Plant Physiol. (1978) 61, 803–805

Factors That Influence the Activity of 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one on *Erwinia* Species in Growth Assays[1]

Received for publication September 7, 1977 and in revised form December 18, 1977

MICHAEL D. WOODWARD,[2] LUIS J. CORCUERA,[3] JOHN P. HELGESON,[4] ARTHUR KELMAN, AND CHRISTEN D. UPPER[4]
Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Factors affecting the inhibitory activity of 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) against *Erwinia carotovora*, a nonpathogen of *Zea mays* L., and against a maize pathovar of *Erwinia chrysanthemi* (ECZ) were examined. Most experiments were performed with DIMBOA dissolved in a bacterial growth medium containing 10 g/liter of sucrose, inorganic salts, and 1 g/liter of casamino acids at pH 6.75. When temperature and pH were held constant, inhibition of *E. carotovora* varied linearly with the logarithm of the initial cell population. By altering temperatures, assays with constant pH and initial cell populations were performed under conditions of varying DIMBOA stability. When *E. carotovora* was grown at 24, 28, 32, and 36 C in the presence of 0.1 to 0.5 mM DIMBOA, the inhibition of bacterial growth was maintained long after DIMBOA had decomposed in the medium to levels which, if added initially, would not have been inhibitory. When assays were performed at pH 5.5, the pH of aqueous maize extracts, *E. carotovora* was more inhibited than at pH 6.75; however, ECZ was substantially less inhibited at the lower pH.

The cyclic hydroxamate, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, is the principal component in extracts of corn (*Zea mays* L.) tissue that is inhibitory to soft rotting *Erwinia* (1). The nature of the inhibition of bacterial growth caused by DIMBOA[5] is a prolongation of lag phase, with no substantial effect on log phase growth rate (1). DIMBOA is unstable in our bioassay medium and decomposes to 6-methoxy-2-benzoxazolinone (MBOA) and other uncharacterized compounds (6). The half-life of DIMBOA (5.3 hr at 28 C, pH 6.75) is substantially less than the duration of inhibition at initial DIMBOA concentrations greater than 0.2 to 0.3 mM (1, 6). Similarly, when DIMBOA was incubated in our bacterial growth medium before addition of bacterial cells, inhibitory activity also was lost with a half-life of about 5 hr (6). MBOA and the other decomposition products were not sufficiently active to account for the observed inhibition (6). Furthermore, other measurements of the biological effects of DIMBOA have extended over periods as long as 21 days (1–5). Clearly then, the relationship between the stability of DIMBOA and its biological activity needs to be examined. In this paper, we present results of tests with variation in pH, incubation temperature, and cell populations.

MATERIALS AND METHODS

Bacterial isolates used in this study were *Erwinia carotovora* (EC, SR-53) and *Erwinia chrysanthemi* pathovar *zeae* (ECZ, SR-120) (see Table I in ref. 1). The former is nonpathogenic on corn, whereas the latter causes a bacterial stalk rot of corn. The bacterial growth assay medium and activity assays were as previously described (1) and inhibition was measured as Δ lag or relative inhibition (RI) as illustrated in Figure 2 of that reference.

DIMBOA (prepared as in ref. 6) was dissolved in the medium by shaking at room temperature for a few min. Solutions were then filter-sterilized (pore size 0.2 μm, Sybron Corp., Rochester, N.Y.)[6] and portions of the sterile medium were diluted with fresh, sterile medium to obtain the desired DIMBOA concentration. DIMBOA concentrations were determined by UV spectrophotometry (ϵ_{273} = 9,300 at pH 6.75). To start assays, known numbers of bacterial cells (in 0.1 ml) were added to 4 ml of the appropriate sterile medium in 25-ml Erlenmeyer flasks. Where appropriate, DIMBOA degradation rates were measured as described by Woodward *et al.* (6).

RESULTS AND DISCUSSION

DIMBOA prolongs lag phase without substantial effect on log phase of bacterial growth (1), and is unstable in aqueous solution (6). Among the possible explanations for the activity of DIMBOA on bacterial cells might be that cells cannot grow in medium containing more than some threshold concentration of DIMBOA. This hypothesis is consistent with the observed activity of DIMBOA. In preliminary experiments, the highest concentration of DIMBOA that would not inhibit growth of *E. carotovora* was about 80 μM. If decomposition of DIMBOA to about 80 μM is the condition necessary to release *E. carotovora* from lag phase, then conditions that alter the rate of degradation of DIMBOA should alter the duration of lag phase in a parallel fashion; conversely, conditions that do not affect the rate of degradation should have

[1] Research cooperative with the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and the Agricultural Research Service, United States Department of Agriculture.

[2] Present address: Division of Plant Industry, CSIRO, P.O. Box 1600, Canberra City, ACT 2601, Australia.

[3] Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile.

[4] Plant Disease Resistance Research Unit, ARS, U.S. Department of Agriculture.

[5] Abbreviations: DIMBOA: 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one; ECZ; strains of *Erwinia chrysanthemi* causing bacterial stalk rot in *Zea mays* L.; MBOA: 6-methoxy-2-benzoxazolinone; RI: relative inhibition; CFU: colony-forming units.

[6] Mention of companies or commercial products does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

no effect on the length of lag phase at a given concentration of DIMBOA. Accordingly, three such conditions were examined: (*a*) variation of inoculum density; (*b*) variation of temperature; and (*c*) variation of pH.

Effect of Inoculum Density. If DIMBOA disappearance is solely due to nonenzymic degradation then inoculum density should not affect the duration of lag phase. However, when inoculum density was varied from 10^4 to 10^8 CFU/ml, there was a marked change in the extent of inhibition caused by a DIMBOA concentration of 0.3 mM (Fig. 1). The increase in duration of lag phase (Δ lag) caused by 0.3 mM DIMBOA was 11.9 hr for an initial inoculum of 10^8 CFU/ml. About two half-lives (10.6 hr, see ref. 6) would be required to reduce the 0.3 mM DIMBOA initially present to 80 μM. Thus, for the high inoculum level, the observed and predicted values fit quite well. However, the 3-fold greater Δ lag observed for the lowest inoculum density is inconsistent with the hypothesis. That the inconsistency is not due to biological (enzymic) acceleration of DIMBOA degradation is apparent in that biological degradation would be expected to increase with increasing bacterial population; yet it is at the highest initial bacterial population that Δ lag comes closest to predicting the degradation rate for DIMBOA in medium alone.

Effect of Assay Temperature. The rate at which DIMBOA decomposes in bacterial growth medium is dependent on temperature (6). By varying the temperature of the bioassay, the time (t) necessary for decomposition of any initial concentration (c_0) of DIMBOA to the threshold concentration (c) for bacterial growth (assumed to be 80 μM for all temperatures) will vary considerably and can be determined by equation 1:

$$t = \frac{\ln (c_0/c)}{k} \qquad (1)$$

where k is the first order rate constant for decomposition of DIMBOA at a given temperature (from Fig. 5 in ref. 6). On the basis of the time required for the control to reach 100 Klett units (X) and the time (t) calculated for a given concentration of DIMBOA to decompose to 80 μM at the test temperature, the RI can be predicted by equation 2:

$$\mathrm{RI} = 1 + \frac{t}{X} \qquad (2)$$

Data for observed RI (——) at 24, 28, 32, and 36 C are compared to predicted RI (. . . . .) in Figure 2. Since stability of DIMBOA decreases with increasing temperature, the predicted RI decreases as temperature is increased. However, the observed RI increased with increasing temperature. At 36 C, in fact, the RI of 4.6 observed for 0.4 mM DIMBOA was nearly three times the predicted value of 1.65. For these conditions, the residual DIMBOA concentration in the medium at the time logarithmic growth finally began (calculated by solving equation 1 for c with t set equal to Δ lag and using $k = 0.433$ hr^{-1} [ref. 6]) was 0.06 μM, more than three orders of magnitude below the expected threshold of 80 μM. Thus, a model which considers only chemical instability of DIMBOA and a threshold concentration at which growth can occur is not sufficient to account for the observed data.

The shapes of the curves in Figure 2 differ (linear for observed data, curved for calculated data). If temperature affects the threshold concentration that allows logarithmic growth to begin, the positions, but not the shapes, of the calculated curves would change. Thus, correcting for variations in threshold concentration

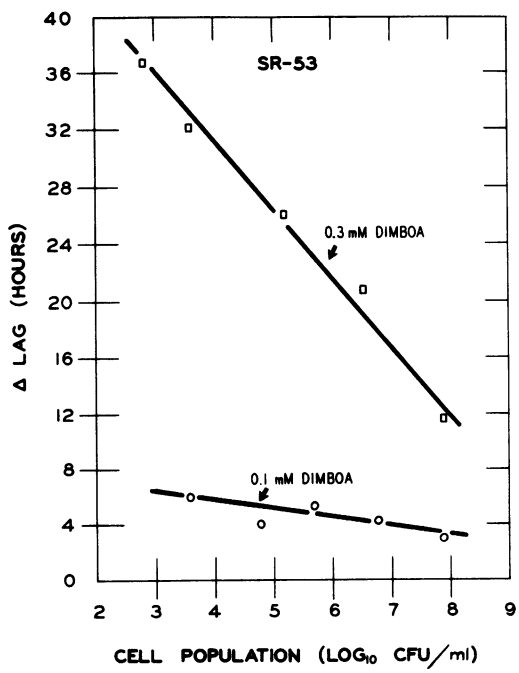

FIG. 1. Relationship between bacterial inhibition due to DIMBOA and population density of *E. carotovora*. Bacterial populations were measured by dilution plating every 4 hr for treatments below 10^7 CFU/ml and every 2 hr for treatments at 10^7 and 10^8 CFU/ml. The cell populations plotted are the lowest experimentally determined populations (by viable cell count) before logarithmic growth began. Data points are averages for two flasks.

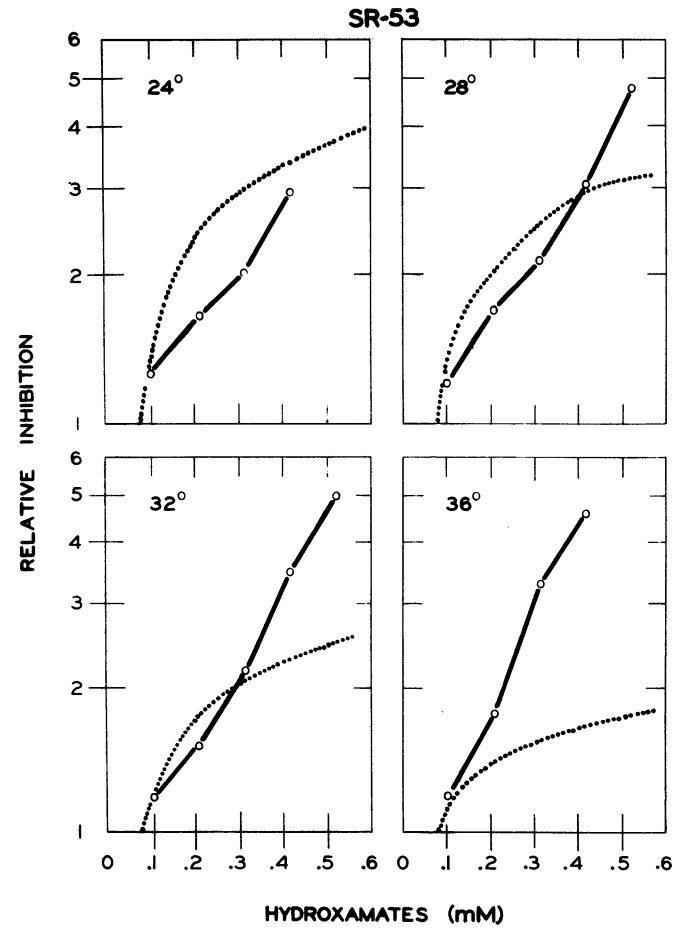

FIG. 2. Comparison of predicted (· · · · ·) and observed (——) inhibition of *E. carotovora* at 24, 28, 32, and 36 C. The times for controls to reach a turbidity of 100 Klett units were 8.9, 6.2, 5.5, and 5.7 hr at 24, 28, 32, and 36 C, respectively. First order rate constants (k) for DIMBOA degradation at pH 6.75 were 0.077, 0.131, 0.231, and 0.433 hr^{-1} at 24, 28, 32, and 36 C, respectively (from ref. 6).

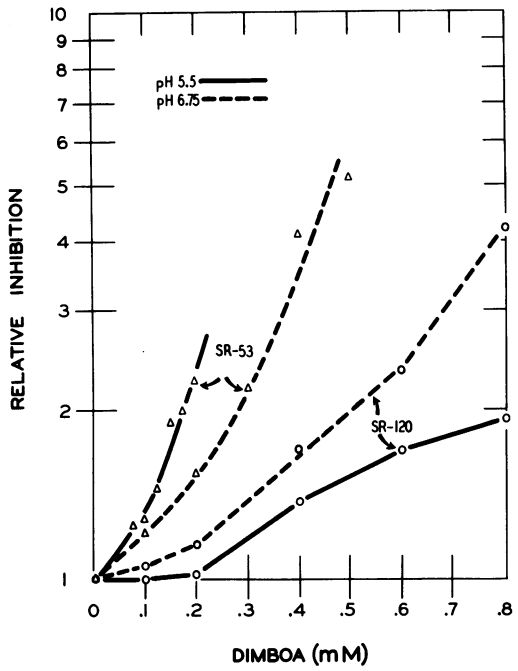

FIG. 3. Comparison of the inhibitory effect of DIMBOA at pH 5.5 (——) and pH 6.75 (- - -). The bacteria used were *E. carotovora* (SR-53) and ECZ (SR-120). For the assays at pH 5.5, a 0.1 M succinic acid-NaOH buffer was used. In all other respects, the media were identical. The controls attained 100 Klett units in 8 hr (pH 5.5) and 6.5 hr (pH 6.75) for SR-53 and 5.6 hr (pH 5.5 and pH 6.75) for SR-120.

with temperature would not produce a good "fit" to the observed data.

Effect of Medium pH. The stability of DIMBOA is strongly affected by the pH of the solution (Fig. 6 in ref. 6). Thus, by comparing the activity of DIMBOA at pH 5.5 (half-life 24 hr) to its activity under our "standard" assay condition of pH 6.75 (half-life 5.3 hr), the activity can be compared to its stability under conditions of constant temperature and inoculum density. Because of the increased stability of DIMBOA at the lower pH, a parallel increase in inhibitory activity would be expected. As predicted, *E. carotovora* was significantly more sensitive to DIMBOA at the lower pH (Fig. 3). This result is consistent with the hypotheses that the increased stability of DIMBOA does increase the apparent biological activity of this compound, and that DIMBOA itself is an inhibitor in this system. The results obtained with the corn pathogen are not consistent with the increased stability-increased activity hypothesis. ECZ was much less sensitive to DIMBOA at the lower pH than at pH 6.75 (Fig. 3). However, the decreased sensitivity of ECZ at the lower pH may be relevant to pathogenesis since the pH of the juice from crushed corn plants is about 5.5.

One hypothesis concerning the action of DIMBOA appears to have been disproven—*E. carotovora* is not always released from inhibition when DIMBOA has decayed to some threshold concentration in the medium. This is apparent from the experiments in which inoculum size and temperature were varied. The possibility that degradation products of DIMBOA function to extend lag phase in these experiments appears unlikely. MBOA, a principal degradation product of DIMBOA, did not cause a prolongation of lag phase, nor was MBOA synergistic with DIMBOA in this system (1). Further, relatively little inhibition due to other degradation products was detected when DIMBOA was degraded in medium prior to addition of bacteria and then tested for activity (6). It is possible that an inhibitory degradation product does exist, but that it escaped detection in our experiments (6). For example, we would not have detected a toxic product that is produced within the bacterial cells. At the present time we cannot explain the rather intriguing property of DIMBOA of being able to inhibit bacterial cells after disappearing from the medium.

Acknowledgment—Assistance of S. Vicen in preparation of figures is gratefully acknowledged.

LITERATURE CITED

1. CORCUERA LJ, MD WOODWARD, JP HELGESON, A KELMAN, CD UPPER 1978 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, inhibitor from *Zea mays* with differential activity against soft rotting *Erwinia* species. Plant Physiol 61: 791–795
2. COUTURE RM, DG ROUTLEY, GM DUNN 1971 Role of cyclic hydroxamic acids in monogenic resistance of maize to *Helminthosporium turcicum*. Physiol Plant Pathol 1: 515–521
3. DAWE DH 1973 The relation of 1,4-benzoxazolinones and related compounds in *Zea mays* to *Helminthosporium maydis* resistance. PhD thesis. Iowa State University
4. KLUN JA, CL TIPTON, TA BRINDLEY 1967 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), an active agent in the resistance of maize to the European corn borer. J Econ Entomol 60: 1529–1533
5. LONG BJ, GM DUNN, JS BOWMAN, DG ROUTLEY 1977 Relationship of hydroxamic acid content in corn and resistance to the corn leaf aphid. Crop Sci 17: 55–58
6. WOODWARD MD, LJ CORCUERA, JP HELGESON, CD UPPER 1978 Decomposition of 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one in aqueous solutions. Plant Physiol 61: 796–802