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THE SYSTEMIC ACTION OF DIMETHYLDITHIOCARBAMATES ON CUCUMBER SCAB CAUSED BY *CLADOSPORIUM CUCUMERINUM* AND THE CONVERSION OF THESE COMPOUNDS BY PLANTS

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GENERAL INTRODUCTION

At present plant disease control by fungicides depends mainly on protection of the host surface against infection. With the use of protective fungicides one has succeeded in combating numerous diseases. There are, however, many cases in which protective fungicides are not effective. Plant parts missed at the time of application of the fungicide and young growing parts formed after application are not protected. Moreover, in general protectants do not affect established infections or prevent infections through the root systems which cause root diseases and vascular wilts. These limitations as well as the discovery of insecticides which are translocated within the plant (systemic insecticides) about twenty years ago, gave new impetus to the study of 'chemotherapy' of fungus diseases.

HORSFALL & DIMOND (1951) defined chemotherapy as disease control by chemicals which are introduced into the host and which through their effect upon the host or pathogen reduce or nullify the effect of the pathogen after it has entered the plant. HORSFALL (1956) distinguishes two different types of chemotherapy:

1. 'topical chemotherapy' in which the chemotherapeutant penetrates only over a small distance into the plant and has a local effect. Dodine for the control of *Venturia inaequalis* is an example of a topical chemotherapeutant (HAMILTON & SZKOLNIK, 1958).

2. 'systemic chemotherapy' which involves the use of systemic chemotherapeutants, compounds which also penetrate into the plant but then become distributed in its inner parts.

Strictly speaking, however, systemic chemotherapy suggests cure of disease by a chemical which is distributed in the hosts. In this sense the term systemic chemotherapeutant can only be used if a compound is distributed and is effective against the disease *after* the host has already been penetrated by the pathogen. According to the terminology of HORSFALL & DIMOND (1951), however, an effective compound, which is distributed *before* penetration of the host has taken place, must also be classified as a systemic chemotherapeutant. In that case the compound does not cure but protects the plants. In general the protective action of systemic chemotherapeutants is better than their curative action.

This ambiguity of the word therapy may account for the fact that not all authors use the term systemic chemotherapy. Instead, the term 'systemic control' (JONES & SWARTHOUT, 1961) or 'systemic protection' (FAWCETT, SPENCER & WAIN, 1957) is used. To avoid the term systemic chemotherapeutants OORT & VAN ANDEL (1960) introduced the term 'systemics'; VAN DER KERK (1957), KAARS SIJPESTEIJN (1961) and WAIN (1961) use, the term 'systemic compounds' which shall be used in this paper also.

Systemic compounds can be divided into different groups:

1. Compounds which are fungitoxic *in vitro*. Usually these compounds are called 'systemic fungicides' (CREMLYN, 1961). Their systemic activity may be due to a direct toxic action on the pathogen within the plant.

2. Compounds which are not fungitoxic *in vitro*. Regarding their mode of action the following possibilities can be given:

a. They are converted into fungitoxic compounds in plants. In this connection dimethyldithiocarbamates which are not fungitoxic *in vitro* can be mentioned. Their action will be considered in detail in chapter V of this paper.

b. They inhibit enzymes of the pathogen which are essential for the attack of the host plant, as for instance pectolytic enzymes (GROSSMANN, 1962; KAARS SIJPESTEIJN & PLUIJGERS, 1961). Obviously such inhibitors of enzymes will not inhibit growth of the fungus *in vitro* as these enzymes are not essential for growth on ordinary nutrient media.

c. They act by interference with host metabolism which results in an increase of resistance of the host against the pathogen. Growth-regulating compounds may belong to this group (DAVIS & DIMOND, 1953; OORT & VAN ANDEL, 1960; VAN ANDEL 1962).

It will be clear that the mechanism of systemic activity may also depend on a cumulative effect caused by different actions. When, for example, a compound is fungitoxic the possibility is not excluded that at the same time it increases resistance of the host.

For more examples of each group of systemic compounds the reader is referred to recent papers of CREMLYN (1961), KAARS SIJPESTEIJN (1961) and of DIMOND (1962).

The cases in which systemic activity of dimethyldithiocarbamates has been reported will now be dealt with in more detail.

Systemic dimethyldithiocarbamates. Dimethyldithiocarbamates belong to the most active fungicides. Systemic activity of compounds from this group of fungicides has been reported by several authors. GROSSMANN (1957) showed some effect against *Fusarium oxysporum* on tomatoes after uptake of sodium dimethyldithiocarbamate (NaDDC) or of tetramethylthiuramdisulfide (thiram = TMTD) by the roots. Bioassay of the guttation fluid of treated plants revealed no fungitoxicity and therefore it was suggested that the systemic activity was effected by interference with host metabolism.

Seedlings of *Pinus silvestris* grown from seed treated with TMTD were less seriously attacked by *Rhizoctonia spec.* and *Pythium spec.* than seedlings grown from untreated seed (VOLGER, 1959). In a bioassay test fungitoxicity could be demonstrated in the above-ground parts of such treated seedlings. Similar results were obtained with related dimethyldithiocarbamates (VOLGER, 1960).

CORBAZ (1962) found systemic activity of NaDDC against *Peronospora tabacina* on tobacco plants after leaf application.

VAN RAALTE (reported by PLUIJGERS, 1959) found a slight systemic protection of cucumber seedlings against attack by *Cladosporium cucumerinum* after uptake of NaDDC by the roots. Still more important was the discovery of the systemic activity against cucumber scab by some dimethyldithiocarbamates which are not fungitoxic *in vitro* (VAN RAALTE, KAARS SIJPESTEIJN, VAN DER KERK, OORT & PLUIJGERS, 1955). Moreover, the latter compounds were less phytotoxic than NaDDC. No definite conclusions could, however, be drawn regarding the mode of action of NaDDC and of the non-fungitoxic dimethyldithiocarbamates.

To gain insight into the mode of action the uptake, translocation, distribution and the fate of NaDDC and related compounds within cucumber seedlings have been studied. Most of the experiments have been carried out with a special paper-chromatographic bioassay. The results obtained with this technique will be described and discussed in this paper.

This study forms part of the programme of the Institute for Organic Chemistry T.N.O., Utrecht. It has been carried out at the Laboratory of Phytopathology, Agricultural University, Wageningen, The Netherlands, in connection with the activities of the T.N.O. Research Unit for Internal Therapy of Plants.

CHAPTER I

THE DEMONSTRATION OF NaDDC IN SAP OF CUCUMBER SEEDLINGS

I. INTRODUCTION

As already mentioned, VAN RAALTE found (reported by PLUIJGERS, 1959) that NaDDC

$$(CH_3)_2N\text{-}C(=S)\text{-}S\text{-}Na$$

after uptake by the roots of cucumber seedlings is slightly systemically active against *Cladosporium cucumerinum* Ell. & Arth. This fungus is very sensitive to NaDDC, since the minimum concentration which completely inhibits its growth *in vitro* is 20 ppm. It thus seems logical to explain the systemic activity of NaDDC by assuming an uptake of dimethyldithiocarbamate ions (DDC-ions) and subsequent inhibition of the penetrating hyphae. If, however, this holds true it might be expected that a paper-disk dipped in sap expressed from cucumber seedlings which have been treated with NaDDC causes an inhibition zone on an agar plate seeded with *C. cucumerinum*. Sap did, however, not show fungicidal activity on this fungus. Neither VAN ANDEL (1958) nor DEKHUIJZEN (1961b) found activity of this sap on *Glomerella cingulata*, a fungus which is more sensitive to NaDDC than *C. cucumerinum* since its growth *in vitro* is completely inhibited by a minimum concentration of 0.2 ppm (KAARS SIJPESTEIJN & JANSSEN, 1959). The failure to demonstrate a fungitoxic compound in sap of NaDDC treated cucumber seedlings by means of the paper-disk bioassay raises doubt whether the systemic activity of NaDDC is a result of the presence of DDC-ions inside the plants. On the other hand the question can be put whether this bioassay is suitable for the demonstration of small amounts of DDC-ions in plant sap. The present chapter deals with this question.

2. MATERIALS AND METHODS

a. Cultivation of cucumber seedlings

Cucumber seedlings (*Cucumis sativus* L., variety 'Lange gele tros') were used in the experiments. The seedlings were grown in pots with sterilized sand at 28°C. After three days the pots were placed in a room at 20°C and exposed to light for 12 hours daily (PHILIPS T.L. 40 W/33). The plants received 5.3 mW per sphere of 1 cm^2 cross section of the spherical radiation meter developed by WASSINK & VAN DER SCHEER (1951). After seven to ten days, when the cotyledons had been fully developed but leaves were not yet visible, the seedlings were used for the experiments.

b. Expressed sap, the alcohol-soluble fraction and the alcohol-insoluble fraction of sap of cucumber seedlings

Cucumber seedlings were frozen, thawed and squeezed by hand pressing. An equal volume of ethanol (96%) was added to the sap and the solution was centrifuged for 5 minutes at 4500 rpm. The alcohol-soluble fraction was ob-

tained by evaporating the supernatant liquid *in vacuo* at 30–40° C to the original volume of sap. To obtain the alcohol-insoluble fraction of sap the precipitate was suspended in water, centrifuged and resuspended. This washing procedure in which the precipitate was freed from water-soluble compounds was repeated three times. Subsequently the precipitate was suspended in a volume of water equal to the original volume of sap.

c. Paper-disk bioassay

The following basal medium was used: 1% glucose, 0.5% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.05% NaCl, 0.05% $MgSO_4 \cdot 7\,H_2O$, 2% agar, tap water. The pH after autoclaving was 6.3. The glucose was sterilized by Seitz filtration and added to the autoclaved agar. The agar was seeded with conidia of *G. cingulata* (1 ml agar containing 20.000 conidia). 30 ml agar was poured in a petri dish with a diameter of 15 cm. Unless otherwise indicated the paper-disks (SCHLEICHER & SCHÜLL no. 2208, diameter 0.9 cm) were impregnated with 0.1 ml of the expressed sap which was to be tested for fungitoxicity. After drying in an air-current at 30° C the disks were sterilized by exposure to chloroform vapour for 16 hours and placed on an agar plate. Growth was assessed after two days incubation at 24° C.

d. Paper-chromatographic bioassay

WELTZIEN (1958) developed a simple paper-chromatographic method for the demonstration of small amounts of various fungicides on chromatograms. He chromatographed the fungicide and after being dried the strip was placed on a glass plate. It was then sprayed with a conidial suspension in a nutrient solution. After incubation in a damp atmosphere the paper appeared to be grown over by the fungus except for one spot. This spot indicates the presence of a fungicide. Regular growth only occurs after spraying with a dense conidial suspension. Moreover, a dense conidial suspension has the advantage of suppressing the development of other micro-organisms.

This method of WELTZIEN has one disadvantage; during spraying the strip becomes soon saturated with the nutrient solution. At the same moment, the solution will penetrate between the paper and the glass plate. This can result in the diffusion of a water-soluble compound in the liquid between the paper and the glass plate. To prevent this, the following procedure was employed; I made use of a rather thick paper namely WHATMAN 3 or WHATMAN 3MM instead of SCHLEICHER & SCHÜLL 2043b. Sap to be tested for fungitoxicity was applied across 1 cm of the starting line. The width of the strip was 4 cm. The solvent-mixture propanol-water (85:15, v/v) was allowed to run down the paper for 16 hours at 22° C. The paper was dried at 30° C. The strips were then placed on a metal frame stringed with nylon threads and sprayed with a conidial suspension of *G. cingulata* in a glucose-mineral salts medium (4.10^6 conidia/ml). The composition of the nutrient solution was 0.7% KH_2PO_4, 0.3% $Na_2HPO_4 \cdot 2H_2O$, 0.4% KNO_3, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl and 5% glucose in tap water; final pH 6.3. Though the distance between the nylon threads is 4 cm, the strips sprayed with the conidial suspension are lying flat on this support. The frame with the strip was placed in a moist glass box and incubated at 27° C. The glass box was covered with a glass plate and to prevent condensation-water from dripping on the strips, the inner side of the glass plate was

covered with filter paper. The condensation-water evaporated from the filter paper by those parts which projected from the glass box. After two days the strips showed a light red colour caused by growth of the fungus except on the spots which contained a fungitoxic compound. When necessary for quantitative purposes the inhibition zones were measured by a planimeter.

e. Paper-spot bioassay

Sap to be tested for fungitoxicity was added to one spot of a strip of WHATMAN 3MM paper. The diameter of the spot was about 0.5 cm. The spot was dried in an air-current at 30° C. The paper, placed on a metal frame, was sprayed with a conidial suspension of *G. cingulata* and incubated as described under d. The areas of the inhibition zones were measured by a planimeter.

f. Dimethyldithiocarbamates

All dimethyldithiocarbamates used were prepared by the fungicides team of the Institute for Organic Chemistry, T.N.O. Utrecht, under the direction of Dr. C. W. PLUIJGERS. DDC-ions easily chelate with copper to form copper dimethyldithiocarbamate ($CuDDC_2$). For that reason NaDDC was always dissolved in glass-distilled water and not in tap water.

3. RESULTS

a. The inadequacy of the paper-disk bioassay for the demonstration of small amounts of NaDDC and other dimethyldithiocarbamates in sap of plants

To investigate whether the paper-disk bioassay was suitable to demonstrate low concentrations of DDC-ions in sap of cucumber plants, the fungitoxicity of 10, 20 and 30 ppm NaDDC dissolved in sap or water was compared. It was found that, whereas the lowest concentration of NaDDC in water (10 ppm) caused a clear inhibition zone, growth was undisturbed around the disk impregnated with the same concentration of NaDDC in sap (Plate IA). A small inhibition zone was formed if the concentration was raised to 20 ppm. Fifty ppm NaDDC in sap also gave a smaller zone of inhibition than in water; moreover, in the presence of sap inside the inhibition zone a ring of mycelial growth was visible.

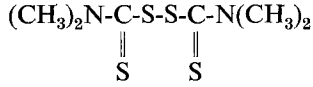
It is known from the work of GOKSØYR (1955), KAARS SIJPESTEIJN, JANSSEN & VAN DER KERK (1957), KAARS SIJPESTEIJN & JANSSEN (1959), JANSSEN & KAARS SIJPESTEIJN (1961) that NaDDC with copper easily forms a chelate (copper dimethyldithiocarbamate, $CuDDC_2$) which is fungitoxic to *G. cingulata*. According to GOKSØYR (1955) and THORN & RICHARDSON (1962) $CuDDC_2$ forms a complex with proteins and this should lead to a decreased fungitoxicity. For this reason most of the proteins were precipitated with ethanol (96 %) as described before. To the alcohol-soluble and the alcohol-insoluble fraction thus obtained, NaDDC was added. It appeared that in the first case the fungitoxicity was the same as when the total sap was used; on the other hand NaDDC dissolved in the alcohol-insoluble fraction was as active as when the fungicide was dissolved in water. From this result it is clear that the reduction of the fungitoxicity of NaDDC by sap is not due to the presence of proteins.

As will be shown in section 3 d of this chapter sap and the alcohol-soluble

fraction, in contrast to the alcohol-insoluble fraction, contain compounds which antagonize the fungitoxicity of NaDDC.

It must be emphasized that the reduction of the fungitoxic activity of NaDDC is not only caused by sap of cucumbers but also by sap expressed from potato tubers, broad bean plants or tomato plants.

Some experiments were carried out with related dimethyldithiocarbamates. It appeared that the fungitoxicity of tetramethylthiuram disulphide,



and of tetramethylthiuram monosulphide,

also decreased in the presence of sap of cucumber plants and of potato tubers.

b. The inadequacy of the paper-spot bioassay for the demonstration of small amounts of NaDDC in sap of plants

A disadvantage of the paper-disk bioassay is the fact that bacteria often grow around the disks on the agar in spite of their exposure to chloroform vapor. This difficulty can be avoided by making use of a dense conidial suspension, thus suppressing development of other microorganisms. Much work is saved, however, if the experiments are carried out on paper instead of on an agar plate. Moreover, the paper-spot bioassay has the advantage over the paper-disk bioassay that after killing the mycelium with ethanol (96%) the paper can be kept for comparison with results of other experiments.

In making use of the paper-spot bioassay NaDDC dissolved in sap revealed again smaller inhibition zones than those caused by the same concentration in water (table 1, column 1 and 2). This table also shows that the inhibition zones formed by the alcohol-soluble fraction of NaDDC dissolved in sap are larger than those formed by the same concentrations in the whole sap (column 2 and 3).

TABLE 1. The difference between the fungitoxicity of NaDDC dissolved in water and in sap of cucumber seedlings against *Glomerella cingulata*. Paper-spot bioassay, 0.1 ml of the liquid added to the paper.

Column	1	2	3
Concentration of NaDDC in ppm	Area of inhibition zone in cm²		
	NaDDC dissolved in		Alcohol-soluble fraction of NaDDC dissolved in sap[1]
	water	sap	
4	3.5	0.0	1.3
10	6.5	0.8	1.7
20	7.2	6.3*	6.5*

[1] NaDDC dissolved in sap. Subsequently an equal volume of ethanol (96%) was added to the sap. After centrifugation for 5 minutes at 4500 rpm the supernatant was evaporated *in vacuo* at 30°C to the original volume of sap.

* Ring of mycelial growth is present inside the inhibition zone.

NaDDC directly added to the alcohol-soluble fraction also caused a larger inhibition zone than the same concentration dissolved in sap. In contrast to what was found with the paper-disk bioassay, the fungitoxicity of NaDDC was slightly reduced when dissolved in the alcohol-insoluble fraction, If, however, this NaDDC-containing solution was centrifuged for 5 minutes at 4500 rpm the inhibition zone formed by the supernatant liquid was equal to the inhibition zone formed by the same concentration in water. The results obtained do not support the idea of a binding of DDC-ions on proteins and other cell constituents. They rather point to an impediment of the diffusion of DDC-ions by the bulky deposit of proteins and perhaps other cell constituents on the paper.

c. The demonstration of small amounts of NaDDC in sap of plants by the paper-chromatographic bioassay

From the results of the experiments described above, it is clear that small amounts of NaDDC in water can be determined with greater sensitivity than NaDDC in sap by the paper-disk or paper-spot bioassay.

How to explain the inactivation of the fungitoxicity by plant sap? It is well known from the work of KAARS SIJPESTEIJN & VAN DER KERK (1952) that L-histidine and to a much smaller extent also L-cysteine and DL-methionine antagonize the fungitoxicity of NaDDC. Therefore it was investigated whether NaDDC could be demonstrated in sap or in the alcohol-soluble fraction after separation from possible antagonists. For this reason paper chromatography was applied.

When chromatograms were made of 0.1 ml of a solution of 10 ppm NaDDC in glass-distilled water an inhibition zone with an R_F value of 0.87 was detected (Plate I B). An inhibition zone with the same R_F value was found when NaDDC was dissolved in sap. Mycelial growth inside the inhibition zone was never observed. Without addition of NaDDC, sap did not cause an inhibition zone.

The high R_F value of a hydrophilic compound like NaDDC seems surprising. In co-operation with Dr. M. J. JANSSEN of the Institute for Organic Chemistry T.N.O., Utrecht it was demonstrated that in neither of these cases the inhibition zones contained NaDDC. Instead, the presence of the copper salt, $CuDDC_2$ could be demonstrated spectrophotometrically after chloroform extraction of the zone with an R_F value of 0.87. As already described DDC-ions easily chelate with copper to form $CuDDC_2$ and this compound is almost insoluble in water and highly fungitoxic to *G. cingulata* (KAARS SIJPESTEIJN & JANSSEN, 1959). Apparently NaDDC reacts immediately with traces of copper always present in the paper to form $CuDDC_2$. This chelate causes the inhibition zone with an R_F value of 0.87.

Although NaDDC dissolved in sap as well as in water can be detected on chromatograms, the areas of inhibition zones were not of equal size in both cases. Sap (0.1 ml) in which NaDDC was dissolved showed less inhibition than 0.1 ml of the same concentration of the fungicide in water (table 2).

Much better results were obtained, however, when an equal volume of ethanol (96%) was added to sap in which NaDDC was dissolved. The area of the inhibition zone formed by the supernatant was now found to be of the same size as the zone formed by NaDDC in water (table 2). Thus the conclusion can be drawn that NaDDC was not decomposed by sap.

Since the total amount of NaDDC in sap could be detected only after precipi-

TABLE 2. Demonstration of NaDDC dissolved in water or in sap of cucumber seedlings by the paper-chromatographic bioassay. 0.1 ml added to the starting line. Solvent: descending propanol-water (85:15, v/v) for 16 hours. Test fungus *Glomerella cingulata*. All zones of inhibition have an R_F value of 0.87.

Column	1	2	3
Concentration of NaDDC in ppm	Area of inhibition zone in cm^2		
	NaDDC dissolved in		Alcohol-soluble fraction of NaDDC dissolved in sap[1]
	water	sap	
4	4.5	0.0	3.8
10	9.5	4.5	9.0
20	12.2	9.8	12.6

[1] NaDDC dissolved in sap. Subsequently an equal volume of ethanol (96%) was added to the sap. After centrifugation for 5 minutes at 4500 rpm the supernatant was evaporated *in vacuo* at 30°C to the original volume of sap. 0.1 ml of the liquid was chromatographed.

tation of most of the proteins by addition of ethanol to the sap, it is highly probable that the presence of these proteins is responsible for the incomplete recovery of the fungicide. This fact is not surprising since it has often been reported that addition of large amounts of different substances on the starting point can result in an irregular separation of the compounds when the solvent-mixture runs through the paper (*cf.* LINSKENS, 1959).

Thus the results show that, whereas not all NaDDC in plant sap can be recovered by the paper-disk or paper-spot bioassay, the total amount of NaDDC in sap can be detected by application of the paper-chromatographic bioassay after removing most of the proteins and other alcohol-insoluble cell constituents.

The fact that chromatography is required to demonstrate the presence of NaDDC in sap suggests that during this procedure the fungicide is separated from one or more antagonists.

d. The demonstration of compounds which antagonize the fungitoxicity of NaDDC in sap

Our next aim was to investigate whether sap of cucumber seedlings in fact contains compounds which are able to antagonize the fungitoxic activity of NaDDC. To gain information on this point 0.2 ml of sap was subjected to paper chromatography. Descending propanol-water (85:15, v/v) was used as solvent-mixture for 16 hours. The strip was dried and sprayed with a conidial suspension of *G. cingulata* in the nutrient medium to which NaDDC had been added (0.5 ppm). One can expect growth only on those spots where compounds are present which are able to antagonize the fungitoxic action of NaDDC. In this way two spots were detected with R_F values of 0.08 and 0.5, respectively.

A similar experiment carried out with 0.2 ml of the alcohol-insoluble fraction did not reveal any zones of mycelial growth. When, however, 0.2 ml of the alcohol-soluble fraction was chromatographed two zones of growth were detected. The R_F values were again 0.08 and 0.5.

As already mentioned L-histidine and to a smaller extent L-cysteine and DL-methionine are known to antagonize the fungitoxic action of dimethyldithio-

carbamates and this suggested that the zones of growth on the chromatograms might be due to the presence of these compounds. To gain information on this point 20 μg L-histidine, 100 μg L-cysteine or 100 μg L-methionine were chromatographed instead of sap. After spraying the NaDDC containing conidial suspension of *G. cingulata* it was found that both histidine and cysteine give rise to a zone of growth with an R_F value of 0.08, whereas no zone of growth was visible after chromatography of methionine. Therefore it seemed possible that the antagonistic activity of sap towards NaDDC was due to histidine and possible to cysteine as well. This possibility was studied more in detail by looking for a solvent-mixture in which histidine and cysteine could be separated. Descending butanol-acetic acid-water (4:1:1, v/v/v) for 16 hours appeared to be a suitable solvent-mixture. To evaporate this solvent-mixture sufficiently from the paper it appeared necessary to dry the paper for 24 hours at room temperature. Histidine, cysteine as well as the alcohol-soluble fraction were chromatographed. Subsequently the strips were sprayed with a conidial suspension of *G. cingulata* to which NaDDC was added (0.5 ppm). Four different zones of growth with R_F values of 0.04, 0.13, 0.23, 0.35, could be detected on the strip after chromatographing the alcohol-soluble fraction (Plate I C). L-histidine and L-cysteine formed zones of growth at an R_F value of 0.13 and 0.35, respectively. Addition of L-histidine or L-cysteine to sap led to the development of larger zones of growth at R_F values of 0.13 and 0.35, respectively. Thus it seems very likely that the alcohol-soluble fraction of sap contains both histidine and cysteine. By comparison of the areas of the zones of growth formed by known amounts of histidine or cysteine with the zones formed by different volumes of the alcohol-soluble fraction of sap the concentration of free histidine and free cysteine was estimated to be about 400 and 500 ppm, respectively.

From these results it is highly probable that the presence of histidine and cysteine in sap or its alcohol-soluble fraction are to a great extent responsible for the fact that NaDDC cannot easily be detected in these liquids without previous chromatography. Yet, the antagonistic activity of sap cannot be completely explained by the presence of histidine and cysteine. From table 3 it can be seen that the antagonizing property of the alcohol-soluble fraction is much stronger than that of water in which histidine and cysteine were dissolved. Moreover, a ring of mycelial growth inside the zone of inhibition is only

TABLE 3. The antagonistic activity of L-histidine, L-cysteine and the alcohol-soluble fraction of sap of cucumber seedlings against NaDDC. The different solutions (0.1 ml) were tested for fungitoxicity by the paper-spot bioassay.

Concentration NaDDC in ppm	Area of the inhibition zone in cm[a]				
	NaDDC dissolved in				
	water	solution of histidine in water (400 ppm)	solution of cysteine in water (500 ppm)	solution of histidine (400 ppm) + cysteine (500 ppm) in water	alcohol-soluble fraction of sap
4	3.9	2.1	1.8	1.7	1.2
10	6.8	5.0	4.8	4.7	1.8
20	7.6	7.2	7.0	6.4	6.7*

* Ring of mycelial growth is present inside the inhibition zone.

formed in the presence of the alcohol-soluble fraction. The fact that besides histidine and cysteine, sap still contains other compounds capable of antagonizing NaDDC suggests that these compounds in one way or another are responsible for the formation of the zone of mycelial growth inside the inhibition zone.

4. Discussion

In the preceding paragraphs it was demonstrated that the paper-spot bioassay is a more suitable method than the paper-disk bioassay for the demonstration of low concentrations of DDC-ions in water or in sap expressed from cucumber seedlings. From table 4 it appears that the amounts of NaDDC which can just be demonstrated by the paper-disk bioassay and by the paper-spot bioassay are 0.2 μg and 0.01 μg, respectively. This difference in sensitivity will be due to the fact that the thickness of the agar plate is about 4 mm, whereas that of the WHATMAN 3 paper is about 0.4 mm. For this reason the amount of diffusing DDC-ions which causes a small inhibition zone on the paper may become too much diluted after diffusing into the agar. Application of the paper-disk bioassay as well as of the paper-spot bioassay clearly showed that sap strongly reduces the fungitoxicity of NaDDC. Both methods led, however, to different results after precipitation of proteins and other cell constituents. Only in the case of the paper-spot bioassay the minimum concentration of NaDDC in sap which can be demonstrated after testing 0.1 ml decreases still further (table 4). The difference can be explained by the fact that the content of a paper-disk is about 13 times the content of the spot on the WHATMAN 3 paper to which NaDDC and sap is added. Therefore the spot on the WHATMAN 3 paper is already saturated with proteins and other cell constituents after addition of 0.1 ml sap, whereas a paper-disk can be impregnated with about 1 ml sap before the point of saturation is reached. Hence it may be concluded that the bulky deposit of substances from 0.1 ml sap impedes the diffusion of DDC-ions into the paper whereas these substances do not interfere with the diffusion of the ions into the agar. If this holds true, it can be expected that a larger quantity of sap will also impede the diffusion of

TABLE 4. The minimum concentration (ppm) of NaDDC dissolved in water or in sap of cucumber seedlings which can be demonstrated by three different methods. 0.1 ml of the liquids tested for fungitoxicity (0.1 ml, 2 ppm contains 0.2 μg NaDDC).

Column	1	2	3
Method	Minimum concentration (ppm) causing an inhibition zone		
	NaDDC dissolved in		Alcohol-soluble fraction of NaDDC dissolved in sap[1]
	water	sap	
Paper-disk bioassay	2	20	20
Paper-spot bioassay	0.1	5	1
Paper-chromatographic bioassay	2	5	2

[1] NaDDC dissolved in sap. Subsequently an equal volume of ethanol (96%) was added to sap. The supernatant of the centrifuged sap (5 min., 4500 rpm) was evaporated *in vacuo* at 30°C to the original volume of sap.

DDC-ions into the agar. This could be confirmed in an experiment in which 1 ml of a solution of NaDDC in sap was tested for fungitoxicity by the paper-disk bioassay. Precipitation of the proteins was now also followed by the formation of larger inhibition zones.

Although the alcohol-insoluble fraction of sap influences the fungitoxicity of NaDDC on paper, a comparison of columns 1, 2 and 3 from table 1 shows that the problem of the reduction of the fungitoxic action of NaDDC by sap is only partially solved by precipitation of most of the alcohol-insoluble substances. Further comparison of table 4, columns 1 and 3, confirms this observation by showing that NaDDC in water is ten times more effective than NaDDC in the alcohol-soluble fraction when using the paper-disk or paper-spot bioassay. This difference can be overcome by subjecting the alcohol-soluble fraction of sap to paper chromatography. From table 2, columns 1 and 3, it is clear that in that case NaDDC can be completely recovered from sap.

In a previous publication (DEKHUIJZEN, 1961a) it was already suggested that sap contains antagonists of NaDDC which are separated from the fungicide by the chromatographic technique. Evidence for this hypothesis was now gained from experiments in which sap or the alcohol-soluble fraction of sap was chromatographed and NaDDC was added to the conidial suspension of *G. cingulata*. It appeared that NaDDC, as $CuDDC_2$, is separated from histidine, cysteine and some other antagonists.

Table 4 shows that for the demonstration of NaDDC in water the paper-spot bioassay is twenty times more sensitive than the paper-chromatographic bioassay. Without doubt this will be due to the fact that a concentration of NaDDC which just causes an inhibition zone by application of the paper-spot bioassay is too much diluted to cause an inhibition zone after a solvent-mixture has run through the paper.

5. SUMMARY

Sap expressed from cucumber seedlings reduces strongly the fungitoxicity of sodium dimethyldithiocarbamate (NaDDC). For this reason small amounts of NaDDC in sap cannot be determined quantitatively by means of the paper-disk or paper-spot bioassay. Experiments have shown that the reduction of the fungitoxicity of NaDDC by plant sap is due first of all to the fact that sap contains compounds able to antagonize the fungitoxic activity of NaDDC. The fact that proteins and perhaps other cell constituents interfere with the diffusion of NaDDC into the medium is of secondary importance.

These difficulties in the direct determination of NaDDC in sap can be overcome by removing most of the proteins and other alcohol-insoluble cell constituents from the sap and by subsequent application of paper chromatography. Application of the latter method leads to a separation of NaDDC, as copper dimethyldithiocarbamate ($CuDDC_2$), from the antagonists.

CHAPTER II

THE TRANSFORMATION OF DIALKYLDITHIOCARBAMATES INTO OTHER FUNGITOXIC COMPOUNDS BY PLANTS

1. INTRODUCTION

As shown in the previous chapter the systemic activity of NaDDC against *Cladosporium cucumerinum* might be explained by the presence of the fungitoxic DDC-ions within the cucumber seedlings. Since DDC-ions can be easily detected in small amounts by the paper-chromatographic bioassay, this method renders it possible to investigate the presence of DDC-ions in sap expressed from seedlings after uptake of NaDDC by the roots.

2. MATERIALS AND METHODS

a. Application of the compounds to plants

Cucumber seedlings were grown as described in Chapter I. Cucumber plants were grown in soil under equal conditions as the seedlings and used when two or three leaves had developed.

Broad beans (*Vicia faba* L., variety 'Con Amore'), tobacco plants (*Nicotiana tabacum* L., variety 'White Burley') and French beans (*Phaseolus vulgaris* L., variety 'Processor') were grown in soil in a greenhouse. The plants were used when two or three leaves had fully developed.

Ten cucumber seedlings were placed with their roots in beakers containing an aqueous solution (40 ml) of the compound under investigation. If older plants were used, two plants of each species were placed with their roots in beakers containing 50–100 ml of the solution. After two days the seedlings or plants were rinsed in tap water. The roots were separated from the above-ground parts and subsequently both plant parts were frozen at –15° C.

The temperature during the uptake of the compounds was kept constant at 20° C. The plants were exposed to light for 12 hours daily (Philips TL 40W/33) and received about 5.3 mW per sphere of 1 cm^2 cross section of the spherical meter. The relative humidity of the air varied between 40 and 80 per cent.

b. Paper-chromatographic bioassay

The experiments were carried out as described in Chapter I. In some experiments conidia of *Aspergillus niger* (4×10^6/ml) or *C. cucumerinum* (4×10^6/ml) were used instead of *Glomerella cingulata*. In the former case biotin (0.002 μg/ml) and aneurin (0.2 μg/ml) were added to the nutrient solution to get optimal growth. In the latter case only biotin was supplied to the solution.

Since it was shown that proteins interfere with the chromatographic separation, in most experiments an equal volume of ethanol 96% was added to the expressed sap after the different plant parts had been thawed. The alcohol-soluble fraction was chromatographed (see chapter I, section B 2).

c. Ultra-violet irradiation of the glucoside of dimethyldithiocarbamic acid

The glucoside of dimethyldithiocarbamic acid (DDC-glucoside), a transformation product of NaDDC in plants, cannot be detected directly by the paper-

chromatographic bioassay because this compound is low fungitoxic. As will be described, it was found that in the presence of copper sulphate and UV irradiation, DDC-glucoside is transformed into $CuDDC_2$. Based on this observation the following procedure was employed to detect small amounts of DDC-glucoside after it was chromatographed; the strip was sprayed with a solution of copper sulphate in water (10 ppm). Subsequently the moist paper was irradiated with UV light by means of a Philips UV irradiation apparatus (270 W, 220 V) for 5 minutes. Maximum intensity occurred at 3700 Å. The distance between the lamp and the chromatograms was 18 cm and the paper received 18 mW per cm^2 cross section of the spherical meter (WASSINK & VAN DER SCHEER, 1951). After irradiation the chromatograms were dried and sprayed with a conidial suspension of *G. cingulata.*

d. Separation of the L*-alanine derivative of dimethyldithiocarbamic acid from cucumber plants by column chromatography*

For the separation of the L-alanine derivative of dimethyldithiocarbamic acid (L-DDC-alanine), another transformation product of NaDDC, rooted cucumber plants (300) with 4 or 5 fully developed leaves were placed in an aqueous solution of 800 ppm NaDDC for 4 days. After plants were expressed 1600 ml ethanol (96%) were added to 1600 ml sap. This mixture was centrifuged for 5 minutes at 4500 rpm. The supernatant was evaporated to 5 ml at 30–40° C *in vacuo* and subjected to chromatography on a column of starch according to STEIN & MOORE (1948). For this purpose 200 ml starch was suspended in 400 ml butanol-water (96:4, v/v) and added to a column of 40 cm long and 3 cm wide. After the starch had settled down the solvent-mixture, butanol-water (85:15, v/v) was run through the starch.

Since the second solvent-mixture contains relatively more water than the first one, the starch swells and the rate of flow decreases. Therefore it appeared preferable to run the second solvent-mixture through the column under pressure. The pressure was obtained by two communicating open vessels which were placed on an unequal level according to LEDERER & LEDERER (1957). The top vessel was filled with water which flowed slowly into the lower vessel. The current of air which comes from the lower vessel was led to the bottle filled with the solvent-mixture on top of the column.

When the swelling of the starch was finished the concentrated, supernatant portion of the sap (5 ml) was added to the column and butanol-water (85:15, v/v) ran again through the column under pressure. The filtrate was collected in 8 ml fractions by the use of an automatic fraction collector; 0.05 ml of each fraction was tested on fungitoxicity by the paper-spot bioassay. Subsequently equal volumes of those fractions which revealed a zone of inhibition were chromatographed on paper to ascertain, by comparing the R_F value with that of pure DL-DDC-alanine, which fraction contained DDC-alanine. Those fractions containing the highest amounts of DDC-alanine were once more collected and evaporated to 2 ml at 40° C *in vacuo* and again chromatographed on a starch column. In this case, however, starch was suspended in acetone-water (94:6, v/v) and allowed to swell by running a mixture of acetone-water (85:15, v/v) through the column under pressure. The fractions were again tested on fungitoxicity by the paper-spot and paper-chromatographic bioassay. Those fractions containing DDC-alanine were collected and evaporated to

dryness at 40° C *in vacuo* and dissolved in 10 ml distilled water. The paper-chromatographic bioassay showed that this solution contained DDC-alanine but no DDC-glucoside, $CuDDC_2$ or fungicide X. KASLANDER *et al.* (1962) demonstrated DDC-alanine in potato tubers treated with NaDDC. As will appear in the near future from a publication by KASLANDER this compound is indeed the L-isomer. It may be assumed that the solution from cucumbers also contained the L-isomer of DDC-alanine.

When the solution was chromatographed and ninhydrin was sprayed on the paper it appeared to contain several amino acids. It can be assumed that still other sap impurities were present.

By means of the quantitative determination method described in the next chapter it was found that the solution contained an amount of L-DDC-alanine which was as fungitoxic as about 19 mg DL-DDC-alanine dissolved in the same volume of distilled water.

3. RESULTS

a. The transformation of NaDDC into DDC-glucoside, DDC-alanine and into other compounds by plants

Sap (0.015 ml) as well as the alcohol-soluble fraction of sap (0.015 ml) expressed from hypocotyls and cotyledons of cucumber seedlings treated with NaDDC (150 ppm) showed only vague inhibition zones using the paper-spot bioassay. When, however, the same volumes were subjected to paper chromatography in descending propanol-water (85:15, v/v) for 16 hours the presence of a compound fungitoxic to *G. cingulata* was detected on both strips (R_F 0.27). The inhibition zones were of the same size. When, moreover, the alcohol-soluble fraction was concentrated about 40 times, two other fungitoxic compounds were visible as well (R_F 0.03 and 0.58; Plate I B). The same fungitoxic products could be detected after chromatographing 0.6 ml of the alcohol-soluble fraction of sap expressed from the roots.

It is surprising that none of the observed inhibition zones has an R_F value equal to that of the copper salt of NaDDC (R_F 0.87). When, however, 250 ppm instead of 150 ppm NaDDC was applied to the roots then 0.6 ml of the alcohol-soluble fraction of the above-ground parts as well as of the roots did in fact show a small inhibition zone with an R_F value of 0.87. These results suggest that most of the NaDDC taken up by the seedlings has been transformed into three other fungitoxic compounds.

The three transformation products were also found in sap of cucumber plants, broad beans, tobacco plants and French beans after addition of NaDDC (400 ppm) to the roots for two days or after leaf application (1000 ppm).

It is noteworthy that these products have also been detected in sap of cucumber and tobacco plants four days after spraying an aqueous suspension (1000 ppm) of tetramethylthiuram disulphide (TMTD) on the leaves.

Using *A. niger* or *C. cucumerinum* as test organisms instead of *G. cingulata* the three inhibition zones were also visible on the chromatograms.

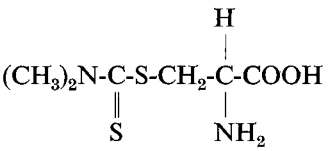
When slices of potato tubers were incubated for two days at 20° C under continuous aeration in a solution of 400 ppm NaDDC, fungitoxic compounds with an R_F value of 0.27 and of 0.58 could be detected also after chromatographing of 0.6 ml of the alcohol-soluble fraction of sap. The fact that potato

slices boiled for 5 minutes did not give rise to the compounds after incubation with NaDDC strongly indicates that the compounds are formed enzymically.

The fungitoxic action of the three unknown compounds could be counteracted by addition of L-histidine (0.15%) or potassium dibutyldithiocarbamate (0.003%) to the conidial suspension of *G. cingulata* before spraying. Since it is known (KAARS SIJPESTEIJN & JANSSEN, 1959) that histidine and dibutyldithiocarbamate are able to antagonize the fungitoxic action of NaDDC, this indicated that the three fungitoxic compounds in plants had the same mode of action as NaDDC itself. Therefore it was already suggested in previous papers that the three unknown compounds are derivatives of dimethyldithiocarbamic acid which had been formed enzymically (DEKHUIJZEN, 1961 a, b).

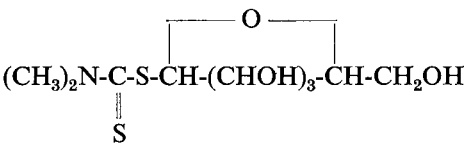
At this stage of these investigations it was clear that further progress could only be made by identifying the unknown fungitoxic compounds. This part of the work was undertaken by Drs. J. KASLANDER and will be dealt with separately in his dissertation which will appear shortly. The main results of his study have been published in the mean time (KASLANDER *et al.* 1961, 1962).

The hypothesis that the fungitoxic products might be derivatives of dimethyldithiocarbamic acid could be confirmed for the compounds with R_F 0.27 and R_F 0.58. They have been isolated from potato slices treated with NaDDC. The compound with R_F 0.27 could be characterized as the alanine derivative of dimethyldithiocarbamic acid, *viz.* β-(N, N-dimethylthiocarbamoylthio)-alanine:



A publication by KASLANDER in the near future will show that the compound is the L-isomer. Henceforth this compound will be designated as L-DDC-alanine.

The compound with R_F 0.58 was found to be 1-(N, N-dimethylthiocarbamoylthio)-1-desoxy-D-glucose, presumably the β-form:



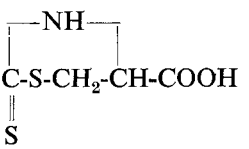
This glucoside of dimethyldithiocarbamic acid will be designated as DDC-glucoside. The fungitoxic compound with the lowest R_F value has not yet been isolated. It will be described henceforth as fungicide X.

The three transformation products of NaDDC in plants can also be demonstrated on chromatograms if descending butanol-acetic acid-water (4:1:1, v/v/v) or pyridine-water (8:2, v/v) are used as solvent-mixture. To evaporate the solvent-mixtures sufficiently from the paper it appeared to be necessary in these cases to dry the paper for 24 hours at room temperature before spraying the conidial suspension of *G. cingulata.* The R_F values are summarized in table 5.

TABLE 5. R_F values of the three fungitoxic transformation products of NaDDC in plants in different descending solvent-mixtures. Cucumber seedlings treated with NaDDC (250 ppm) for two days. 0.2 ml of the alcohol-soluble fraction of the above-ground parts added to the starting line. Paper-chromatographic bioassay. Test fungus *G. cingulata*.

	R_F values		
	Propanol-water (85:15, v/v)	Butanol-acetic acid-water (4:1:1, v/v/v)	Pyridine-water (8:2, v/v)
Fungicide X	0.03	0.27	0.16
DDC-alanine	0.27	0.45	0.55
DDC-glucoside	0.58	0.58	0.78

From potato slices incubated in a solution of NaDDC yet a third compound has been isolated by KASLANDER *et al.* (1962). Similar to DDC-alanine this compound gave a positive reaction with the iodine-sodium azide reagent (FEIGL, 1960). This indicated the presence of a C = S or a C-S-H group in the molecule. Further investigation revealed the compound to be optically active thiazolidine-2-thione-4-carboxylic acid (TTCA):



In contrast to DDC-alanine this compound is not fungitoxic and could therefore not be detected by the paper-chromatographic bioassay. KASLANDER *et al.* (1962) suppose that the presence of TTCA is due to a non-enzymic decomposition of DDC-alanine. TTCA and DDC-alanine have the same R_F value in propanol-water (85:15, v/v). As is shown in table 6 the compounds can, however, be separated in descending butanol-acetic acid-water (4:1:1, v/v/v).

TABLE 6. R_F values of compounds in sap of above-ground parts of cucumber seedlings after uptake of NaDDC (250 ppm) for two days. Solvent-mixture descending butanol-acetic acid-water (4:1:1, v/v/v) for 16 hours. Chromatograms sprayed with iodine-sodium azide reagent or a conidial suspension of *Glomerella cingulata*.

	Reference compounds (20 µg) after spraying the strips with		Sap (0.2 ml) of treated plants after spraying the strips with	
	Iodine-sodium azide R_F	*G. cingulata* R_F	Iodine-sodium azide R_F	*G. cingulata* R_F
	–	–	0.27*	0.27*
DDC-alanine	0.45	0.45	0.45	0.45
DDC-glucoside	0.58	0.58	0.58	0.58
TTCA[1]	0.74	–	0.74	–

[1] thiazolidine-2-thione-4-carboxylic acid
* Most probably the R_F value of fungicide X

The isolation of TTCA from potato tubers treated with NaDDC raised the question if this decomposition product of DDC-alanine is also present in cucumber seedlings after uptake of NaDDC by the roots. To investigate this, cucumber seedlings were placed in an aqueous solution of NaDDC (250 ppm) for two days. The alcohol-soluble fraction of sap (0.2 ml) from the hypocotyls and cotyledons was chromatographed in descending butanol-acetic acid-water (4:1:1, v/v/v) for 16 hours (table 6).

Decolorization occurred on four different spots (R_F 0.27, 0.45, 0.58 and 0.74) after spraying with the iodine-sodium azide reagent. In order to identify the spots, pure DL-DDC-alanine, DDC-glucoside and TTCA were also chromatographed. Table 6 shows that the spots at R_F 0.45 and 0.58 are caused by DDC-alanine and DDC-glucoside, respectively. The non-fungitoxic compound at R_F 0.74 must be attributed to the presence of TTCA in sap of treated plants, since sap of untreated plants did not decolorize the reagent at that R_F value. The fungitoxic compound at R_F 0.27 reacts also with the iodine-sodium azide reagent which suggests the presence of fungicide X. The results leave little doubt that DDC-alanine, enzymically formed from NaDDC, has been partially decomposed into TTCA.

TTCA (400 ppm) applied to the roots of cucumber seedlings was recovered in the above-ground parts with the iodine-sodium azide reagent, whereas the paper-chromatographic bioassay revealed no inhibition zones. These results led to the conclusion that TTCA is translocated in cucumber seedlings but that the compound is not convertible into DDC-alanine.

b. The demonstration of small amounts of DDC-glucoside on chromatograms after ultra-violet irradiation

It has already been mentioned by KASLANDER *et al.* (1961) that DDC-glucoside is only a weak fungicide. The minimum concentration causing complete inhibition of the germination of the conidia of *G. cingulata* and of some other fungi *in vitro* is about 500 ppm, whereas 20 μg DDC-glucoside is the minimum amount which can be demonstrated by the paper-chromatographic bioassay. Therefore a low concentration of DDC-glucoside in plant sap can only be detected if a rather high volume of sap is chromatographed. For that reason a more sensitive method was sought. An indication for such a method was obtained when we observed that a glass tube filled with a colourless solution of DDC-glucoside in water (250 ppm) to which copper sulphate ($CuSO_4 \cdot 5H_2O$, 20 ppm) was added slowly changed into a yellow suspension if exposed to daylight in the laboratory. After a few hours a brown precipitate was present on the bottom of the glass tube. If the experiment was carried out in a dark room this phenomenon was not observed. Since $CuDDC_2$ has a brown colour and is nearly insoluble in water, it was obvious to assume a fission of the DDC-glucoside between the thiol sulphur atom and the C_1-atom of the glucoside group under the influence of light. Subsequently the free DDC-ions form $CuDDC_2$ with the copper-ions present in the solution. Evidence that $CuDDC_2$ is formed was obtained by comparison of the absorption spectrum of an 1:1 aqueous acetone solution of the brown precipitate in the glass tube and an 1:1 aqueous acetone solution of $CuDDC_2$ (5 ppm). Both solutions revealed the same absorption spectrum with a maximum wave length of 430 mμ.

Copper appeared not to be necessary for the fission of the DDC-glucoside molecule since $CuDDC_2$ was also formed if a solution of DDC-glucoside in glass distilled water was exposed to light before addition of copper sulphate.

Subsequently it was found that ultra-violet (UV) irradiation leads to a much quicker formation of $CuDDC_2$ from a solution of DDC-glucoside in the presence of copper sulphate. $CuDDC_2$ is very fungitoxic to *G. cingulata* (KAARS SIJPESTEIJN & JANSSEN, 1958) and, consequently it was investigated whether small amounts of the slightly fungitoxic DDC-glucoside could be demonstrated as $CuDDC_2$ on chromatograms after UV irradiation. For that reason 2 μg or 4 μg DDC-glucoside (monohydrate) was applied to a series of strips and chromatographed in descending propanol-water (85:15, v/v) for 16 hours. After drying the strips were sprayed with a solution of copper sulphate in water (10 ppm) and exposed to UV light for different times. To obtain a maximum effect of the UV irradiation both sides of the strips were exposed for half of the total exposure time. Subsequently the chromatograms were dried and sprayed with the conidial suspension of *G. cingulata*. The presence of copper sulphate was found to be harmless to mycelial growth on the paper.

A similar experiment was carried out with different amounts of $CuDDC_2$. In fig. 1 the results of both experiments are summarized. The areas of the inhibition zones are plotted against the period of UV irradiation. In the case of DDC-

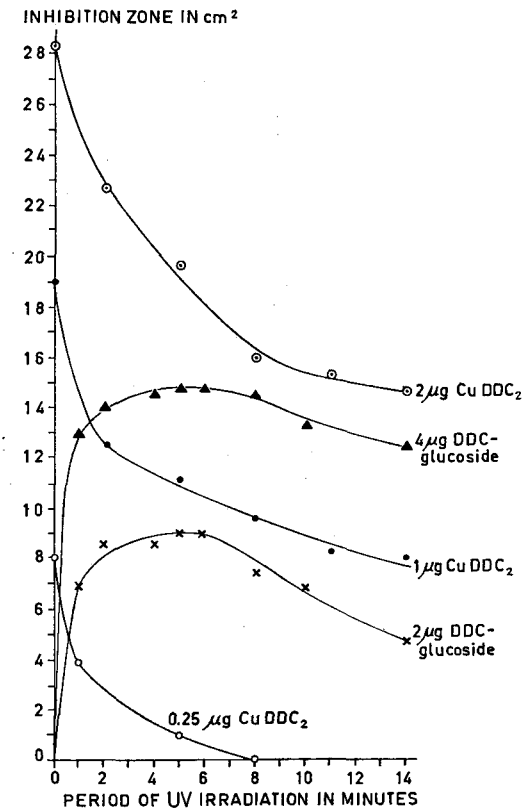


FIG. 1.
Influence of different periods of UV irradiation on the fungitoxicity of DDC-glucoside (monohydrate) and $CuDDC_2$ against *Glomerella cingulata* in the presence of copper sulphate (10 ppm) on chromatograms.

glucoside the areas of the inhibition zones reach their maximum if the chromatograms are exposed to UV light for about 5 minutes. If $CuDDC_2$ is exposed to UV light for different periods the areas of the inhibition zones decrease (fig. 1). The rate of reduction of the fungitoxicity of $CuDDC_2$ appears to coincide rather well with the rate of reduction of the fungitoxicity of DDC-glucoside after UV irradiation for 6 minutes and longer periods. Apparently the rate of formation of $CuDDC_2$ from DDC-glucoside and copper sulphate is higher than the rate of decomposition of $CuDDC_2$ in the periods up to 6 minutes. After 6 minutes the amount of DDC-glucoside has decreased to such a low level that the rate of decomposition of $CuDDC_2$ determines the course of the declining part of the curve.

Theoretically 2 μg DDC-glucoside (monohydrate) can maximally yield 1 μg $CuDDC_2$. From fig. 1, however, it follows that the maximum inhibition zone caused by 2 μg DDC-glucoside is equal to that of about 0.25 μg $CuDDC_2$. Thus a maximum of 25 per cent of the DDC-glucoside is transformed into $CuDDC_2$ by UV irradiation in the presence of copper sulphate. The remaining 75 per cent will be decomposed into non-fungitoxic compounds.

The minimum amount of DDC-glucoside which can be demonstrated after UV irradiation for 5 minutes appeared to be about 1 μg, which is 20 times lower than without application of UV irradiation. The fungitoxicity of DDC-alanine appeared, however, to decrease by UV irradiation. If therefore DDC-alanine and DDC-glucoside in plant sap had to be demonstrated, two different chromatograms had to be made in which only one of the strips is exposed to UV irradiation for 5 minutes. To demonstrate both compounds on one chromatogram, only that part of the chromatogram has to be irradiated where DDC-glucoside can be expected to be present. After running descending propanol-water (85:15, v/v) as a solvent-mixture for 16 hours the distance between the starting line and the front is about 48 cm. Since the R_F value of DDC-glucoside is 0.58, it is sufficient to irradiate that part of the chromatograms which is situated between 21 and 35 cm from the starting line. Moreover, this has the advantage of preventing the decomposition of $CuDDC_2$ by the UV light since this compound, if present, would be found on that part of the strip which is situated between 35 and 48 cm from the starting line.

c. The interconvertibility of DDC-glucoside and DDC-alanine in cucumber seedlings

The fact that NaDDC in plants can be transformed enzymically into DDC-alanine and DDC-glucoside, led to the question whether DDC-alanine can be converted into DDC-glucoside and whether plants are able to synthesize DDC-alanine from DDC-glucoside. To this end ten cucumber seedlings were placed in an aqueous solution of DDC-glucoside (500 ppm) or DL-DDC alanine (100 ppm) for two days. The alcohol-soluble fraction of sap (0.05 ml) expressed from roots or from above-ground parts was chromatographed in propanol-water (85:15, v/v) for 16 hours. It appeared that both parts of the plants contained a fungitoxic compound with an R_F value of fungicide X (0.03) and another with an R_F value equal to that of DDC-alanine (0.27) irrespective of the fact whether these plants had stood in DL-DDC-alanine or in DDC-glucoside. If, moreover, the alcohol-soluble fractions were concentrated 6 times, small inhibition zones with an R_F value equal to that of $CuDDC_2$ (0.87) were also visible.

UV irradiation for 5 minutes yielded a fourth inhibition zone with an R_F value equal to that of DDC-glucoside (0.58) in the alcohol-soluble fraction of sap of the roots and the above-ground parts (0.05 ml) of plants treated with DDC-glucoside as well as with DL-DDC-alanine.

The impure aqueous solution of L-DDC-alanine as well as pure D-DDC-alanine gave rise to exactly the same products as DL-DDC-alanine. These results show that L- and D-DDC-alanine are convertible into DDC-glucoside in cucumbers. DDC-glucoside is converted into DDC-alanine, presumably the L-form. Moreover, DDC-alanine and DDC-glucoside give rise to the formation of $CuDDC_2$ and fungicide X.

d. The transformation of sodium diethyldithiocarbamate into other fungitoxic compounds by cucumber seedlings

In the foregoing it was recorded that plants are able to convert NaDDC into DDC-alanine, DDC-glucoside and the fungicide X. It seemed of interest to investigate whether cucumber seedlings could transform also a higher dialkyldithiocarbamate into its corresponding alanine and glucose derivative. Therefore the fungicide sodium diethyldithiocarbamate (250 ppm) was applied to the roots for two days. The alcohol-soluble fraction of sap expressed from the above-ground parts (0.1 ml) or 1.5 μg sodium diethyldithiocarbamate were added to a series of strips and chromatographed in propanol-water (85:15, v/v) for 16 hours. The strips were incubated with *G. cingulata*. The R_F value of sodium diethyldithiocarbamate was found to be 0.9. In analogy to dimethyldithiocarbamate it can be assumed that the spot at R_F 0.9 is due to the copper chelate of diethyldithiocarbamate.

According to KAARS SIJPESTEIJN & JANSSEN (1959) the solubility of the 1:2 complex of diethyldithiocarbamate in water is just high enough to cause a slight inhibition of *G. cingulata*. Thus the observation that 1.5 μg sodium diethyldithiocarbamate and 0.5 μg NaDDC cause inhibition zones of about the same size after chromatographing can be explained by the fact that the 1:2 complex of diethyldithiocarbamate and copper is less fungitoxic than the 1:2 complex of NaDDC and copper.

From the chromatograms of sap it appeared that almost no diethyldithiocarbamate is present but that the seedlings had transformed this compound into two other fungitoxic compounds with R_F values of 0.29 and 0.5, respectively. If one of the strips was irradiated with UV light as described for the detection of DDC-glucoside, a third compound could be detected (R_F 0.75). This strongly suggests the presence of the glucoside of diethyldithiocarbamic acid on the chromatogram.

Most probably the other fungitoxic compounds are also diethyldithiocarbamic acid derivatives. This supposition gains support by their positive reaction with the iodine-sodium azide reagent and by the observation that their toxic action can be antagonized by sodium dibutyldithiocarbamate (30 ppm) added to the conidial suspension of *G. cingulata* used for spraying the strips. Although strict proof is lacking, it is very probable that the fungitoxic compound with an R_F value of 0.5 is the alanine derivative of diethyldithiocarbamic acid, whereas the compound with an R_F value of 0.29 is the diethyl homologue of fungicide X.

4. Discussion

The experiments carried out showed that DDC-ions taken up by the roots of cucumber seedlings are transformed into three fungitoxic compounds. It was found, moreover, that DDC-ions are able to penetrate into the leaves of cucumbers, broad beans, tobacco plants, and French beans since these transformation products were detected in sap of leaves which were sprayed with NaDDC. TMTD sprayed on the leaves of cucumbers and tobacco plants gave also rise to the formation of these products.

It is interesting to note that Massaux (1963) detected compounds with the same R_F values in sap of cucumber leaves after application of ^{35}S labeled TMTD on the leaves. Thus much evidence has been obtained for the view that TMTD penetrates into the leaves and gives rise to DDC-ions which are converted into the fungitoxic compounds.

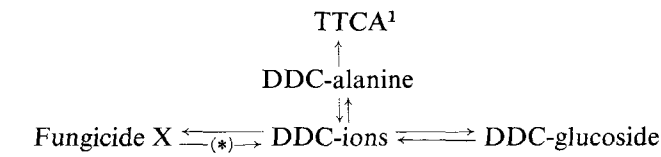
Darpoux, Halmos & Leblanc (1958) investigated sap of the above-ground parts of different plants after root application of TMTD. They found an inhibition zone by means of the paper-disk bioassay and using *Ustilago zeae* as test organism. From our results it may be concluded that all three transformation products have contributed to the formation of the inhibition zone on the agar plate.

Kaslander *et al.* (1961, 1962) succeeded in isolating and characterizing two of the compounds *viz.* the β-glucoside and the alanine derivative of dimethyldithiocarbamic acid. The third transformation product (fungicide X) could also be demonstrated by the iodine-sodium azide reagent which indicates the presence of C = S or C-S-H in the molecule. The fungitoxic action of this compound was also antagonized by dibutyldithiocarbamate added to the conidial suspension before spraying, indicating that fungicide X has the same mode of action as dimethyldithiocarbamate itself (Kaars Sijpesteijn & Janssen, 1959). Therefore most probably fungicide X is also a dimethyldithiocarbamic acid derivative.

With regard to the formation of DDC-glucoside by plants it can be noticed that β-glucosidation in plants is also known for other compounds for instance for phenolic compounds (Hutchinson, Roy & Towers, 1958; Winter, Peuss & Schönbeck, 1959; Winter, Schönbeck-Peuss & Schönbeck, 1959) and for indole-3-acetic acid (Zenk, 1961).

DDC-alanine and DDC-glucoside are interconvertible in cucumber seedlings. The presence of small amounts of $CuDDC_2$ on chromatograms after uptake of DDC-alanine or DDC-glucoside suggests that plants liberate DDC-ions from these compounds. It is, however, not known whether $CuDDC_2$ has been formed from free DDC-ions with copper present in the plant or in the Whatman paper.

Although strict proof is lacking it may be supposed that liberation of DDC-ions is an intermediate step in the interconvertibility of DDC-alanine and DDC-glucoside. If, furthermore, one assumes that fungicide X can be converted into DDC-alanine and DDC-glucoside after liberation of DDC-ions a schematic picture can be given for the pathways of the conversion of DDC-ions and their transformation products in cucumber seedlings (fig. 2):



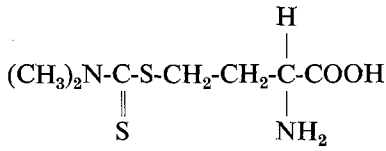
[1] thiazolidine-2-thione-4-carboxylic acid

FIG. 2. Pathways of the conversion of DDC-ions and their transformation products in cucumber seedlings. The line marked with (*) represents a reaction for which no experimental evidence is available.

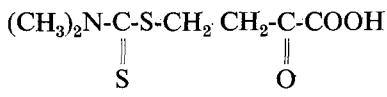
It must be noticed that the scheme is correct for L-DDC-alanine but only partially for D-DDC-alanine, since it is highly improbable that DDC-glucoside is converted into D-DDC-alanine by higher plants. As a result of the transformation of D-DDC-alanine into DDC-glucoside the possibility cannot be ruled out that DDC-ions liberated from D-DDC-alanine are converted into L-DDC-alanine.

Diethyldithiocarbamate is most probably converted into the diethyl homologue of DDC-alanine, DDC-glucoside and fungicide X. Therefore it can be assumed that the scheme of fig. 2 also holds true for diethyldithiocarbamate ions.

According to KAARS SIJPESTEIJN, KASLANDER & VAN DER KERK (1962) washed suspensions of microorganisms such as *Saccharomyces cerevisiae* and *Bacterium coli* as well as mycelial pellets of *G. cingulata*, *A. niger* and *C. cucumerinum* do not convert NaDDC into the same compounds as higher plants. These authors proved, however, that microorganisms are able to convert NaDDC into the α-aminobutyric acid derivative of dimethyldithiocarbamic acid *viz.*, γ-(N,N-dimethylthiocarbamoylthio)-α-aminobutyric acid, presumably the L-form.



In the following pages this compound will be designated as DDC-α-aminobutyric acid. Still another, unstable, fungitoxic compound could be detected in the culture medium. Most probably this compound is the corresponding α-keto acid of DDC-α-aminobutyric acid:



KAARS SIJPESTEIJN, DEKHUIJZEN, KASLANDER, PLUIJGERS & VAN DER KERK (1963) connected the formation of DDC-α-aminobutyric acid with the biosynthesis of methionine from cysteine and homoserine by microorganisms according to the following scheme (fig. 3):

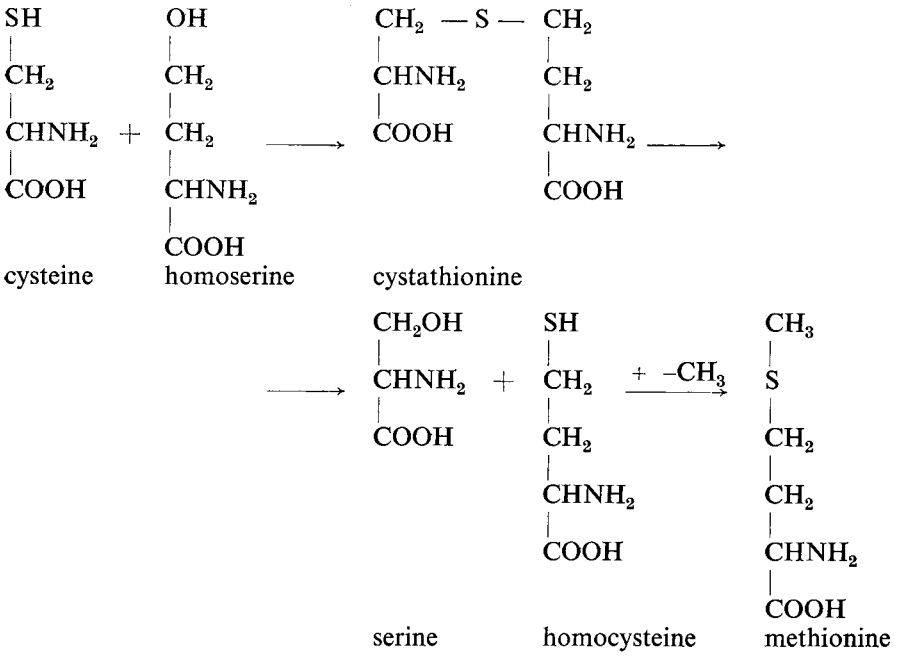
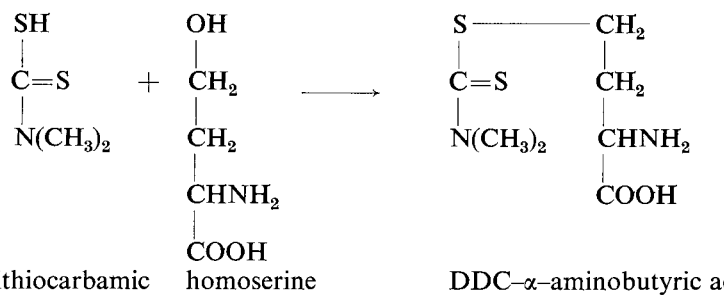


FIG. 3. Scheme for the biosynthesis of methionine from cysteine by microorganisms.

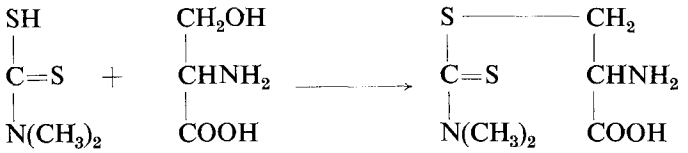
It is supposed that the enzyme which combines cysteine with homoserine to cystathionine can also combine homoserine with dimethyldithiocarbamate to DDC-α-aminobutyric acid according to:



Most probably DDC-α-ketobutyric acid is formed by transamination or by deamination of DDC-α-aminobutyric acid. DDC-α-aminobutyric acid is toxic to several fungi for instance *G. cingulata*. The toxic action was found to be due to the liberation of DDC-ions from the aminobutyric acid derivative. This could be explained by the fact that the conversion of cysteine into methionine by the way of cystathionine is reversible in microorganisms.

With regard to the formation of DDC-alanine KAARS SIJPESTEIJN *et al.* (1963) point to the possibility that in higher plants the prevailing reaction may

proceed under experimental conditions from cystathionine to cysteine. They suppose that homocysteine might be replaced by DDC-ions which leads to the formation of DDC-alanine instead of cystathionine according to:



dimethyldithiocarbamic acid | serine | DDC-alanine

It is assumed that the fungitoxicity of DDC-alanine is caused by DDC-ions split from the molecules by microorganisms. The mode of action of DDC-ions can be explained in the following ways. It is known that low concentrations of DDC-ions inhibit mycelial growth by the formation of toxic $CuDDC_2$ with copper from the medium (KAARS SIJPESTEIJN & JANSSEN, 1959). On the other hand it might be possible that the fungitoxicity of higher concentrations of DDC-ions is due to their interference with methionine synthesis. It must, however, be emphasized that additional evidence for this suggestion is not available.

In contrast to microorganisms higher plants are not able to convert DDC-ions or DDC-alanine into DDC-α-aminobutyric acid. DDC-α-aminobutyric acid applied to the roots of cucumber seedlings has been recovered in the above-ground parts. Moreover, the compound was found to give rise to small amounts of DDC-alanine, DDC-glucoside, fungicide X and to $CuDDC_2$ in the seedlings. The presence of $CuDDC_2$ indicates that, similar to what has been found for DDC-alanine, DDC-α-aminobutyric acid is split in the plant. Subsequently the liberated DDC-ions are transformed according to the pathways given in the schema of fig. 4.

From the data given by KAARS SIJPESTEIJN *et al.* (1962, 1963) and from the results mentioned in this chapter a schematic picture can be given for the pathways of the conversion of DDC-ions and their transformation products in higher plants and fungi.

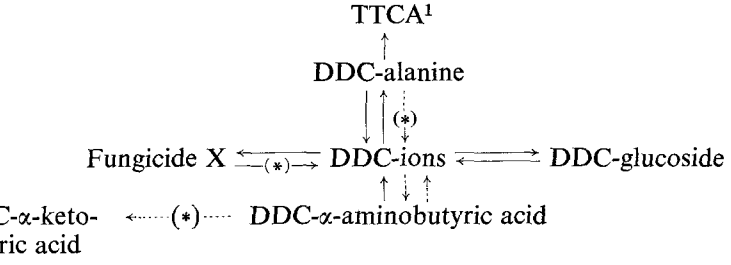


FIG. 4. Pathways of the conversion of DDC-ions and their transformation products in higher plants and fungi. The solid lines indicate transformations occurring in higher plants, the dotted lines those occurring in fungi. The lines marked with (*) represent reactions for which no experimental evidence is available.

[1] thiazolidine-2-thione-4-carboxylic acid

With regard to the systemic effect of NaDDC against *C. cucumerinum* it is clear that this is not caused by the toxic action of free DDC-ions taken up by the plants on the penetrating hyphae since only very little of this compound could be detected in sap of treated plants. It can be supposed, however, that the systemic action of NaDDC is caused by the fungitoxic action of its three transformation products.

DDC-glucoside is only a weak fungicide in comparison with DDC-alanine. For instance, 500 ppm DDC-glucoside is the minimum concentration giving complete growth inhibition of conidia of *C. cucumerinum in vitro* whereas of DL-DDC-alanine only 20 ppm is required (KASLANDER *et al.*, 1961, 1962).

Nothing can be said with certainty about the fungitoxicity of fungicide X with regard to that of DDC-alanine and of DDC-glucoside. From chromatograms sprayed with conidia of *C. cucumerinum* or of *G. cingulata* it can, however, be seen that after uptake of NaDDC the area of the inhibition zone caused by fungicide X, similar to that of the zone caused by DDC-glucoside, is much smaller than the area of the inhibition zone formed by DDC-alanine (Plate I B). As already mentioned in section 2d of this chapter the L-isomer of DDC-alanine is most probably present in the plant. This observation indicates that, if the systemic action of NaDDC against *C. cucumerinum* is due to a toxic action of its transformation products on the penetrating hyphae, L-DDC-alanine plays the most important role.

5. SUMMARY

Application of the paper-chromatographic bioassay as described in chapter I showed that dimethyldithiocarbamate ions are transformed into three other fungitoxic compounds in plants. The compounds are formed enzymically. KASLANDER *et al.* (1961, 1962) succeeded in isolating and characterizing two of these compounds *viz.* the glucoside and the alanine derivative of dimethyldithiocarbamic acid (DDC-glucoside and DDC-alanine, respectively). The third compound is most probably also a derivative of dimethyldithiocarbamic acid (fungicide X). Moreover, a non-fungitoxic decomposition product of DDC-alanine, thiazolidine-2-thione-4-carboxylic acid (TTCA) was found.

Small amounts of DDC-glucoside cannot be detected directly on chromatograms using *Glomerella cingulata* as test organism since this compound is only slightly fungitoxic. Chromatography of DDC-glucoside and subsequent UV irradiation of the strips in the presence of copper sulphate yielded, however, $CuDDC_2$ which is highly fungitoxic to *G. cingulata.* In this way small amounts of DDC-glucoside have been demonstrated as $CuDDC_2$ by means of the paper-chromatographic bioassay.

DDC-glucoside and DDC-alanine are interconvertible in plants. Moreover, both compounds give rise to the formation of $CuDDC_2$ and fungicide X.

According to KAARS SIJPESTEIJN *et al.* (1963) the formation of DDC-alanine might be connected with the biosynthesis of cysteine in higher plants.

Results indicate that diethyldithiocarbamate ions give rise to similar fungitoxic transformation products as dimethyldithiocarbamate ions in plants.

CHAPTER III

A QUANTITATIVE STUDY ON THE FATE OF DL-DDC-ALANINE IN CUCUMBER SEEDLINGS

1. INTRODUCTION

In the preceding chapter attention shifted from NaDDC to L-DDC-alanine since the results suggested that the latter compound is responsible for the systemic activity of NaDDC against cucumber scab. Preliminary results showed that L-DDC-alanine itself acts systemically against *Cladosporium cucumerinum*. This chapter describes the results of a quantitative study on uptake, translocation and degradation of DDC-alanine in cucumber seedlings. Since pure L-DDC-alanine was not available we had to use the racemic mixture. The systemic activity will be dealt with in more detail in the next chapter.

2. MATERIALS AND METHODS

a. Hoagland solution

In some experiments cucumber seedlings treated initially with DL-DDC-alanine were transferred to a Hoagland solution. One liter of this solution contained, tap water, 410 mg $Ca(NO_3)_2$; 253 mg KNO_3; 246 mg $MgSO_4 \cdot 7H_2O$; 68 mg KH_2PO_4; 30 mg iron sodium salt of sequestrene; 0.61 mg H_3BO_3; 0.234 mg $MnSO_4 \cdot 4H_2O$; 0.055 mg $CuSO_4 \cdot 5H_2O$; 0.055 mg $ZnSO_4 \cdot 7H_2O$; 0.055 mg $Al_2(SO_4)_3$; 0.0333 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 0.028 mg KJ; 0.028 mg KBr.

b. Quantitative determination of DDC-alanine, DDC-glucoside and thiazolidine-2-thione-4-carboxylic acid (TTCA)

In analogy to the work of FISHER, PARSON & MORRISON (1948), who quantitatively determined amino acids on chromatograms after spraying ninhydrin, we estimated the concentration of DDC-alanine, DDC-glucoside and of TTCA in sap of plants.

To this end the areas of the zones on the chromatograms caused by known amounts of the reference compounds were compared with those caused by various volumes of the alcohol-soluble fraction of sap by measuring the areas with a planimeter. The reference compound as well as the alcohol-soluble fraction were simultaneously chromatographed and sprayed with a conidial suspension of *Glomerella cingulata* or with the iodine-sodium azide reagent as described in chapter II. For the quantitative determinations the use of *C. cucumerinum* was restricted to a few experiments since the border of the inhibition zones is not as sharp as after spraying a conidial suspension of *G. cingulata*. Moreover, growth of *C. cucumerinum* within the zones of inhibition is often not completely suppressed.

Determination of DDC-alanine. In the experiments in which DL-DDC-alanine was applied to the roots of cucumber seedlings the concentration of this compound in sap is calculated as DL-DDC-alanine. In fact evidence is lacking whether the D- and the L-isomer of the compound are equally well translocated and metabolized. We assume, however, that both isomers are present in the ratio 1:1 in sap of seedlings treated with DL-DDC-alanine.

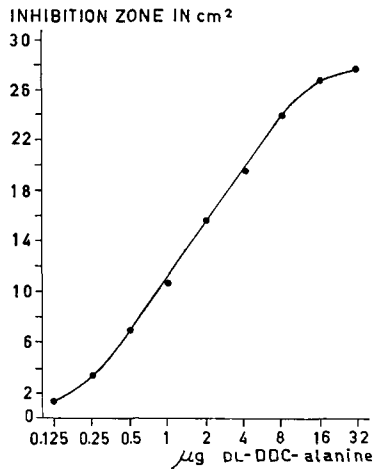


FIG. 5.
Relation between doses of DL-DDC-alanine and areas of inhibition zones on chromatograms. Solvent-mixture descending propanol-water (85:15, v/v). Paper-chromatographic bioassay with *Glomerella cingulata*. Doses on log scale.

The dosage-response curve between 0.125 and 32 µg DL-DDC-alanine is S-shaped (fig. 5) if the doses are plotted logarithmically on the abscissa and the areas of the inhibition zones linearly on the ordinate. Between 0.5 and 8 µg the points tend to be located along a straight line (fig. 6A). The alcohol-soluble fraction of sap of plants after uptake of DL-DDC-alanine (100 ppm) for two days reveals also a linear regression line of y on w within certain limits (fig. 6B).

The concentration of DL-DDC-alanine in sap is determined from a comparison of the regression line of sap (test line) with that of DL-DDC-alanine (standard line). The equations of the lines are calculated from the dosages and their responses (fig. 6A, B).

From a theoretical point of view the test line is expected to run parallel with the standard line if sap does not interfere with the fungitoxicity of DDC-alanine. In our experiments we found that these regression lines fulfilled satisfactorily this expectation.

A response of about 50% in fig. 5 is found at y = 14, since at y = 28 the

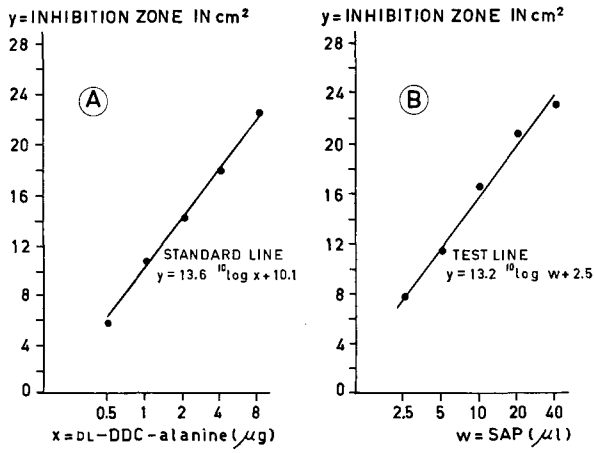


FIG .6.
A. Regression of areas of inhibition zones on doses of DDC-alanine. Doses on log scale. Solvent-mixture descending propanol-water (85:15, v/v). Paper-chromatographic bioassay with *Glomerella cingulata*.
B. Corresponding regression on DDC-alanine in the alcohol-soluble fraction of sap of cucumber seedlings.

response is at its maximum. Therefore we chose $y = 14$ as the value at which the dosages w and x contain the same amount of DL-DDC-alanine.

The amount of DL-DDC-alanine in w µl sap can be calculated from substitution of $y = 14$ in the equations.

The average concentration ($\bar{x}$) of DL-DDC-alanine in the same sample of sap appeared to be $\bar{x} = 240$ ppm in five different experiments. Designating the standard deviation by s, the coefficient of variation $V = \frac{s}{\bar{x}} = 4.7\%$.

If, instead of 5 dosages per regression line only 0.5, 2 and 8 µg DL-DDC-alanine and 2.5, 10 and 40 µl of the alcohol-soluble fraction of sap are involved in the calculation, the average concentration of DL-DDC-alanine in sap was found to be 230 ppm. In that case $V = 9\%$. This accuracy was considered to be sufficient and was also found when a known concentration of DL-DDC-alanine in sap of untreated plants was chromatographed. Therefore the concentration of DL-DDC-alanine in sap of treated plants is determined at $y = 14$ after simultaneous chromatography of 0.5, 2 and 8 µg DL-DDC-alanine and three different volumes of the alcohol-soluble fraction of sap in the ratio of 1:4:16.

Determination of DDC-glucoside. The concentration of DDC-glucoside in sap of cucumber seedlings treated with 1000 ppm of this compound was determined from areas of the inhibition zones after chromatograms were irradiated with UV light for 5 minutes as described in chaper II. The standard line and test line are assessed in the same experiment (fig. 7A, B). The average concentration of DDC-glucoside in the same sample of the alcohol-soluble fraction of sap in five different experiments was found to be 534 ppm for $y = 14$, whereas $V = 13.1\%$.

A known concentration of DDC-glucoside in sap of untreated plants could be assessed with the same accuracy. Henceforth the concentration of DDC-glucoside in sap is determined at $y = 14$ after simultaneous chromatography of 2, 4, 8, 16 and 32 µg DDC-glucoside and of five different volumes of the alcohol-soluble fraction of sap in the ratio of 1:2:4:8:16.

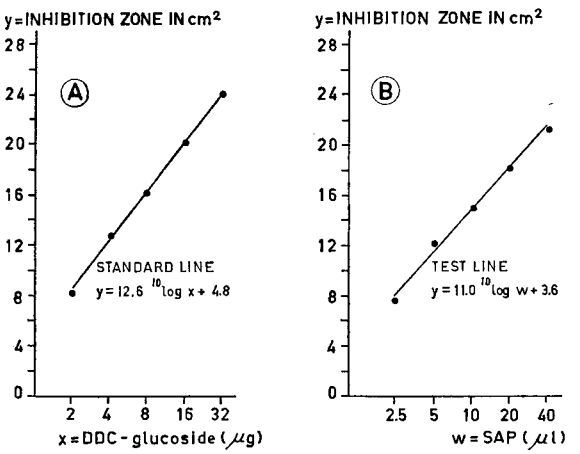


FIG. 7.
A. Regression of areas of inhibition zones on doses of DDC-glucoside. Doses on log scale. Solvent-mixture descending propanol-water (85:15, v/v). Paper-chromato-graphic bioassay with *Glomerella cingulata* after UV irradiation of the strips.
B. Corresponding regression on DDC-glucoside in the alcohol-soluble fraction of sap of cucumber seedlings.

Determination of thiazolidine-2-thione-4-carboxylic acid. To estimate the concentration of thiazolidine-2-thione-4-carboxylic acid (TTCA) in sap of plants the same procedure is followed as described for DDC-alanine and DDC-glucoside. In this case, however, butanol-water-acetic acid (4:1:1, v/v/v) is used as solvent-mixture and the chromatograms are sprayed with iodine-sodium azide reagent as described in chapter II. The regression lines are calculated from spots formed by 5, 10, 20, 40 and 80 μg TTCA and from areas of spots formed by different volumes of the alcohol-soluble fraction of sap. The volumes of sap are in the proportion of 1:2:4:8:16 (fig. 8). Since the spots reach their maximum at y = 24 the average concentration of TTCA in the same sample of the alcohol-soluble fraction in five different experiments was determined at y = 12. X was found to be 239 ppm whereas V = 13.8 %.

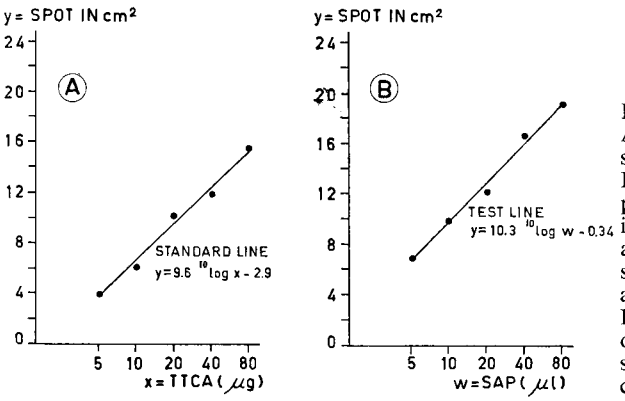


FIG. 8.
A. Regression of areas of spots on doses of TTCA. Doses on log scale. The compound is chromatographed in descending butanol-water-acetic acid (4:1:1, v/v/v) and sprayed with iodine-sodium azide reagent.
B. Corresponding regression on TTCA in the alcohol-soluble fraction of sap of cucumber seedlings.

3. RESULTS

a. The ratio between the fungitoxicity of DL-, L- and D-DDC-alanine in vitro

A comparison between the standard lines obtained after chromatographing equal amounts of DL-DDC-alanine and D-DDC-alanine in propanol-water (85:15, v/v) in the same experiment showed that DL-DDC-alanine is about 1.7 times more fungitoxic against *G. cingulata* and *C. cucumerinum* than D-DDC-alanine. A calculation shows that the fungitoxicity of DL-DDC-alanine is about 0.7 times that of L-DDC-alanine while L-DDC-alanine is about 2.4 times as effective as the D-form.*

b. The accumulation of DDC-alanine in cucumber seedlings during the uptake period

The quantitative paper chromatographic method renders it possible to study more in detail the fate of DDC-alanine in cucumber seedlings after this compound has been administered to the roots for different periods of time. For this purpose ten seedlings were placed with their roots in beakers containing 40 ml of an aqueous solution of 100 ppm DL-DDC-alanine for 8, 24, 48 or 72 hours. After this period roots and above-ground parts were frozen separately.

* Note added in proof. The same values were obtained in experiments in which the fungitoxicity of pure L-DDC-alanine was compared with that of the D-isomer and the racemic mixture.

The volumes of expressed sap, collected after thawing were measured by assessing their weights. Subsequently the concentrations of DDC-alanine, DDC-glucoside and TTCA in the sap were determined. Moreover, the remaining volume of the solution in the beakers and its DDC-alanine concentration were determined by the same methods. Fig. 9 illustrates the general pattern of the increase of DDC-alanine and its transformation products during the period of uptake. The concentration of DDC-alanine increases in the roots as well as in the above-ground parts. Transformation products were found only in the roots. The rate of accumulation of DDC-alanine and its transformation products decreases after uptake for 48 hours. Whether the reduction of the rate of accumulation should be attributed to a saturation of the tissues or to phytotoxicity is not clear.

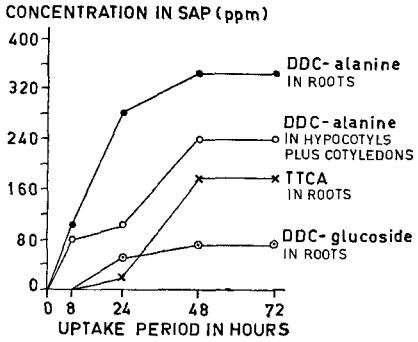


FIG. 9.
Accumulation of DDC-alanine and its transformation products in cucumber seedlings during the uptake period. 100 ppm DL-DDC-alanine applied to the roots. Concentration of DDC-alanine in sap calculated as the DL-form.

Root exudate from cucumber seedlings, which were placed for two days in tap water did not excert any influence on the fungitoxicity of DL-DDC-alanine. Therefore the amount of DL-DDC-alanine, which disappeared from the solution, must have been taken up by the plants.

Theoretically one mole of DDC-alanine yields one mole of DDC-glucoside or TTCA. On this basis the amount of the applied compound can be calculated which was used for the formation of DDC-glucoside and TTCA in the plant. This can be added to the amount of DDC-alanine recovered in sap (table 7).

To determine the amounts of DDC-alanine and its transformation products present in the residue which remains after expressing the thawed plant tissues, 4 ml of a mixture of ethanol (96%)-water (1:1, v/v) were added to the residue. The liquid was centrifuged for 5 minutes at 4500 rpm. The concentrations of DDC-alanine and of the transformation products in the supernatant liquid were determined after application of chromatography. The total amounts of the compounds calculated as DL-DDC-alanine in the residues are presented in table 7. It appears that the supernatant liquid of the residues contains about 10% of the compounds present in expressed sap. The total amount of the compounds calculated as DL-DDC-alanine is found by adding the values obtained for the residues to the values obtained for sap expressed from the roots and the above-ground parts of the seedlings.

From data presented in table 7 it can be seen that about 50% of the compound which disappeared from the solution surrounding the roots is recovered in the seedling. The other 50% must at least partly have been transformed into the unknown fungicide X.

TABLE 7. Balance-sheet for DL-DDC-alanine after different periods of uptake. For each object ten cucumber seedlings were placed in 40 ml of 100 ppm DL-DDC-alanine. Data derived from the same experiment as illustrated in fig. 9.

	Roots				Above-ground parts				Whole plant				Solution			
Period of uptake in hours	8	24	48	72	8	24	48	72	8	24	48	72	8	24	48	72
Total concentration of the compounds in sap in ppm[1]	104	341	627	590	84	103	243	238								
Sap in ml expressed from roots or above-ground parts	0.9	1.1	1.1	1.1	1.8	2.0	1.5	2.3								
Total amount of the compounds in sap in mg[1]	0.09	0.38	0.69	0.65	0.15	0.21	0.37	0.55	0.24	0.59	1.06	1.20				
Total amount of the compounds in the residues in mg	0.01	0.04	0.06	0.06	0.01	0.03	0.03	0.05	0.02	0.07	0.09	0.11				
Total amount of the compounds in sap plus residues in mg (A)	0.10	0.42	0.75	0.71	0.16	0.24	0.40	0.60	0.26	0.66	1.15	1.31				
Remaining concentration of DL-DDC-alanine in the applied solutions in ppm													96	82	48	43
Remaining volume of solutions in ml													36.5	33.9	28.2	24.1
Amount of DL-DDC-alanine taken up from solution in mg (B)													0.50	1.22	2.65	2.96
A/B in %									52	54.1	43.4	44.3				

[1] Concentration and amounts of DDC-alanine and its transformation products have been calculated as DL-DDC-alanine.

c. Transformation and decomposition of DDC-alanine in cucumber seedlings after the uptake period

It seemed of interest to establish the fate of DDC-alanine in the tissues after an initial treatment of cucumber seedlings with this compound. For each object ten seedlings were placed in 40 ml of an aqueous solution of 100 ppm DL-DDC-alanine for two days. Subsequently the seedlings were transferred to a Hoagland solution. The seedlings were frozen directly, 7, 14 or 21 days later. The results are given in fig. 10. It must be remarked that the lines in these figures have to be considered as the resultants of translocation, formation and decomposition of the various compounds. Turning attention first of all to the roots it can be seen that DDC-alanine decreased rapidly in the first seven days after application of the compound. About 19% of the amount present in the roots at the end of the period of uptake could be recovered in the Hoagland solution after seven days. Apparently some of the DDC-alanine has diffused from the roots into the surrounding Hoagland solution.

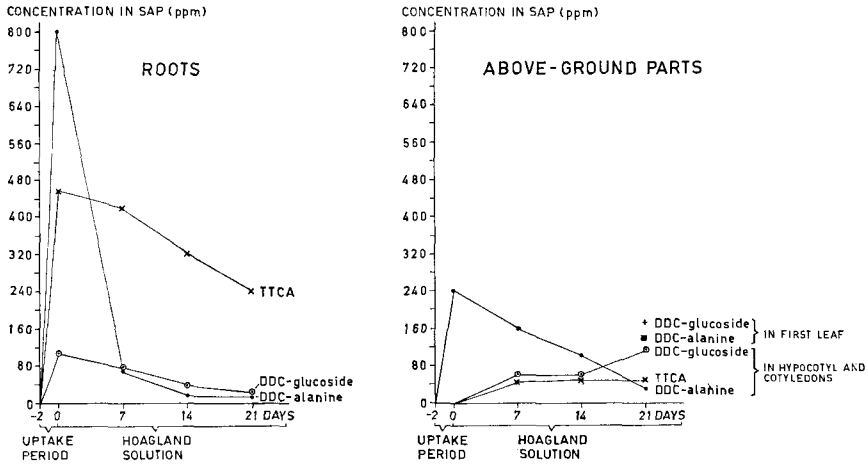


FIG. 10. Transformation and decomposition of DDC-alanine in cucumber seedlings after the uptake period. Concentration of DDC-derivatives in sap of seedlings after DL-DDC-alanine (100 ppm) has been applied to the roots. Uptake period two days. Subsequently plants were transferred to Hoagland solution. Concentration of DDC-alanine in sap calculated as the DL-form.

DDC-glucoside and TTCA were found in the roots directly after the period of uptake but not in the Hoagland solution 7 days later. Probably the concentration of these compounds was too low.

The concentration of DDC-glucoside and TTCA in the roots decreased less rapidly than that of DDC-alanine in the first 7 days after the uptake period. Fig. 11 shows, however, that the amount of TTCA in the roots increased still during 14 days. This indicates that part of the DDC-alanine which disappeared from the roots has been transformed into TTCA after the uptake period. On the other hand DDC-alanine may be decomposed into unknown compounds or has been translocated from the roots to the above-ground parts.

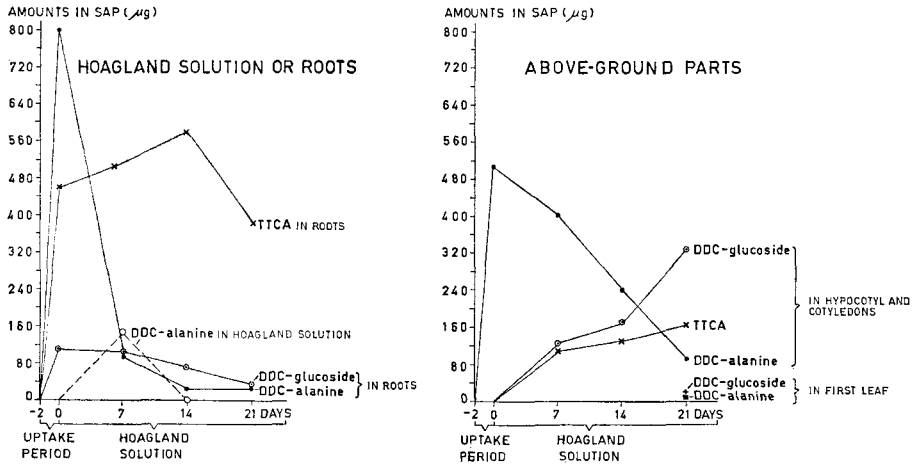


FIG. 11. Transformation and decomposition of DDC-alanine in cucumber seedlings after the uptake period. Amounts of DDC-derivatives in sap of ten seedlings and in 40 ml of the Hoagland solution after DL-DDC-alanine (40 ml of 100 ppm) has been applied to the roots. Uptake period two days. Subsequently plants were transferred to Hoagland solution. Amount of DDC-alanine calculated as the DL-form.

DDC-alanine decreased rather slowly in the above-ground parts, but it is surprising that the concentrations of DDC-glucoside as well as of TTCA increased (fig. 10). From fig. 11 it can be seen that in 21 days the amounts of DDC-glucoside and TTCA in the roots decreased with 0.078 mg and with 0.076 mg, respectively. In sap of hypocotyls and cotyledons DDC-glucoside and TTCA increased with 0.330 mg and 0.162 mg, respectively, during the same period. Thus the increase of DDC-glucoside and TTCA in hypocotyls and cotyledons cannot only be attributed to a transport of these compounds from the roots to the above-ground parts. The accumulation of DDC-glucoside and TTCA must be partly due to a conversion of DDC-alanine into these compounds in the above-ground parts of the seedlings.

Clear differences were observed between the sizes of the inhibition zones formed by the fungicide X directly or 21 days after the period of uptake. A volume of sap yielded a larger zone after 21 days than directly after the uptake period. This result suggests that, apart from its transformation into DDC-glucoside and TTCA, degradation of DDC-alanine is connected with a conversion into fungicide X in the seedlings. It must be pointed out, however, that fungicide X can also be derived from DDC-glucoside.

Moreover, it appeared that DDC-alanine and DDC-glucoside are translocated from the hypocotyls and cotyledons to the developing first leaf. The possibility, however, cannot be ruled out that DDC-glucoside has arisen from DDC-alanine which is translocated to this leaf. Alternatively, DDC-alanine might be formed from DDC-glucoside translocated to that leaf.

Finally the loss of DDC-alanine during the period in which the plants were standing in the Hoagland solution has been given in table 8. It can also be seen from this table that the percentage of DDC-alanine which could be recovered decreased continuously. This might be due to a continuous increase of unknown decomposition products of DDC-alanine, DDC-glucoside and TTCA.

TABLE 8. Balance-sheet for DDC-alanine. For each object ten cucumber seedlings were placed in 40 ml DL-DDC-alanine (100 ppm) for two days. Thereafter plants were transferred to Hoagland solution. The whole plant was analysed. Data derived from the same experiment as illustrated in fig. 11.

Days in Hoagland solution	Amount of DDC-alanine in mg[1]	Loss of DDC-alanine in mg (A)	Loss of DDC-alanine in % of the amount present after the uptake period	Part of (A) recovered in %[2]
0	1.304			
7	0.409	0.895	69	47
14	0.312	0.992	76	41
21	0.131	1.173	90	25

[1] DDC-alanine present as such in the plant.
[2] Amount of DDC-alanine in Hoagland solution added to amount converted into DDC-glucoside and TTCA in the plant.

d. The distribution of DDC-alanine in cucumber seedlings after uptake by the roots

In the foregoing experiments hypocotyls and cotyledons were expressed together and analysed on the presence of DDC-alanine. Evidence is lacking, however, whether DDC-alanine is really translocated into the cotyledons or is mainly present in the hypocotyls. To get information on this point the amounts and concentrations of DDC-alanine and DDC-glucoside have been assessed separately in sap of the roots, hypocotyls, and cotyledons. The results presented in table 9 show that DDC-alanine is translocated into the cotyledons and that the concentration of the compound in this tissue is somewhat lower than that in the hypocotyls. The absolute amount of DDC-alanine is, however, higher in the cotyledons than in the hypocotyls because less sap has been expressed from the latter tissues than from the former ones.

DDC-glucoside has also been found in the different tissues.

TABLE 9. Amount and concentration of DDC-alanine and DDC-glucoside in sap of roots, hypocotyls and cotyledons after uptake of DL-DDC-alanine (100 ppm) by ten cucumber seedlings for two days. DDC-alanine has been calculated as the DL-form. Volumes of sap expressed from roots, hypocotyls and cotyledons were 1.9, 0.9 and 2.1 ml, respectively.

	Roots		Hypocotyls		Cotyledons	
	Amount (mg)	Concentration (ppm)	Amount (mg)	Concentration (ppm)	Amount (mg)	Concentration (ppm)
DDC-alanine	0.703	370	0.216	240	0.411	196
DDC-glucoside	0.140	74	0.014	16	0.048	23

4. DISCUSSION

Quantitative experiments showed that about 50% of the DDC-alanine which disappeared from a solution in which cucumber seedlings were placed with their roots could be recovered as DDC-alanine or its transformation products DDC-glucoside and TTCA. The part which could not be recovered after uptake has been transformed to a certain extent into fungicide X. On the other hand DDC-ions are liberated from DDC-alanine by plants (chapter II). According to KLÖPPING & VAN DER KERK (1951) these ions easily give rise to

volatile carbon disulphide and to dimethylamine. Thus it seems reasonable to assume that DDC-alanine has been partly decomposed into these volatile compounds. Moreover, DDC-alanine might have been converted into compounds which are not detectable with the paper-chromatographic bioassay or with the iodine-sodium azide reagent. It is also possible that DDC-alanine is incorporated in the alcohol-insoluble fraction which has not been analysed.

DDC-alanine was found to be translocated from the roots to the hypocotyls, to the cotyledons, and to the developing first leaf (table 9 and fig. 10).

RUDD JONES (1956) investigated the persistance of sulphanilamide in broad beans. After the compound had been taken up by the roots the plants were transferred to a Hoagland solution. He found a loss of 40 and 60% of sulphanilamide from the plants after 7 and 13 days, respectively. In our experiments, cucumber seedlings were found to lose 69, 76 and 90% of DDC-alanine after 7, 14 and 21 days, respectively (table 8). RUDD JONES (1956) observed also a disappearance of some of the compound from the roots into the Hoagland solution. This occurred with 25% of the compound present in the roots at the end of the uptake period whereas we found a value of 19% for DDC-alanine.

As in the case of DDC-alanine, sulphanilamide is converted into a non-fungitoxic compound. In this case, however, detoxification occurs by acetylation of the amino group and the amido group (CROWDY & RUDD JONES, 1958). Acetylation by plants of the amino group of DDC-alanine was not found in our experiments since sap of cucumber seedlings and broad beans treated with DL-DDC-alanine did not yield a compound with an R_F value equal to that of the synthetic acetylation product.

5. SUMMARY

Application of a quantitative paper-chromatographic bioassay showed that about 50% of DL-DDC-alanine which disappeared from the solution surrounding the roots of cucumber seedlings could be recovered in the plants as such, as DDC-glucoside, and as TTCA.

The amount of DDC-alanine present in the cucumber seedlings after uptake decreased rather slowly during a period of three weeks. During this period the compound was partly converted into DDC-glucoside and TTCA.

CHAPTER IV

THE SYSTEMIC ACTION OF NaDDC, DDC-ALANINE AND DDC-GLUCOSIDE ON SOME PLANT DISEASES

1. INTRODUCTION

It has already been suggested in chapter II that the systemic effect of NaDDC against *Cladosporium cucumerinum* is caused by the fungitoxic action of L-DDC-alanine on the hyphae within the host.

Similar to what has been found for NaDDC, DDC-glucoside acts systemically against cucumber scab. The activity of DDC-glucoside could, however, only be observed when relatively high concentrations of about 1000 ppm were applied to the roots of cucumber seedlings. As described in chapter II, DDC-glucoside is partly transformed into L-DDC-alanine by the plant. Therefore protection by DDC glucoside may also be based on L-DDC-alanine. If this holds true a correlation may be expected between the degree of protection of the seedlings treated with NaDDC, DDC-glucoside or L-DDC-alanine and the level of L-DDC-alanine in the above-ground parts. Evidence for this correlation will be given in this chapter. Moreover, the systemic activity of DDC-alanine against some other plant diseases will be described.

2. MATERIALS AND METHODS

a. Tests for evaluating the systemic activity of dimethyldithiocarbamates

All plants used in the experiments were grown at a temperature of about 20° C. After an uptake period of two days (chapter II, section 2a) plants were transferred to water or Hoagland solution (see chapter III, section 2a), inoculated and kept in a greenhouse at about 20° C in a moist atmosphere until disease symptoms were clearly visible on the untreated plants. After inoculation with *Erysiphe cichoracearum* DC., however, no special measures were taken with regard to the relative humidity of the air.

Cladosporium cucumerinum test. Cucumber seedlings were grown as described in chapter I, section 2a. The roots were rinsed with water to remove adhering sand particles. Ten plants were placed with their roots and the lower parts of the hypocotyls in beakers containing 40 ml of water or 40 ml of an aqueous solution of the compound to be tested for systemic activity. After two days the solution was replaced by tap water and the seedlings were inoculated with a conidial suspension of *C. cucumerinum* by means of a DE VILBISS sprayer. One drop of an adhesive agent (Tween 20) was added to 20 ml of the suspension. One drop of orange juice was added to assure optimal germination. Disease symptoms were clearly visible after 4–6 days. The hypocotyls could be seriously affected, showing necrotic spots. In the final stage the seedlings collapse. The cotyledons were not often affected. The effectiveness of the systemic compound is given in % protection of the control plants (see below).

Colletotrichum lagenarium test. This test proceeded in exactly the same way as the *C. cucumerinum* test. Disease symptoms were visible four to five days after inoculation with *Colletotrichum lagenarium* (Pass.) Ell. &

HALST. Hypocotyls as well as cotyledons are liable to attack by this pathogen. The affected areas were pale green, water-soaked, and within 24 hours the plants collapsed.

Erysiphe cichoracearum test. Cucumber plants, variety 'Lange gele tros', were grown on Hoagland solution under the same conditions as described for seedlings. When the third leaf was about 1/5 fully developed, for each experiment two plants were placed for two days with their roots in beakers containing 200 ml of water or 200 ml of an aqueous solution of the compound under investigation. The plants were transferred to a Hoagland solution and inoculated with the conidia of *E. cichoracearum*. Symptoms became visible after eight days. Disease was rated on the twelfth day. At that time younger as well as older leaves of untreated plants were covered with mycelium and conidia of powdery mildew.

Colletotrichum lindemuthianum test. French beans (*Phaseolus vulgaris* L.), variety 'Processor', were grown on Hoagland solution in a greenhouse. When the first trifoliate leaf was about 1/3 fully developed, for each experiment three plants were placed with their roots in beakers containing 75 ml of water or 75 ml of an aqueous solution of the compound to be tested for systemic activity. After two days the solution was replaced by Hoagland solution and the plants were inoculated with the conidia of *Colletotrichum lindemuthianum* (Sacc. & Magm.)Bri. & Cav. Disease was graded five days later when black necrotic spots on hypocotyls, epicotyls, stems and leaves could be observed.

Botrytis fabae test. Broad beans (*Vicia faba* L.), variety 'Con Amore', were grown in soil in a greenhouse. For each experiment stems of 3 plants with four pairs of leaves were cut and placed in beakers containing 100 ml of an aqueous solution of the compound to be tested. After two days the solution was replaced by water. The upper part of the leaves was inoculated with a conidial suspension of *Botrytis fabae* Sard. After incubation for twenty-four hours black spots were visible on the inoculated leaves and the disease was graded.

Alternaria solani test. Tomato plants (*Solanum lycopersicum* L.), variety 'Tuckqueen', were grown in Hoagland solution in a greenhouse. After development of three leaves, for each experiment two plants were placed with their roots in beakers containing 100 ml of an aqueous solution of the compound. Two days afterwards the plants were transferred to a Hoagland solution and the leaves were inoculated with the conidia of *Alternaria solani* Ell. & Martin. To obtain strong sporulation, the fungus growing on potato-glucose agar was scratched and exposed to ultra-violet light for 20 minutes (Philips HPW, 125 W) two days before inoculation. Disease was graded six days after inoculation when leaves showed brown spots.

Degree of protection. Disease symptoms were rated on an arbitrary scale of 0 to 3 in which 0 = no symptoms, 1 = slightly visible symptoms, 2 = clearly visible symptoms and 3 = plants or plant parts killed or as in the case of powdery mildew covered with mycelium. The mean disease index for every treatment was obtained by the sum of the products of every disease rating and the number of plants having that rating and dividing this sum by the total number of plants. The mean disease index of control plants was put at 0%

protection, and the effect of the compound applied was calculated as % protection of the control plants. It will be clear that the estimation of the degree of protection itself is not very accurate and differences of less than 10% may not be significant.

b. Fungitoxicity in vitro (roll-culture test method)

The fungitoxicity of different dimethyldithiocarbamates against some fungi on glucose-mineral salts agar has been assessed by the roll-culture test method of MANTEN, KLÖPPING & VAN DER KERK (1950). The glucose-mineral salts agar, later designated as a minimal medium, contained; 1% glucose, 0.1% $(NH_4)_2SO_4$, 0.25% K_2HPO_4, 0.25% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 2% agar(KAARS SIJPESTEIJN & VAN DER KERK, 1952). Only in case of *C. cucumerinum* biotin was added (0.002 µg/ml).

3. RESULTS

a. The systemic activity of NaDDC, DDC-alanine and DDC-glucoside against Cladosporium cucumerinum on cucumber seedlings

An investigation of the systemic activity of NaDDC, DDC-glucoside and L-DDC-alanine against cucumber scab in relation to the concentration of L-DDC-alanine found in the seedlings should be carried out with the pure L-isomer. We had, however, only an impure aqueous solution of L-DDC-alanine at our disposal (chapter II, section 2d). This solution, when fed to the roots, protected seedlings against cucumber scab. Thus, there can be little doubt that L-DDC-alanine itself acts systemically.

Pure DL-DDC-alanine reduced disease development more than the D-isomer.

The difference between the fungitoxicity of the L-isomer and that of DL-DDC-alanine *in vitro* is small (chapter III, 3a). For this reason the systemic activity of NaDDC and DDC-glucoside was compared with that of DL- and not with that of L-DDC-alanine. The concentrations of DDC-glucoside and DDC-alanine (calculated as the DL-form) were determined after estimating the degree of protection. The results are illustrated in Plate II A and summarized in table 10.

TABLE 10. Systemic activity of NaDDC, DDC-glucoside and DL-DDC-alanine against *Cladosporium cucumerinum* and the concentration of DDC-alanine and DDC-glucoside in sap of above-ground parts of cucumber seedlings 5 days after inoculation. Concentration of DDC-alanine in sap has been calculated as the DL-form.

	NaDDC				DDC-glucoside			DDC-alanine				
Conc. of compounds applied to the roots (ppm)	0	62.5	125	250	250	500	1000	12.5	25	50	100	200
Protection in %	0	10	39	49	25	43	55	12	43	65	76	93
Phytotoxicity[1]	–	–	±	±	–	–	–	–	–	–	–	–
Conc. DDC-glucoside in sap (ppm)	0	18	38	102	72	110	172	0	17	40	80	103
Conc. DDC-alanine in sap (ppm)	0	50	90	114	62	103	125	50	110	207	292	337

[1] –, no visible phytotoxic symptoms.
±, partial loss of root turgescence.
+, total loss of root turgescence.

The increase of the concentrations of the different compounds applied to the roots results in an increase of DDC-alanine in the above-ground parts of the seedlings and in an increase of protection. Only the highest concentrations of NaDDC appeared to be phytotoxic to the roots. The systemic activity of NaDDC, DDC-glucoside and DL-DDC-alanine is correlated with the concentration of DDC-alanine found in sap of the above-ground parts. This correlation finds clear expression in fig. 12A in which the concentration of DDC-alanine is plotted against the degree of protection. The points found for plants which have taken up NaDDC or DDC-glucoside are situated close to the line found after uptake of DL-DDC-alanine.

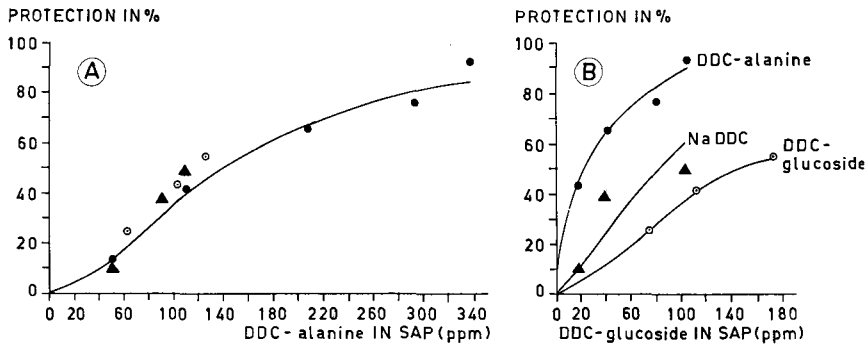


FIG. 12. Systemic activity of NaDDC, DDC-glucoside and DL-DDC-alanine against *Cladosporium cucumerinum*.
A. Concentration of DDC-alanine in sap of cucumber seedlings plotted against degree of protection. The line has been drawn through the points found for the plants which have taken up DL-DDC-alanine. The concentration of DDC-alanine in sap of the above-ground parts has been calculated as the DL-form.
B. Concentration of DDC-glucoside in sap of cucumber seedlings plotted against degree of protection.
Plants treated with: DL-DDC-alanine •, NaDDC ▲, DDC-glucoside ⊙.

In fig. 12B the concentration of DDC-glucoside in sap is plotted against the degree of protection. It can be seen that the lines for plants which have taken up NaDDC or DL-DDC-alanine do not coincide with the line which is found after uptake of DDC-glucoside. Thus the systemic activity of NaDDC, DL-DDC-alanine and DDC-glucoside is not correlated with the concentration of DDC-glucoside in sap.

These results strongly support the idea that the systemic effect of NaDDC and DDC-glucoside against *C. cucumerinum* is caused by L-DDC-alanine and that DDC-glucoside present in the cucumber seedlings is not directly involved in this activity.

VAN ANDEL (1958) made the interesting observation that the systemic activity of NaDDC against cucumber scab increases if D-serine is added simultaneously to the roots of cucumber seedlings. One might expect that in that case the concentration of DDC-alanine in sap of the seedlings would be higher than in sap of those seedlings which only received NaDDC. Our results, however, did not confirm this possibility. Thus it can be concluded that in the case of a simultaneous addition of NaDDC and D-serine, the concentration of

DDC-alanine in sap is not correlated with the degree of protection. One must, however, not forget that D-serine alone shows some protection and therefore the increasing systemic activity can be explained by a cumulative effect caused by D-serine itself and by L-DDC-alanine formed after uptake of NaDDC.

b. The fungitoxicity of DL-DDC-alanine against Cladosporium cucumerinum in vitro

As shown in fig. 12A at least 100 ppm DDC-alanine must be present in sap of cucumber seedlings to reduce an attack by *C. cucumerinum* by about 60%. According to KASLANDER *et al.* (1962) 20 ppm DL-DDC-alanine completely inhibits growth of conidia of this fungus on a minimal medium containing salts, glucose and biotin. Cucumber sap is a far richer medium and therefore the influence of DL-DDC-alanine on growth of the fungus was investigated in sap expressed from untreated cucumber seedlings. For this purpose an equal volume of alcohol (96%) was added to sap and centrifuged at 4500 rpm for 5 minutes. The supernatant liquid was evaporated at 40°C to about 1/10 of the original volume of the sap. This sap which is nearly free from proteins and cell wall material, was made up to the original volume of sap by adding distilled water and sterilized by Seitz filtration. Conidia (20.000/ml) and 50, 100, 200 or 400 ppm DL-DDC-alanine were added to 40 ml. of the sap. The dry weight of the mycelium was assessed after shaking the culture at 20°C for 3 days.

From fig. 13 it appears that the fungitoxicity of DL-DDC-alanine against *C. cucumerinum* in sap is much lower than in a minimal medium, since 100 ppm inhibited growth of the fungus in sap only by about 50%. A comparison of fig. 12A with fig. 13 shows that the relationship between the concentration range of DDC-alanine in sap of treated seedlings and the degree of protection corresponds within certain limits with the relationship between the same concentration range and growth inhibition of the fungus *in vitro*. This correspondence between both curves suggests that the systemic activity of DDC-alanine depends on its toxic action on the hyphae within the plant.

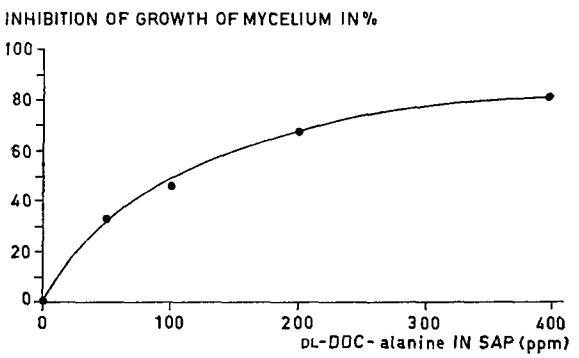


FIG. 13.
Influence of DL-DDC-alanine on growth of *Cladosporium cucumerinum* in a shake culture of sap expressed from untreated cucumber seedlings.

The reduction of the fungitoxicity of DL-DDC-alanine by sap strongly reminds one of the reduction of the fungitoxicity of NaDDC by antagonists present in sap of plants (chapter I). For this reason it was investigated whether or not sap also contains compounds able to antagonize the fungitoxic activity of DL-DDC-alanine.

In order to gain information on this point the same method was applied that was used for the detection of antagonists of NaDDC in sap. Sap of untreated seedlings (0.1 ml) was added to two different strips and chromatographed in butanol-acetic acid-water (4:1:1, v/v/v) for 16 hours. After drying, one of the strips was sprayed with a conidial suspension of *G. cingulata* to which was added 1 ppm DL-DDC-alanine. Growth of the mycelium was just inhibited by that concentration except on 8 spots (R_F values 0.013, 0.1, 0.17, 0.28, 0.35, 0.49, 0.52 and 0.66). The first five zones of growth were not completely separated. On the other strip these spots reacted ninhydrin positive, suggesting that sap contains L-amino acids which antagonize the fungitoxic activity of DL-DDC-alanine against *G. cingulata*. Similar zones of growth were obtained when 4 ppm DL-DDC-alanine were added to a conidial suspension of *C. cucumerinum* and sprayed on the chromatograms.

The influence of some L-amino acids on the fungitoxicity of DDC-alanine against *C. cucumerinum* was investigated more in detail by means of the paper-spot bioassay. The results summarized in table 11 show that most L-amino acids, with the exception of L-cysteine and L-tyrosine, antagonize the fungitoxicity of DL-DDC-alanine and of the D- and L-isomer separately.

Although the cause of the antagonistic effect of so many different amino acids has not yet been elucidated, the results strongly indicate that these compounds are responsible for the observed reduction of the fungitoxicity

TABLE 11. Antagonistic activity of L-amino acids (50 µg) against the fungitoxicity of DL-DDC-alanine (2 µg), D-DDC-alanine (2 µg), and L-DDC-alanine (about 2 µg). Paper-spot bioassay. Test fungus *Cladosporium cucumerinum*.

\- Area of inhibition zone equal to that formed by the fungicide alone.
± Area of inhibition zone slightly smaller than that formed by the fungicide alone.
\+ Area of inhibition zone much smaller than that formed by the fungicide alone.
\+ + Activity of the fungicide completely antagonized by amino acid.

L-amino acids added to the paper[1]	Fungicide added to the paper		
	L-DDC-alanine	DL-DDC-alanine	D-DDC-alanine
histidine	+	+	+ +
α-alanine	+ +	+ +	+ +
valine	+ +	+ +	+ +
leucine	+ +	+ +	+ +
isoleucine	+ +	+ +	+ +
aspartic acid	±	±	±
glutamic acid	±	±	±
ornithine	+ +	+ +	+ +
arginine	+ +	+ +	+ +
threonine	+	+	+
tyrosine	-	-	±
phenylalanine	+ +	+ +	+ +
serine	+ +	+ +	+ +
methionine	+ +	+ +	+ +
cysteine	-	-	-
proline	+	+	+
tryptophane	+	+	+ +

[1]Compounds were obtained from S. A. F. Hoffmann-La Roche & Co. Ltd. Basle (Switzerland).

of DL-DDC-alanine in sap. Most probably the toxic action of DDC-alanine on hyphae of *C. cucumerinum* in cucumber seedlings is also antagonized by amino acids. If this holds true, one might expect that the systemic activity of DL-DDC-alanine (100 ppm) would be reduced if for instance L-alanine (1000 ppm) was added simultaneously to the roots of cucumber plants. In fact, addition of L-alanine decreases the systemic activity of DL-DDC-alanine. This suggested that the fungitoxic action of DDC-alanine was antagonized by L-alanine which had been taken up. It appeared, however, that L-alanine interferes with the uptake of DL-DDC-alanine by the seedlings since beakers filled with both compounds contained at the end of the period of uptake more DL-DDC-alanine than beakers which had only been filled with DL-DDC-alanine. Moreover, the above-ground part contained less DDC-alanine when the roots of the seedlings were placed in a solution containing both compounds than when the roots only received DL-DDC-alanine.

c. Anatomical observations on cucumber seedlings systemically protected by DL-DDC-alanine against Cladosporium cucumerinum

As shown in table 10, DL-DDC-alanine is systemically active against cucumber scab. In these experiments disease development was evaluated 5 or 6 days after inoculation. It often happens, however, that a systemic compound only retards growth of the pathogen, so that the difference between disease development of untreated and treated plants disappears after some days. For this reason it was decided to follow the disease symptoms over a longer period of time and to investigate how far the hyphae advance into the tissues of the hypocotyls of seedlings treated with DL-DDC-alanine. Growth of hyphae in untreated seedlings has not been involved in the experiments since this has already been studied in detail by EL-DIN FOUAD (1956).

In previous experiments the roots of the cucumber seedlings were placed in water during the incubation period. It will be clear that an experiment over a longer period of time can only be successful when the seedlings receive nutrients. For this reason seedlings were treated with 50, 100 or 200 ppm DL-DDC-alanine for two days, and those which had not collapsed six days after inoculation were transferred to a Hoagland solution.

Although six days after inoculation no phytotoxicity could be observed, some weeks later it became obvious that concentrations of 100 and 200 ppm DL-DDC-alanine seriously inhibited growth of the plants and that the first leaves were misshapen. These phenomena were also observed on treated but non-inoculated plants (Plate IIC and Plate IVA). Furthermore D-DDC-alanine appeared to be less phytotoxic than the equimolar concentration of DL-DDC-alanine.

Infection seemed to be checked in seedlings which had not collapsed 6 days after inoculation, since the small necrotic spots which could be observed on the hypocotyls of all treated plants did not extend further (Plate IIB).

The occurrence of hyphae was investigated on free hand cross sections from hypocotyls of treated plants 40 days after inoculation. The sections were stained in cotton blue-lactophenol. Epidermis cells were often filled with a yellow granular substance whereas the cell walls were yellow in colour. Non-inoculated plants did not show these changes. In many cases mycelium was found in the epidermis. Frequently, however, not only the epidermis but also

some layers of the underlying cells were affected. The cells showed yellow walls and contained a dark yellow-brown granular substance. The infected area was often found to be surrounded by actively dividing cells (Plate III A, B). Hyphae have been observed on both sides of the dividing cells. Plate III C, D shows hyphae which have penetrated far into the parenchymatic tissue between two vascular bundles. The vascular bundles were never found to be affected.

These observations raise the question whether the pathogen has been killed or whether there are still living hyphae in the tissues. To answer this question hypocotyls were cut from plants which had been treated with 50, 100 or 200 ppm DL-DDC-alanine in the seedling stage and which had been standing for 40 or 60 days in a Hoagland solution. These hypocotyls were immersed in a solution of 0.2% mercuric chloride for 30 seconds. To prevent penetration of mercuric chloride into the tissues, the cut ends were smeared with vaseline. Subsequently the hypocotyls were rinsed with sterilized water and cut into parts of 0.5 cm length and placed at 23° C on cherry agar. To suppress bacterial growth terramycin (25 ppm) was added to the agar.

To our surprise some days afterwards a dark green mycelium was growing from many parts of all hypocotyls of seedlings previously treated with DL-DDC-alanine and inoculated with *C. cucumerinum*. The mycelium was subcultured on cherry agar without terramycin. No morphological difference could be observed between this mycelium and that of *C. cucumerinum*. Cucumber seedlings inoculated with conidia of mycelium isolated from the hypocotyls of treated plants, showed typical disease symptoms of cucumber scab. Mycelium of *C. cucumerinum* was not isolated from untreated, non-inoculated seedlings. To ensure that conidia or germinated conidia were not living on the outside of the disinfected hypocotyls 60 days after inoculation, conidia of *C. cucumerinum* were streaked on hypocotyls of untreated cucumber seedlings. Then the hypocotyls were disinfected with mercuric chloride immediately after inoculation or twelve hours after germination of the conidia in a moist chamber. In both cases the hypocotyls did not yield growth of *C. cucumerinum* on agar plates. Thus the conclusion is justified that even hypocotyls of plants previously treated with 200 ppm DL-DDC-alanine contain living mycelium of *C. cucumerinum* up to 60 days after inoculation. Most probably, however, many hyphae were killed after penetration since mycelium was not growing from all parts of the hypocotyls in which hyphae were observed microscopically.

It seems remarkable that living mycelium is still present in hypocotyls of cucumber plants 60 days after inoculation, whereas the plants are not killed. From the work of EL-DIN FOUAD (1956) it is known, however, that *C. cucumerinum* can only succesfully attack young growing tissues, whereas older tissues become less susceptible (see also table 12). The nature of this resistance of mature plant parts is not known.

In the light of these data the following picture can be given of the mode of systemic action of DDC-alanine against *C. cucumerinum*: the hyphae within the hypocotyls are killed, or growth is retarded by the presence of DDC-alanine. The DDC-alanine concentration decreases but in the meantime the inoculated plant parts grow older and reach a stage in which the tissues acquire natural resistance against the pathogen.

d. Period during which DL-*DDC-alanine remains systemically active against cucumber scab*

In the experiments cucumber seedlings were always inoculated directly at the end of the period of uptake. Since the concentration of DDC-alanine in the above-ground parts of cucumber seedlings decreased rather slowly after the period of uptake (fig. 10), it is to be expected that the compound is also systemically active after a longer interval between uptake and inoculation. Therefore cucumber seedlings which had been treated with 100 ppm DL-DDC-alanine for two days, were transferred to a Hoagland solution and inoculated 0, 3, 6 or 12 days later. Table 12 illustrates that hypocotyls growing older are less susceptible to *C. cucumerinum*. It can be seen, moreover, that DL-DDC-alanine is systemically active against an attack on the hypocotyls even 12 days after treatment. The degree of protection, however, decreases slowly and seems to be correlated with the decrease of DDC-alanine in the hypocotyls.

TABLE 12. Period during which DL-DDC-alanine is systemically active against cucumber scab. Seedlings were treated with 100 ppm DL-DDC-alanine for two days, transferred to Hoagland solution and inoculated 0, 3, 6 or 12 days later. Concentration of DDC-alanine in sap is calculated as the DL-form.

Plants treated with	Interval in days between the end of the period of uptake and moment of inoculation	Disease index[1]		Protection in %		DDC-alanine in sap 5 days after inoculation (ppm)	
		Hypocotyls + cotyledons	First leaf	Hypocotyls + cotyledons	First leaf	Hypocotyls + cotyledons	First leaf
Water	0	2.6	–	0	–	–	–
100 ppm DDC-alanine	0	0.7	–	73	–	290	–
Water	3	1.8	2.1	0	0		
100 ppm DDC-alanine	3	0.6	0.0	67	100	197	791
Water	6	1.2	2.4	0	0		
100 ppm DDC-alanine	6	0.5	0.2	58	93	128	525
Water	12	0.6	2.6	0	0		
100 ppm DDC-alanine	12	0.3	0.5	50	81	60	260

[1] Maximum disease index is 3.

In accordance with the results described in chapter III, DDC-alanine is translocated to the developing first leaf. The highest degree of protection of the first leaf was obtained in plants which were inoculated three days after the end of the period of uptake. This maximum coincides with a maximum concentration of DL-DDC-alanine in sap of the first leaf.

e. The systemic activity of DL-*DDC-alanine against Colletotrichum lagenarium and Erysiphe cichoracearum on cucumbers*

After the systemic activity of DL-DDC-alanine against *C. cucumerinum* had been studied in detail it seemed of interest to investigate its activity against some other pathogens on the same host. Fig. 14 illustrates that, although a considerable amount of DL-DDC-alanine was found in the above-ground parts, the systemic activity against *C. lagenarium* is rather low in comparison to that against *C. cucumerinum*. Moreover, in contrast to an infection by cucumber scab, plants inoculated with *C. lagenarium* collapsed finally. These results seem remarkable since *in vitro C. lagenarium* is more sensitive to DL-DDC-alanine

than *C. cucumerinum* (table 13, first column). It is, however, well known that, in contrast to *C. cucumerinum*, *C. lagenarium* attacks older plant parts as well as younger tissues. From this fact the following picture can be given of the mode of systemic action of DDC-alanine against *C. lagenarium*: DDC-alanine retards growth of this pathogen within the cucumber seedlings. The DDC-alanine concentration decreases and the seedlings are growing older in the meantime, but their susceptibility does not decrease as in the case of cucumber scab. Therefore, the seedlings collapse only a few days after the control plants.

DL-DDC-alanine was also found to be systemically active against *E. cichoracearum* but protected only the young developing leaf and not the older leaves of cucumbers. Sap of the youngest leaf contained a considerably higher concentration of DDC-alanine than sap of the older leaves (fig. 14).

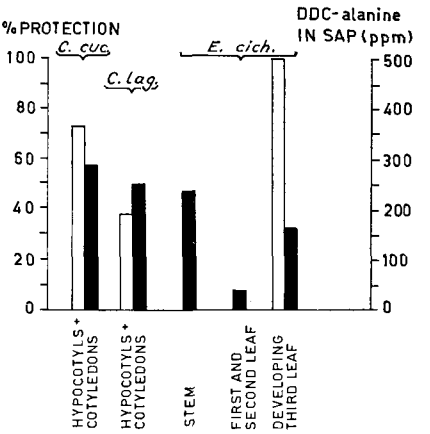


FIG. 14.
Systemic activity of DL-DDC-alanine against some pathogens on cucumbers. Activity of 100 ppm tested against *Cladosporium cucumerinum* (data derived from table 12) and *Colletotrichum lagenarium* on seedlings. Activity of 200 ppm tested against *Erysiphe cichoracearum* on older plants. □, protection in % (stem not inoculated, leaf one and two not protected against *E. cichoracearum*) ■, concentration of DDC-alanine in sap (calculated as the DL-form) 5 days after inoculation.

TABLE 13. Influence of casamino acids on growth inhibiting activity of DL-DDC-alanine against some plant pathogens *in vitro*. Roll-culture test method.

Column	1	2
	Minimum concentration of DL-DDC-alanine in ppm causing complete growth inhibition	
	without casamino acids	in the presence of 3% casamino acids[1]
Cladosporium cucumerinum	20	400
Colletotrichum lagenarium	1	200
Colletotrichum lindemuthianum	5	400
Botrytis fabae	1	200
Alternaria solani	2	200

[1] Casamino acids (vitamine free), Difco Laboratories Detroit U.S.A.

f. The systemic activity of DL-DDC-alanine against pathogens on other plant species

The systemic activity of DL-DDC-alanine (200 ppm) has also been tested against pathogens on other plant species. The results are given diagramatically in fig. 15. Infection of hypocotyl, epicotyl, stem and first trifoliate leaf of

French beans by *C. lindemuthianum* is far more reduced than that of the primary leaves. This result appears to correlate with the concentration of DDC-alanine in sap of these plant parts.

Similar to the results with cucumbers (fig. 14) and French beans, sap of the stems and youngest parts of broad beans and tomatoes yielded the highest DDC-alanine concentrations (fig. 15).

It is noteworthy that 0.05 ml of the alcohol-soluble fraction of sap of the leaves of untreated tomato plants revealed an inhibition zone with an R_F value of 0.42 in descending propanol-water (85:15, v/v). This inhibition zone was not detected after chromatographing the same volume of sap expressed from the roots or from the stem. Apparently a fungitoxic compound occurs in the leaves of untreated plants.

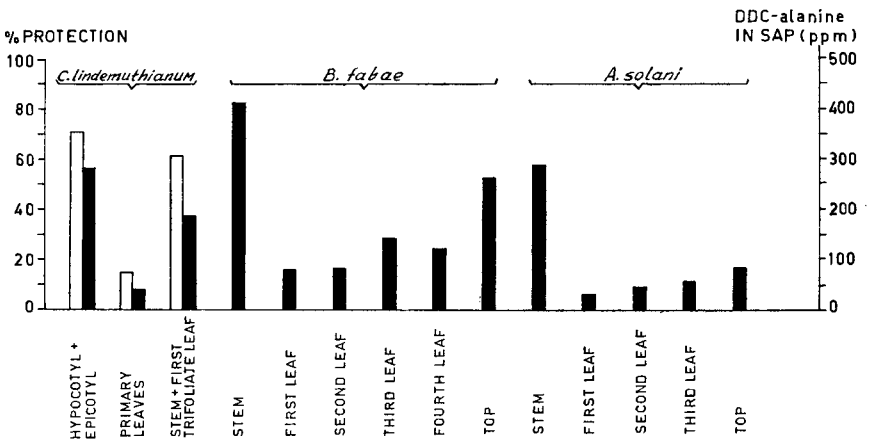


FIG. 15. Systemic activity of DL-DDC-alanine (200 ppm) against *Colletotrichum lindemuthianum* on French beans, *Botrytis fabae* on broad beans and *Alternaria solani* on tomatoes. □, protection in % (no protection observed on broad beans and tomatoes). ■, concentration of DDC-alanine in sap (calculated as the DL-form) 5, 1, and 6 days after inoculation, respectively.

DL-DDC-alanine did not exert a systemic activity against *B. fabae* and *A. solani* on broad beans and tomatoes, respectively. This is remarkable because relatively high concentrations of this compound were found in sap of different parts of broad beans and tomatoes. Moreover, these pathogens are very sensitive *in vitro* to DL-DDC-alanine (table 13, column 1). Column 2 of table 13 shows, however, that *in vitro* the fungitoxicity of DL-DDC-alanine against *B. fabae* and *A. solani* is antagonized by L-amino acids. Thus it may be assumed that the L-amino level in broad beans and tomatoes is high enough to antagonize completely the fungitoxic action of DDC-alanine on the hyphae of *B. fabae* and *A. solani*.

4. DISCUSSION

The systemic activity of NaDDC, DDC-glucoside and DL-DDC-alanine against *C. cucumerinum* was found to correlate with the concentration of DDC-alanine in sap of the above-ground parts of cucumber seedlings (fig. 12A). Such a correlation was not found between the degree of protection and the concentra-

tion of DDC-glucoside. L-, D- as well as DL-DDC-alanine reduced disease development. It is highly probable that L-DDC-alanine, which is formed in the plant is responsible for the systemic activity of NaDDC and DDC-glucoside against cucumber scab because L- and DL-DDC-alanine are about equally fungitoxic.

It is very difficult to prove whether the systemic activity of DDC-alanine is based exclusively on its toxic action on the hyphae within the plant or on an interference with host metabolism resulting in an increase of resistance. An approach to this problem has been made by adding the fungicide to sap in a shake culture. As shown in fig. 12A and 13 the relationship between the concentrations of DDC-alanine in sap of treated seedlings and the degree of protection corresponds within certain limits with the relationship between the same concentration range and growth inhibition of *C. cucumerinum in vitro*. This correspondence suggests that the systemic activity of NaDDC, DDC-glucoside and DL-DDC-alanine is most probably due to a toxic action of DDC-alanine on the pathogen within the host. Evidence is lacking, but it can be assumed that the mode of systemic action of DDC-alanine against some other plant pathogens, for instance *C. lindemuthianum* and *E. cichoracearum*, is based on the same principle. The possibility can, however, not be ruled out that these compounds have also a certain effect on plant metabolism resulting in an increase of resistance.

In general, infection of plant parts appeared to be reduced only when the concentration of DDC-alanine was at least 100–200 ppm in sap. These concentrations are much higher than the concentrations which completely inhibit growth of the investigated pathogens on a minimal medium *in vitro* (table 13, column 1). It has been shown that L-amino acids counteract the toxicity of L-, D- and DL-DDC-alanine against many fungi (table 13, column 2). In this way it can be understood that the DDC-alanine level in the tissues must be high to compete successfully with the amino acids in the tissues. Relatively high concentrations are reached in hypocotyls, epicotyls, stems, growing leaves but not in older leaves. There can be little doubt that these facts are connected with the general tendency of a compound to be translocated to young growing tissues (ARISZ, 1952).

The best systemic effect with DL-DDC-alanine was obtained against *C. cucumerinum*. Yet, proof has been given that the compound does not kill all hyphae in the hypocotyl. It seems more likely that in this particular case the compound helps the growing seedlings to overcome a temporary stage of natural sensitivity to the pathogen. In this way it is also clear that DL-DDC-alanine is less effective against *C. lagenarium* since cucumber seedlings which are growing older do not build up a natural barrier against this parasite. On the other hand the possibility cannot be ruled out that the quantity of hyphae of *C. cucumerinum* is reduced to a level which is harmless for the plant, whereas for *C. lagenarium* this level is still high enough to kill the host.

From the results obtained so far it is clear that neither the isomers nor the racemic mixture of DDC-alanine offer prospects for practical application. This is due to their phytotoxicity and to the antagonistic action of L-amino acids in the plants. DL-DDC-alanine is less phytotoxic than an equivalent concentration of NaDDC (table 10). In comparison with DL-DDC-alanine, D-DDC-alanine is less phytotoxic to cucumber seedlings but also less fungitoxic to *C.*

cucumerinum and most probably therefore less systemically active against cucumber scab. Phytotoxic symptoms are not clearly visible shortly after uptake of the compounds but only some weeks later. Then growth appears to be retarded and morphological abnormalities can be observed on the oldest leaves (Plate IIC and IVA).

DL-DDC-alanine does not bring about cell elongation in the *Avena* cylinder test (personal communication from Dr. O. M. VAN ANDEL). Tomato plants which respond to the application of growth substances with epinasty of the leaves (VAN ANDEL, 1962) did not show this effect after uptake of DL-DDC-alanine by the roots although growth of the plants was inhibited. Therefore it is very likely that DL-DDC-alanine does not possess any true growth substance activity but can be called a growth regulator inducing only formative effects (ZIMMERMANN, 1951).

Finally a remark about the fungitoxic compound found in untreated tomato plants. FONTAINE *et al.* (1948) isolated a fungitoxic compound from tomato plants which they called tomatine. The structure of tomatine, a glycosidal alkaloid, has been elucidated by KUHN, LÖW & TRISCHMANN (1956). Below pH 7 the compound is soluble in water. It is possible that the fungitoxic compound found in these experiments on chromatograms of sap expressed from untreated leaves is identical with tomatine.

5. SUMMARY

DL-, D- as well as L-DDC-alanine have a high systemic activity against *Cladosporium cucumerinum* on cucumber seedlings. L-DDC-alanine formed in these plants is responsible for the observed systemic activity of NaDDC and DDC-glucoside against this pathogen, because a correlation was found between the concentrations of DDC-alanine in sap of cucumber seedlings and the degree of protection. Most probably, the mechanism of the systemic activity of DDC-alanine is based on its toxic action on the hyphae within the host. Yet, not all hyphae are killed in the hypocotyl, but growth is retarded. As only young hypocotyls are susceptible to *C. cucumerinum* it is assumed that DDC-alanine helps the seedlings to overcome a temporary stage of natural sensitivity to this pathogen, or that the quantity of hyphae is reduced to a level which does not cause much damage.

L-amino acids reduce the fungitoxicity of DDC-alanine against many fungi *in vitro*.

In young growing parts of cucumber plants and French beans development of *Colletotrichum lagenarium*, *Erysiphe cichoracearum* and *Colletotrichum lindemuthianum*, respectively, was inhibited after uptake of DL-DDC-alanine by the roots. It can be assumed that the DDC- alanine level in these plant parts was high enough to compete successfully with L-amino acids.

DL-, D- as well as L-DDC-alanine are not useful for practical application since they inhibit plant growth and induce morphological abnormalities.

CHAPTER V

THE SYSTEMIC ACTION OF SOME GROWTH-REGULATING DIMETHYLDITHIOCARBAMATES ON *CLADOSPORIUM CUCUMERINUM*

1. INTRODUCTION

VAN RAALTE *et al.* (1955) showed that carboxymethyl-DDC

$$(CH_3)_2N\text{-}\overset{\overset{S}{\|}}{C}\text{-}S\text{-}CH_2COOH$$

is systemically active against cucumber scab. This fact is highly remarkable since this compound is a weak fungicide *in vitro* (growth of *Cladosporium cucumerinum* is completely inhibited by a minimum concentration of 500 ppm). For matters of convenience the compound henceforth is designated as non-fungitoxic.

A plausible explanation for the systemic activity of carboxymethyl-DDC has been given by VAN DER KERK (1956). He suggested that the compound is translocated as such and is decomposed inside the plant. The decomposition was expected to give rise to fungitoxic DDC-ions which reduce disease development. The carboxymethyl group might then be regarded as a 'carrier' for the fungitoxic dimethyldithiocarbamate part of the molecule.

A quite different explanation is also possible. VAN RAALTE *et al.* (1955) demonstrated that tomatoes treated with carboxymethyl-DDC showed strong epinastic curvatures suggesting growth-regulating activity. Further studies using the *Avena* cylinder test showed that carboxymethyl-DDC has indeed auxin activity (VAN DER KERK *et al.* 1955). The idea that the action of systemic compounds might be connected with their growth-regulating activity had been discussed already by DAVIS & DIMOND in 1953. Therefore, a study was started to investigate the connection between the systemic activity and growth-regulating activity of carboxymethyl-DDC and related compounds (VAN RAALTE *et al.* 1955; PLUIJGERS, 1959; PLUIJGERS & VAN DER KERK, 1961). The systemic activity appeared to be retained when the H-atoms of the C_1-atom of the carboxymethyl side chain were substituted by methyl groups, whereas the auxin activity was lost. According to VAN ANDEL (1962), however, this compound (1-methyl-1-carboxyethyl-DDC) has antiauxin activity, as demonstrated by its effect on the root growth of cucumber seedlings (table 14).

Substitution of only one H-atom by a methyl group did not seem to alter the auxin activity nor the systemic activity. ÅBERG (1960) and VAN ANDEL (1962) demonstrated, however, that of the two optical isomers of this compound the (+) isomer acts as an auxin and the (–) isomer as an antiauxin. Systemic activity was only found with the (–) isomer (table 14).

These results supported the view that the mode of systemic action of carboxymethyl-DDC and related compounds might depend on an interference with the auxin metabolism resulting in an increase of resistance against the pathogen.

In view of the results described in chapter II, however, it seemed of interest to investigate more in detail the explanation given by VAN DER KERK (1956). If, DDC-ions are liberated they will be converted into DDC-alanine and DDC-

TABLE 14. Systemic activity and growth-regulating activity of some dimethyldithiocarbamates according to VAN ANDEL (1962).

Name	Abbreviation	Formula	Growth-regulating activity		
			Auxin activity[1]	Antiauxin activity[2]	Systemic activity[3]
S-carboxymethyl-N,N-dimethyldithiocarbamate	carboxymethyl-DDC	$(CH_3)_2N\text{-}C(=S)\text{-}S\text{-}CH_2COOH$	+	−	+
(+) S-(1-carboxyethyl)-N, N-dimethyldithiocarbamate	(+) 1-carboxyethyl-DDC	$(CH_3)_2N\text{-}C(=S)\text{-}S\text{-}CH(CH_3)COOH$	+	−	−
(−) S-(1-carboxyethyl)-N, N-dimethyldithiocarbamate	(−) 1-carboxyethyl-DDC	$(CH_3)_2N\text{-}C(=S)\text{-}S\text{-}CH(CH_3)COOH$	−	+	+
S-(1-methyl-1-carboxyethyl)-N, N-dimethyldithiocarbamate	1-methyl-1-carboxyethyl-DDC	$(CH_3)_2N\text{-}C(=S)\text{-}S\text{-}C(CH_3)_2COOH$	−	+	+

[1] Tomato epinasty test.
[2] Root growth test with cucumber.
[3] Against *Cladosporium cucumerinum*.

glucoside. This reasoning may also hold true for 1-carboxyethyl-DDC and for 1-methyl-1-carboxyethyl-DDC. In fact preliminary experiments showed that these compounds do give rise to the formation of DDC-alanine and DDC-glucoside in cucumber seedlings. Since the presence of fungitoxic DDC-alanine in plants might explain the systemic activity of the non-fungitoxic dimethyldithiocarbamates, the fate of the latter compounds in cucumbers was investigated quantitatively. The results are described in this chapter.

2. Materials and methods

a. Qualitative and quantitative determination of carboxymethyl-DDC and related compounds on chromatograms

Carboxymethyl-DDC, 1-carboxyethyl-DDC and 1-methyl-1-carboxyethyl-DDC can be demonstrated on chromatograms in exactly the same way as described for DDC-glucoside in chapter II (Section 3b). In this case UV irradiation leads also to a liberation of DDC-ions. In the presence of copper ions they form $CuDDC_2$ which can be detected after incubation with *Glomerella cingulata*. Table 15 summarizes the R_F values and the minimum amounts of the compounds which can be detected on chromatograms without or with UV irradiation in presence of copper sulphate (10 ppm).

For the quantitative determination of the concentrations of the compounds in plant sap we followed the same method as described for DDC-glucoside in

Table 15. R_F values of some slightly fungitoxic dimethyldithiocarbamates and minimum amounts which cause just visible inhibition zones on chromatograms. Solvent-mixture descending propanol-water (85:15, v/v) for 16 hours.

	R_F value	Minimum amount (μg) which can be detected with the paper-chromatographic bioassay	
		without UV irradiation	with UV irradiation
Carboxymethyl-DDC	0.37	20	0.5
1-carboxyethyl-DDC	0.50	20	0.3
1-methyl-1-carboxyethyl-DDC	0.66	10	0.4

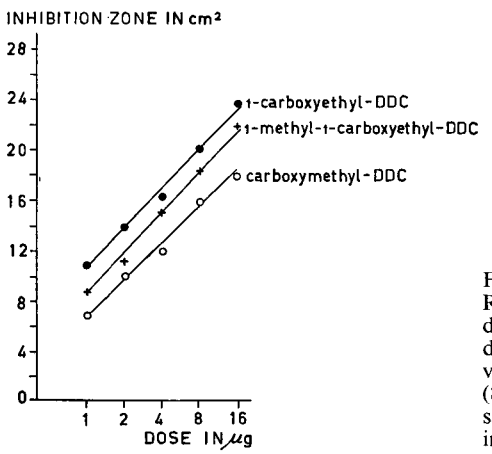


Fig. 16.
Regression of areas of inhibition zones on doses of some growth-regulating dimethyldithiocarbamates. Doses on log scale. Solvent-mixture descending propanol-water (85:15, v/v). Paper-chromatographic bioassay with *Glomerella cingulata* after UV light irradiation of the strips for 5 minutes.

chapter III. The standard lines of carboxymethyl-DDC, 1-carboxyethyl-DDC and 1-methyl-1-carboxyethyl-DDC are given in fig. 16. For y = 14 the coefficients of variation were found to be 15.4, 10.1 and 13.2%, respectively.

b. Test for evaluating the systemic activity

The systemic activity of the compounds is evaluated with the *C. cucumerinum* test as described in chapter IV.

3. Results

a. The fate of carboxymethyl-DDC and related compounds in plants

The alcohol-soluble fraction of sap of the roots and the above-ground parts of cucumber seedlings contained DDC-alanine, DDC-glucoside, fungicide X and $CuDDC_2$ after uptake of carboxymethyl-DDC (250 ppm) for two days. Whereas for the detection of the former three transformation products only 0.05 ml of the fraction needed to be chromatographed, detection of $CuDDC_2$ required at least 0.5 ml of the fraction. The formation of $CuDDC_2$ suggests that carboxymethyl-DDC gives rise to DDC-ions which are converted into DDC-alanine, DDC-glucoside and fungicide X.

When slices of potato tubers were incubated for two days at 20° C under continuous aeration in a solution of 1000 ppm carboxymethyl-DDC the transformation products and $CuDDC_2$ could be detected in the same way. The fact that potato slices boiled for 5 minutes, did not yield these compounds after incubation with carboxymethyl-DDC strongly indicates that the compounds are formed enzymically.

1-carboxyethyl-DDC or 1-methyl-1-carboxyethyl-DDC (250 ppm) applied to the roots of cucumber seedlings were found to be transformed into the same compounds as carboxymethyl-DDC.

Addition to cucumber seedlings of some other non-fungitoxic dimethyldithiocarbamates of the type

$$(CH_3)_2N\text{-}\underset{\underset{S}{\|}}{C}\text{-}S\text{-}(CH_2)_nCOOH$$

in which n of the side-chain with the carboxylic group is two, three or four, yielded also DDC-alanine, DDC-glucoside, fungicide X and $CuDDC_2$.

It was thought of interest to compare quantitatively the fate of carboxymethyl-DDC, 1-carboxyethyl-DDC and 1-methyl-1-carboxyethyl-DDC with that of DL-DDC-alanine. For this reason equimolar concentrations of the different compounds (960 μM) were applied to cucumber seedlings in the same experiment. The results are calculated in ppm since the molecular weights of the compounds applied did not differ very much (table 16).

All compounds proved to be translocatable to the above-ground parts. On application of the non-fungitoxic compounds, however, lower concentrations of these compounds were recovered from sap than of DDC-alanine after uptake of this compound (table 16). It could be demonstrated also that in general the non-fungitoxic compounds yielded lower concentrations of DDC-glucoside in sap than after uptake of DDC-alanine. This raises the question whether there is a quantitative difference in the uptake of the compounds. For information on this point the decrease of the compounds in the solutions has been

protection. After uptake of the non-fungitoxic compounds sap contained about 30 ppm DDC-alanine. According to experiments described in chapter IV (fig. 12A) such a concentration gives only very little protection. Therefore it can be concluded that the systemic effect of the non-fungitoxic compounds is not caused by DDC-alanine in the plant. This conclusion is confirmed in experiments carried out with both isomers of 1-carboxyethyl-DDC.

VAN ANDEL (1962) showed that the (–) isomer of 1-carboxyethyl-DDC in contrast to the (+) isomer gives systemic protection (table 14). Plants treated with the same concentrations, yielded about the same concentrations in expressed sap (table 19).

Assuming that the formation of DDC-alanine after uptake of the (–) isomer plays a role in the systemic activity, a higher concentration of DDC-alanine must be expected in sap expressed from the above-ground parts of the seedlings treated with the (–) isomer than in those treated with the (+) isomer. As this was not found to be true (table 19) the hypothesis that the systemic activity of the racemic mixture and of the (–) isomer of 1-carboxyethyl-DDC is based on

TABLE 19. Systemic activity of isomers of 1-carboxyethyl-DDC against *Cladosporium cucumerinum* and concentration of these compounds and DDC-alanine in sap of cucumber seedlings. The isomers were kindly provided by Dr. M. MATELL at Göteborg, Sweden.

		Concentration of compounds in sap of above-ground parts of seedlings in ppm[3]	
Compound applied to the roots	Systemic activity[1]	DDC-alanine[2]	1-carboxyethyl-DDC
1-carboxyethyl-DDC (racemic mixture) .	+	44	23
(+) isomer . . .	–	35	19
(–) isomer . . .	+	36	18

[1] For each object ten seedlings in 193 ppm (40 ml) of the compound for two days. Data according to VAN ANDEL (1962).
[2] Calculated as the DL-form.
[3] For each object ten seedlings in 250 ppm (40 ml) of the compound for two days. Concentration determined directly after the uptake period.

the presence of fungitoxic DDC-alanine formed in the plant must be rejected. It can be safely assumed that the hypothesis neither holds true for carboxymethyl-DDC nor for 1-methyl-1-carboxyethyl-DDC, because sap of plants contained about the same concentration of DDC-alanine after uptake of these compounds as after uptake of 1-carboxyethyl-DDC (table 18). The conclusion can be drawn that an interference of the non-fungitoxic compounds with host metabolism resulting in an increase of resistance is far more probable.

To investigate whether or not the hyphae have been killed within the tissues, cucumber seedlings protected by a treatment with 250 ppm carboxymethyl-DDC were transferred to a Hoagland solution 6 days after inoculation with *C cucumerinum*. Disease development was found to be checked, but forty day later living mycelium of the pathogen has been isolated frequently fro several parts of the hypocotyls. As in the case with DDC-alanine (chapter I

in cross sections lesions surrounded by dividing cells and hyphae were visible within the cortex.

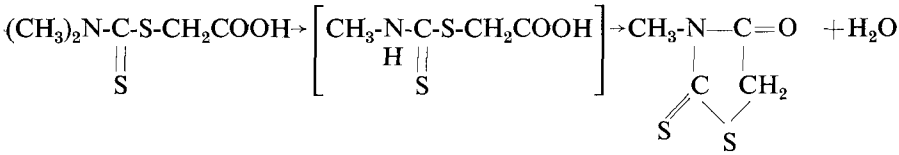
Similar to our findings with DDC-alanine the systemic protection of carboxymethyl-DDC against *C. cucumerinum* proved to be persistent whereas an attack by *C. lagenarium* could only be delayed.

Finally we will draw attention to the auxin activity of carboxymethyl-DDC and 1-carboxyethyl-DDC on cucumbers. Although cucumber seedlings are less sensitive than tomatoes, a close examination shows downward curvatures of the cotyledons when a concentration of 960 μM is applied to roots. Seedlings treated with the same concentration of DL-DDC-alanine or 1-methyl-1-carboxyethyl-DDC in the same experiment did not show this effect. Furthermore, inhibition of growth and morphological abnormalities, including a thickening of the leaf petiole were visible on the oldest leaves of cucumber plants which had been treated with carboxymethyl-DDC during the seedling stage (Plate IVB). On a cross section such a petiole has the form of a horseshoe which reminds of the response of plants treated with growth-regulating substances (BEAL, 1951). Morphological abnormalities were also caused by DL-DDC-alanine (Plate IVA). In this case, however, a thickening of the leaf petiole was not observed.

4. DISCUSSION

Proof has been given in this chapter that carboxymethyl-DDC and related non-fungitoxic dimethyldithiocarbamates are converted into fungitoxic DDC-alanine and fungicide X by cucumber seedlings. DDC-glucoside is formed as well. The results indicated that formation of the three compounds is preceded by an enzymic liberation of DDC-ions from carboxymethyl-DDC, 1-carboxyethyl-DDC and 1-methyl-1-carboxyethyl-DDC. This was also found to be true if the side chain with the carboxylic group bears two, three or four CH_2-groups. It is interesting to note that PLUIJGERS & VAN DER KERK (1961) and GARRAWAY & WAIN (1962) found β-oxidation in this side chain. Whether β-oxidation precedes the formation of DDC-ions or whether DDC-ions are liberated directly is not known.

As shown in table 17 the ratios between the amount recovered in the plants and the amount of carboxymethyl-DDC, 1-carboxyethyl-DDC and 1-methyl-1-carboxyethyl-DDC taken up are lower than that in the case of DL-DDC-alanine. It is not known whether the differences between the ratios would be affected very much if fungicide X could be involved in the calculation. The differences might be caused also by another type of conversion of carboxymethyl-DDC and related compounds as was found by KASLANDER in broad beans and cucumbers (VAN DER KERK, 1961). Demethylation of one of the methyl groups of the dimethylamino group occurs, possibly by enzymic oxidation. This is followed by ring closure which leads to the formation of the non-fungitoxic N-methylrhodanine:



An analogous conversion has been demonstrated for 1-carboxyethyl-DDC but not for 1-methyl-1-carboxyethyl-DDC.

According to results which will soon be published by KASLANDER the amount of rhodanine is about one third of the amount of carboxymethyl-DDC which is present in broad beans. If we assume the same ratio in cucumber seedlings a calculation shows that the ratio of carboxymethyl-DDC which can be recovered will not exceed about 13%. Thus rhodanine formation only explains to some extent the difference between the ratio recovered after uptake of carboxymethyl-DDC and of DDC-alanine. The same conclusion holds true for 1-carboxyethyl-DDC, but not for 1-methyl-1-carboxyethyl-DDC since for this compound rhodanine formation was not found by KASLANDER.

The differences might be due to the formation of unknown compounds, but a quite different explanation may also be given. It is often stated that growth-substances, for instance IAA and naphthalene acetic acid, are strongly bound to proteins (SIEGEL & GALTSON, 1953; GUPTA & SEN, 1962). In general the growth-substances can be liberated by proteolytic enzymes or by alkaline hydrolysis (GORDON, 1946). Although evidence is completely lacking it may be possible that growth-regulating dimethyldithiocarbamates are bound to proteins in the plants.

In view of this it might be worthwhile to subject homogenates of plants treated with growth-regulating dimethyldithiocarbamates, to proteolytic hydrolysis or to alkaline hydrolysis before application of paper chromatography.

Let us turn now to the systemic activity of carboxymethyl-DDC, 1-carboxyethyl-DDC and 1-methyl-1-carboxyethyl-DDC against *C. cucumerinum*. Table 18 showed that in comparison with seedlings which have taken up DL-DDC-alanine, those treated with the non-fungitoxic compounds contain 8–10 times less DDC-alanine whereas they show nearly the same degree of protection. Therefore it was concluded that the systemic action of the non-fungitoxic compounds is not caused by fungitoxic DDC-alanine formed in the plant. Yet, against this conclusion one objection can be made. In sap all cell contents are pooled. The micro-distribution of DDC-alanine in the plants after uptake of this compound and of the non-fungitoxic compounds has not been investigated.

Plants treated with the same concentration of the (+) and (–) isomer of 1-carboxyethyl-DDC yielded the same concentration of these compounds in sap (table 19). Therefore it may be assumed that the micro-distribution of the isomers in the plants did not differ very much. Neither, in this experiment a correlation was found between the degree of protection and a concentration of DDC-alanine in sap of seedlings treated with the (+) or the (–) isomer.

The results presented are incompatible with the hypothesis put forward in the introduction of this chapter that the systemic effect of the non-fungitoxic compounds might be caused by fungitoxic DDC-alanine formed in the plant. In search for another explanation one might assume that the compounds act by an interference with host metabolism by which development of the pathogen is inhibited. In this connection attention must be paid to the lowering of the sugar level in cucumber seedlings after uptake of carboxymethyl-DDC. KAARS SIJPESTEIJN (1961) considers it possible that the decrease of the sugar level might influence susceptibility. VAN ANDEL (1962) offered a very different explanation for the systemic action of the non-fungitoxic growth-regulating dimethyldithiocarbamates. She suggested that the compounds by their auxin

or anti-auxin activity interfere with the action of IAA resulting in a change of composition of the cell wall which in turn affects fungus infection.

In conclusion it must be stated that in comparison with the mechanism of the systemic action of DDC-alanine the mechanism of carboxymethyl-DDC and related compounds is far from elucidated. The only thing known with certainty is that, although infection by *C. cucumerinum* is checked, not all hyphae are killed within the tissues after uptake of either carboxymethyl-DDC or DDC-alanine by cucumber seedlings. Restriction of disease development, however, is reached in different ways by both compounds. In the case of DDC-alanine a toxic action of the compound on the hyphae was found while in the case of carboxymethyl-DDC protection is obtained in an indirect way, most probably by increasing the resistance of the host.

5. Summary

Uptake and transformation of carboxymethyl-DDC and some other non-fungitoxic growth-regulating dimethyldithiocarbamates in cucumber seedlings have been investigated. The compounds applied can be detected after UV irradiation and incubation with *Glomerella cingulata* of chromatograms of sap of the above-ground parts. The compounds were found to be converted into DDC-alanine, DDC-glucoside and fungicide X.

It is most unlikely that the mechanism of the systemic action of carboxymethyl-DDC and related compounds against *Cladosporium cucumerinum* is based on DDC-alanine formed in the seedlings because the level of this compound is far too low. The mechanism of the non-fungitoxic, growth-regulating compounds is attributed to an interference with host metabolism resulting in an increase of resistance of the plant.

GENERAL DISCUSSION

The experiments reported in the preceding chapters yielded information on the systemic action of NaDDC and related compounds against *Cladosporium cucumerinum* on cucumber plants. Evidence has been given for the view that the mechanism of checking the infection by NaDDC and DDC-glucoside is based mainly on the presence of their transformation product DDC-alanine within the plant.

GROSSMANN (1957) showed a slight systemic activity against *Fusarium oxysporum* on tomatoes after root uptake of TMTD. He was, however, not able to detect a fungitoxic compound in the guttation fluid of these plants by the paper-disk bioassay. Therefore the systemic activity was assumed to be effected by interference with host metabolism. Although this conclusion cannot be completely rejected, it may be asked in the light of the data presented in chapter II whether the guttation fluid in fact did not contain DDC-alanine. Moreover, according to VAN DIE (1961) guttation fluid of tomatoes may contain amino acids and, as shown in my experiments (chapter IV), amino acids mask the fungitoxicity of DDC-alanine when the paper-disk bioassay is used.

CORBAZ (1962) in a recent publication showed systemic activity of NaDDC against *Peronospora tabacina* on tobacco. Although we did not work with this pathogen, it is highly probable that the effect is caused by DDC-alanine, since this compound has been demonstrated in sap of tobacco plants after spraying NaDDC on the leaves (chapter II).

Considering the different types of systemic compounds discussed in the General Introduction, DDC-alanine can be designated as a 'systemic fungicide' since the results presented in chapter IV strongly indicate a toxic action on the pathogen within the plant. Whether DDC-alanine interferes also with host metabolism resulting in an increase of resistance of the plant against the pathogen cannot be established as there is no experimental evidence for this mode of action.

Non-fungitoxic compounds such as carboxymethyl-DDC and related compounds also give rise to DDC-alanine. Yet, the results did not indicate that protection against *C. cucumerinum* by these compounds is based on a toxic action of DDC-alanine on the pathogen, because the concentration of DDC-alanine in sap of the plants was too low. The compounds have growth-regulating properties and we already pointed to their possible action on host metabolism resulting in an increase of resistance of the plant. Systemic activity of non-fungitoxic, growth-regulating, compounds has been reported earlier by DAVIS & DIMOND (1953) and CORDEN & DIMOND (1959), but it must be emphasized that no proof has been obtained for a causal relationship between growth-regulating effects and systemic activities.

Although protection of cucumber seedlings against *C. cucumerinum* by DDC-alanine and the non-fungitoxic dimethyldithiocarbamates is obtained in totally different ways both have one property in common: in both cases not all penetrated hyphae are killed. The results suggest a retardation of growth of the pathogen. It is possible that the quantity of hyphae in the plant is reduced to a harmless level. On the other hand sensitivity of the host which is growing older decreases in the mean time. Whether growth of the hyphae in the tissues finally

has stopped or proceeded very slowly without causing much damage is not known. Growth of a pathogen within a plant without causing visible damage has also been reported by other authors. DEKKER (1957) found living mycelium of *Ascochyta pisi* in pea plants which were raised from infected seeds and on which no disease symptoms could be observed.

In general these investigations have shown that systemic control of diseases in mature plants is far from simple. A systemic compound may be transformed or its action may be antagonized by compounds in the plants. Moreover, they may be distributed unequally over stem, fully grown leaves and still growing leaves.

VOLGER (1959, 1960) obtained some very interesting results with seedlings of *Pinus silvestris* grown from seeds treated with TMTD or some other dimethyldithiocarbamates. The seedlings were less attacked by *Rhizoctonia spec.* and *Pythium spec.* than seedlings grown from untreated seeds. Phytotoxicity was not observed, and sap expressed from seedlings two weeks after treatment of the seeds was fungitoxic against *Botrytis cinerea in vitro.* We did not investigate transformation of NaDDC by gymnosperms but it can be assumed that the compounds penetrated into the seedlings and have been transformed into DDC-alanine resulting in a protection of the seedlings against soil-inhabiting fungi. Another example of effective systemic control against soil-inhabiting fungi has been given by LEACH, GARBER & TOLMSOLFF (1960). Sugar beet seedlings grown from seeds treated with the fungicide 'Dexon' (p-dimethylaminobenzenediazo sodium sulfonate) were protected against *Pythium ultimum* and against *Aphanomyces cochlioides.* Results obtained by HILLS (1962) indicate that Dexon can be taken up by the roots of sugar beet seedlings and is translocated in sufficient quantities to the hypocotyls to contribute to disease control.

In the light of our results with cucumber scab it is possible that the favourable results obtained by VOLGER and by LEACH *et al.* are due to the fact that in general seedlings are only temporarily sensitive to soil-inhabiting fungi. Therefore, the control of soil-inhabiting fungi may offer more prospects for practice than the systemic combat of fungi which attack young as well as older parts of fully grown plants. Up till now none of the organic synthetic systemic fungicides has reached the stage of practical application in the control of diseases of fully grown plants. Phytotoxicity always forms one of the most important obstacles for such application. This is certainly true for DDC-alanine and other dimethyldithiocarbamates which indicates that the compounds act both on metabolism of the host and on that of the pathogen. The search for more specific compounds is hampered, however, by the lack of knowledge on essential differences in fungal and plant metabolism. Therefore up till now the search for systemic compounds mostly has been one of trial and error.

Yet, those compounds which under experimental conditions display a certain systemic activity against fungi can lead to a better understanding of the metabolic processes which are involved in pathogenicity. In this way DEKKER (1962), working on the systemic activity of 6-azauracil against *Erysiphe cichoracearum* on cucumber plants, obtained evidence for an essential role of the ribonucleic acid synthesis in the relationship between cucumber and powdery mildew. GROSSMANN (1962), who found that rufianic acid acts systemically against *Fusarium oxysporum* on tomato plants, turns the attention to the role of pecto-

lytic enzymes of the pathogen during infection. Systemic activity of growth substances and their action on plant metabolism is another field for fruitful investigations on the interaction between pathogen and host (VAN ANDEL, 1962).

It is hoped that investigations of this type will contribute to the fundamental knowledge of the way in which the pathogen fights its way through the plant. This work may bring us a step nearer to new ways of controlling plant diseases. So far, it has taught us that selective interference in a complex of biological processes has its limitations.

SUMMARY

1. Investigations have been carried out to clarify the mechanism of the systemic action of sodium dimethyldithiocarbamate (NaDDC) and related compounds on *Cladosporium cucumerinum* after the compounds have been taken up by cucumber seedlings.

2. The paper-disk bioassay appeared to be inadequate for the detection of small amounts of NaDDC in sap of plants since sap contains compounds able to antagonize this fungicide.

3. A technique was developed by which NaDDC could be detected on chromatograms after incubation with a conidial suspension of *Glomerella cingulata* (paper-chromatographic bioassay). Small amounts of NaDDC in sap can be determined quantitatively by means of the paper-chromatographic bioassay, because this procedure leads to a separation of the fungicide from antagonists which are present in plant sap.

4. The paper-chromatographic bioassay and the work presented by KASLANDER *et al.* (1961, 1962) showed that plants transform enzymically DDC-ions into the L-alanine derivative (DDC-alanine), the β-glucoside (DDC-glucoside) and an unknown fungitoxic derivative (fungicide X) of dimethyldithiocarbamic acid. DDC-alanine is highly fungitoxic. Most probably this action is due to enzymic cleavage of the compound leading to the liberation of the fungitoxic DDC-ions.

5. DDC-glucoside is a weak fungicide. Therefore small amounts of this compound could not be detected on chromatograms. Chromatography of DDC-glucoside and subsequent UV irradiation of the strips in the presence of copper sulphate yielded, however, copper dimethyldithiocarbamate ($CuDDC_2$) which is highly fungitoxic to *G. cingulata*. In this way small amounts of DDC-glucoside have been demonstrated as $CuDDC_2$ by means of the paper-chromatographic bioassay.

6. DDC-glucoside and DDC-alanine were found to be interconvertible in plants.

7. By means of a quantitative paper-chromatographic bioassay it was found that about 50% of DL-DDC-alanine which disappeared from the solution surrounding the roots of cucumber seedlings could be recovered in the plants as such, as DDC-glucoside and as thiazolidine-2-thione-4-carboxylic acid (TTCA). TTCA is a non-fungitoxic decomposition product of DDC-alanine.

8. The amount of DDC-alanine present in cucumber seedlings after uptake decreased rather slowly during a period of three weeks. During this period the compound was partly converted into DDC-glucoside and TTCA.

9. L-DDC-alanine is responsible for the observed systemic activity of NaDDC and DDC-glucoside against *C. cucumerinum*. Most probably the protection is caused by the toxic action of DDC-alanine on the hyphae within the plant. As only young hypocotyls are susceptible to *C. cucumerinum* it is assumed that DDC-alanine either helps the seedlings to overcome a temporary stage of

natural sensitivity to this pathogen, or reduces the quantity of hyphae to a level which does not cause much damage.

10. The fact that DL-, D- as well as L-DDC-alanine cannot be used for practical application is due to the presence of L-amino acids in plants which antagonize the fungitoxicity of these compounds. Therefore the effective dose of DDC-alanine in plants for systemic control must be high. Unfortunately high amounts of DDC-alanine inhibit plant growth and induce morphological abnormalities (chapter IV).

11. An attempt was made to throw light on the mode of the systemic action of S-carboxymethyl-N, N-dimethyldithiocarbamate (carboxymethyl-DDC) and related non-fungitoxic, growth-regulating compounds against cucumber scab. Although the compounds are non-fungitoxic they can easily be demonstrated on chromatograms after UV irradiation in the presence of copper sulphate. In a manner similar to that found for DDC-glucoside the formed $CuDDC_2$ can be demonstrated on the strips after incubation with *G. cingulata.*

12. Carboxymethyl-DDC and related compounds are transformed into DDC-alanine, DDC-glucoside and the fungicide X by plants. Thus NaDDC as well as carboxymethyl-DDC are converted into DDC-alanine. It is, however, most unlikely that the mechanism of the systemic action of carboxymethyl-DDC and related compounds against *C. cucumerinum* is based on DDC-alanine formed in cucumber seedlings, because the level of this fungitoxic compound is far too low. It is assumed that the systemic action of the non-fungitoxic growth-regulating dimethyldithiocarbamates is caused by interference with host metabolism which results in an increase of the resistance of the plant against the pathogen.

SAMENVATTING

De systemische werking van dimethyldithiocarbaminaten op het vruchtvuur van de komkommer veroorzaakt door Cladosporium cucumerinum en de omzetting van deze verbindingen door planten

INLEIDING

Voor Nederlandse literatuur over de ontwikkeling van bestrijdingsmiddelen die door de plant worden opgenomen en getransporteerd wordt de lezer verwezen naar artikelen van GROSJEAN (1950); VAN RAALTE (1952); OORT (1959, 1963); KAARS SIJPESTEIJN (1960) en DEKKER (1963). Men spreekt hierbij van 'systemische bestrijding' van planteziekten waarbij men gebruik maakt van 'systemica' of 'systemische middelen'.

De aanleiding tot het onderzoek vormden de literatuurgegevens over de systemische werking van natrium dimethyldithiocarbaminaat (NaDDC) en verwante verbindingen tegen het vruchtvuur van de komkommer veroorzaakt door *Cladosporium cucumerinum* en tegen enkele andere schimmelziekten. Om meer inzicht te krijgen in het mechanisme van deze systemische werking werd de opname, het transport en het lot van deze stoffen in de planten nader onderzocht. Er werd een speciale papierchromatografische methode ontwikkeld waardoor het mogelijk is kwantitatief fungicide verbindingen in plantesap te bepalen.

Tenzij anders aangeduid is er gewerkt met komkommerkiemplanten. Toediening van de stof heeft altijd in water plaats gevonden. Hiertoe werden de kiemplanten gedurende 2 dagen in de oplossing geplaatst.

HOOFDSTUK I

HET AANTONEN VAN NaDDC IN SAP VAN KOMKOMMERKIEMPLANTEN

De systemische werking van NaDDC zou kunnen berusten op de aanwezigheid van fungitoxische dimethyldithiocarbaminaat ionen (DDC-ionen) in de plant waardoor mycelium van het pathogeen gedood of in zijn ontwikkeling geremd wordt. Getracht werd DDC-ionen direct in plantesap aan te tonen met behulp van een agarplaat methode die de 'paper-disk bioassay' werd genoemd. Hoewel *Glomerella cingulata* zeer gevoelig is voor DDC-ionen werd met deze methode in perssap van met NaDDC behandelde planten geen stof gevonden die fungitoxisch is voor deze schimmel. Deze methode is echter weinig geschikt gebleken voor het aantonen van lage DDC-ionen concentraties in perssap van de plant aangezien de fungitoxiciteit van DDC-ionen ten opzichte van *G. cingulata* vermindert door het toedienen van perssap van onbehandelde planten (Plaat I A). Het zelfde verschijnsel werd gevonden indien in plaats van agar, papier werd gebruikt als medium waarop de conidiën van *G. cingulata* bij aanwezigheid van voedingsstoffen zich konden ontwikkelen (tabel 1). Deze methode werd de 'paper-spot bioassay' genoemd.

De vermindering van de fungitoxiciteit van NaDDC door plantesap wordt in de eerste plaats veroorzaakt door de aanwezigheid van stoffen zoals b.v. histidine en cysteine die de fungitoxische werking van NaDDC antagoneren.

In de tweede plaats belemmeren eiwitten en andere sapbestanddelen de diffusie van DDC-ionen in het medium.

Deze moeilijkheden bij een directe bepaling van DDC-ionen in sap kunnen opgeheven worden door eerst eiwitten neer te slaan met aethanol en dan de bovenstaande vloeistof te chromatograferen (tabel 2). DDC-ionen, in de vorm van het fungitoxische koper dimethyldithiocarbaminaat ($CuDDC_2$), kunnen dan op de chromatogrammen worden aangetoond door conidiën van *G. cingulata* op het papier te spuiten ('paper-chromatographic bioassay'). Tijdens het chromatograferen worden de DDC-ionen gescheiden van hun antagonisten in het perssap. Dit kon duidelijk aangetoond worden door sap van onbehandelde planten te chromatograferen en een geringe hoeveelheid NaDDC aan de sporensuspensie toe te voegen. Hierdoor wordt de ontwikkeling van het mycelium op het papier geremd, behalve op die plaatsen waar stoffen aanwezig zijn die de fungitoxische werking van NaDDC antagoneren (Plaat IC).

In tabel 4 komt de gevoeligheid van de drie methoden bij het aantonen van NaDDC in water of in sap tot uiting.

HOOFDSTUK II

DE OMZETTING VAN DIALKYLDITHIOCARBAMINATEN IN ANDERE FUNGITOXISCHE VERBINDINGEN DOOR PLANTEN

Verrassende resultaten werden verkregen na het uitpersen en chromatograferen van komkommerkiemplanten die met de wortels gedurende twee dagen in een waterige oplossing van NaDDC stonden. NaDDC, in de vorm van $CuDDC_2$, werd slechts in zeer geringe hoeveelheden in de bovengrondse delen gevonden. Er bleken echter wel drie fungitoxische omzettingsprodukten aanwezig te zijn (Plaat IB). Twee van deze verbindingen werden geïdentificeerd als het glucosederivaat (DDC-glucoside) en het alaninederivaat (DDC-alanine) van dimethyldithiocarbamine zuur (KASLANDER *et al.*, 1961; 1962). De derde fungitoxische verbinding aangeduid met 'fungicide X' is nog niet in zuivere toestand verkregen. DDC-alanine werd door KASLANDER nader geïdentificeerd als de L-vorm. Bovendien werd aangetoond dat DDC-alanine kan worden omgezet in het niet-fungitoxische thiazolidine-2-thion-4-carbonzuur (TTCA). In tegenstelling tot de eerste twee omzettingen verloopt de laatste niet enzymatisch (KASLANDER *et al.*, 1962).

DDC-glucoside is in vergelijking tot DDC-alanine weinig fungitoxisch en kan dus alleen bij aanwezigheid van relatief grote hoeveelheden op het chromatogram aangetoond worden. Relatief kleine hoeveelheden DDC-glucoside kunnen echter wel aangetoond worden wanneer na het chromatograferen de stroken worden bespoten met kopersulfaat (10 mg/ml). Door nu deze stroken met UV licht te bestralen worden de DDC-glucoside moleculen gesplitst. De vrijkomende DDC-ionen vormen met de aanwezige koper-ionen het meer fungitoxische $CuDDC_2$ dat vervolgens door bespuiten met *G. cingulata* kan worden aangetoond. De vorming van $CuDDC_2$ is volgens fig. 1 optimaal na een belichtingsperiode van vijf minuten.

DDC-glucoside, maar niet het TTCA, wordt na toediening aan de wortels omgezet in DDC-alanine (fig. 2). Diethyldithiocarbaminaat-ionen worden waarschijnlijk ook omgezet in hun alaninederivaat en hun glucoside.

Volgens onderzoek van KAARS SIJPESTEIJN *et al.* (1962, 1963) worden DDC-ionen in micro-organismen niet omgezet in DDC-alanine, maar in het α-aminoboterzuurderivaat van dimethyldithiocarbaminaat (DDC-α-aminoboterzuur). Toevoeging van deze stof aan planten leidt tot de vorming van DDC-alanine, DDC-glucoside, fungicide X en van $CuDDC_2$. In fig. 4 zijn de verschillende omzettingen van DDC-ionen in planten en schimmels samengevat.

HOOFDSTUK III

EEN KWANTITATIEF ONDERZOEK OVER HET LOT VAN DL-DDC-ALANINE IN KOMKOMMERKIEMPLANTEN

Gezien de uiterst geringe hoeveelheden DDC-ionen in perssap van komkommerkiemplanten na opname van NaDDC was het waarschijnlijk dat de systemische werking van deze stof niet berust op een directe werking van de opgenomen DDC-ionen op het binnendringende mycelium maar op die van de fungitoxische omzettingsprodukten. Waarschijnlijk speelt L-DDC-alanine hierbij de belangrijkste rol want DDC-glucoside is een zwak fungicide terwijl fungicide X in vergelijking met L-DDC-alanine slechts een zeer kleine remmingszone veroorzaakt (Plaat I B).

Het leek daarom interessant kwantitatief de opname, verdeling en afbraak van L-DDC-alanine in de plant verder na te gaan. We beschikten echter niet over zuiver L-DDC-alanine maar wel over D- en DL-DDC-alanine. De fungitoxiciteit van L- en DL-DDC-alanine verschilt weinig van elkaar. Daarom werd het lot van het racemische mengsel kwantitatief nader onderzocht. Hiertoe werden verschillende hoeveelheden perssap en bekende hoeveelheden DL-DDC-alanine en DDC-glucoside tegelijkertijd gechromatografeerd. De oppervlakken van de remmingszones werden gemeten met een planimeter. Door vergelijking van de oppervlakken kan de hoeveelheid DDC-alanine of DDC-glucoside in het sap bepaald worden (fig. 6A, B en 7A, B).

Door een mengsel van jodium en natrium azide op de chromatogrammen te spuiten kan op de zelfde wijze de hoeveelheid TTCA in perssap bepaald worden (fig. 8 A, B).

Van het toegediende DL-DDC-alanine kon direct na de opname periode ongeveer 50% teruggevonden worden in de planten als DDC-alanine, DDC-glucoside en als TTCA (fig. 9 en tabel 7).

De hoeveelheid DDC-alanine in de planten werd ook na de opname periode bepaald. Deze bleek in de loop van drie weken af te nemen (fig. 10, 11 en tabel 8). Gedurende deze periode waarin de planten in een Hoagland oplossing stonden werd DDC-alanine omgezet in DDC-glucoside en TTCA. Bovendien diffundeerde een kleine hoeveelheid DDC-alanine vanuit de wortels in de Hoagland oplossing. Tabel 9 laat de verdeling van DDC-alanine en DDC-glucoside in de wortels, hypocotylen en cotylen zien na opname van DL-DDC-alanine.

HOOFDSTUK IV

DE SYSTEMISCHE WERKING VAN NaDDC, DDC-ALANINE EN DDC-GLUCOSIDE OP ENIGE PLANTEZIEKTEN

L-, D- en DL-DDC-alanine zijn systemisch werkzaam tegen *C. cucumerinum*. Het racemische mengsel reduceert de ziekteaantasting sterker maar is ook phytotoxischer dan D-DDC-alanine.

Er werd een duidelijke correlatie gevonden tussen de mate van bescherming na opname van NaDDC, DDC-glucoside of van DL-DDC-alanine en de hoeveelheid gevormd DDC-alanine in sap van hypocotylen en cotylen (Plaat IIA, tabel 10 en fig. 12A). Een dergelijke correlatie werd niet gevonden tussen de systemische werking van die verbindingen en de hoeveelheid gevormd DDC-glucoside (fig. 12B). Aangezien de fungitoxiciteit van L- en DL-DDC-alanine weinig van elkaar verschilt is het zeer waarschijnlijk dat de systemische werking van NaDDC en DDC-glucoside berust op de uit deze verbindingen gevormde L-DDC-alanine.

Verder bleek dat de betrekking tussen de concentratiereeks van DDC-alanine in sap van behandelde planten en de mate van bescherming binnen zekere grenzen overeenkomt met de betrekking tussen de zelfde concentratiereeks en de groeiremming van *C. cucumerinum* in perssap *in vitro* (fig. 12A en 13). Dit resultaat maakt het waarschijnlijk dat de bescherming veroorzaakt door DDC-alanine en dus ook door NaDDC en DDC-glucoside berust op een toxische werking van DDC-alanine op het binnendringende pathogeen.

De fungitoxiciteit van DL-DDC-alanine tegen *C. cucumerinum* in perssap *in vitro* is veel geringer dan in een minimaal medium. Dit kan verklaard worden door het feit dat L-aminozuren de fungitoxiciteit van DDC-alanine antagoneren (tabel 11 en 13).

Hoewel DL-DDC-alanine de komkommerkiemplanten zeer goed beschermt tegen een aantasting door *C. cucumerinum* kon anatomisch mycelium worden aangetoond in de hypocotylen van komkommerplanten die deze verbinding in het kiemplantstadium hadden opgenomen en daarna geïnoculeerd (Plaat III).

Door de hypocotylen van deze planten uit te leggen op agarplaten bleek dat er zelfs 60 dagen na inoculatie nog levend mycelium in de weefsels aanwezig was.

Enerzijds is het mogelijk dat de hoeveelheid schimmel in de plant door de DDC-alanine verschoven wordt naar een niveau dat niet schadelijk is voor de plant. Anderzijds moet rekening gehouden worden met het feit dat alleen jonge delen van komkommerplanten vatbaar zijn voor *C. cucumerinum*. In dit verband kan dus gedacht worden aan een vertraging van de myceliumgroei in de hypocotylen door het aanwezige DDC-alanine en een gelijktijdig ouder en dus minder vatbaar worden van deze weefsels (tabel 12). Dit verklaart ook waarom DL-DDC-alanine na opname door komkommerkiemplanten een aantasting door *Colletotrichum lagenarium* alleen maar vertraagt. In dat geval wordt de myceliumgroei wel geremd, maar omdat hierbij geen 'ouderdomsresistentie' optreedt en de hoeveelheid DDC-alanine afneemt in de loop van de tijd wordt de plant tenslotte toch gedood.

Vooral in jonge groeiende delen van komkommer respectievelijk boon (*Phaseolus vulgaris*) wordt de ontwikkeling van *C. lagenarium* en *Erysiphe*

cichoracearum, respectievelijk *Colletotrichum lindemuthianum* geremd na wortelopname van DL-DDC-alanine (fig. 14 en 15).

Waarschijnlijk is juist in die delen de hoeveelheid DDC-alanine hoog genoeg om te kunnen concurreren met de aanwezige L-aminozuren.

Op grond van de antagonerende werking van L-aminozuren in de plant, maar vooral ook door het veroorzaken van groeiremmingen en morphologische veranderingen (Plaat II C en Plaat IV A) komt DDC-alanine niet in aanmerking van een praktische toepassing.

HOOFDSTUK V

DE SYSTEMISCHE WERKING VAN ENIGE GROEIREGULERENDE DIMETHYLDITHIOCARBAMINATEN OP *Cladosporium cucumerinum*

VAN RAALTE *et al.* (1955), PLUIJGERS (1959) en VAN ANDEL (1962) hebben aangetoond dat carboxymethyl-DDC en enige andere groeiregulerende, maar *in vitro* niet-fungitoxische dimethyldithiocarbaminaten, systemisch werkzaam zijn tegen *C. cucumerinum* (tabel 14). Naar aanleiding hiervan werd nagegaan of deze werkzaamheid berustte op de vorming van het fungitoxische DDC-alanine uit die verbindingen door de plant.

De toegediende verbindingen kunnen met behulp van papierchromatografie en UV-belichting op dezelfde wijze als DDC-glucoside worden bepaald (fig. 16, tabel 15 en 16). Gedeeltelijk worden ze omgezet in DDC-alanine, DDC-glucoside en het fungicide X (tabel 16).

De hoeveelheden van de opgenomen verbindingen die in de komkommerkiemplanten kunnen worden teruggevonden staan vermeld in tabel 17.

Het is zeer onwaarschijnlijk dat de systemische werking van carboxymethyl-DDC en verwante verbindingen berust op het gevormde DDC-alanine, omdat de hoeveelheid van de fungitoxische verbinding in de plant veel te laag is (tabel 18). De systemische werking van de verbindingen zou evenwel kunnen berusten op een verhoging van de resistentie van de plant waardoor de ontwikkeling van de parasiet vertraagd wordt. Hierbij kan gedacht worden aan een mogelijk causaal verband tussen de groeiregulerende werking van de verbindingen (zie Plaat IV B) en hun systemische werking.

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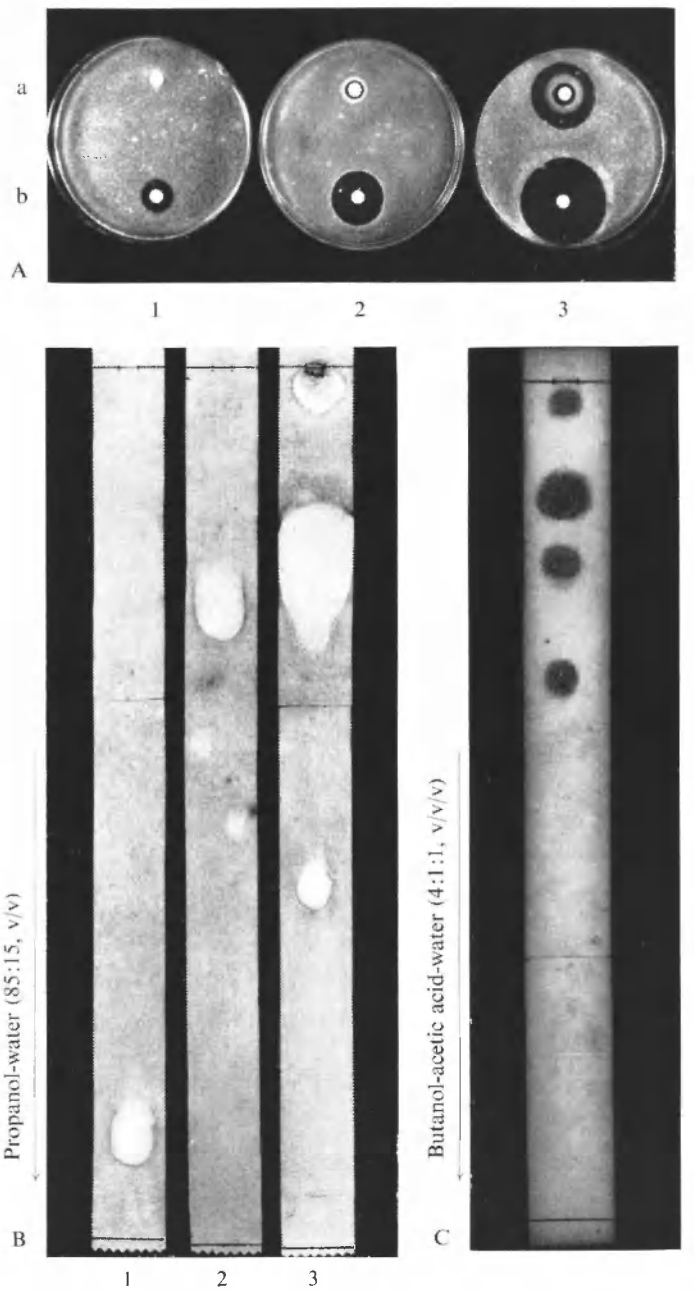
PLATE I

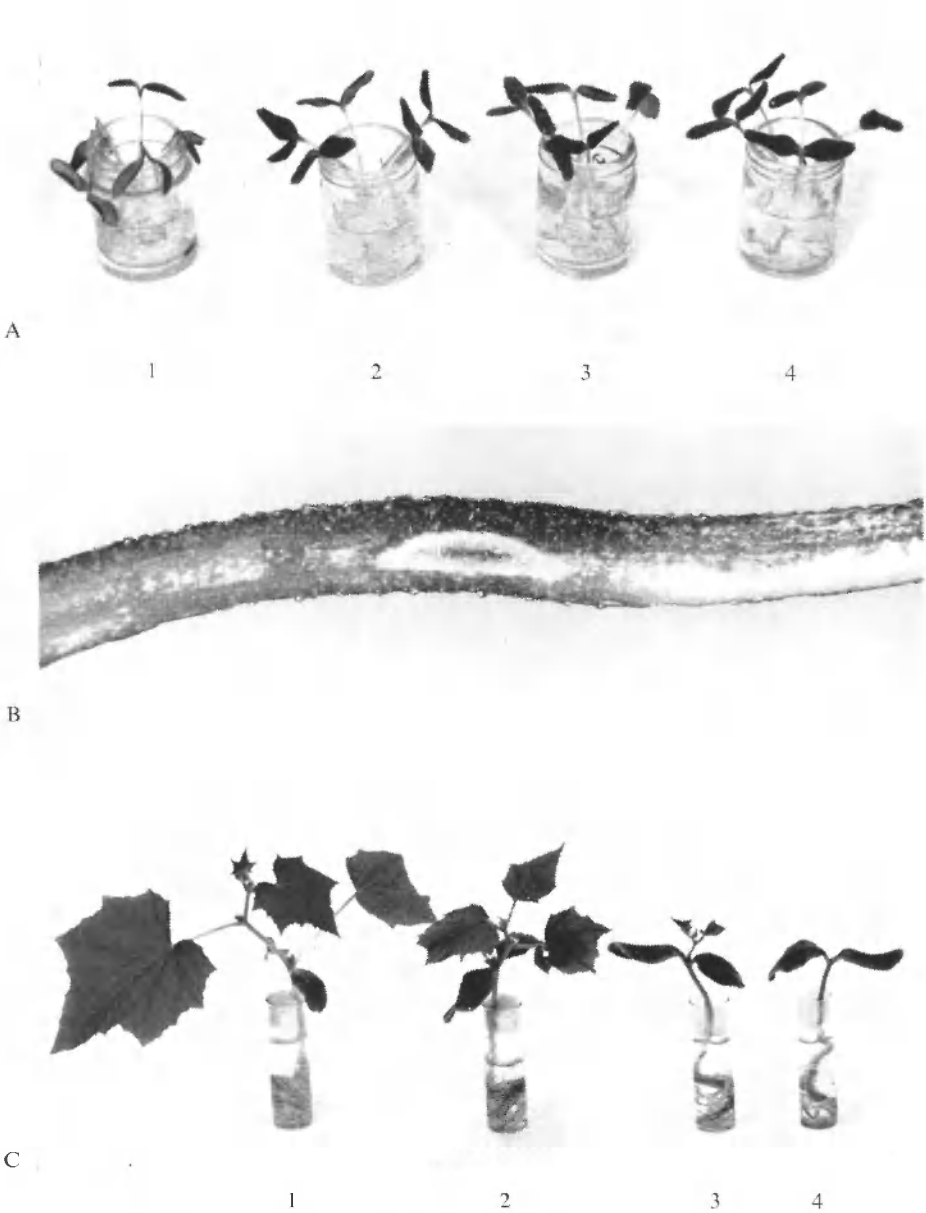


A. Difference between the fungitoxicity of NaDDC dissolved in water and in sap expressed from cucumber seedlings against *Glomerella cingulata*. Paper-disk bioassay. Paper-disks impregnated with 0.1 ml of the liquid. a: NaDDC dissolved in sap; b: NaDDC dissolved in water. 1: 10 ppm; 2: 20 ppm; 3: 50 ppm.

B. Demonstration of fungitoxic compounds by the paper-chromatographic bioassay. Test fungus *G. cingulata*. 1: 1 µg NaDDC; 2: 0.15 ml of the alcohol-soluble fraction of sap from hypocotyls and cotyledons of cucumber seedlings after uptake of NaDDC for two days by the roots (150 ppm); 3: 0.6 ml of the same fraction.

C. Demonstration of compounds in the alcohol-soluble fraction of sap (0.2 ml) of cucumber seedlings which antagonize the fungitoxicity of NaDDC. Zones of mycelial growth formed after the strip was incubated for two days with a conidial suspension of *G. cingulata* to which was added 0.5 ppm NaDDC.

PLATE II

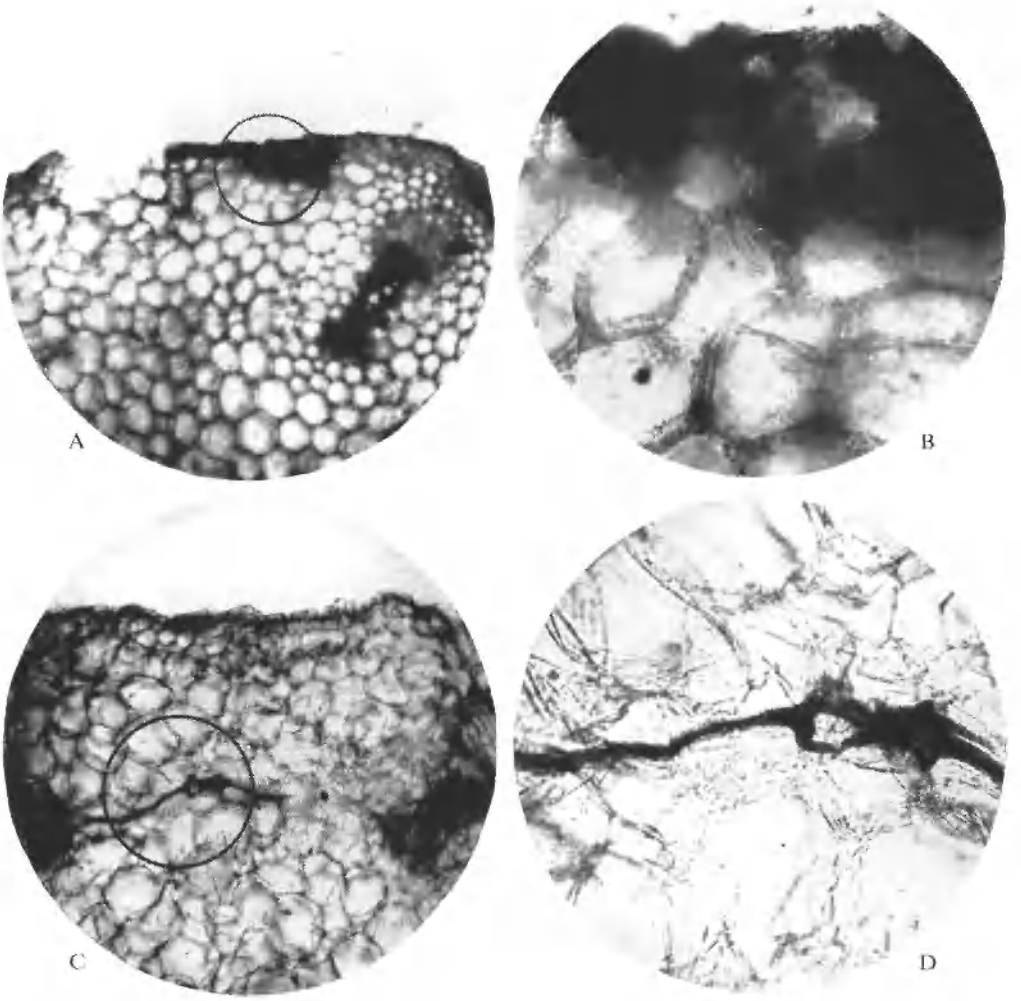


A. Systemic effect of NaDDC, DDC-glucoside and DL-DDC-alanine on *Cladosporium cucumerinum* on cucumber seedlings. Root application 1: water; 2: NaDDC (250 ppm); 3: DDC-glucoside (1000 ppm); 4: DL-DDC-alanine (50 ppm). Seedlings photographed 5 days after inoculation.

B. Necrotic spot caused by *C. cucumerinum* on a hypocotyl of a cucumber seedling. Seedling treated with 100 ppm DL-DDC-alanine for two days before inoculation. Seedling transferred to Hoagland solution 6 days after inoculation and photographed 18 days later. × 4.

C. Growth-inhibition of cucumber plants caused by DL-DDC-alanine. Seedling treated with DDC-alanine for two days, and transferred to Hoagland solution. Plants photographed 26 days after transfer. 1: untreated; 2: DDC-alanine (50 ppm); 3: DDC-alanine (100 ppm); 4: DDC-alanine (200 ppm).

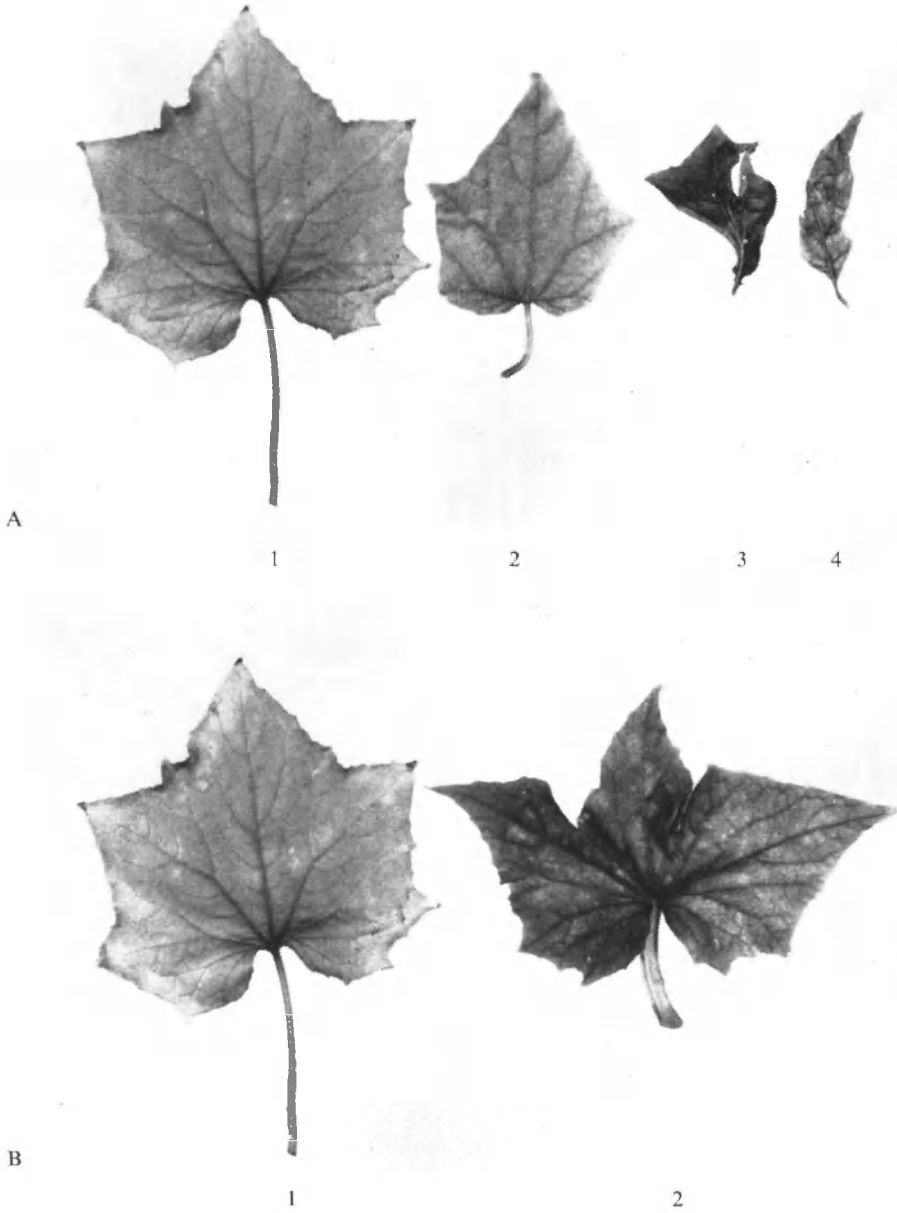
PLATE III



Cross section of a hypocotyl of a cucumber seedling treated with 100 ppm DL-DDC-alanine for two days before inoculation. Plants transferred to Hoagland solution 6 days after inoculation with *Cladosporium cucumerinum*. Section made 34 days later.

A. Lesion surrounded by dividing cells (enclosed by circle). × 60.
B. The same lesion at higher magnification. × 270. Mycelium was not clearly visible in this section.
C. Hyphae (enclosed by circle) stained with cotton blue-lactophenol, present in the parenchymatic tissue between two vascular bundles. × 60.
D. The same hyphae at higher magnification. × 240.

PLATE IV



Morphological abnormalities on the first leaf of cucumbers caused by DL-DDC-alanine and carboxymethyl-DDC. Seedlings treated for two days with the compounds and transferred to Hoagland solution. First leaves photographed 60 days after transfer.

A. Plants treated with DL-DDC-alanine. 1: untreated; 2: DDC-alanine (50 ppm); 3: DDC-alanine (100 ppm); 4: DDC-alanine (200 ppm).
B. Plants treated with carboxymethyl-DDC (250 ppm). 1: untreated; 2: treated.