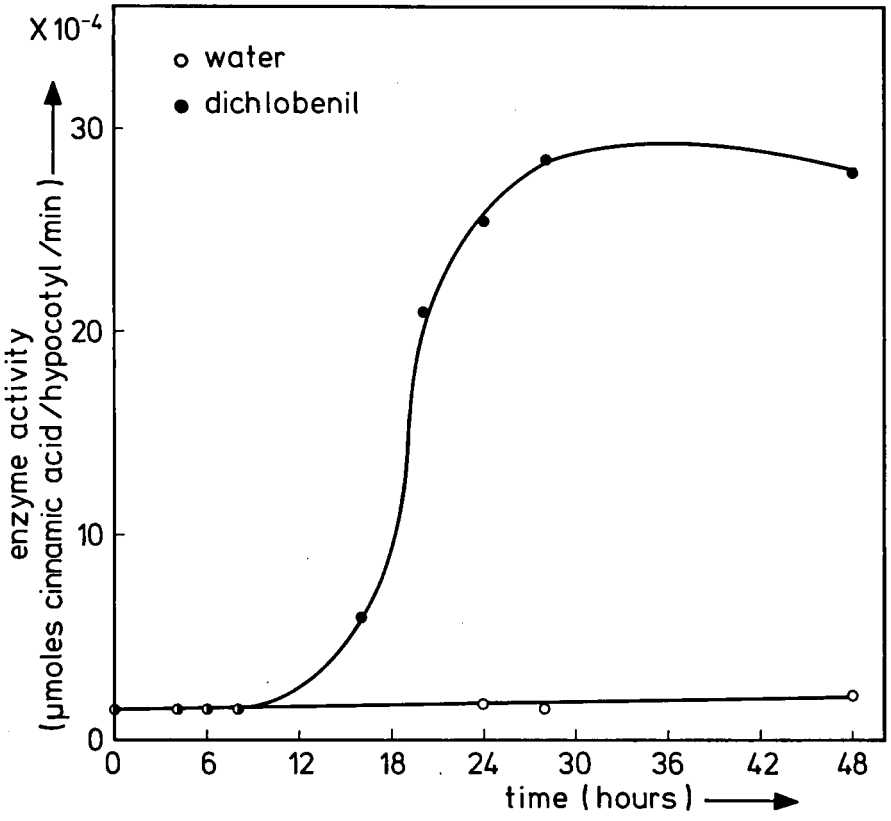

Fig. 1. Development of PAL activity in the hypocotyl of gherkin seedlings that were incubated in a 10^{-4} M dichlobenil solution in darkness at 25°.

Table 1. The effect of dichlobenil on the level of various enzymes and on the amounts of free and lignin-like phenolics. The plants were incubated in a 10^{-4} M dichlobenil solution for 24 hours in darkness at 25°. Values in percent of the controls from plants maintained on water.

peroxidase	122
invertase	119
PAL	1750
free phenolics (hydroxycinnamic acids)	130
lignin-like phenolics	225

to the induction of PAL. In agreement with the results of PRICE (1969) we found that the dichlobenil treatment raised the amount of water insoluble phenolics in the gherkin hypocotyl. According to PRICE (1969) the deposition of lignin-like material is the result of an increase in permeability of the tonoplast in seedlings treated with dichlobenil. Soluble phenolics, originally separated from compartments with peroxidases and phenoloxidases, would now become available for oxidation by these enzymes. Whether this applies to gherkin seedlings, too, was tested in an experiment in which the leakage of hydroxycinnamic acids from gherkin hypocotyl segments floated on a dichlobenil solution was compared with that from segments floated on water. *Fig. 2* shows that dichlobenil does not affect the release of hydroxycinnamic acids from the segments during the first six hours of incubation but that thereafter it has a pronounced effect. From these results it appears likely that the treatment with dichlobenil results in a

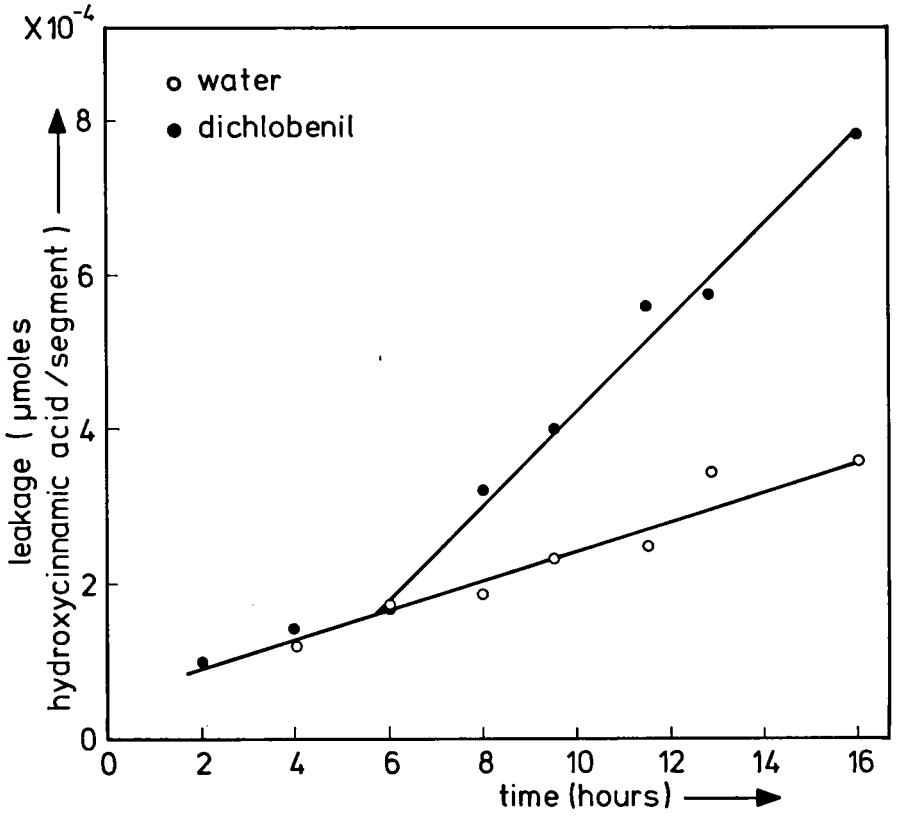

Fig. 2. The influence of dichlobenil (10^{-4} M) on the leakage of hydroxycinnamic acids from gherkin hypocotyl segments (25°).

(temporary) decrease in the concentration of hydroxycinnamic acids in certain cell compartments. This might then lead to derepression of PAL synthesis and/or diminished inactivation of PAL. Thereafter the increase in PAL activity may cause the rate of synthesis of the hydroxycinnamic acids to increase which would compensate for the loss of hydroxycinnamic acids due to the conversion into lignin-like material. Thus it can be explained that eventually a new equilibrium with a more or less constant PAL level is reached.

The increase in the PAL level in Mn-treated seedlings has been explained as well by a decrease in the concentration of hydroxycinnamic acids in certain cell compartments (ENGELSMA 1972). The mechanism by which this is achieved seems to be different from that causing the effect of dichlobenil. Mn causes the total disappearance of ferulic acid from the gherkin hypocotyl whereas in seedlings treated with dichlobenil the ratio *p*-coumaric acid/ferulic acid is the same as in the controls. The phenolic reaction products in Mn-treated seedlings cause a brownish-red discolouration of the hypocotyl whereas there is no staining of this organ of seedlings treated with dichlobenil.

Evidence has been obtained with a number of plants that the photoreversible pigment phytochrome is involved in the photoinduction of PAL (ATTRIDGE & SMITH 1967; SMITH & ATTRIDGE 1970; ENGELSMA 1970; HUAULT et al. 1971; WEIDNER et al. 1968). Much of the information available about phytochrome seems to indicate that it acts by changing the membrane permeability (HENDRICKS & BORTHWICK 1967; SMITH 1970). At present we are investigating whether there is a similarity between the induction mechanism of PAL mediated by phytochrome and that caused by dichlobenil.

ACKNOWLEDGEMENT

The author wishes to express his thanks to Miss M. J. G. Peeters and Mr. J. M. H. van Bruggen for technical assistance.

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