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EFFECT OF BENOMYL ON SPORES OF *FUSARIUM OXYSPORUM*

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Key Word Index—*Fusarium oxysporum*; Fungi; fungicides; benomyl; spore germination; respiration; RNA; protein; enzymes.

Abstract—Benomyl, methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate, inhibited germination of *Fusarium oxysporum*. The fungicide was found to be very rapidly absorbed by the conidia. Benomyl decreased the RNA content and at high concentrations the respiration rate was inhibited. Among various respiratory enzymes that have been tested, L-malate:NAD oxidoreductase was the most sensitive to the fungicide. Protein content and amino acid uptake did not appear to be significantly altered by treatment with benomyl.

INTRODUCTION

IN A PREVIOUS report,[1] the main diseases cured by benomyl have been reviewed and its fungitoxic activity has been demonstrated *in vivo* on *Fusarium oxysporum*. So far little is known[2] about the mechanism of action of this fungicide, however it has been reported recently[3,4] that in aqueous solution, benomyl yields a decomposition product, identified as methyl-2-benzimidazole carbamate (MBC), whose activity is either equal or less than the original compound, according to the organism used to test the activity of MBC.[5]

This paper reports the effects of benomyl on several metabolic processes in microconidia of *F. oxysporum*: germination rate, RNA and protein synthesis and a few steps of the respiratory chain. Some preliminary results have already been published.[6]

RESULTS

In most experiments the fungicide was added to ungerminated conidia. The inhibitory effect of benomyl in concentrations in the range of 1–50 μg/ml on the rate of germination after 7 hr incubation appears to be in a logarithmic relationship with the fungicide concentration as has been reported for other fungicides.[7] The same concentrations of benomyl are more effective against the spores which are allowed to germinate either on agar or in static liquid medium than on spores which are grown in liquid shaken conditions.[6] Therefore care is needed in comparing results which have been obtained under different experimental conditions. Benomyl is concentrated in the conidia very rapidly and using methanol extracted material, it can be seen that more than 46% of the fungicide was recovered from the spores treated with 10 μg/ml for 60 min (Fig. 1).

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[7] A. P. TRINCI and K. GULL, *J. Gen. Microbiol.* **60**, 287 (1970).

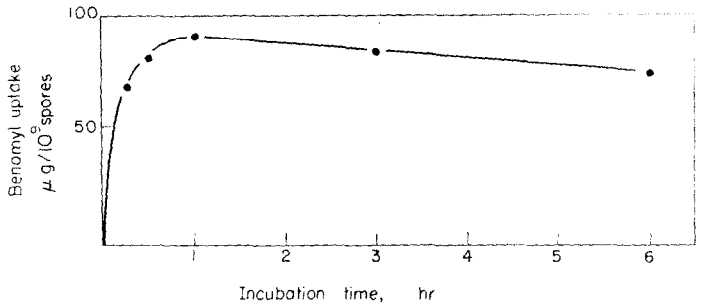

FIG. 1. BENOMYL UPTAKE BY SPORES OF *Fusarium oxysporum.*

The respiration rate of germinating conidia of *F. oxysporum* was decreased by a 50 μg/ml benomyl treatment (Table 1). The percentage inhibition was 38·4% after 60 min, but using increased incubation periods this inhibition became progressively reduced, and it was only 28·6% after 7 hr. At lower fungicide concentrations, this effect on respiration is

TABLE 1. EFFECT OF BENOMYL ON THE RESPIRATION OF GERMINATING *F. oxysporum* SPORES

	Differential O_2 uptake (μl/mg dry wt/min)	
Incubation time (hr)	Control	Benomyl-treated (50 μg/ml)
1	20·1(2·0)*	13·9(1·4)
3	28·2(2·8)	19·6(2·0)
4	32·3(3·2)	21·8(2·2)
5	37·4(3·5)	27·3(2·7)
6	46·6(4·7)	36·9(3·7)
7	61·3(6·1)	43·8(4·4)

* Standard deviation.

not observed. It must be noted that some other authors,[8] working with *P. omnivorum* failed to observe any respiration decrease after benomyl treatment. As far as *F. oxysporum* is concerned, this reduction of respiration did not appear to be related to any decrease of the

TABLE 2. EFFECT OF BENOMYL ON CYTOCHROME OXIDASE ACTIVITY

	Absorbance at 550 nm after indicated incubation time						
Time (sec)	0	30	60	90	120	150	180
Control	0·15	0·12	0·11	0·09	0·07	0·05	0·04
Benomyl 50 μg/ml	0·15	0·13	0·12	0·09	0·06	0·05	0·04

cytochrome oxidase activity as has been reported[9] for benzimidazole. Indeed it was found that the oxidation rate of reduced cytochrome *C* by crude spore extracts remained unmodified in the presence of 50 μg/ml benomyl (Table 2). In the same way, there was no correlation

[8] S. D. LYDA and E. BURNETT, *Phytopathol.* **60**, 726 (1970).

TABLE 3. RELATIONSHIP BETWEEN THE SUCCINATE DEHYDROGENASE ACTIVITY AND BENOMYL TREATMENTS DURING SPORE GERMINATION

	Succinate dehydrogenase activity (μl O_2 consumed/hr 100 μg dry wt)			
Germination time (hr)	Untreated	Benomyl conc. (μg/ml) 10	20	50
0	2·61(0·36)*	2·55(0·21)	2·83(0·29)	2·76(0·19)
4	2·75(0·27)	2·49(0·30)	2·85(0·27)	2·74(0·23)
7	2·69(0·26)	2·59(0·33)	2·86(0·31)	2·71(0·24)

* Standard deviation.

between the decrease of respiration and any variation in the succinate dehydrogenase activity, as has been suggested for some other systemic fungicides.[10] When spores were treated with benomyl concentrations ranging from 10 to 50 μg/ml, no difference could be detected for this dehydrogenase activity between control and treated material (Table 3). However,

TABLE 4. EFFECT OF BENOMYL ON L-MALATE:NAD OXIDOREDUCTASE ACTIVITY

	Absorbance $\times 10$ at 340 nm	
Incubation time (min)	Control	Benomyl-treated (50 μg/ml)
1	0·25	0·04
2	0·35	0·10
3	0·50	0·12
4	0·60	0·14
5	0·78	0·15

malate dehydrogenase was found to be very sensitive to the fungicide, since as soon as benomyl was added to a mitochondrial extract, it prevented the reduction of NAD^+, using Na malate as substrate (Table 4).

TABLE 5. EFFECT OF A 20 μg/ml BENOMYL TREATMENT ON RNA AND PROTEIN CONCENTRATION DURING SPORE GERMINATION

	RNA concentration (mg/100 mg dry wt)		Protein N (mg/100 mg dry wt)	
Incubation period (hr)	Control	Benomyl-treated	Control	Benomyl-treated
0	3·06(0·18)*	3·00(0·21)	5·32(1·02)	5·26(0·93)
4	3·90(0·19)	3·24(0·20)	6·67(1·23)	5·63(1·09)
7	4·26(0·19)	3·42(0·19)	8·03(1·37)	7·63(1·15)

* Standard deviation.

[9] A. M. VERHULST and A. A. SELS, *Arch. Int. Physiol. Biochim.* **77**, 973 (1969).
[10] D. E. MATHRE, *Phytopathol.* **60**, 671 (1970).

Because it has been suggested elsewhere[2] that benomyl could be converted into a nucleotide, interfering with the normal metabolism of the cell, this possibility has been examined. The results reported in Table 5 show that the total RNA content of the spores, calculated on a dry weight basis, increased from 3·06 to 4·26% after 7 hr germination in untreated material, while the net RNA increase was significantly reduced in the samples treated with 20 μg/ml benomyl. This inhibition of RNA synthesis was further confirmed by using a RNA radioactive precursor, since under the same experimental conditions, the incorporation rate of uracil-^{14}C into RNA was significantly lower when compared to the control (Table 6). Therefore, it appears that benomyl prevents at least partly *de novo* RNA

TABLE 6. EFFECT OF A 20 μg/ml BENOMYL TREATMENT ON THE URACIL-2-^{14}C AND LYSINE-^{14}C UPTAKE BY SPORES

	Net cpm incorporation/10^6 spores			
	Uracil-2-^{14}C		L-Lysine-^{14}C	
Incubation period (hr)	Control	Benomyl-treated	Control	Benomyl-treated
0·25	3·226	3·146	60·318	61·990
4	166·768	134·532	76·006	74·084
7	261·574	232·563	95·480	94·768

formation. Such an effect has previously been reported[11] for benzimidazole which was shown to interfere with RNA synthesis by competition with orotic acid for the use of orotidine-5′-phosphate pyrophosphorylase. Although the structure of benomyl is closely related to that of benzimidazole, we failed to observe any competition between the fungicide and orotic acid (Table 7). This suggests that benzimidazole and benomyl would not act in

TABLE 7. EFFECT OF BENOMYL ON OROTIDINE-5′-PHOSPHATE PYROPHOSPHORYLASE ACTIVITY

	Absorbance at 295 nm after indicated time (min)					
	0	1	2	3	4	5
Control	1·17	0·93	0·89	0·88	0·88	0·87
Benomyl-treated	1·17	0·92	0·90	0·87	0·87	0·87

the same way. We also attempted to determine whether the fungicide would affect the protein content of the spores during their germination as was reported[12,13] for the closely related fungicide 2-(4′-thiazolyl) benzimidazole (TBZ). Although there is some decrease in the protein content on benomyl treatment, it probably has no statistical significance (Table 5). Similarly, we failed to find any significant variation in the L-lysine-^{14}C uptake after a fungicide treatment (Table 6).

[11] M. KAPOOR and E. R. WAYGOOD, *Can. J. Biochem.* **43**, 153 (1965).
[12] T. STARON and C. ALLARD, *Phytiatrie-Phytopharmacie* **13**, 163 (1964).
[13] P. M. ALLEN and D. GOTTLIEB, *Appl. Microbiol.* **20**, 919 (1970).

DISCUSSION

In liquid shaken conditions, benomyl reduces the rate of germination for *F. oxysporum* conidia but does not prevent it, since most of the conidia are finally able to form a germ tube. The fungicide is absorbed by the spores rather rapidly since the greatest part of the compound entered into the cells within 60 min (Fig. 1). Such a rapid uptake has also been shown for TBZ.[14] It remains however to show where benomyl is located in the cell and how long it persists in an active form. The decrease of respiration rate induced by benomyl, when used at high concentration (Table 1) is in good agreement with the results obtained with TBZ.[13] Both fungicides prevent activity of NAD-linked enzymes, but whereas TBZ seems able to act on a few other respiratory enzymes, the reduction of the oxygen uptake by benomyl appears to be restricted mainly to a decrease of the activity of malate dehydrogenase (Table 4). The action of benomyl on respiratory enzymes could thus be more specific.

The reduction of RNA synthesis (Table 5) is similar to that previously reported for TBZ[13] and benzimidazole,[11] though it is likely that the mechanism of action of benomyl, which does not compete with orotic acid, would not be the same as for benzimidazole. The possibility of a similarity between the modes of action of benomyl and TBZ is more likely, though some differences seem to occur, namely as far as their effect on protein synthesis is concerned. Some authors[12] have reported that it was possible to diminish the effect of TBZ by a guanine treatment. This gave rise to the assumption that both TBZ and benomyl would act only by preventing the nucleic acid metabolism without any action on respiration and energy production.[15] Further experimental data[13] have not supported this hypothesis for TBZ and, as far as benomyl is concerned, we were never able to observe any prevention of its effects by applying a prior treatment either with guanine or with any other nucleic acid base. As a conclusion of the present work, it seems that benomyl is able to inhibit simultaneously different parts of the metabolism of the conidia. The decrease of the respiration rate from the very beginning of the treatment could be related mainly to an inhibition of the malate dehydrogenase activity which appears to be decreased as soon as the fungicide is added. On the other hand, this respiration inhibition seems to decrease slightly as the incubation time progresses, at least up to 7 hr. However, we do not believe actually that this reduction of respiration would be only transient, since by applying benomyl treatments longer than 7 hr, there is a sharp increase in the rate of death of the cells as will be shown in another paper. However, it seems likely that the respiration inhibition is not the main effect (the so-called 'primary effect') resulting in the inhibition of growth of the germ tubes since the benomyl concentration that has to be used in order to observe such a respiration decrease is somewhat high. With regard to RNA synthesis, the inhibition produced by the fungicide is probably more significant and in relation with this the influence of benomyl on DNA metabolism undoubtedly deserves investigation.

EXPERIMENTAL

An isolate of *Fusarium oxysporum* f.sp.*elaeidis*(R 24) was cultured in 500 ml Erlenmeyer flasks containing 100 ml of minimal medium (20 g glucose, 1 g KH_2PO_4, 500 mg $MgSO_4.7\,H_2O$, 1·6 g NH_4NO_3, 2·6 ppm Fe^{3+}, 1·5 ppm Zn^{2+}, 0·6 ppm Cu^{2+}, 0·6 ppm B^{3+}, 0·5 ppm Mo^{5+} per litre of distilled H_2O) and the incubations were carried out at 26° on a rotary shaker for 3–4 days. The spores were obtained by filtering, washed with distilled H_2O, resuspended in fresh minimal medium and stored at 4° until use.

[14] D. Gottlieb and K. Kumar, *Phytopathol.* **60**, 1451 (1970).
[15] A. K. Siupestein, *World Rev. Pest Control* **8**, 85 (1970).

For the germination studies, benomyl (1–50 μg/ml) was added to minimal medium with 10^6 ungerminated spores/ml. The flasks were incubated on a rotary shaker at 26° for 7 hr. Spores with germs tubes at least as long as their diameter after swelling were considered as germinated. At least 200 spores were examined for each sample in order to determine the percentage of germination.

For determining fungicide uptake, flasks containing 100 ml minimal medium were inoculated with *F.oxysporum* to obtain a concentration of 50×10^6 ungerminated conidia/ml. The fungicide was added to some cultures at 10 μg/ml initial concentration and after benomyl had been allowed to act for different incubation times, the conidia were collected, washed by centrifugation with H_2O until no absorption at 286 nm could be detected in the wash. Each fraction was then added to 20 ml MeOH and the conidia were broken by grinding with acid-washed sand.[16] The sand that had been used for grinding was further extracted twice with 10 ml MeOH and after centrifugation the soluble parts were added to the spore extract. Each crude extract was centrifuged twice at 2900 *g* for 15 min and the pellet was discarded. The absorptivity of this supernatant was then measured at 286 nm. The uptake of benomyl could be calculated by using the difference between the absorbances from treated and non-treated fractions. A spectrophotometric procedure has already been used for benomyl uptake determination,[17] and also for TBZ.[14]

Respiration rates were determined by manometry. Each Warburg vessel contained 1·5 ml germination medium, 1 ml 0·05 M phosphate buffer at pH 6·8; 0·25 M sucrose and 10^7 ungerminated spores. The system was equilibrated for 60 min and 0·5 ml of a benomyl suspension was added to the reaction mixture in order to obtain a final concentration of 50 μg/ml.

The activity of cytochrome C. O_2 oxidoreductase was determined in a crude spore extract. By measuring the decrease in the absorbance at 550 nm.[18] Aliquots of 0·05 ml spore extract were added to 3 ml cytochrome *C* (Fe^{2+}) ($1{\cdot}7 \times 10^{-5}$ M) and the readings were taken at 30 sec intervals. Benomyl (50 μg/ml final concentration) was added to the spore extract 30 sec prior to the first reading.

The activity of succinate. PME oxidoreductase was measured with an O_2 Clark electrode. The reaction mixture contained 3·2 ml 0·06 M phosphate buffer at pH 7·6; 0·25 M sucrose, 0·1 ml phenazine methosulfate solution (PMS: 40 mg/ml), 0·1 ml 0·03 M KCN and 0·5 ml spore extract. The reaction was started by adding 0·1 ml of 0·2 M Na succinate and the fungicide was added at the beginning of the experiment.

The activity of L-*malate.* NAD oxidoreductase was measured by the increase of absorptivity at 340 nm caused by the decrease of NAD^+. For this purpose, 30 g of fresh material was suspended in 40 ml 0·01 M phosphate buffer at pH 7·2; 0·25 M sucrose and the cells were broken with 30 g acid-washed sand in a Sorvall omni-mixer. The homogenate was centrifuged at 700 *g* for 15 min and the supernatant centrifuged again at 20 000 *g* for 10 min in order to pellet the mitochondria. The pellet was then resuspended in 2 ml 0·01 M phosphate buffer. The entire procedure was carried out between 0 and 4°. The reaction mixture was 2·5 ml of 0·1 M Tris-HCl buffer at pH 8·8, 0·1 ml 0·01 M NAD^+, 0·05 ml mitochondrial suspension and 0·25 ml H_2O. The reaction was started by adding 0·1 ml 0·1 M Na malate. For the benomyl treatment, the fungicide was added as 0·1 ml of a concentrated solution (final concentration: 50 μg/ml), prior to the addition of Na malate.

The total RNA content of the spores was estimated after phenol extraction by UV spectrophotometry. The proteins were precipitated with cold 5% CCl_3COOH after homogenization of the spores, and the protein-N content of the ppt. was measured by the micro-Kjeldahl technique.

For the radioactive isotopes studies, uracil-2-^{14}C (sp. act.: 50 mCi/mM) and L-lysine-^{14}C (sp. act.: 117 mCi/mM) were added separately to the germination medium (8·33 μCi of uracil-^{14}C or 4·17 μCi of lysine-^{14}C per $2{\cdot}5 \times 10^9$ ungerminated spores). Both benomyl and radioactive precursors were added as soon as germination commenced. After incubation, the spores were centrifuged, washed 3× with cold distilled H_2O and extracted with 20 ml of 80% EtOH for 60 min. After centrifugation, the precipitated material was treated with 10 ml CCl_3COOH at 4° for 60 min and recentrifuged. The radioactivity was then determined by liquid scintillation counting.

The determination of the activity of orotidine-5′-phosphate: pyrophosphate phosphoribosyl transferase was based on the absorptivity decrease at 295 nm following the formation of orotidine monophosphate.[19] The spectrophotometric cell contained 1 ml Tris-HCl buffer pH 8·5 (60 μmol), 0·5 ml $MgCl_2$ (6 μmol), 0·5 ml Na orotate (0·3 μmol) and 0·3 ml spore extract. The reaction was started by adding 0·5 ml of 5′-phosphoribosyl-1′-pyrophosphate (PRPP) (0·34 μmol). For the benomyl treatment, the fungicide was added (0·34 μmol) prior to the addition of the PRPP solution.

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