DEVELOPMENT OF TOLERANCE TO THE PHYTOALEXIN 6-METHOXYMELLEIN IN MICROORGANISMS

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6-Methoxymellein (6MM), a phytoalexin of carrot, exerted a static effect on the growth of fungi, yeast and bacteria at low concentrations. It retarded the spore germination of *Chaetomium globosum* and extended the lag phase in the growth of *Bullera alba*, *Candida albicans* and *Staphylococcus aureus*. When *B. alba* and *C. albicans* were first treated with 6MM and then a second time their growth was not inhibited by the second dose, suggesting that they become tolerant to 6MM through some induced factor. By contrast, *S. aureus* appeared to tolerate 6MM in a constitutive manner, since the bacterial growth was inhibited by the second dose of 6MM as well as the first. In *S. aureus* culture, however, the duration of growth inhibition by 6MM was dependent on the inoculum size, and the inhibitory effect was negligible when the bacterial concentration in the culture was sufficiently high. *Chaetomium globosum* and *B. alba* incorporated appreciable amounts of 6MM, but no metabolite of the phytoalexin appeared in the culture. The results suggest that 6MM tolerance occurred in these microorganisms independently of its degradation or transformation.

Many higher plants produce antimicrobial compounds when they are challenged by microorganisms. These compounds known as phytoalexins, are believed to be involved in the defense reactions of plants (1, 2). Phytoalexin-mediated resistance to pathogens may be influenced by a number of factors which involve the biosynthesis and degradation of the phytoalexin by plant cells and acquisition of tolerance by microorganisms. Phytoalexin tolerance by microorganisms has been studied by many workers, but how pathogens tolerate phytoalexin is not fully understood. Some fungi and bacteria have the ability to detoxify phytoalexins by converting them to less inhibitory compounds (3–8). Cases have also been reported in which microorganisms become tolerant to phytoalexin by a non-degradative mechanism (7, 9–11).

We recently reported the toxicity of a carrot (*Daucus carota*) phytoalexin, 6-methoxymellein (6MM), to some fungi, yeasts and bacteria, and showed that the phytoalexin has a broad antimicrobial spectrum (12). In this paper, we wish to report the effect of 6MM on the growth of a filamentous fungus (*Chaetomium globosum*), two yeasts (*Bullera alba* and *Candida albicans*) and a bacterium (*Staphylococcus aureus*) and also the tolerance of these microorganisms to the phytoalexin.

MATERIALS AND METHODS

Isolation of 6MM. Crystalline 6MM was prepared from carrot root slices infected by *C. globosum* according to the method of KUROSAKI and NISHI (12).

Microorganisms and cultivation. *Chaetomium globosum* and *B. alba* were obtained from Dr. K. TSUBAKI, Institute of Biological Sciences, Tsukuba University. *Candida albicans* was obtained from Dr. K. TANAKA, Faculty of Medicine, Nagoya University. These fungi and yeasts were maintained on an yeast-malt agar medium (yeast extract 0.4%, malt extract 1% and glucose 0.4%). *Staphylococcus aureus* (ATCC 25923) was obtained from Dr. K. KONISHI, Faculty of Medicine of this university, and maintained on a heart infusion agar (Difco). Spore suspension of *C. globosum* were prepared by adding distilled water to a 3 week-old culture and filtering through one layer of gauze to remove large mycelial fragments. The spores were collected by centrifugation at $1,000 \times g$ for 10 min, and stored at 4° until use. For the germination assay, the spores were resuspended in the yeast-malt liquid medium at a concentration of 2×10^5 spores/ml and agitated on a reciprocal shaker (72 strokes/min) at 27°. *Bullera alba* and *C. albicans*, kept on agar slants, were transferred to the yeast-malt liquid medium and similarly shaken at 27°. *Staphylococcus aureus* was cultured at 37° in the heart infusion liquid medium with shaking. Prior to the experiments, these yeasts and bacteria had been pre-cultured in the respective liquid media for a period in which they were subcultured at least 3 times.

Determination of microbial growth. Microorganisms were cultured in test tubes (14 mm diameter) containing 5 ml of medium. For use in bioassay, the 6MM was dissolved in ethanol and added to the culture to give a final solvent concentration of 2%. Spore germination of *C. globosum* was observed under a microscope. The growth of other microorganisms was followed by comparing the turbidity at 540 nm with that of an uninoculated medium.

Preparation of ^{14}C -labelled 6MM. ^{14}C -Acetate was used as the radioactive precursor of 6MM, since KUĆ (1) suggested that the acetate-malonate pathway is the most likely route for the biosynthesis of 6MM. Carrot slices (200 g) were inoculated with a 2 day-old culture of *C. globosum*, and after 3 hr, 300 μCi of 2- ^{14}C -sodium acetate (specific activity, 55 $\mu\text{Ci}/\mu\text{mol}$) in 40 ml water was sprinkled over them. Another 300 μCi of ^{14}C -acetate was added to the culture after 6 hr

incubation. Further incubation was carried out at 27° for 24 hr, then the infected carrot slices were immersed in acetone. The extraction and purification procedures for radioactive 6MM were the same as for the unlabelled compound. The radioactivity of the product was measured by a liquid scintillation spectrometer using a commercial scintillation cocktail (Amersham ACS II). The amount of ^{14}C -6MM was determined by a thin layer chromatography (TLC) scanning method at λ_s 265 nm and λ_R 400 nm, as reported previously (12), and the specific activity was adjusted to 185,000 dpm/mg by diluting the ^{14}C -6MM with unlabelled 6MM.

Metabolism study. Loss of 6MM from culture media was measured by determining the radioactivity of ^{14}C -6MM added to the cultures. *Bullera alba*, *C. albicans* and *S. aureus* were inoculated into 1 ml of the respective media which contained 0.2 mM ^{14}C -6MM for *B. alba* and *C. albicans* and 1.0 mM for *S. aureus*. A spore suspension (1 ml) of *C. globosum* was also supplemented with 1.0 mM ^{14}C -6MM. At various intervals, 100 μl -aliquots were removed from the cultures, and 6MM was extracted from the culture fluid by blending with 100 μl *n*-butanol for 15 sec. After standing for 1 min, 50 μl of *n*-butanol extract was applied to a silica gel TLC plate (Merck, silica gel 60 F 254) and the plate was developed for 15 cm in benzene-methanol (100:2, v/v). The region containing 6MM was detected under an ultraviolet lamp, and scraped off. ^{14}C -6MM was extracted overnight from silica gel with 0.5 ml ethanol and the radioactivity of the ethanol extract was measured.

A possible metabolite of 6MM was studied by TLC analysis after the fungi were fed with ^{14}C -6MM. A spore suspension of *C. globosum* in 1 ml medium was incubated with 1.0 mM ^{14}C -6MM for 12 hr, and the medium and resulting mycelia were separated by filtration through a 0.45 μm millipore filter. The mycelia were transferred to 1 ml of ethanol and the ^{14}C -labelled compounds were extracted by boiling for 30 min. The ^{14}C -labelled compounds in the medium were also extracted by blending with 1 ml *n*-butanol. From these extracts, 100 μl -aliquots were applied to a silica gel plate, and the plate was developed as mentioned above. The silica gel was scraped off in 1 cm widths, and the radioactivity of each region was measured. *Bullera alba* was incubated with 0.2 mM ^{14}C -6MM for 24 hr, and the experiment was carried out in the same manner.

RESULTS

Effect of 6MM on germination of spores of C. globosum

Under the present conditions, about 70% of the spores of *C. globosum* have emerged germ-tubes after 6 hr of cultivation (Fig. 1). In the presence of 1.0 mM 6MM, spore germination was almost completely prevented for the first 3 hr. The inhibitory effects were transient, however, and the phytoalexin simply lengthened the lag period before the germ-tube emission occurred. After cultivation

Fig. 1. Effect of 6MM on spore germination of *C. globosum*.

The symbols represent 0(○), 0.1(●), 0.5(■) and 1.0(▼) mM 6MM. Points and bars are the means and standard deviations, respectively, obtained from five replicate experiments.

Fig. 2. Effect of 6MM on growth of *B. alba* (a), *C. albicans* (b) and *S. aureus* (c).

The symbols represent 0(○), 0.1(●), 0.2(▲), 0.5(■) and 1.0(▼) mM 6MM. The growth of microorganisms was followed by turbidity measurements.

for 6 hr, the percentages of germination had reached a level similar to that of the control culture.

Effect of 6MM on microbial growth

Addition of 6MM to the cultures of yeasts and bacteria caused an extension of the lag period before active growth commenced (Fig. 2). The duration of 6MM-induced growth inhibition was roughly proportional to the concentration of 6MM added to the culture. After the lag period, microbial growth resumed

Fig. 3. Effect of successive additions of 0.2 mM 6MM on cultures of *B. alba* (a) and *C. albicans* (b).

The first addition of 0.2 mM 6MM was made at time zero (■); upward arrows indicate the time of the second addition to the same culture. To one of the control cultures (●), 0.2 mM 6MM was added at the time indicated by downward arrows, and another culture (○) received only ethanol at the same time.

at the same rate as in the control culture. In the culture of *B. alba* and *C. albicans* treated with 0.5 mM 6MM, however, the cell densities at the stationary phase were obviously lower than that in the absence of 6MM (Fig. 2a, b). Viable cells in these cultures, as determined by a colony count, was 40–50% of the control. These observations suggest that in these microorganisms, tolerance occurred after a transient period of sensitivity at a low concentration of 6MM, but the phytoalexin appears to exert a fungicidal effect when applied at a higher concentration.

A further experiment was set up to test whether the microorganisms become tolerant to 6MM following exposure to it (Fig. 3). The yeasts were inoculated to the culture medium supplemented with 0.2 mM 6MM. When the exponential growth resumed after the 6MM-induced lag phase, 6MM was again added to the culture. Addition of the second dose of 6MM (0.2 mM) did not show any discernible effect on the growth of the yeasts. On the other hand, *B. alba* and *C. albicans* which had not been previously treated with 6MM were sensitive and the addition of the phytoalexin resulted in an abrupt, but transient cessation of the increase in turbidity. It is likely that the tolerance of *B. alba* and *C. albicans* for 6MM was induced by the first dose of the phytoalexin.

The inhibitory effect of 6MM on *S. aureus* appeared to be influenced by the bacterial cell density. When an exponentially growing culture of *S. aureus* was inoculated at various cell density, the delay of the beginning of active growth was

Fig. 4. Effect of 6MM on *S. aureus* at various bacterial cell densities. *S. aureus* culture in the exponential phase was inoculated into a 5 ml medium containing 0.2 mM 6MM. Initial cell density; (●), 0.2×10^4 ; (▲), 1.6×10^4 ; (■), 1.0×10^5 ; (▼), 1.6×10^6 /ml. Open symbols represent respective control cultures without 6MM. Insert figure shows the effect of 6MM on the culture of *S. aureus* pre-treated with 0.2 mM 6MM for 12 hr.

inversely proportional to the number of bacteria in the culture (Fig. 4). The culture of *S. aureus* pre-treated with 0.2 mM 6MM was also tested in this series of experiments (Fig. 4). When the bacterial density was low, *S. aureus* showed a sensitivity to the second dose of 6MM similar to the first one. The tolerance of *S. aureus*, in contrast to *B. alba* and *C. albicans*, seemed to be caused in a manner other than an inducible factor.

Loss of 6MM from culture fluid

The limited duration of growth inhibition caused by 6MM might be a consequence of enzymic degradation or transformation of the phytoalexin. To test this, changes in the 6MM concentration in the medium was followed during culture. When the culture medium was inoculated with the spores of *C. globosum*, the concentration of 6MM in the medium decreased gradually during the first 5–6 hr, and then rapidly after spore germination had been completed (Fig. 5a). In *B. alba*, 6MM in the medium decreased after a short lag period, and reached a constant level of about 50% of the initial concentration (Fig. 5b). The level of 6MM in the media of *C. albicans* and *S. aureus* decreased only slightly (Fig. 5c, d). The restoration of growth of these microorganisms in a few hours after the addition of the phytoalexin implies that they acquired the tolerance to 6MM while the culture medium still contained enough phytoalexin to inhibit their growth.

Fig. 5. Loss of 6MM from culture medium.

Cultures of *C. globosum* (a) and *S. aureus* (d) were supplemented with 1.0 mM ^{14}C -6MM, and *B. alba* (b) and *C. albicans* (c) with 0.2 mM ^{14}C -6MM. Aliquots of the culture (0.1 ml) were removed at the time indicated and 6MM was extracted from the culture medium with *n*-butanol. The radioactivity of ^{14}C -6MM was measured after being separated by TLC. Data presented are the percentages of 6MM left to that added at time zero.

As the concentration of 6MM in the culture fluid of *C. globosum* and *B. alba* decreased appreciably during cultivation, possible metabolites of 6MM were investigated after incubation with these two microorganisms. In these experiments, *C. globosum* was cultured in the presence of ^{14}C -6MM and the extracts obtained from mycelia and medium were developed on a silica gel plate. About 70% of the 6MM added to the culture was recovered in the mycelia, and no ^{14}C -labelled compound other than 6MM occurred in the extract from either the mycelia or culture fluid. This suggests that the mycelium of *C. globosum* possesses an ability to take up 6MM and deactivate it in a way other than degradation or transformation. The radioactivity in the cells was easily removed by washing with several portions of water or buffer (40 mM Tris-HCl, pH 7.2) indicating that the 6MM associated only loosely with the cellular components. The experiment on *B. alba* gave a result similar to that described for *C. globosum*.

DISCUSSION

The results reported in this paper show that 6MM is a static antimicrobial compound at a low concentration. Pterocarpin phytoalexins have been shown to be static agents against fungi, yeasts and bacteria (13, 14). DENNY and VANETTEN (7), however, reported that medicarpin, an isoflavonoid phytoalexin from the jack bean, showed a fungicidal effect when applied at a high concentration. Spores treated with 90 μ g of the phytoalexin could not germinate even after being washed. The inhibitory effect of 6MM also depended on its concentration. *Chaetomium globosum* and *S. aureus* were transiently inhibited by 0.2 mM 6MM (Figs. 2 and 3). At a concentration greater than 0.5 mM, however, the yields of these yeasts when the maximum growth was attained and their colony forming activities were obviously decreased compared to the control, suggesting that the phytoalexin exerts a fungicidal effect at a high concentration.

The microorganisms tested in this experiment expressed phytoalexin tolerance after a period during which growth or germination was inhibited. *B. alba* and *C. albicans* pre-treated with 6MM were no longer sensitive to the phytoalexin when the second dose was added to the culture (Fig. 3). In these cells, the phytoalexin tolerance appears to be induced by the first dose of 6MM. On the other hand, it is likely that *S. aureus* tolerates 6MM by a constitutive manner because the second dose of 6MM resulted in a growth inhibition similar to the first. WEINSTEIN et al. (15) reported that *Erwinia carotovora* detoxifies glycinol, one of the phytoalexins produced by soybeans, by a constitutive factor. By contrast, DENNY and VANETTEN (7) showed that an isolate of *Nectria haematococca* has the ability to adapt to a pterocarpin phytoalexin. These findings indicate that phytoalexins are tolerated by microorganisms in different ways.

The metabolism of phytoalexins has been studied by several workers (3-8), and cases have been reported in which microorganisms became insensitive to phytoalexin without loss of the compound from the culture medium (7, 9-11). Tolerance may occur in the following alternative ways: 1) *C. globosum* and *B. alba* incorporated an appreciable amount of 6MM, but no degradation product or metabolite was detected in these cultures. This suggests that these microorganisms acquire phytoalexin tolerance by a mechanism without biochemical modification of the compound. 2) If 6MM blocks an essential metabolic step, cells could become tolerant by forming an alternate pathway. 3) Tolerance may also occur by a compartmentation of the toxic compound or binding it to a certain cellular component. Transferring a substance from cytoplasm to vacuoles, however, generally requires its transformation to a more hydrophilic compound (e.g. hydroxylation, glycosylation or conjugation with amino acid). The inducible nature of the tolerance mechanism in *B. alba* and *C. albicans* may favor the first hypothesis, though no direct evidence for this is available. However, that hypothesis can not explain the tolerance in *S. aureus* in which the duration

of growth inhibition was dependent on the size of the bacterial inoculum. It is possible that the phytoalexin lost its toxicity by being adsorbed to cellular components or cell-surface structures when a sufficient number of attachment sites were present. Inactivation of some chemotherapeutic agents, such as sulfanyl-amide-derivatives and β -lactum antibiotics, by binding to serum protein has been recognized for many years (16, 17). A similar dependence of the inhibitory activity of phytoalexin on bacterial concentration has been reported by GNANAMANICKAN and PATIL (18) and WEINSTEIN et al. (15).

6MM affected microbial growth in both the exponential and stationary phases. The site of action of 6MM is, on this account, presumed to be a process not characteristically altered according to the growth phase. The broad antimicrobial spectrum previously reported and the phase-independent effect of 6MM suggest that this phytoalexin interferes with a common process in the metabolism of these microorganisms.

The present experiments showed that many fungi are initially sensitive to 6MM but tolerance is rapidly developed in these microorganisms. If the phytoalexin plays a role as a defense substance, it should be accumulated at an infection site to a concentration sufficient for the complete inhibition of microbial growth. In some host-parasite systems, it was shown that phytoalexins accumulations in diseased plants exceeded the concentration required for the restriction of fungal growth. Cytological studies at the site of phytoalexin accumulation combined with in vitro experiments would help to solve the problem (19, 20).

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