ACTA CHEMICA SCANDINAVICA 12 (1958) 124—128

On the Antifungal Effect of Benzoxazolinone and 6-Methoxybenzoxazolinone, Respectively, on *Fusarium nivale*

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The decomposition of benzoxazolinone and 6-methoxybenzoxazolinone by *Fusarium nivale*, the adaptation of this fungus to benzoxazolinone, and the fungistatic or fungicidic action of benzoxazolinone on it has been investigated. The mode of inhibition of growth of *F. nivale* in media containing benzoxazolinone is discussed in relation to the results. The results suggest that benzoxazolinone has a fungistatic influence in a concentration < 0.5 mg per ml. In a concentration > 0.6 mg per ml the fungicidic influence is already prevailing and the growth of *Fusarium* is totally inhibited.

The growth curves, shown in Fig. 1, are obtained when the biological activity of benzoxazolinone (BOA), the antifungal substance found in rye seedlings, is determined in an oat-glycerol solution or on agar plates, using different BOA concentrations and *F. nivale* as test organism.

A pronounced lag period is thus observed which is the longer the higher the BOA content. Even after a long period of time no growth was observed when the BOA concentration was higher than 0.5 mg/ml.

Some experiments were performed in order to find out if the influence of BOA and 6-methoxybenzoxazolinone (MBOA) is fungistatic or fungicidic and to study the reactions underlying the lag period. In this paper these experiments and the results are described and discussed.

DECOMPOSITION OF BOA BY *F. nivale*

40 mg of BOA were added to 100 ml of Czapek's nutrient solution containing 0.63 g of glycerol per litre and sterilized for 15 min under 0.8 atm pressure. Subsequently an amount corresponding to about 7 mg dry weight of the mycelium of *F. nivale*, grown for 7 days at 20°C on oat-glycerol-agar, was added aseptically. The suspension in a 1 litre Erlenmeyer flask, closed by a cotton

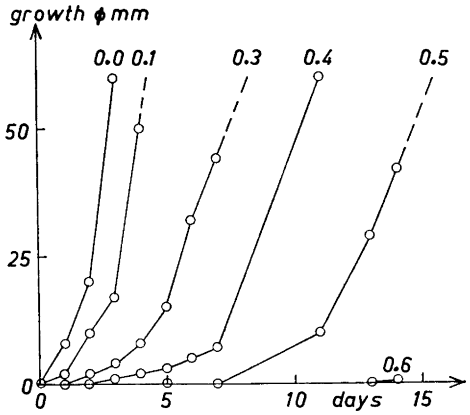

Fig. 1. Growth of the mycelium of *F. nivale* in a BOA containing oat-glycerol-medium. Curve numbers indicate the BOA content, mg/ml.

plug, was shaken in an automatical shaker at a frequency of 50 oscillations per min. A corresponding suspension without BOA was used as control. At intervals 1 ml samples were taken with a sterile pipette. The samples were diluted to 100 ml, and the absorption at 230 to 300 mμ was determined with a Beckman spectrophotometer. The BOA content was calculated from the

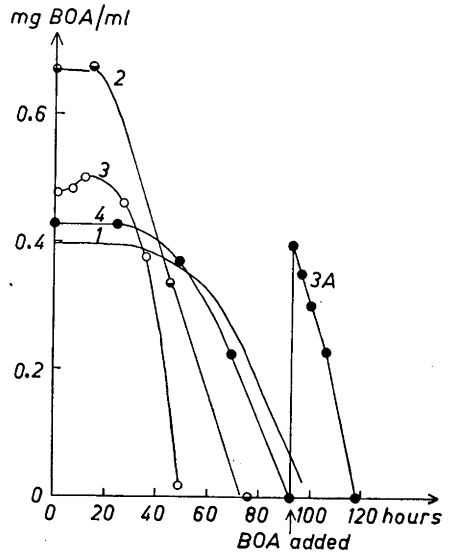

Fig. 2. Decomposition of BOA in a suspension of *F. nivale* in: 1 and 2 : Czapek solution ; 3 and 4 : oat-glycerol solution. The numbers of curves 3 and 4 have changed places.

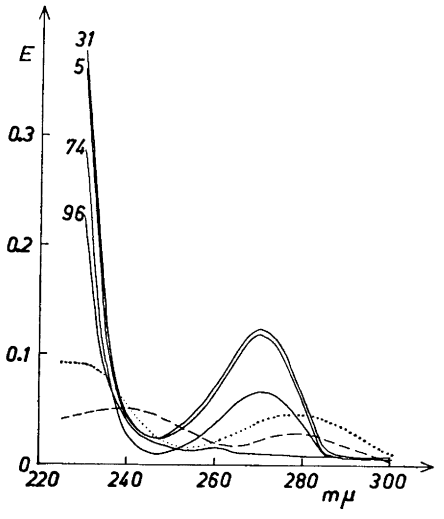

Fig. 3. UV-Spectra of the suspension of *F. nivale* in a Czapek solution containing BOA. Solid lines: vigorously shaken suspension. Curve numbers indicate test time in hours. Broken line: The spectrum of an unshaken suspension after decomposition of BOA. Dotted line: The spectrum of *o*-aminophenol in the same suspension.

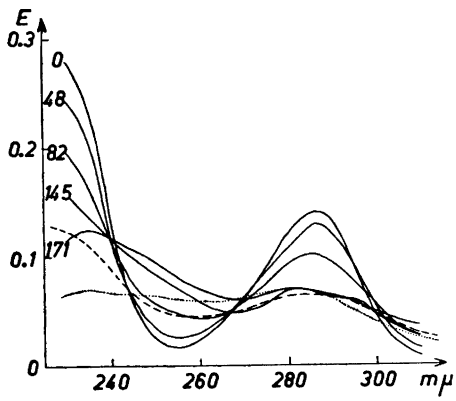

Fig. 4. Solid lines: The breakdown of MBOA effected by the mycelium of *F. nivale* in a vigorously shaken Czapek solution. Curve numbers indicate test time in hours. Broken line: 2-amino-5-methoxyphenol, partly oxidized in air. Dotted line: 2-amino-5-methoxyphenol, further oxidized in air.

absorption maximum at 270 mμ. The weight of the mycelium was determined in 5 ml samples. The results are shown in Fig. 3 and curve 1 in Fig. 2.

In another experiment, in which the BOA content was 0.67 mg per ml, and the amount of mycelium corresponded to about 70 mg dry weight per 100 ml solution, the BOA content decreased by 50 % in 45 h; after 76 h no BOA could be found in the test solution (Fig. 2, curve 2).

After 120 h a determination of BOA was made on the mycelium in 50 ml test solution; 0.041 mg of BOA was found, which is about 1/500 of the amount of BOA added at the beginning of the experiment.

It is noticeable that in all experiments it took 20 to 30 h before the decomposition of BOA started. Does this lag period depend on an adaptive formation of a BOA-decomposing enzyme? To test this possibility a new portion of BOA was added to a suspension in which the originally added BOA was decomposed. As can be seen from curve 3 A in Fig. 2 the decomposition of BOA has now started immediately.

DECOMPOSITION OF 6-METHOXYBENZOXAZOLINONE BY *F. nivale*

The experiment was performed as above. The MBOA content of the solution was 0.4 mg per ml, and the amount of mycelium corresponded to about 90 mg dry weight per 100 ml solution.

After 170 h practically all MBOA had disappeared from the solution.

CHANGES IN THE UV SPECTRUM DURING THE DECOMPOSITION OF BOA AND MBOA BY *F. nivale*

The UV spectrum of the BOA solution with the mycelium of *F. nivale* had during the experiment the same shape as the spectrum of pure BOA solution, from which fact it can be concluded that aromatic decomposition products of BOA, possibly formed during the experiment, were hardly enriched in the solution (Fig. 3). When practically all BOA had disappeared a very weak maximum at 260 mμ could be noticed. It disappeared rapidly, however.

On performing the experiment without shaking, two new maxima (at 240 and 280 mμ) were observed when BOA had disappeared (Fig. 3).

In the MBOA solution with *F. nivale* mycelium two new maxima were observed at 235 mμ and 282 mμ after 125 h as well as an inflexion point at 295 mμ which disappeared after 170 h when all MBOA was already decomposed (Fig. 4). In addition there was a maximum at 460 mμ. The change in the spectrum cannot be due to pH-variations, since the pH remained constant during the experiment. It thus seems that one or more new compounds containing a benzene ring had been formed from MBOA by *F. nivale*.

In Figs. 3 and 4 are also seen the spectra of *o*-aminophenol and 2-amino-5-methoxyphenol oxidized in the air. The latter compound also showed a maximum at 460 mμ. It is seen that the spectra do not quite correspond to the maxima observed in *F. nivale* suspensions during and after the disappearance of BOA and MBOA though there are certain similarities.

The rate of decomposition seems to depend on the amount of mycelium as well as on the original BOA concentration of the solution. MBOA seems to decompose slower than BOA. It ought to be mentioned that in an unshaken mycelium suspension BOA was no longer decomposed in a concentration of 0.5 to 0.6 mg/ml.

ADAPTATION OF *F. nivale* TO BOA

The lag period might thus be the time required by *F. nivale* to decompose BOA around the inoculate. Another possibility might be the adaptation of *F. nivale* to BOA. Therefore the following experiment was performed.

F. nivale mycelium was transferred to 20 oat-glycerol-agar plates containing 0.6 mg of BOA per ml. After 9 days growth could be seen on 12 plates. The mycelium grown on these plates was cultivated during 4 transfers on plates containing 0.6, 1.0, and 1.25 mg of BOA per ml. As early as during the second transfer *F. nivale* grew in the concentration 1 mg per ml. Even after the fourth transfer *F. nivale* did not grow in a higher concentration than this.

The observed length of the lag period at concentrations of 0.1 to 0.5 mg BOA per ml can be supposed to depend on this weak adaptation.

THE FUNGICIDIC INFLUENCE OF BOA AND MBOA ON *F. nivale*

Nine days old mycelium of *F. nivale* was transferred to sterile 0.9 % NaCl solutions containing different amounts of BOA. Each test tube contained 5 ml solution and about 5 mg dry weight of mycelium. At the contact times, shown in Table 1, samples of the mycelium were separated, washed three times with sterile 0.9 % NaCl solution and then transferred to an oat-glycerol-agar. Growth was followed during 7 days.

The results shown in Table 1 indicate that BOA has a fungicidic influence on *F. nivale* from a concentration of 0.5 mg per ml upwards. In concentrations higher than this the fungicidic influence is comparatively rapid. MBOA also exerts a fungicidic influence on *F. nivale* at least in the concentration 0.5 mg per ml (contact time 6 days).

Table 1. Growth of the mycelium of *F. nivale* after contact times of 1 to 33 days. Substrate in the growth tests oat-glycerol-agar.

mg BOA/ml	Growth Contact time, days 1	2	3	4	8	17	33
0 (control)	+	+	+	+	+	+	(+)
0.3	+	+	+	+	+	+	+
0.5	+	+	+	+	(+)	(+)	−
0.75	+	(+)	−	−	−	−	−
1.0	+	(+)	−	−	−	−	−
1.4	+	−	−	−	−	−	−

Marking of growth: + = normal growth, (+) = poor growth, − = no growth.

DISCUSSION

On the basis of the observations reported above the growth of *F. nivale* in a nutrient solution or on agar plates containing BOA or MBOA could perhaps be explained in the following way: in a concentration up to 0.4 mg per ml the influence is fungistatic, and the lag period depends on the adaptation of *F. nivale* to these substances. When growth has started, it at first proceeds slowly, and then accelerates to reach about the same rate as in a solution without BOA (Fig. 1). BOA determinations from growth flasks indicate that at this point BOA had practically disappeared from the media. In 0.5 mg per ml there is already a weak fungicidal influence, the fungistatic one still prevailing, however. When the concentration rises to 0.6 or 0.7 mg per ml, the fungicidic influence prevails, and growth is totally inhibited.

The chemical mechanism of the fungistatic and fungicidic influence of BOA and MBOA is still unknown.

As regards the way of the decomposition of BOA and MBOA by *F. nivale* it is possible that the corresponding aminophenols occur among the products, though, at least in the case of BOA, the decomposition probably goes further.

The support given by the *Rockefeller Foundation* is gratefully acknowledged.

Received September 25, 1957.