Cyanide Tolerance in Stemphylium loti

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ABSTRACT

Stemphylium loti was more tolerant of cyanide than were three other *Stemphylium* spp. Cyanide tolerance was measured by colony growth on liquid and solid media for all four species, and by germination of conidia and oxygen uptake of conidia for *S. loti* and *S. sarcinaeforme*. *Stemphylium loti* adapted to cyanide. Adaptation was characterized by stimulated oxygen uptake upon exposure to cyanide, by an ability to remove or transform cyanide, and by increased germinability of conidia when incubated in cyanide. *Stemphylium sarcinaeforme* developed an ability to respire in low levels of cyanide, but not to remove or transform cyanide, nor did conidia exhibit increased germinability in cyanide upon prior exposure to cyanide. Oxygen uptake by mitochondria from adapted *S. loti* cells was not stimulated by cyanide. Phytopathology 61:494-500.

Additional key words: Lotus corniculatus, Stemphylium botryosum, Stemphylium consortiale.

Stemphylium loti is the principal pathogen of birdsfoot trefoil (*Lotus corniculatus* L.) in New York state. Birdsfoot trefoil is a cyanogenic plant (3), and cyanide is released from the β-glucosides linamarin and lotaustralin upon mechanical injury or infection by *S. loti* (10). Both the plant and the pathogen possess enzymes which can effect the release of cyanide from trefoil tissue (10). *Stemphylium loti* is more tolerant of cyanide than are eight other fungi (10).

Reynolds suggested (14) that a pathogen's tolerance of cyanide might be a significant factor in determining its pathogenicity to a cyanogenic plant. A number of investigators have considered the role of cyanide in resistance of cyanogenic plants to pathogens, and most have found no correlation between cyanide content of the host and disease resistance (9, 13, 14, 16, 17). On the basis that trefoil is a cyanogenic plant which is relatively free of fungal pathogens, and that *S. loti* is tolerant of cyanide, Millar & Higgins (10) postulated that cyanide tolerance in this fungus might be a significant factor in its pathogenesis of trefoil. Consequently, in this study we have focused on the pathogen rather than on the plant, and have examined the nature of cyanide tolerance in *S. loti*. *Stemphylium sarcinaeforme* was also studied extensively because it is morphologically similar to *S. loti*, is pathogenic in nature to red clover but not to trefoil, and under highly favorable greenhouse conditions can cause lesions on trefoil.

MATERIALS AND METHODS.—*Stemphylium botryosum* Wallr., *S. loti* Graham, and *S. sarcinaeforme* (Cav.) Wilt. were obtained from J. H. Graham, USDA, Beltsville, Md. Virulence was maintained by passing each fungus periodically through its suscept; i.e., alfalfa (*Medicago sativa* L.), trefoil (*Lotus corniculatus* L.), and red clover (*Trifolium pratense* L.), respectively. *Stemphylium consortiale* was obtained from S. W. Braverman, USDA, Geneva, N.Y. Cultures were maintained on V-8 juice agar (11) under continuous fluorescent light at 21-23 C.

Growth measurements.—Mycelial dry wt were determined for the different *Stemphylium* spp. grown in several concentrations of cyanide in a medium consisting of 20% V-8 juice (supernatant of whole juice centrifuged 10 min at 12,000 *g*) and 0.05 M potassium phosphate buffer adjusted to a final pH of 6.5. Twenty-seven ml of medium were dispensed into 125-ml Erlenmeyer flasks and sterilized by autoclaving for 15 min at 15-lb. pressure. Three ml of filter-sterilized KCN solution and then a 3-mm agar disc bearing the fungus were added aseptically to each flask. Each cyanide treatment was replicated 3 times for each fungus. Cultures were incubated at 21-23 C under fluorescent light for 4 days. The contents of each flask were then filtered onto a tared filter paper, dried in an oven at 70 C for 24 hr, and weighed.

Radial growth of the different *Stemphylium* spp. on V-8 juice agar which contained different concentrations of KCN was also determined. Filter-sterilized KCN was added to cooled (ca. 50 C) liquid agar, which was then dispensed aseptically into petri plates. An agar disc (3 mm) bearing the appropriate fungus was placed on the KCN medium so that the mycelium was in contact with the medium. Each cyanide treatment was replicated 3 times. Parafilm was wrapped around the petri plates to retard loss of cyanide. Growth was recorded as average colony diam less the diam of the original agar disc.

At pH 6.5, cyanide exists as HCN which is volatile. Consequently, the concentration of cyanide in liquid or agar media decreased with time. After 4 days' incubation, the cyanide content of the sterile liquid medium was 20-30%; that of the sterile agar medium was 40-50% of the initial concentrations.

Oxygen uptake by Stemphylium spp.—Oxygen uptake by the fungi for periods up to 6 hr was measured manometrically with a Gilson Differential Respirometer, Model GRP 20; for short-time intervals, oxygen uptake was measured polarographically with a Gilson Oxygraph Model KM equipped with a Clark electrode. Treatments in the respirometer were equilibrated 20 min before measurements were initiated.

In most experiments, the oxygen uptake was determined for spores in suspension. Spores from 10- to 20-day-old cultures were harvested in water, filtered through two layers of cheesecloth, washed 3 times with distilled water by centrifuging (1 min at ca. 1,500 *g* in

an International Clinical centrifuge), then suspended in 0.1 M phosphate buffer at pH 6.5. The pH was 6.5 ± 0.2 after 6-hr incubation. Spore concentration was determined by measuring the absorbance of the suspension at 450 nm. The absorbance was then converted to mg dry wt per ml from a standard curve. About 1 mg spores/ml were used in most experiments. Temperature was 25 C in all experiments.

When cyanide was used in an experiment, $Ca(CN)_2$ in 10% $Ca(OH)_2$ was placed in the center wells of respirometer vessels to prevent distillation of cyanide from the reaction mixture into the alkali in the center wells (15). For 0.1, 0.46, 1.0, and 2.2 mM cyanide in the reaction mixture, the $Ca(CN)_2$ concentrations in the center wells were 0.051, 0.21, 0.42, and 0.78 M, respectively.

For experiments which required removal of spores from the vessels, reaction mixtures were made 0.05% with Tween 20 (polyoxyethylene sorbitan monolaurate). This concentration of Tween 20 had no observable effect on oxygen uptake or on germination of the spores. Spores used following a given incubation period in the respirometer were washed several times in water, then suspended in 0.1 M phosphate buffer pH 6.5.

Results of tests in which the cyanide effect was measured with the oxygen electrode are expressed as percentage of the control. The control rate of oxygen uptake was that of spores in the absence of cyanide. After the control rate was established, cyanide was added to the reaction mixture and the experimental rate determined.

Spore germination studies.—Spore germination during incubation in the respirometer was determined at the conclusion of some experiments. In these tests, the medium contained 0.1 M phosphate pH 6.5, 0.05 M glucose, 0.5% yeast extract, and cyanide (0-2.2 mM). Shaking of the respirometer vessels caused the germinated spores to aggregate in such a way that germ tubes from individual spores could not be measured. Germ tubes at the margin of an aggregation of spores could be measured, however, and the lengths of these germ tubes were measured with an ocular micrometer. The average length of 20 germ tubes was determined.

Estimation of cyanide concentration.—Estimation was by means of alkaline sodium picrate (picric acid, 5 g; Na_2CO_3, 25 g; H_2O, 2 liters) which with cyanide produces a red chromogen. Absorbance of the chromogen was measured at 480 nm (10). One ml of cyanide solution was added to 4 ml of picrate solution, and color was developed by heating the mixture in a boiling water bath for 5 min. Absorbance was measured in a Bausch and Lomb spectronic 20 colorimeter. Absorbance values were converted to cyanide concentrations by means of a standard curve.

Cyanide in spore suspensions was estimated by adding 1 ml of spore suspension to 4 ml picrate solution. The spores settled out and did not interfere with the assay. Glucose, however, produced a chromogen with the picrate solution; hence, acetate (0.05 M) was used as the carbon source for spore suspensions in which cyanide concentrations were measured.

In some experiments, cyanide concentrations in spore suspensions were measured concurrently with oxygen uptake. Fourteen respirometer vessels were prepared in such a way that each contained spores suspended in 0.1 M phosphate pH 6.5, 0.05 M acetate, and 0.46 mM cyanide. Two additional control vessels did not contain cyanide. Oxygen uptake of the spores in each vessel was recorded as described above. At time 0, and at hourly intervals thereafter, two vessels were removed from the respirometer and the cyanide content was determined.

The intracellular cyanide content of *S. loti* spores, which previously had been incubated in buffer or in cyanide solution from which the cyanide had disappeared, was determined. After incubation, the spores were washed several times in distilled water, then resuspended in 10 ml 0.1 M phosphate pH 9.5. This spore suspension was shaken 1 min at 4,000 cycles/min with 15 ml 0.45-0.50 mm glass beads in a Bronwill MSK cell homogenizer. Cell breakage was about 99%. The cyanide concentration of the resulting homogenate was determined with the picrate technique.

Sensitivity of cell-free particles to hydrogen cyanide.—Cell free particles obtained from mycelium grown 36-48 hr in liquid shake culture at 25 C were tested for their sensitivity to hydrogen cyanide. Cultures were started by transferring, aseptically, 5 ml of a dense spore suspension of the fungus to a 500-ml Erlenmeyer flask containing about 100 ml of medium (20% V-8 juice and 0.05 M phosphate adjusted to pH 6.5). Mycelium was harvested, washed with distilled water, suspended in 50 ml 0.1 M phosphate pH 7.0, then fragmented for 10 sec at high speed in a Waring Blendor. The mycelial suspension was divided into two equal portions. One portion was incubated 2 hr in 1% glucose and 0.1 mM KCN in a 250-ml Erlenmeyer flask; a cup with 10% $Ca(OH)_2$ containing 0.051 M $Ca(CN)_2$ was suspended over the suspension. The other portion was incubated in 1% glucose. At the end of 2 hr, mycelium from each treatment was washed several times, and the respiratory response of the cells to 0.46 mM cyanide was determined in the respirometer.

Three g wet wt of mycelium exposed or not exposed to cyanide were added to 15 ml of isolation medium (0.75 M mannitol, 0.15% bovine serum albumin [BSA], and 1.0 mM EDTA at a final pH of 7.0). All subsequent treatments were at 4 C. Cells were separated by grinding at full speed for 2 min in a Virtis 45 homogenizer. The resulting suspension was shaken in a Bronwill MSK cell homogenizer for 10 sec (4,000 cycles/min) with 15 ml of 0.45-0.50 mm diam glass beads and 10 ml additional isolation medium. The resulting suspension was centrifuged at 2,000 *g* for 7 min, and the pellet discarded. The supernatant was centrifuged at 10,000 *g* for 7 min; the resulting pellet was resuspended in 2 ml of isolation medium and centrifuged at 10,000 *g* for 7 min. This latter pellet was suspended in 1.0 ml of isolation medium, and the supernatant centrifuged 10 min at 30,000 *g*. The 30,000 *g* pellet was suspended in 2 ml of isolation medium which then was centrifuged at 30,000 *g*, and the resulting pellet was suspended in 1.0

ml of isolation medium. Particles were sedimented in a Lourdes "Beta-Fuge" Model A with a 9 RA rotor.

The different pellets were tested by means of the oxygraph for their ability to take up oxygen in the absence or presence of cyanide. The reaction medium contained 1.3 ml of 0.75 M mannitol, 0.06 M 2 (N-Morpholino) ethane sulfonic acid (MES), and 0.15% BSA, at a final pH of 7.0, and 0.3 ml of the appropriate resuspended pellet.

Effect of 2,4-dinitrophenol on spores.— The effect of 2,4-dinitrophenol (DNP) on spores was determined in the medium described for germination and oxygen uptake studies. In studies with cyanide and DNP, DNP was added from the side arm after a 30-min incubation of the spores in cyanide. All experiments were repeated at least once.

RESULTS.—*Growth and respiratory responses of Stemphylium spp. to cyanide.*—As measured both by dry wt increase and radial extension, *S. loti* had greater tolerance to cyanide than did the other three *Stemphylium* species (Fig. 1-A, B). Of the four fungi, *S. sarcinaeforme* appeared to be the most sensitive to cyanide. *Stemphylium consortiale* was nearly as tolerant to cyanide as *S. loti* when growth was measured by radial extension (Fig. 1-A)

Stemphylium loti and *S. sarcinaeforme* spores, which previously had not been exposed to cyanide, responded similarly to cyanide (Fig. 2-A, B) in experiments in which the oxygen electrode was used to measure respiratory responses. When oxygen uptake by each fungus was measured during 6 hr, however, they exhibited differences in their responses to cyanide (Fig. 3-A, B).

Oxygen uptake by *S. loti* in 1.0 mM cyanide initially was inhibited. After about 3 hr, however, the fungus regained the ability to respire (Fig. 3-A). *Stemphylium loti* cells whose oxygen uptake was initially inhibited by cyanide, but which subsequently was stimulated by cyanide, are referred to as adapted cells. Nonadapted cells are those which had never been exposed to cyanide. Oxygen uptake by adapted *S. loti* spores was greater in the presence than in the absence of cyanide (Fig. 2-A, 4).

Adaptation of *S. loti* was effected by incubating spores for 2 hr in 0.1 M phosphate pH 6.5, 0.05 M glucose, and 0.1 mM cyanide. *Stemphylium sarcinaeforme* spores were also previously incubated in phosphate, glucose, and cyanide (0.022 mM). Spores incubated 2 hr in the same medium, but without cyanide, served as nonadapted controls. After incubation, all spores were washed with distilled water by centrifugation.

Initially, oxygen uptake by *S. sarcinaeforme* was inhibited by cyanide, but upon further incubation in the presence of cyanide, it developed an ability to take up oxygen. This development, however, was not nearly so striking as was adaptation of *S. loti* to cyanide. *Stemphylium loti* and *S. sarcinaeforme* differed in yet another respect. For experiments in which spores of each fungus were exposed to cyanide for 6 hr, oxygen uptake by *S. loti* was stimulated by cyanide concentrations which were at least 100-fold greater than those which inhibited oxygen uptake by *S. sarcinaeforme* (Fig. 3-A, B).

The respiratory response to cyanide of adapted *S. loti* spores, and *S. sarcinaeforme* spores previously incubated in cyanide, was determined with the oxygen electrode (Fig. 2-A, B) and with the respirometer (Fig. 3-A, B). Initial oxygen uptake by spores of either fungus previously incubated in cyanide was stimulated by cyanide (Fig. 2), but with time, oxygen uptake of previously incubated *S. sarcinaeforme* spores was inhibited by cyanide (Fig. 3-B). The oxygen uptake of adapted *S. loti* spores exposed to high cyanide concentrations remained at a rate greater than that for similar spores not exposed to cyanide (Fig. 3-A).

Effect of cyanide on spore germination.—Adapted *S. loti* spores germinated better at high HCN concentrations than did nonadapted spores. For example, at 0.1 and 2.2 mM cyanide, the average germ tube length of adapted spores was 55 and 35 μ, respectively. At the same cyanide concentrations, average germ tube length of nonadapted spores was 15 and 0 μ, respectively. In the absence of cyanide, the average germ tube length of both adapted and nonadapted *S. loti* spores was 125 μ.

Previous incubation in cyanide did not enhance the ability of *S. sarcinaeforme* spores to germinate in cyanide; rather, germinability was adversely affected. In the absence of cyanide, average germ tube length was 60 and 30 μ for spores previously not exposed and exposed to cyanide, respectively. In 0.001 and 0.0046 mM cyanide, germination of previously incubated *S. sarcinaeforme* spores was completely inhibited, whereas average germ tube length of previously nonexposed spores was 15 and 0 μ, respectively.

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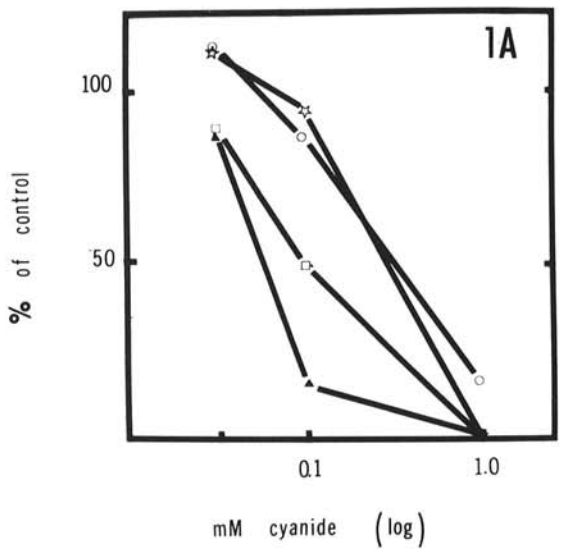
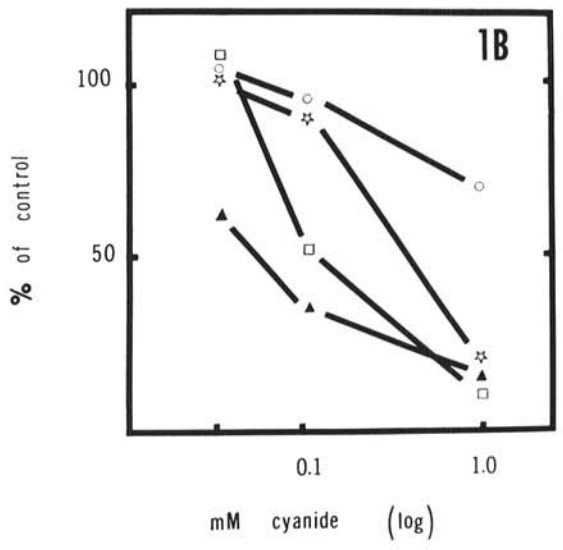
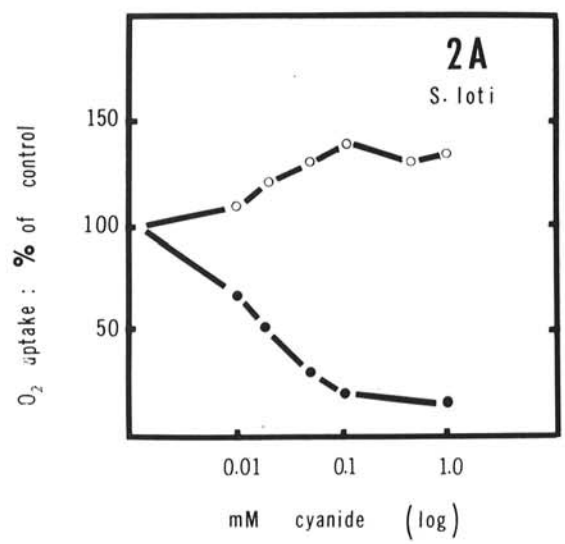
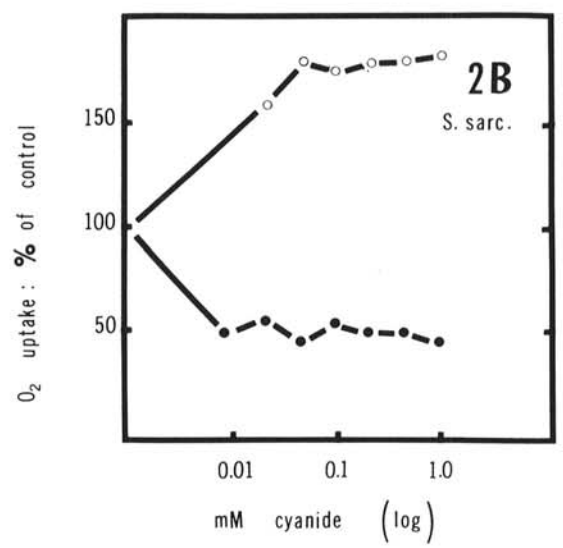
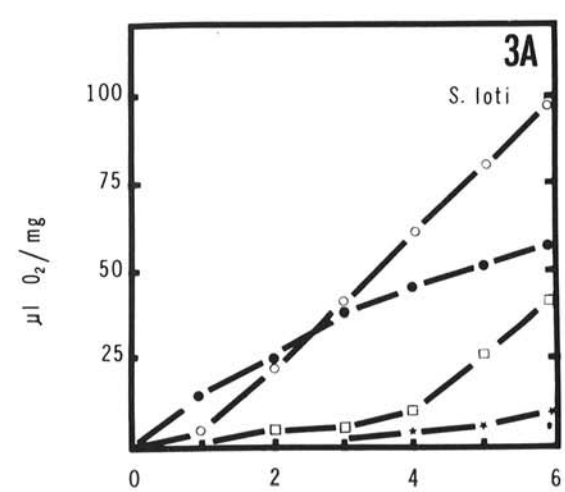
Fig. 1-3. **1)** Growth responses of four *Stemphylium* spp. to different concentrations of cyanide. Growth was measured after 4 days by **A)** increase in colony diam on agar medium; and **B)** increase in colony dry wt in liquid medium. Results are presented as percentage of control (growth in the absence of cyanide). Control values for the different fungi in **A, B),** respectively, were: *S. botryosum,* 9 mm, 54 mg; *S. consortiale,* 21 mm, 120 mg; *S. loti,* 6 mm, 48 mg; *S. sarcinaeforme,* 6 mm, 55 mg; *S. botryosum* is represented by (□ —— □), *S. consortiale* by (☆ —— ☆), *S. loti* by (○ —— ○), and *S. sarcinaeforme* by (▼ —— ▼). **2)** Effect of cyanide on the rate of oxygen uptake of spores previously exposed (○ —— ○) and unexposed (● —— ●) to cyanide, as measured with the oxygen electrode. Results are presented for **A)** *S. loti* and **B)** *S. sarcinaeforme* spores. Rates were measured within 2 min of cyanide addition. Control rates (rate of oxygen uptake in the absence of cyanide) for *S. loti* varied from 6.1 to 9.0 μmoles/min/mg. Control rates for *S. sarcinaeforme* varied from 4.4 to 7.5 μmoles/min per mg. Reaction medium in the chamber of the oxygen electrode consisted of 0.1 M phosphate pH 6.5, 0.05 M glucose, and 2.0-2.5 mg/ml spores. **3)** Effect of different cyanide concentrations on oxygen uptake of **A)** *S. loti* and **B)** *S. sarcinaeforme* spores, as measured with the respirometer at 0, 2, 4, and 6 hr. Reaction medium was 0.08 M phosphate pH 6.5, 0.05 M glucose, spores (about 1 mg/ml dry wt), and different concentrations of cyanide; center well contained 10% $Ca(OH)_2$ or $Ca(CN)_2$ in 10% $Ca(OH)_2$. Oxygen uptake was measured for spores incubated in no cyanide (● —— ●); or in 0.1 mM (○ —— ○), 1.0 mM (□ —— □), or 4.6 mM (★ —— ★) cyanide.

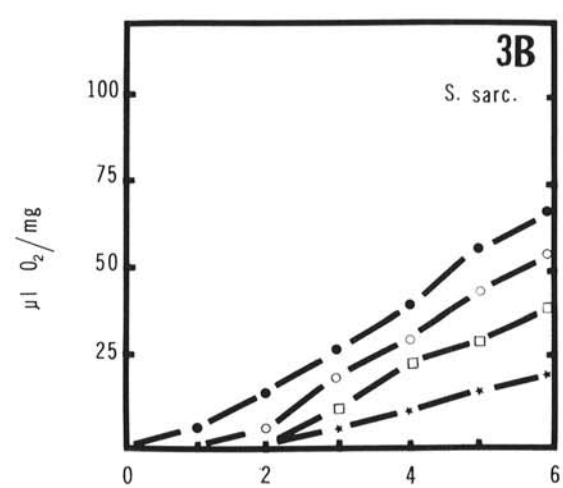

1A
100
50
% of control
0.1
1.0
mM cyanide (log)


1B
100
50
% of control
0.1
1.0
mM cyanide (log)


2A
S. loti
150
100
50
O2 uptake : % of control
0.01
0.1
1.0
mM cyanide (log)


2B
S. sarc.
150
100
50
O2 uptake : % of control
0.01
0.1
1.0
mM cyanide (log)


3A
S. loti
100
75
50
25
µl O2/mg
0
2
4
6


3B
S. sarc.
100
75
50
25
µl O2/mg
0
2
4
6

Association of adaptation of S. loti with loss of cyanide from solution.—In a typical experiment in which the concentration of cyanide was measured during adaptation of *S. loti* to cyanide, cyanide disappeared from solution and the loss was correlated with the ability of the fungus to take up oxygen (Fig. 5). Cyanide concentration in the respirometer vessels was maintained if either autoclaved spores or phosphate buffer alone constituted the reaction mixture. We tested the possibility that *S. loti* produced a compound which interfered with the method to determine cyanide. Adapted *S. loti* spores (about 1 mg/ml) were incubated in 0.1 M phosphate buffer pH 6.5, 0.05 M acetate pH 6.5, and 1.0 mM cyanide. Within 30 min, the cyanide was not detectable. The suspension was once again made 1.0 mM by adding cyanide, and all of the added cyanide was detectable when assays were made immediately.

From these data we concluded that *S. loti* does not produce a compound which interferes with the assay

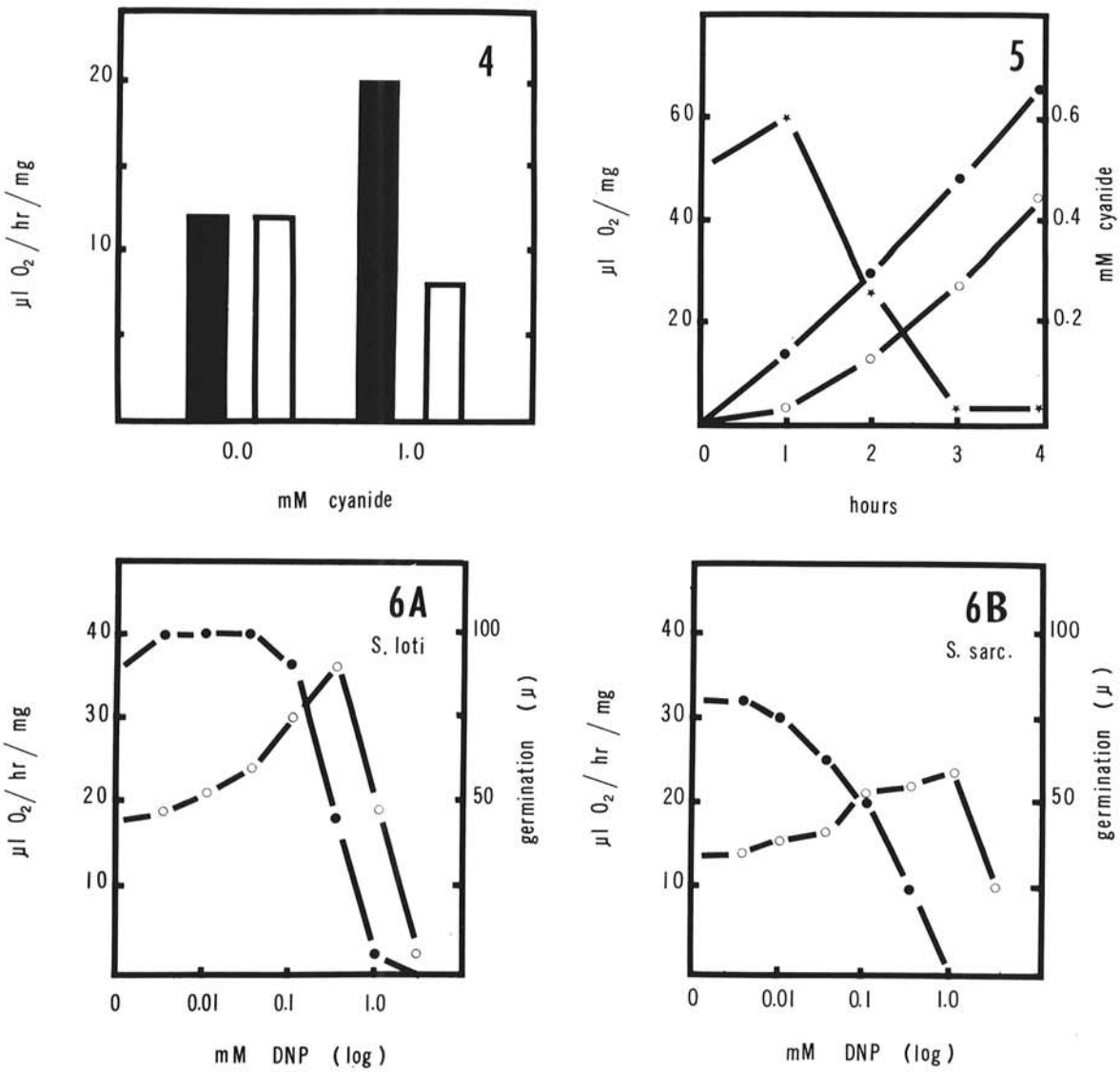


Fig. 4-6. **4)** Effect of 1.0 mM cyanide on oxygen uptake of adapted *Stemphylium loti* spores (solid bars) and adapted *S. sarcinaeforme* spores (open bars), as measured with the respirometer; *S. loti* spores were adapted by incubating them 3 hr in 0.46 mM cyanide; *S. sarcinaeforme* spores were adapted by incubating them 2 hr in 0.022 mM cyanide. **5)** Oxygen uptake of *S. loti* spores in 0.5 mM cyanide (○——○), or, in the absence of cyanide (●——●) and cyanide concentration (★——★), in a spore suspension of *S. loti*. Reaction mixture contained 0.97 mg/ml spores, 0.05 M acetate pH 6.5, 0.08 M phosphate pH 6.5, and the indicated concentration of cyanide. **6)** Effect of 2,4-dinitrophenol (DNP) on oxygen uptake (○——○) and germination (●——●) of **A)** *S. loti* and **B)** *S. sarcinaeforme* spores. Reaction mixture in respiration studies consisted of 0.05 M glucose, 0.08 M phosphate pH 6.5, and 1 mg/ml dry wt spores. Reaction mixture for germination studies was the same except that it contained 0.5% yeast extract. Rate of oxygen uptake was determined after 1 hr in DNP, and germination was measured after 6-hr incubation.

for cyanide. Moreover, cyanide was not present in spores incubated in a solution from which the cyanide had disappeared. Homogenates of spores incubated in 50 ml of 1.0 mM cyanide did not contain any detectable cyanide, even though the cyanide had disappeared.

Previously incubated *S. sarcinaeforme* spores did not effect a disappearance of cyanide from solution, and there was no loss of cyanide from solution during incubation of this fungus in cyanide.

In order to determine the ability of adapted *S. loti* spores to effect the removal of cyanide from solution, aliquots of reaction mixtures that contained 0.1 M phosphate pH 6.5, 0.05 M acetate, and 1 mg/ml of (i) viable, adapted *S. loti* spores; (ii) viable, nonadapted *S. loti* spores; (iii) autoclaved, adapted *S. loti* spores; or (iv) no spores, were assayed for cyanide at intervals following addition of cyanide. Cyanide concentration decreased from 1.1 to 1.0 mM in 30 min when the reaction mixture contained nonadapted *S. loti* spores, autoclaved adapted *S. loti* spores, or only buffer. When viable, adapted *S. loti* spores were incubated in the presence of cyanide, however, the cyanide concentration decreased from 1.0 to 0.0 mM in 30 min. Thus, only adapted *S. loti* spores were capable of removing or transforming cyanide.

Sensitivity of cell-free fractions of adapted and nonadapted S. loti to cyanide.—We attempted to determine whether the particulate fractions from adapted *S. loti* also were resistant to cyanide. Adaptation of intact mycelium was determined by measuring its respiratory response to cyanide. In one such test, adapted mycelium took up 7 μliters O_2/hr per mg dry wt in the presence of 0.46 mM cyanide. Nonadapted mycelium took up 7 μliters O_2/hr per mg dry wt in the absence of cyanide, but in the presence of 0.46 mM cyanide, nonadapted mycelium took up 2 μliters O_2/hr per mg dry wt.

Two particulate fractions from adapted and two from nonadapted mycelium were analyzed for their respiratory response to cyanide. The first fraction contained particles which sedimented at 10,000 *g*. The second fraction contained particles which did not sediment at 10,000 *g* but did sediment at 30,000 *g*. The resuspended pellets in each case had low rates of endogenous oxygen uptake and utilized succinate (3.2 mM), but neither pyruvate (3.2 mM) and malate (3.2 mM) nor citrate (4.0 mM), as carbon sources. The increased rate of oxygen uptake due to 3.2 mM succinate was 10-30 fold for the 30,000 *g* pellet, and about 2-fold for the 10,000 *g* pellet. The particles were without respiratory control, since they were not stimulated by ADP (3 mM) or DNP (0.3 mM).

In a typical experiment, particles from nonadapted mycelium spun down at 30,000 *g* took up 5.4 mmoles oxygen/min in the absence of cyanide, whereas an equivalent quantity of particles from adapted mycelium took up 6.0 mmoles oxygen/min. Particles from adapted cells, as well as those from nonadapted cells, took up oxygen in 0.10 mM cyanide at a rate about 20% of the rate determined for particles in the absence of cyanide. Particles which sedimented at 10,000 *g* responded similarly. In 0.1 mM cyanide, the 10,000 *g* pellet from adapted and nonadapted *S. loti* took up oxygen at 43 and 35%, respectively, of the control rate. The identity of these particles was not definitely established, but presumably they consisted of mitochondria (10,000 *g*), microsomes, and mitochondrial fragments (30,000 *g*).

Interaction of cyanide and 2,4-dinitrophenol.—At concentrations of DNP which normally stimulated oxygen uptake by *S. loti* and *S. sarcinaeforme,* germination of the spores was almost completely inhibited (Fig. 6-A, B). This result indicates that DNP (a classical uncoupler) probably uncoupled oxygen uptake from energy production in *S. loti.* In respirometer studies, DNP added to adapted *S. loti* whose rate of oxygen uptake had previously been stimulated by 4.6 mM cyanide caused little further increase in rate of oxygen uptake. The interaction between cyanide and DNP is not at all clear, however, since 0.3 mM DNP initially inhibited oxygen-uptake stimulated by 0.1 mM cyanide (Table 1). Conversely, 0.1 mM cyanide initially inhibited the stimulation due to 0.3 mM DNP.

DISCUSSION.—In most aspects, *S. loti* and *S. sarcinaeforme* differed in their responses to cyanide. *Stemphylium loti* adapted to cyanide, and this adaptation was characterized by development of a cyanide-stimulated respiration which was maintained over a period of several hr, by development of an ability to transform cyanide in such a way that it was no longer detectable, and by development of an increased ability of spores to germinate in cyanide. The increased ability to germinate may have been a secondary effect of cyanide transformation or removal. In contrast, *S. sarcinaeforme* did not adapt to cyanide in the same sense as did *S. loti. Stemphylium sarcinaeforme* did develop an ability to take up oxygen in the presence of cyanide, and after development of this ability, initial exposure of *S. sarcinaeforme* to cyanide resulted in a stimulated oxygen uptake; however, the stimulation was not maintained over long time intervals. *Stemphylium sarcinaeforme* did not develop an ability to transform cyanide, and it did not develop a capacity for increased germination in the presence of cyanide.

The fate of cyanide when incubated with *S. loti* has not yet been investigated. However, Allen & Strobel (1) demonstrated that several fungi incorporated labeled cyanide into alanine or asparagine.

The possibility that cyanide might act to uncouple

TABLE 1. Effect of 0.3 mM 2,4-dinitrophenol (DNP), and 0.1 mM cyanide on oxygen uptake of adapted and nonadapted *Stemphylium loti* spores as measured in the oxygen electrode

Treatment	Rate as % of control[a]	
	Adapted	Nonadapted
Cyanide	134	35
Cyanide + DNP	88	35
DNP	138	129
DNP + Cyanide	71	35

[a] Control rate was that in the absence of either DNP or cyanide; it varied from 6.9 to 11 μmoles oxygen taken up/mg per min. Reaction medium consisted of 0.1 M phosphate pH 6.5, 0.05 M glucose, and spores in concentration of 2.2 mg/ml (adapted), 3.05 mg/ml (nonadapted).

oxygen uptake from growth in *S. loti* has been suggested (5). An uncoupling effect caused by cyanide or its transformation product could explain the stimulated respiration observed when adapted cells in cyanide were incubated. In potato tissue, cyanide did act as an uncoupler (8). DNP caused little additional stimulation of *S. loti* oxygen uptake previously stimulated by cyanide. This result does not necessarily implicate cyanide as an uncoupler in *S. loti,* because in addition to uncoupling, cyanide or its product might have stimulated electron transport maximally so that uncoupling by DNP did not further stimulate oxygen uptake.

We were unable to demonstrate a cyanide-stimulated respiration in mitochondria from adapted *S. loti* cells. A comparable situation has been reported for *Myrothecium verrucaria* (4). However, cyanide tolerance has been demonstrated for both whole cells and for isolated mitochondria of skunk cabbage, *Symplocarpus foetidus* (7). In *Achromobacter,* cyanide resistance and cyanide-resistant respiration are associated with the formation of cytochrome a_2, which is a cyanide-resistant oxidase (2). Our inability to demonstrate cyanide stimulated respiration in mitochondria from adapted *S. loti* may have resulted from any of several possibilities. First, the product(s) of cyanide transformation and not cyanide itself might have caused the stimulated respiration. Second, the factor causing increased oxygen uptake might not have resided in the mitochondria. Third, the mitochondria might have been severely damaged in the isolation process, so that they did not function normally.

Stemphylium loti, when adapted to cyanide, became tolerant of cyanide. Adaptation was a complex response of the fungus to cyanide, and was observable by different criteria. We believe that the main cause of this tolerance is an ability of the adapted cells to remove or transform cyanide, and the tolerance is probably not effected by a cyanide-resistant respiration in the mitochondria. The mechanism by which the removal or transformation is accomplished is under investigation.

Pierre & Millar (12) established that each of *S. loti, S. botryosum,* and *S. sarcinaeforme* can penetrate trefoil leaves, but that only *S. loti* develops beyond the primary hypha stage to produce secondary hyphae which ramify in the tissues and cause necrosis. Infection of trefoil results in release of cyanide (10). Millar & Higgins (10) postulated that this release of cyanide as a consequence of penetration and injury of cells may constitute a resistance mechanism precluding further invasion of tissues by cyanide-sensitive fungi such as *S. sarcinaeforme,* but not preventing further invasion by *S. loti* which rapidly adapts to cyanide. The findings reported here and elsewhere (6) provide some understanding of the nature of the differential response of these fungi to cyanide. Cyanide released in response to penetration may effect in both *S. sarcinaeforme* and *S. loti* an uncoupling of oxygen uptake from energy production, as was indicated by a marked stimulation of respiration of spores of both fungi. This may act to halt growth by *S. sarcinaeforme,* which does not have the capacity to transform cyanide or maintain a cyanide-stimulated respiration. On the other hand, *S. loti* apparently rapidly synthesizes a metabolic system capable of transforming cyanide and maintaining a cyanide-stimulated respiration. The development of this capacity in *S. loti* thus may provide it the means of continuing its invasion of trefoil in spite of the release of cyanide.

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