Changed Metabolic Pathways and the Germination of Alternaria solani Spores

Paul E. Waggoner and J. -Y. Parlange

The Connecticut Agricultural Experiment Station, New Haven, CT 06504.
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ABSTRACT

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Antimycin A slows the germination of *Alternaria solani* spores, and further slowing is caused by a concentration of salicyl hydroxamic acid (SHAM) that is ineffective alone. Germination is slowed more when antimycin and SHAM are applied some time after wetting of the spores than when they are applied at the time of wetting. A brief exposure of the spores to the chemicals at the time of wetting did not slow germination, a brief exposure later caused significant slowing, and additional exposures did not cause additional slowing.

Additional key words: mathematical model.

The simplest way of calculating the development of an organism in a changing environment is to consider it as a series of constant environments and merely add the development that would be made in each of the constant environments. When the rate of development increases linearly with temperature and temperature is the controlling factor, this simple means of calculation is the same as the well-known method of degree days. In the case of the germination of *Alternaria solani* spores, we found that this simple method worked over a wide range of temperatures (4). We considered germination to be the process of development that begins with the wetting of the spores and ends with the production of a germ tube.

In two cases, however, change in the state of the environment slowed germination and delayed the appearance of germ tubes. If the temperature fluctuated above and below the optimum, germination was slower than expected from the rates at constant temperatures (5). Or, if the hydration of the spores was changed by changing the osmotic potential of the environment, germination was slower than expected from the rates in constant osmotic potentials (6). In both of these cases, we proposed that development was proceeding along different pathways at the different temperatures or osmotic potentials and that the delay was caused by the change in pathway forced by a change in the environment.

These results and our proposal of pathways for development suggested to us that we should next examine whether a change between biochemically distinct metabolic pathways would also slow germination. We chose to examine the alternative oxidase systems that are sensitive to cyanide or antimycin A, in one case, and to salicyl hydroxamic acid (SHAM), in the other (1). The discoverers of antimycin A (2) found that it would control tomato early blight, but would not completely inhibit the growth of *A. solani*, an observation that suggests an alternative oxidase pathway that *A. solani* could switch to

when exposed to antimycin.

In the experiments reported here we show that adding SHAM to antimycin slows the germination of *A. solani* spores and that exposing the spores to antimycin during part of the germination process slows germination as much as continuous exposure, which suggests that changing pathways slows development.

MATERIALS AND METHODS

The *A. solani* isolate that we used and methods for production and storage of the spores in a cool, dry environment have been described previously (3). We have also described the manner of brushing of spores onto filters, the changing of solutions that moisten the filters and spores, and the observation of germination of spores on the filters (6). The formation of germ tubes was observed at times chosen to give a range of percentages. The half-time $t_{1/2}$ is the estimated time for half the spores to produce germ tubes, s is the standard deviation of these times for individuals, and together $t_{1/2}$ and s represent the cumulative normal curve that can be fitted to the increase with time in the percentage of spores that have produced germ tubes (3). The speed of germination is defined as the reciprocal of $t_{1/2}$, and the speed of germination in the presence of an inhibitor can be normalized by dividing it by the speed in the standard solution.

The standard solution for moistening the spores was 0.25 M sucrose, which provides the optimum osmotic pressure for germination (6). The medium was buffered at pH 5.8 by 0.01 M di-potassium phosphate adjusted with mono-potassium phthalate. Antimycin solutions were prepared by adding the requisite amount of antimycin A dissolved in acetone to the standard solution. The dilute acetone alone did not affect germination. At 4 to 64 μg/ml, cloudiness showed that some precipitation took place and that the actual concentration may have been less than its nominal value. Solutions of SHAM were prepared by dissolving the compound in warm standard solution.

RESULTS AND DISCUSSION

Antimycin.—Filters were moistened with 0 to 64 $\mu g/ml$ (0 to about 0.1×10^{-3} M) antimycin in standard solution. The speed of germination was normalized by dividing the $t_{1/2}$ for no antimycin by the $t_{1/2}$ with antimycin, and the normalized speed was plotted as a function of concentration (Fig. 1).
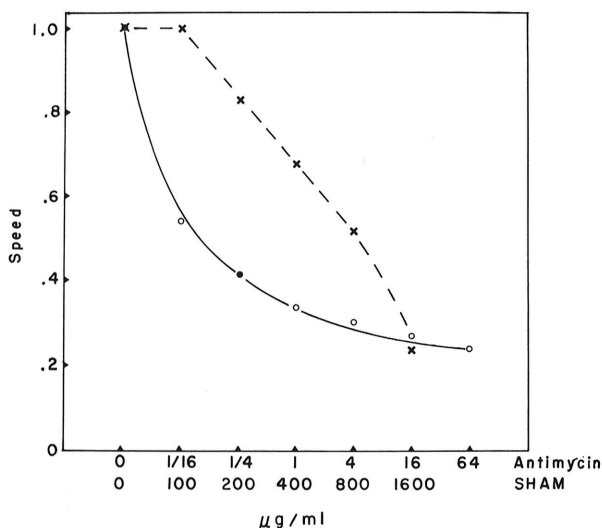
Antimycin clearly slowed germination of *A. solani*

Fig. 1. The effect of antimycin (o) and salicyl hydroxamic acid (SHAM) (X) upon the speed of germination of *Alternaria solani* spores. The speed for the observations of Table 1 is shown here by ●. Speed is $t_{1/2}$ with no inhibitor divided by $t_{1/2}$ with inhibitor.
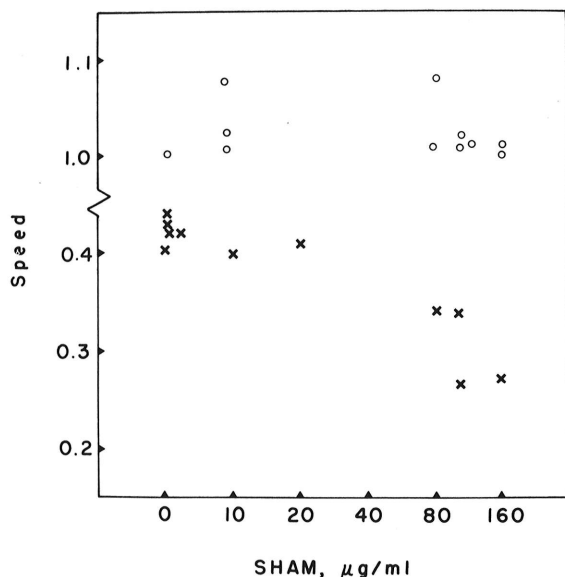

Fig. 2. The effect of 160 $\mu g/ml$ salicyl hydroxamic acid (SHAM) upon the speed of germination of *Alternaria solani* spores, with (X) and without (o) 0.25 $\mu g/ml$ antimycin. Speed is $t_{1/2}$ with no inhibitor divided by $t_{1/2}$ with inhibitor.

spores, but the high concentrations of 4 $\mu g/ml$ or more did not stop germination completely. It will be recalled that antimycin decreased but did not stop the growth of *A. solani* (2). Antimycin also decreased but did not stop the respiration of *Neurospora crassa* (1). Since the latter was attributed to an alternate, SHAM-sensitive respiration, we next tested SHAM.

Salicyl hydroxamic acid.—Filters were moistened with 0 to 1,600 $\mu g/ml$ (0 to about 10^{-2} M) SHAM. The speed of germination was plotted as a function of SHAM concentration in Fig. 1. Although high concentrations of SHAM slowed germination of *A. solani*, 100 $\mu g/ml$ or 650×10^{-6} M SHAM had no observable effect on germination, whereas 2×10^{-6} M antimycin slowed it substantially.

Antimycin plus salicyl hydroxamic acid.—Filters were moistened with 0 to 160 $\mu g/ml$ SHAM, with or without 0.25 $\mu g/ml$ antimycin. Concentrations of SHAM that were ineffective alone slowed germination when antimycin was present (Fig. 2). This is consistent with the hypothesis i) that an antimycin-insensitive, SHAM-sensitive system is present in *A. solani* as an alternative to the normal system inhibited by antimycin and ii) that inhibition of these systems inhibits germination.

Changing solutions.—Experiments were designed to force germination from the usual, antimycin-sensitive system to another. Spores were exposed continuously to antimycin by flushing the spores initially and at 0.5 hr with 5 ml of 0.25 $\mu g/ml$ antimycin. Alternatively, the solution was changed by first moistening the spores with the standard solution, and then after 0.5 hr they were changed to the antimycin solution. In this case, the spores were first flushed with 5 ml of the standard solution and then flushed at 0.5 and 1.0 hr with 5 ml of 0.25 $\mu g/ml$ antimycin. In a third treatment or regime, the spores were not changed to antimycin until 0.75 hr had passed. Germination was observed at 2, 3, and 4 hr in a total of six replicates. A fourth group of spores was simply moistened with the standard solution. We transformed the percentages of germination into angles and performed an analysis of variance of the angles for each time of observation.

In the absence of antimycin, the percentage of spores germinated was 11% at 1 hr, 74% at 1.5 hr, and 89% at 2

TABLE 1. Percentage of *Alternaria solani* spores germinated when exposed to 0.25 $\mu g/ml$ antimycin continuously or when changed from a standard solution[a] to antimycin solution

Exposure to antimycin	Germination (%) at:		
	2 hr	3 hr	4 hr
Continuous	6	44	67
Changed at 0.5 hr	2**[b]	43	70
Changed at 0.75 hr	10*	42	69

[a]Standard solution: 0.25 M sucrose buffered with 0.01 M di-potassium phosphate adjusted to pH 5.8 with mono-potassium phthalate.

[b]Asterisks indicate significantly more or less germination at $P = 0.05$ (*) or 0.01 (**) than continuous exposure as shown by analysis of variance of angular transformation of percentages. Near 5% germination the standard error of the above percentages is 1.3% germination. In the absence of antimycin, 89% of the spores had produced germ tubes after 2 hr.

hr. Continuous treatment with antimycin slowed germination (Table 1). It is striking that the spores germinated little faster when the exposure to antimycin was delayed until 0.75 hr after the spores were wet. Three-quarters of 1 hr is fully half the $t_{1/2}$ in the standard solution, and thus the germination process was well advanced before antimycin was added. Still more striking, adding antimycin 0.5 hr after the spores were wet caused significantly less germination at 2 hr than continuous exposure to antimycin. These results suggest that a change nullified the advantage of an initial 0.5 to 0.75 hr without inhibitor.

Assuming that germination proceeds reasonably well in 0.25 μg/ml antimycin via the SHAM-sensitive pathway, we next sought a more striking slowing of germination by a change into both antimycin and SHAM, which might force respiration into a third pathway even slower than the SHAM-sensitive one. Thus, spores were exposed continuously to the two inhibitors by flushing them initially with 0.1 μg/ml antimycin plus 100 μg/ml SHAM. Alternatively, spores were moistened initially with standard solution and after 0.5 hr were exposed to the antimycin-SHAM solution. In a third regime, exposure was delayed for 0.75 hr. There were six replicates of each treatment.

Germination at all times was significantly less when antimycin and SHAM were applied after 0.5 hr than when they were present continuously (Table 2). Delaying the exposure to 0.75 hr tempered the effect, but even here the change caused somewhat more slowing of germination than did continuous exposure. The six replicates analyzed in Table 2 are shown individually in Fig. 3. Further, data for two additional replicates of continuous application and application after 0.5 hr are shown as well as for three replicates of untreated spores.

A comparison of the effects of *change* to antimycin alone (Table 1) with *change* to antimycin plus SHAM (Table 2), reveals a greater effect of change to the combination than to the single inhibitor. If the combination forces metabolism along a third pathway, then changing to this third pathway caused greater delay than either continuous operation via the third pathway or changing to the SHAM-sensitive pathway.

Germination has been conceived in a model as progressing through f stages at P stages per unit time, and

the transformation of the observed half-time $t_{1/2}$ and the standard deviations of individual germination times into f and P has provided a method for predicting germination in diverse temperature and water environments (3, 4, 5, 6). In steady environments $t_{1/2}$ and s are transformed into f and P by:

$$P = t_{1/2} / s^2, \text{ stages/hour} \qquad (I)$$
$$f = P\,t_{1/2}, \text{ stages} \qquad (II)$$

In a changing environment, f is the sum of the products of rates P and times spent at the different conditions (3):

$$f = P_0\,t_0 + P\,(t_{1/2} - t_0), \text{ stages} \qquad (III)$$

where P_0 and t_0 are the rate and time without inhibitor, P is the rate when changed to inhibitor and $t_{1/2}$ is the total time for half the spores to produce germ tubes. In a changing environment,

$$s^2 = f / P^2, \text{ (hours)}^2 \qquad (IV)$$

where P, as in equation III, is the rate near $t_{1/2}$.

The model allows us to analyze development during the times before and after a change in environment. First, equations I and II are used to estimate P and f during steady conditions and then equations III and IV are used to estimate the parameters during the period after the change in environment. In a steady environment without inhibitor, f was 14 and P was 12 (Table 2), which agree with earlier observations (4). In the steady environment of continuous exposure to antimycin and SHAM, $t_{1/2}$ was 3.9 and s was 1.6, which correspond to f = 6 and P = 1.5.

With the value of 12 for P_0, we next use equations III and IV to estimate P for development after antimycin and SHAM are applied at $t_0 = 0.5$ hr and f for the total development process. After the application, P = 2.1, which is faster than the 1.5 obtained for continuous exposure, and f = 13, which is larger than the value of 6 obtained for continuous exposure. P and f also increase when temperature (5) or osmotic pressure (6) are changed.

When t_0 was 0.75 hr, f and P were again increased, suggesting that the harm of change increased as change was delayed. Delaying warming to a high temperature

TABLE 2. Percentage of *Alternaria solani* spores germinated when exposed to 0.1 μg/ml antimycin plus 100 μg/ml salicyl hydroxamic acid (SHAM) continuously or when changed from a standard solution[a] to antimycin plus SHAM solution, time $t_{1/2}$ for 50% germination, the standard deviation s, and the parameters f and P[b]

Exposure to antimycin and SHAM	Development time			Germination parameters[c]			
	2	3	4	$t_{1/2}$	s	f	P
Continuous	16	29	54	3.90	1.60	6	1.5
Changed at 0.5 hr	5**[d]	18*	39**	4.50	1.70	13	2.1
Changed at 0.75 hr	11	23	45	4.25	1.65	16	2.4

[a]Standard solution: 0.25 M sucrose buffered with 0.01 M di-potassium phosphate adjusted to pH 5.8 with mono-potassium phthalate.

[b]In the absence of the inhibitors, germination at 28 to 30 C was 18% at 1 hr, 82% at 1.5 hr and 96% at 2 hr. These correspond to f = 14 and P = 12.

[c]The observed half-time ($t_{1/2}$, in hours) and standard deviation (s, in hours) of individual germination times have been transformed by means of a model into a rate of progress of P stages per hour through f stages.

[d]Asterisks indicate significantly less germination at $P = 0.05$ or 0.01 than continuous exposure as shown by analysis of variance of angular transformation of percentages.

also increased f and P (3).

The fit of the model to the data is shown in Fig. 4. The time has been normalized by subtracting $t_{1/2}$ and dividing by s. This causes all the observations of germination to fit a single cumulative normal curve, whether the spores were exposed to the inhibitors continuously, after 0.5 or 0.75 hr, or never and whether the $t_{1/2}$ was 1.2 or 4.5 hr. The percentage germination of untreated spores, which was far separated from other observations in Fig. 3, falls on the same curve as the others in Fig. 4. Observations for other treatments are moved horizontally on Fig. 4 by the normalization of the model so that the averages for a given time fall close to the single curve. It is gratifying that the two additional replicates of treated and a replicate of untreated spores not analyzed in Table 2 also fall near the curve.
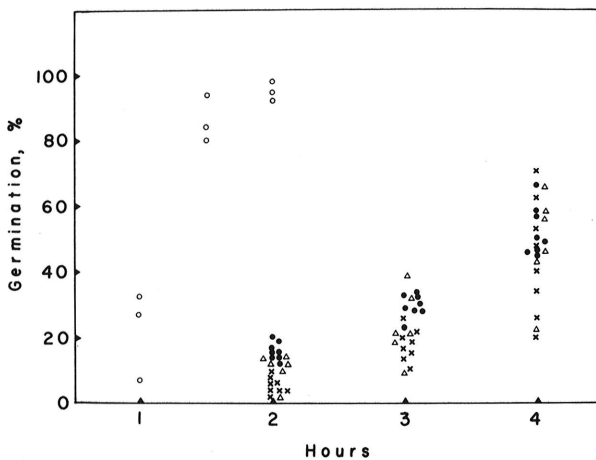

Fig. 3. Germination of *Alternaria solani* spores in the standard solution (o) and when 0.1 μg/ml antimycin plus 100 μg/ml salicyl hydroxamic acid are continuously present (●) or applied 0.5 (×) or 0.75 (△) hour after the spores are wetted. Each point plotted is a mean of three observations of 50 spores on a microscope slide.
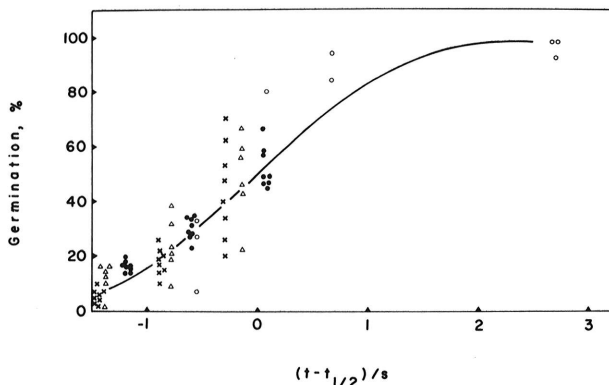

Fig. 4. The observations of Fig. 3 fitted to the model described in the text by subtracting from time t of observation the half-time $t_{1/2}$ and dividing by the standard deviation s (Table 2). The curve is the cumulative normal curve with $t_{1/2}$ equal to 0 and s equal to 1.

Repeated changes.—Because multiple changes in temperature and osmotic pressure were more harmful than a single change (5, 6), a logical extension of the above experiments was to proceed from the single change to repeated changes of inhibitors. Repeated changes from inhibitor to standard solution required that we be able to wash out the inhibitor.

To test whether a change of solution on the filter changed the concentration of inhibitor affecting germination, spores were placed on filters moistened with a variety of solutions of antimycin and SHAM, and then after 0.5 hr they were washed with 5 ml of standard solution. In most experiments the spores were washed repeatedly at half-hour intervals. When spores were first exposed for 0.5 hr to 0.1 or 0.25 μg/ml antimycin, with or without 100 μg/ml SHAM, and then washed several times with the standard solution, they germinated almost as fast as in the absence of inhibitors. When 1 or 16 μg/ml antimycin was applied for 0.5 hr, however, subsequent washing did not remove it fully. These experiments demonstrated an insensitivity of the spores during the first 0.5 hr, and more importantly, they demonstrated that the solution could be largely removed by flushing a new solution through the filter.

Next we tried to slow germination more than in a single change (Table 2) by washing inhibitor away after 0.5 hr and reapplying it after an additional 0.5 hr. Germination was not delayed more than by the single change. The same result was obtained for a variety of concentrations of antimycin, with and without SHAM. Although absence of inhibitor for half the time did not shorten the germination time, it did not lengthen the time either. Thus the single change in pathway produces the maximum effect on germination.

In conclusion, the use of antimycin and SHAM has indicated that there are alternate metabolic pathways, perhaps three, that affect spore germination. In these terms, the increase in pathway length f caused by the change represents stages in shifting from the normal pathway to an alternate pathway. And the new, faster rate P would represent an average of progress through the shift and the later development in the inhibitor. We have observed a surprising increase in inhibition of germination by using inhibitors for a shorter time and have found another use for a model that also analyzes changes in the temperature and water of the environment of germination. The lengthening of the pathway obtained by changing an inhibitor in the environment resembles the results of changes in the temperature and water of the environment of germination that have been analyzed by the same model.

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