Modifications Induced by Benomyl and Related Compounds Into Chloroplasts Spectral Patterns, Photosynthetic Rates and Chlorophyll Contents of *Spinacia oleracea* and *Cucumis melo*

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As some other derivatives of benzimidazole,benomyl has a considerable fungicidal activity which together with its systemic properties has led to its widespread use to control a variety of plant diseases.While the metabolic fate of benomyl in plants is already good documented(SIEGEL 1973,ROUCHAUD et al.1974),there are so far only a few reports dealing with the general effects of the fungicide on plant metabolism,although some particular data concerned with a cytokinin-like action(SCHRUFT 1971,THOMA 1973), morphogenetic malformations(FORSBERG 1969)and reduction of the phenylalanine-ammonia lyase(PAL)activity(SWINBURNE 1975)have been published.This situation is likely to be related to the fact that in most cases no or little phytotoxic effects are observed after treatments with benomyl although in some conditions,namely with particular plant species that are more sensitive to the fungicide or when an overdosis is applied,important phytotoxicity may happen.

In the present work,attempts were made to characterize the effects of benomyl and some metabolic related compounds on plant metabolism through chlorophyll contents determinations,photosynthetic activity and chloroplasts spectral patterns measurements.

EXPERIMENTAL

Material In glasshouse experiments,Cucumis melo(muskmelons) seedlings were grown in sterilized compost at 28°C by day with 16 hours of light(11000-13000 lux)and 24°C at night and a relative air humidity ranging between 60 and 90%.The treatments were applied when the plants had 4 leaves fully expanded.

In vitro experiments were carried out on chloroplasts isolated from Spinacea oleracea(spinach)or melon leaves by the procedure of ARNON et al.(1956).After purification by repeated centrifugations,the chloroplasts were resuspended into 10 ml NaCl 0.35M and these"stock"suspensions were kept in the dark at 4°C.

The fungicides and chemicals used in the assays were: benomyl:(methyl-1-butylcarbamoyl)-2-benzimidazole carbamate; carbendazim:methyl benzimidazol-2yl carbamate(MBC);benzimidazole and 2-aminobenzimidazole(2-AB).These compounds were of analytical grade when used for in vitro experiments with isolated chloroplasts.In glasshouse experiments,benomyl was used as a formulated fungicide(Benlate,50% a.i.).

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Chlorophyll determination Total chlorophyll was determined as chlorophylls a+b by the procedure of ARNON et al.(1949)by adding 4 volumes of ethanol to 1 volume of NaCl 0.35M chloroplasts suspensions.After 60 minutes extraction at room temperature,the suspensions were filtered on glass fibers filters(Gelman type GA) and the optical density at 652 nm of the filtrate was measured. To determine the chlorophyll contents of vegetal tissues from glasshouse experiments,fractions of 5 g(wet weight)were added with 45 ml NaCl 0.35M,the leaves being then homogenized with an omnimixer Sorvall at 4°C and 16000 rpm for 10 minutes.Fractions of 10 ml of the homogenate were added with 40 ml ethanol and chlorophylls were extracted for 60 minutes at 4°C before the samples being centrifuged at 10000 g for 10 minutes.The OD at 652 nm was finally determined on the supernatant.

Photosynthetic activity determinations The photosynthetic activity was measured polarographically with an YSI oxygraph on isolated chloroplasts by a Hill reaction derived from a procedure of SIEGENTHALER and VAUCHER(1971).All the determinations were carried out on a total volume of 5 ml with an incubation medium consisting of NaCl,35 x 10^{-3}M;Tris HCl pH 8.0,0.2 x 10^{-3}M;$MgCl_2$, 5 x 10^{-3}M;KH_2PO_4,5 x 10^{-3}M and $K_3Fe(CN)_6$,0.5 x 10^{-3}M.The final chlorophyll concentration was adjusted to 50 x 10^{-6}g/ml for all measurements.During the illuminated phase of the measurements, the suspensions of chloroplasts were illuminated with two 100 W tungsten lamps.By keeping the stock suspensions of chloroplasts in the dark at 4°C between the measurements,it was verified that their specific photosynthetic activity was rather stable up to 10 hours;in typical experiments,this specific activity ranged between 20 and 30 x 10^{-6}moles evolved O_2/hour x mg chlorophyll.

Spectral changes measurements The modifications of the absorption spectra of diluted chloroplasts suspensions used by INOUE et al.(1972)to characterize the effects of several herbicides was applied here in a simplified version.Before each measurement,aliquots were taken up from the chloroplasts stock suspensions and diluted with NaCl 0.35M to a final chlorophyll concentration ranging between 5 and 10 x 10^{-6}g/ml.These diluted samples were left standing at room temperature for 15 minutes under laboratory illumination conditions(1500-6000 lux)before to be used for spectrophotometry.Because the spectral variations were found to be the most important between 400 and 480 nm,namely at 440 nm,the OD change at this last wavelenght was retained in some assays to measure the spectral changes.With these standardized conditions,there was a progressive decrease of the chloroplasts absorption spectrum due to the ageing of the stock suspension; although this decrease kept rather slight(the OD variation at 440 nm was less than 5% after 8 hours),all the treatments within one experiment were made on suspensions with the same conservation time.

RESULTS

Experiments on melon plants The dosis of 150 mg benomyl/plant was retained for in vivo experiments where melon plants were treated because it rapidly induced phytotoxic effects.The treatment was done through Benlate aqueous suspension(3.5 mg/ml) and only one treatment was made each time on sets of 12 plants that were analyzed dayly from the 5th up to the 9th day after the fungicide was added.Within these conditions,no external symptoms of phytotoxicity could be detected up to the 5th day;from the 6th day,a slight marginal chlorosis of the leaves became visible and from the 8th day,some leaves displayed malformations and their edges begun to show necrotic lesions.

Since previous assays(ROUCHAUD et al.1974)have demonstrated that benomyl quickly concentrated in the leaves of drenched plants,only this fraction was analyzed for chlorophyll contents, chloroplasts absorption spectra and photosynthetic activity.

The determination of chlorophyll contents showed that there was a progressive decrease of the average chlorophyll contents for both the control and the treated plants but it appeared that this reduction was significantly enhanced by the fungicide from the 9th day following the treatment(Table I).

TABLE I

Effect of benomyl on chlorophyll contents of melon plant leaves

	mean chlorophyll contents(mg/g wet weight)	
number of days after treatment	control	benomyl(1 x 150 mg)
5	1.27 ± 0.26[a]	1.33 ± 0.14
6	1.36 ± 0.11	1.23 ± 0.19
7	1.42 ± 0.25	1.09 ± 0.17
8	0.92 ± 0.19	0.95 ± 0.09
9	1.01 ± 0.09	0.69 ± 0.06

[a]standard deviation(n=6)

When chloroplasts were isolated from treated plants,their specific photosynthetic activity was found to be markedly decreased by benomyl 5days after the treatment was begun although, because of the ageing of the control plants,this difference became still progressively less important when the duration of the treatment was prolonged(Table II).

TABLE II

Effect of benomyl on photosynthetic activity of chloroplasts

number of days after treatment	O_2 evolution (10^{-6} moles/hour x mg chlorophyll)	
	control	benomyl (1 x 150 mg)
5	32.57 ± 2.08[a]	23.56 ± 1.80
6	28.13 ± 2.66	24.50 ± 2.53
7	32.49 ± 1.52	25.79 ± 0.48
8	24.49 ± 3.87	22.95 ± 3.40
9	21.80 ± 2.07	22.50 ± 1.97

[a] standard deviation(n=6)

The most striking effect was that on the absorption spectrum of chloroplasts which was found very deeply modified by the fungicide;at the 5th day following the beginning of the treatment a general increase of the absorption pattern was observed that raised further up to the 7th day.After this time,a fast decrease of the spectrum was noticed resulting into negative differential spectra(Figure 1).

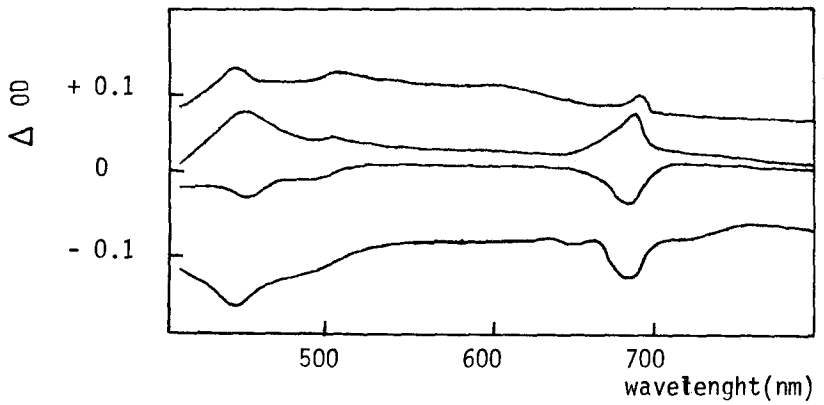

Fig.1.Difference spectra of chloroplasts isolated from control and melon plants treated with 150 mg benomyl/plant,5(A),7(B), 8(C)and 9(D)days after that the fungicide was added to the plants.The final chlorophyll concentration was 5 x 10^{-6}g/ml.

Assays on isolated chloroplasts In relation to the results obtained with treated plants,benomyl and its main metabolic conversion products in the plants vz.MBC,2-AB and benzimidazole,were

assayed in vitro for their effects on photosynthetic activity and chloroplasts absorption spectrum on isolated chloroplasts of S.oleracea.

For the Hill reaction measurements,the treatments were carried out in shaked conditions with a final 50 x 10^{-6} g/ml chlorophyll concentration at 22°C.After that the fungicide or the chemical was added,the chloroplasts suspensions were incubated in the dark for periods ranging between 30 minutes and 5 hours.Each 30 minutes,the photosynthetic activity was determined on 5 ml fractions of the suspensions.Treatments with concentrations ranging from 50 to 250 x 10^{-6}M demonstrated that neither the fungicide nor its metabolites influenced significantly the photosynthetic activity of chloroplasts up to concentrations of 200 x 10^{-6}M.At values of 250 x 10^{-6}M,the effect of benomyl remained rather weak,the most significant difference being due to 2-AB which induced a mean 30% decrease of the O_2 evolution rate of the chloroplasts;there was no effect due to MBC and benzimidazole (Table III).

TABLE III

Effect of benomyl and its metabolites on photosynthetic activity of isolated chloroplasts

treatment[a]	O_2 evolution (10^{-6}moles/hour x mg chlorophyll)
control	28.74 + 3.28[b]
benomyl 250 x 10^{-6}M	22.66 + 3.06
MBC 200 x 10^{-6}M[c]	29.78 + 1.27
2-AB 250 x 10^{-6}M	20.12 + 0.83
benzimidazole 250 x 10^{-6}M	30.07 + 3.33

[a]all the treatments were carried out for 120 minutes

[b]standard deviation(n=9)

[c]the solubility of MBC did not allow a final concentration higher than 200 x 10^{-6}M.

For the determination of the chloroplasts spectral changes, the chemicals were introduced in the suspensions at final concentrations ranging between 50 and 250 x 10^{-6}M as aliquots dissolved in methanol.Most of the treatments were carried out for 30 minutes at the end of which the absorption spectrum was measured between 400 and 750 nm and compared with those of a control suspension that had been added with the same volume of methanol.As for the effects of the different compounds on photosynthetic activity, the spectral changes induced on isolated chloroplasts were not significantly altered at concentrations lower than 250 x 10^{-6}M; at this last value only benomyl and 2-AB displayed a significant

effect on absorption pattern(Table IV).

TABLE IV

Effect of benomyl and its metabolites on absorption spectrum of isolated chloroplasts

treatment	spectral change at 440 nm[a]
benomyl 250 x 10^{-6}M	-0.080 + 0.006[b]
MBC 200 x 10^{-6}M	-0.003 + 0.001
2-AB 250 x 10^{-6}M	+0.077 + 0.005
benzimidazole 250 x 10^{-6}M	+0.015 + 0.003

[a]the control suspension displayed an OD value at 440 nm of 0.750; the spectral changes are expressed as the OD difference at 440 nm between the control suspension and the treated ones.The final chlorophyll concentration was 11 mg/ml.

[b]standard deviation(n=9)

DISCUSSION

It was reported that melon plants treated with 40 to 120 mg benomyl/plant showed growth rates that were temporary reduced for periods proportional to the dosis of the fungicide while afterwards a general growth stimulation was observed(WENSLEY and HUANG 1969,WENSLEY 1972).By applying higher dosis of benomyl,as in this work,this recovery does no more happen and permanent phytotoxic symptoms are displayed by the plants after a few days.

From the results described here,it may be concluded that benomyl when applied at the rate of 150 mg/plant induces firstly considerable changes in the chloroplasts absorption pattern and to a lesser extent a decrease in their photosynthetic activity. Both phenomenons may be measured a few days before that external symptoms can be detected.This property could be used to set up a procedure that would allow a fast determination of phytotoxicity due to benomyl namely by following the changes of chloroplasts absorption patterns of treated plants.These modifications are followed by a decrease of the chlorophyll contents of the leaves but this becomes only significative after that external symptoms are already visible.From the work of INOUE et al.(1972) who demonstrated that intensification of flattening of absorption bands in the chloroplasts absorption pattern resulted from conformational changes,it may be suspected that benomyl first induces a swelling of the chloroplasts up to the 7th day in the plant followed by a shrinkage from the 8th day probably when the fungicide has reached a given level in the chloroplasts.

No attempts were made here to characterize further the effects of benomyl on chloroplasts properties,but it appeared that in vitro the fungicide,up to the concentration of 200 x 10^{-6}M,is

unable to modify directly neither the absorption spectrum, nor the photosynthetic activity of isolated chloroplasts.

These results are to be compared with data reported by KRISTEVA and KRISTEV (1971) who observed an inhibition of the Hill reaction for isolated chloroplasts treated in vitro with benomyl (300×10^{-6}M) but also a reduction of apparent photosynthesis rates of apples leaf discs after spraying the leaves with the fungicide. This last result could not be reproduced by FERREE and HALL (1975) but it must be pointed out that for both experiments the effective fungicide concentration in the leaves was not indicated which makes the comparison difficult.

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