B I O S U R F A C T A N T S

Their Identity and Potential Efficacy in the Biological Control of Zoosporic Plant Pathogens

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Additional strategies to pesticides are needed for the control of agriculturally important pests, including plant pathogens, weeds, and insects. Such strategies will help address public concern regarding pesticide pollution, as well as the perception that pesticide residues on food pose a threat to human health. Further, alarm resulting from the growing incidence of pesticide insensitivity has prompted global efforts directed toward the search for additional pest control strategies. One such strategy is biological control, which is the use of antagonistic microorganisms (primarily bacteria and fungi) to either directly or indirectly control target pests.

Implementation of biological control is fraught with constraints, and successful application depends on a thorough knowledge of the ecology and biology of both the target pest and the biological control agent. Paramount to efficacious employment of biological control is knowledge of the specific mechanism of microbial antagonism involved. Documented biological control mechanisms for plant pathogens include competition, hyperparasitism, predation, and the production of extracellular metabolites such as antibiotics, hydrogen cyanide (HCN), and siderophores (14). Recently, we discovered a novel antimicrobial metabolite, a biosurfactant (17).

This paper reviews the historical evolution of the use of synthetic surfactants, which led to the discovery of biosurfactants as a new antagonistic mechanism for the biological control of zoosporic plant

pathogens (3). Evidence for the potential efficacy of biosurfactants is presented, and some of the research needs for future application of biosurfactant-producing bacteria as well as for biosurfactants themselves are addressed.

Zoosporic Pathogens: The Target Pest

There are approximately 143 species of zoosporic plant pathogens. Although these species occur in taxonomically diverse families and genera (Table 1), they share a common stage in their life cycle: a unicellular, motile, uni- or biflagellate asexual life stage known as a zoospore. Zoospores are produced in either a vesicle or a sporangium (Fig. 1). Upon their release from the latter structures in the presence of free water (rain, dew, or irrigation water), they swim for a period of time, varying from minutes to hours. Ultimately, using primarily chemotactic mechanisms, the zoospores locate and encyst on a suitable host. The zoospore has been implicated as the

primary, if not sole, life stage responsible for both the spread of the pathogen and the location of appropriate infection sites. Of singular importance to disease control is the fact that the zoospore is encased solely by a plasma membrane. This is in contrast to the other life stages of the organism, each of which has a protective cell wall.

Among the more destructive zoosporic pathogens are the downy mildews, which are primarily associated with severe foliar diseases of numerous crops. In addition, numerous species in the genera *Pythium* and *Phytophthora* are destructive pathogens of roots, foliage, and fruits. Two of the more notorious zoosporic pathogens are *Phytophthora infestans*, the cause of late blight of potato, and *Plasmopara viticola*, the cause of downy mildew of grape.

Numerous strategies have been developed for the control of diseases caused by these zoosporic plant pathogens. Such strategies include the use, when available, of resistant cultivars, as well as various cultural, physical, biological, and chemical methods. However, the use of chemical

Table 1. Economically important zoosporic plant pathogens

Class	Order	Family	Genus
<i>Oomycetes</i>	<i>Peronosporales</i>	<i>Albuginaceae</i>	<i>Albugo</i>
		<i>Pythiaceae</i>	<i>Peronophythora</i>
			<i>Phytophthora</i>
			<i>Pythium</i>
		<i>Peronosporaceae</i>	<i>Plasmopara</i>
			<i>Pseudoperonospora</i>
			<i>Sclerophthora</i>
			<i>Sclerospora</i>
			<i>Aphanomyces</i>
			<i>Synchytrium</i>
<i>Chytridiomycetes</i>	<i>Saprolegniales</i>	<i>Saprolegniaceae</i>	
	<i>Chytridiales</i>	<i>Synchytriaceae</i>	
	<i>Spizellomycetales</i>	<i>Olpidiaceae</i>	<i>Olpidium</i>
	<i>Blastocladales</i>	<i>Physodermataceae</i>	<i>Physoderma</i>
<i>Plasmodiophoromycetes</i>	<i>Plasmodiophorales</i>	<i>Plasmodiophoraceae</i>	<i>Plasmodiophora</i>
			<i>Polymyxa</i>
			<i>Spongospora</i>

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pesticides has been, and continues to be, the most effective and economic method of control. Chemical pesticide control began in 1885 following the discovery of the efficacy of copper in the control of grape downy mildew. In 1980, world use of copper for control of zoosporic pathogens accounted for 15% of the fungicide market (3). In 1994, approximately \$4 billion (U.S.) was spent worldwide for fungicides to control a single zoosporic plant pathogen, *Phytophthora infestans* (22). The continued use of and reliance on pesticides, particularly the newer systemic and curative ones, have led to the development and/or selection of pesticide-resistant strains in many species of the major zoosporic plant pathogens. Although numerous and chemically diverse pesticides exhibiting activity toward zoosporic plant pathogens have been employed, one unusual (and lesser known) class of chemicals used to control zoosporic pathogens is the synthetic surfactants. Surfactants are amphiphathic molecules that can modify the properties of a liquid medium at a surface or interface by reducing the surface tension.

Zoospore Control with Synthetic Surfactants

The intentional use of synthetic surfactants for the control of a zoosporic pathogen was pioneered by Tomlinson and

Faithfull (30). During their studies on the control of big vein of hydroponically grown lettuce, which is caused by a virus vectored by zoospores of *Olpidium brassicae*, they observed that disease control was achieved following the addition of a benzimidazole fungicide (Bavistin, BASF) to the recirculating nutrient solution. It was subsequently discovered that the inert ingredients in the fungicide formulation exhibited lytic activity against zoospores of

Olpidium. It was suspected that the inert ingredients contained a synthetic surfactant. Further studies demonstrated the lytic effects of various surfactants, including anionic (Manoxol O/T, Marasperse CB, Sodium lauryl sulfate), nonionic (Agral, Ethylan CPX, Spreadite, Triton X100), and cationic (Cetrimide, Deciquam 222, Hyamine 1622) types, to zoospores of *O. brassicae*. These studies showed differential effects of the surfactants that depended

Fig. 2. Twelve separate hydroponic units in a temperature-controlled greenhouse.

Fig. 1. Zoospores in (A) a sporangium of *Phytophthora palmivora* and (B) a vesicle of *Pythium intermedium*. Bar = 30 μ m.

Fig. 3. Mortality of pepper plants after hypocotyl inoculation of a single plant (marked by an X) on one side of a two-sided recirculating hydroponic unit in the (A) absence or (B) presence of a synthetic surfactant.

on surfactant type and concentration. A nonionic surfactant (Agral 90, ICI) was subsequently chosen and shown to control the disease in commercial lettuce production facilities following the addition of the

surfactant to the recirculating nutrient solution (31). The efficacy of this surfactant was further demonstrated by Tomlinson and Thomas (32) in 1986 for the control of yet another virus disease, melon necrotic

spot of cucumber, which is vectored by zoospores of *Olpidium radicale*.

In 1987, the *in vitro* lytic effect of surfactants toward zoospores was extended to major root-infecting zoosporic plant pathogens. Zoospores of four species of *Pythium* and *Phytophthora parasitica* were shown to lyse rapidly upon exposure to a synthetic surfactant (29). The synthetic surfactant destroyed the permeability of the plasma membrane, resulting in a loss of motility and rapid lysis (death) of the zoospores of all species tested. The same synthetic surfactant had little or no effect on other stages in the pathogen life cycle, which have cell walls (sporangia, encysted zoospores, and hyphae). The potential utility of synthetic surfactants for the control of these major zoosporic root-infecting pathogens, although recognized, was not experimentally demonstrated in this early work (29).

Expanding on the preceding observations, we recently documented the *in vivo* efficacy of some synthetic surfactants in the control of root rot of cucumbers and peppers caused by *Pythium aphanidermatum* and *Phytophthora capsici*, respectively, in a near-commercial hydroponic recirculating cultural system (Fig. 2) (26–28). In the absence of the surfactant, all plants within the recirculating hydroponic system were killed following inoculation of a single plant (Fig. 3A). However, amendment of the recirculating nutrient solution with a nonionic synthetic surfactant (20 µg/ml) resulted in the elimination of the spread of zoospores via the recirculating nutrient solution and 100% control of root rot caused by these two destructive root-infecting pathogens (Fig. 3B). Similar studies also demonstrated the efficacy of surfactants in the control of root rot of lettuce caused by *Plasmopara lactucae-radici* (M. E. Stanghellini, unpublished).

During our investigations, the unexpected happened: no plant mortality occurred in one of the pathogen-inoculated hydroponic units. We discovered a bacterium in the recirculating nutrient solution that produced extracellular metabolites that caused lysis of zoospores *in vitro*. Since the mode of action of cultural supernatants from this bacterium was similar to the mode of action of synthetic surfactants, i.e., rupture of the plasma membrane, we hypothesized that the putative lytic agent was a biosurfactant.

Biosurfactants: General Comments

Biosurfactants of diverse chemical structure are produced by a variety of microorganisms. For example, some isolates from the following bacterial genera are reported to produce surfactants: *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Rhodococcus*, *Acinetobacter*, and *Corynebacterium*. Several fungal genera, including *Candida*

Fig. 4. The rhamnolipid shown is the major monorhamnolipid species, rhamnosyl-β-3-hydroxydecanoyl-3-hydroxydecanoate (also called a C₁₀-C₁₀ rhamnolipid) where R = H. Also commonly isolated are dirhamnolipid species where R = rhamnosyl. Production of rhamnolipid is species specific. For example, some species produce only the mono- or dirhamnolipid, while others produce a mixture of rhamnolipids. In either case, the rhamnolipids produced also vary in length of the lipid chains. We have isolated strains that produce mixtures ranging from C₈-C₁₀ to C₁₂-C₁₂ (R. M. Miller, unpublished). It also has been reported that some strains can produce a nonionic rhamnolipid form where the rhamnolipid carboxyl group is modified to a methyl ester (12,34). The rhamnolipid, as for any surfactant molecule, is composed of a polar head group and one or more nonpolar tail groups (as presented in the schematic).

Fig. 5. Schematic of regulation of rhamnolipid production in *Pseudomonas* spp.

and *Torulopsis*, are also reported to produce surfactants. Several extensive reviews address these surfactant producers (13,24). The production of a specific biosurfactant is generally associated with a specific microbial genus. Although several of these biosurfactants are well-described chemically (e.g., rhamnolipid, surfactin, and trehalose lipid), biosurfactant function in nature is not yet clear. Several potential roles have been proposed for biosurfactants, including (i) enhancement of bioavailability and biodegradation of slightly soluble organic carbon sources such as petroleum hydrocarbons (15); (ii) use as an aid in attachment and detachment of bacteria to surfaces (24); (iii) use in antibacterial defense mechanisms (24); (iv) function as a virulence factor in the pathogenesis of specific microorganisms in both plants (5,10) and animals (23); (v) use as an aid in the colonization of leaf surfaces (4); and (vi) most recently, because they have been shown to efficiently bind metals, may play a role in metal uptake or in reducing metal toxicity (9,16).

Perhaps the best studied of the biosurfactants is a class of glycolipids, the rhamnolipids, which are produced and secreted by *Pseudomonas* spp. Several forms of rhamnolipid have been identified, and these surfactants are usually anionic in nature (Fig. 4) (23). Rhamnolipids are secreted from the cell during the late log and stationary phases of growth, and they can be isolated and purified following the procedures outlined by Zhang and Miller (33,34). There are a number of methodologies available to detect and quantify rhamnolipids, both in pure form and in complex mixtures. The most common approaches for quantitation are surface tension analysis, a rhamnose assay, and high-performance liquid chromatography (18,23). Rhamnolipids can also be qualitatively detected using thin-layer chromatography (33) or an indicator medium (25). High-performance liquid chromatography and thin-layer chromatography separate rhamnolipid types, while the other methods do not.

Studies on the regulation of rhamnolipid synthesis have been conducted to date only with strains of *Pseudomonas aeruginosa* (19–21). These studies have resulted in the identification of a cluster of genes that regulate rhamnolipid synthesis. This cluster includes two genes, *rhlA* and *rhlB*, that encode a rhamnosyltransferase responsible for the transfer of rhamnose from TDP-rhamnose to β -hydroxydecanoyl- β -hydroxydecanoate (19). A regulatory gene, *rhlR*, encodes for a transcriptional activator protein, RhlR, that positively regulates rhamnolipid synthesis (20). Finally, an *rhlI* gene has been identified that encodes for an autoinducer that binds and activates the RhlR protein (21). Figure 5 shows the rhamnolipid biosynthesis gene cluster and the suggested mechanism of regulation.

Table 2. Quantity of rhamnolipid produced by fluorescent *Pseudomonas* isolates obtained from plants

<i>Pseudomonas</i> isolate ^a	Isolate source	Rhamnolipid ^b	Amount produced ^c (µg/ml)
R ₅	Cucumber	Mono, di	1,000
L ₁	Cantaloupe	Mono, di	900
L ₂	Cantaloupe	Mono, di	500
R ₁	Tomato	Mono, di	50
R ₃	Cantaloupe	Mono, di	50
R ₄	Cantaloupe	Mono, di	500

^a *Pseudomonas* isolates were obtained by imprinting leaves (L) or roots (R) from the indicated plants on King's B medium agar. Isolates were identified using Biolog.

^b Each *Pseudomonas* isolate was grown in a mineral salts medium (7) with 2% glucose to the stationary phase at room temperature. Rhamnolipids were extracted from the culture supernatant and identified by thin-layer chromatography (34). Mono = monorhamnolipid, di = dirhamnolipid.

^c The amount of rhamnolipid produced was estimated from the mass of the biosurfactant recovered from a 200-ml culture.

Fig. 6. Rapidity of zoospore lysis of three species of zoospore plant pathogens upon exposure to various concentrations of two biosurfactants (a mono- and a dirhamnolipid) produced by *Pseudomonas* spp. A 30-µl suspension of zoospores of each fungus, which contained not less than 600 zoospores, was mixed with 1 ml of sterile distilled water amended with the biosurfactants. The maximum time for cessation of motility and lysis of the entire zoospore population, timed with a stopwatch and determined microscopically, was recorded for each fungus. Three separate experiments were performed, and the data represent the average and standard deviation of these experiments. In the absence of a biosurfactant, zoospores swam for approximately 20 h.

Rhamnolipids as Biocides

As previously mentioned, we suspected that the zoospore-lytic agent in cultural supernatants of the bacterium, which was subsequently identified as *Pseudomonas*

aeruginosa, was a biosurfactant. To test this hypothesis, the biosurfactants produced in vitro by this bacterium, as well as others shown in Table 2, were isolated and identified by thin-layer chromatography as

rhamnolipid: specifically, a mixture of anionic mono- and dirhamnolipids (Fig. 4). Purified mono- and dirhamnolipids were then tested for zoosporicidal activity against species of three representative genera of zoosporeic plant pathogens: *Pythium aphanidermatum*, *Phytophthora capsici*, and *Plasmopara lactucae-radicis*. As shown in Figure 6, exposure of zoospores of all three genera to each biosurfactant at concentrations ranging from 5 to 30 µg/ml caused cessation of motility and lysis of the entire zoospore population in less than 1 min. The necessary concentration of biosurfactant to cause lysis was a function of both the sensitivity of the zoospore and the type of rhamnolipid (compare Fig. 6A–C). In all cases, the dirhamnolipid was equal to or better than the monorhamnolipid in causing lysis. The proposed mechanism for zoospore lysis is that upon exposure to the rhamnolipid, rhamnolipid intercalates into and disrupts the zoospore plasma membrane (Fig. 7). This can be viewed under the microscope as a time sequence that is shown in Figure 8. After rhamnolipid addition, zoospore motility ceases and then the zoospore explodes. In contrast, in the absence of a biosurfactant, zoospores of each species swam for approximately 20 h. Little is currently known concerning the chemical structure of the zoospore membrane, although work by Haug (8) shows that *Blastocladiella emersonii* zoospore membranes contain 51% protein and 49% lipid. Interestingly, the major lipid components were reported to be glycolipids and neutral lipids, rather than the phospholipids normally found in most eukaryotic and bacterial membranes.

In addition to testing both mono- and dirhamnolipids, we have tested other biosurfactants for activity against zoospores. The surfactants tested to date include two other glycolipids, a sophorose lipid and a trehalose lipid, as well as a lipopeptide, surfactin. They were found to have no activity against zoospores at concentrations less than 1,000 µg/ml, the highest dose evaluated.

Fig. 7. Schematic illustrating (A) lysis of a zoospore in the presence of rhamnolipid, (B) the structure of the zoospore membrane and the similarity of the component membrane molecules to a rhamnolipid molecule, and (C) loss of membrane integrity due to intercalation of the rhamnolipid into the membrane.

Fig. 8. Lysis (arrows) of zoospores of *Phytophthora capsici* after exposure to a rhamnolipid for (A) 1 s, (B) 15 s, and (C) 30 s.

Rhamnolipid-Producing Bacteria on Plants

Subsequent to the discovery of the efficacy of rhamnolipids against zoospores, studies were initiated to determine the occurrence of rhamnolipid-producing bacteria in nature. In particular, this was important because studies to date have focused on rhamnolipid-producing isolates that are opportunistic pathogens, such as *Pseudomonas aeruginosa* ATCC 9027; and these would have drawbacks for field application. Therefore, our objective was to determine whether rhamnolipid-producing fluorescent *Pseudomonads* are naturally occurring rhizoplane and phylloplane organisms. Our limited studies to date indicate that rhamnolipid-producing bacteria can be readily isolated from both roots and leaves of field-grown plants, including cucumber, tomato, and cantaloupe. The isolates obtained were subsequently identified by Biolog. They were found to best fit *Pseudomonas aeruginosa* (R₃, R₄, R₅, L₁, and L₂) and *Pseudomonas corrugata* (R₁), with similarity indexes ranging from 0.559 to 0.937. As shown in Table 2, rhamnolipid production by these isolates varied from 50 to 1,000 µg/ml. We further examined one of the plant isolates, R₄, to determine the effect of carbon source on rhamnolipid production. As shown in Table 3, high levels of rhamnolipid were produced during growth on olive oil, glucose, and soybean oil.

The occurrence of rhamnolipid-producing bacteria on plants is not particularly surprising. Numerous studies have shown that fluorescent *Pseudomonas* spp. are common inhabitants of the rhizoplane and phylloplane (2,14). Additionally, it has been known for some time that some fluorescent *Pseudomonas* spp. produce extracellular surface-active metabolites (i.e., biosurfactants) and that a relatively high, but undocumented, percentage of the bacteria produce biosurfactants (4,10). However, neither the identity of the specific biosurfactants produced by these bacteria nor their possible role and potential employment as a biological control agent was previously recognized.

Rhamnolipids and Biological Control in Greenhouse Studies

Preliminary studies designed to evaluate the biological control potential of rhamnolipid-producing strains of various fluorescent *Pseudomonas* spp. were conducted in a temperature-controlled greenhouse (24 to 32°C) containing 12 recirculating hydroponic units (Fig. 2). Each hydroponic unit consisted of two rock wool slabs connected to a common reservoir containing 50 liters of a nutrient solution (Fig. 9). Peppers were chosen as the susceptible host and *Phytophthora capsici* as the zoosporic pathogen. Four 40-day-old pepper seedlings in rock wool cubes were placed

on each rock wool slab. At times ranging from 1 to 4 weeks after transplant, a single plant on one side of a hydroponic system was hypocotyl-inoculated with the pathogen (Fig. 9). This method of inoculation permitted us to evaluate pathogen spread within a recirculating hydroponic unit subsequent to pathogen colonization and reproduction on a single plant. Treatments included uninoculated units, inoculated units, and inoculated units in which the nutrient solution was amended with various isolates of rhamnolipid-producing bacteria both with and without an added carbon source. (Olive oil was identified in our *in vitro* studies as a probable substrate that would support selective growth of the rhamnolipid-producing bacteria as well as enhance rhamnolipid synthesis. See Table 3.) The use of specific carbon sources to enhance selective growth of a bacterium has previously been established (6). The

final concentration of bacteria was 10⁶ CFU/ml of nutrient solution, and the final concentration of the carbon source (olive oil) was 1,000 µg/ml of nutrient solution. Within 2 weeks following inoculation, all plants in each unit, with or without rhamnolipid-producing bacteria (but with no carbon source), died (Fig. 10A). In units that were amended with both rhamnolipid-producing bacteria and a carbon source, disease control was achieved (Fig. 10B). However, it must be noted that in some units amended with a carbon source, complete control was achieved; whereas in others, complete mortality occurred but was delayed 1 to 3 weeks compared with the inoculated control unit. Thus, control occurred but was not consistent.

Rhamnolipids were detected via surface tension analysis of nutrient solution samples obtained from the reservoirs amended with rhamnolipid-producing bacteria plus a

Table 3. Influence of carbon source in production of rhamnolipid by *Pseudomonas aeruginosa* R4

Carbon source (2%) ^a	Biomass produced dry wt. (mg/ml) ^b	Rhamnolipid produced (µg/ml)
Glucose	1.5 ± 0.2	2,800 ± 100
Glycerol	1.3 ± 0.1	440 ± 40
Sucrose	0.045 ± 0.0045	0
α-Mannitol	0.98 ± 0.11	280 ± 30
Fructose	0.53 ± 0.04	45 ± 4
D-sorbitol	No growth	0
Soybean oil	2.8 ± 0.2	1,100 ± 100
Olive oil	2.8 ± 0.1	3,000 ± 600

^a The carbon source was added to a mineral salts medium (7).

^b Biomass produced is expressed as the average of three replicates ± the standard error.

Fig. 9. Schematic of a recirculating hydroponic unit employed to evaluate the efficacy of rhamnolipid-producing bacteria in the control of root rot of pepper caused by *Phytophthora capsici*. A single plant (marked by an X) on one side of the unit was inoculated with the pathogen. The bacteria were added, either with or without a carbon source, to the recirculating nutrient solution.

carbon source. This assay showed the concentration of rhamnolipid to be approximately 8 to 10 $\mu\text{g/ml}$. The presence of rhamnolipids was confirmed using thin-layer chromatography. The results of these preliminary studies demonstrate: (i) the *in situ* production of rhamnolipids, and (ii) that biological control was achieved but that, as in many biological control studies, results were not consistent in performance and/or sustained control. The reason(s) for inconsistency in disease control will be discussed in the section on problems and solutions.

Foliar Studies

Since many of the zoosporic pathogens listed in Table 1 are foliar pathogens, preliminary studies were conducted to assess the ability of selected rhamnolipid-producing bacteria to colonize and persist on leaves of pepper plants and to assess the *in situ* production of rhamnolipids. A rhamnolipid-producing bacterium from Table 2, specifically R₄, was selected and sprayed (1×10^6 CFU/ml) onto leaves of pepper plants. The distribution of the applied bacteria on inoculated leaves was assessed on day 0 (immediately after application), 7, 14, and 21 by leaf printing on King's B medium agar. Additionally, the presence of rhamnolipid was assessed in the following manner: leaves were removed from the plants on the days indicated, transported to the laboratory, and placed in a humid chamber. Drops of distilled water (100 μl) were then applied to the upper leaf surface. After 5 min, a drop of water (20 μl) con-

taining approximately 300 zoospores was added to the water drops on the leaf surface. After an additional 5 min, sample droplets were removed, placed on a glass slide, and observed for zoospores and zoospore motility. Controls consisted of zoospores added to water droplets on control (uninoculated) leaves. Motile zoospores were consistently observed in all of the control drops, those removed after 5 min as well as those removed after subsequent 30-min intervals over a total 4-h period. No zoospores were observed in any of the droplets removed from the leaves sprayed with R₄, indicating that they were completely lysed. Thus, these results indicate that (i) this bacterium can persist on leaves under greenhouse conditions for at least 21 days (the maximum time studied to date) (Fig. 11), and (ii) biosurfactants were present at sufficient concentrations to lyse all the zoospores. Studies of phylloplane bacterial population dynamics and *in situ* rhamnolipid synthesis are in progress.

Problems and Solutions

Biodegradation of rhamnolipids. As previously mentioned, sustained biological control was not achieved in all cases following the addition of rhamnolipid-producing bacteria to the nutrient solution in our greenhouse studies. Not surprisingly, we subsequently discovered that the surface tension increased in those hydroponic units that did not show complete disease control. This suggested that rhamnolipids produced by the introduced rhamnolipid-producing bacteria were being degraded.

There are two possibilities we have experimentally identified for degradation: (i) the rhamnolipid-producing bacterium can degrade rhamnolipid once all of the substrate (olive oil) is completely utilized, and (ii) rhamnolipid can be degraded by other bacteria present in the recirculating nutrient solution. We do not yet know which of these two possibilities is the more important. Obviously, maintaining a sterile environment is not possible or even realistic. Thus, given the fact that rhamnolipid biodegradation will inevitably occur, strategies need to be developed to offset the rapidity and/or magnitude of biodegradation. This could be accomplished by pre-emptive colonization of the intended site to be protected, by use of a specialized carbon substrate that would support the selective growth and production of specific rhamnolipids, and by repeated application of the substrate, the rhamnolipid-producing bacteria, or the rhamnolipids themselves.

The silver bullet? It is unlikely that any single rhamnolipid-producing strain will provide control of all zoosporic plant pathogens under all environmental conditions. Perhaps regionally adapted strains need to be isolated for biological control of specific zoosporic pathogens. For example, some of our rhamnolipid-producing strains were obtained from melon fields during the hot summer months in Arizona. At the time of collection, ambient temperatures exceeded 40°C. Yet these bacteria were present on the leaf surface. Such strains may, once perfected, provide control of *Pseudoperonospora cubensis*, the causal

Fig. 10. Mortality of pepper plants after hypocotyl inoculation of a single plant (marked by an X) on one side of a two-sided recirculating hydroponic unit. The recirculating nutrient solution was amended with rhamnolipid-producing bacteria in the (A) absence and (B) presence of a substrate (olive oil) that was added to enhance bacterial growth and production of rhamnolipids.

Fig. 11. Photograph of pepper leaf prints on King's B medium illustrating the spatial distribution of (A) a fluorescent rhamnolipid-producing bacterium, R₄, on the upper surface of a pepper leaf 21 days after the bacteria were applied and (B) the resident bacterial flora.

agent of downy mildew of cucurbits. However, it is unlikely that they would or could provide control of a cool-weather pathogen such as *Phytophthora infestans*, the causal agent of late blight of potato, whose zoospores are most active at temperatures below 16°C.

Additionally, it is highly probable that deliberate selection of naturally occurring or genetically altered rhamnolipid-producing strains can be accomplished. These strains may be superior in production of rhamnolipid; they may produce specific and superior types of rhamnolipid; or they may have temporally altered production of rhamnolipid (production during early log phase versus late log and stationary phase).

Development of rhamnolipid-insensitive zoospores. It is our opinion that the probability of developing rhamnolipid-insensitive strains is low. This is because development of resistance would require a major structural change in the chemical makeup of the plasma membrane. Further, to the best of our knowledge, synthetic surfactants have been employed continuously for the past 16 years without the development of resistance in the control of zoospores of *Olpidium brassicae*. This is not to say that development of surfactant-insensitive zoospores is impossible. For example, there could be selection for strains that encyst rapidly, thus avoiding exposure of the plasma membrane to the lytic effects of surfactants. Such selection, however, would impact adversely upon the ability of zoospores to spread and move chemotactically to appropriate (and often highly specific) infection sites on the host plant.

Outlook

The identification of biosurfactant-induced rupture of the plasma membrane as a new type of antagonistic mechanism, coupled with knowledge of the specific target, i.e., zoospores, and the common occurrence of epiphytic biosurfactant-producing bacteria in nature, have significant implications for the biological control of zoosporic plant pathogens. This is especially true for pathogens that cause destructive foliar diseases. The presence of free water, which is essential for zoospore liberation, spread, and motility, would also provide the optimum environment for the timely solubilization and dispersal of in situ produced rhamnolipids. Given the rapidity at which lysis of the zoospores can occur in the presence of a low but sufficient concentration of rhamnolipid, it is reasonable to assume that zoospore lysis will occur prior to encystment and host colonization by the pathogen.

Use of these rhamnolipid-producing bacteria as biological control agents could substantially reduce the use of chemical pesticides. According to a recent publication (1), approximately 80% of the \$5.2

billion spent annually for fungicides in the United States is directed toward control of foliar pathogens, yet 80% of the research on biological control is directed toward seed and seedling diseases caused by soil-borne plant pathogens. This discrepancy in focus is attributed, in part, to the perception that the root environment is more physically amenable to biological control than the leaf surface. This is primarily due to the almost continuous occurrence of sufficient moisture in soil for microbial growth. *Pseudomonas* spp., however, occur widely as epiphytes on leaves, as previously mentioned. Further, one species of *Pseudomonas*, *P. syringae*, has been reported to multiply even on dry leaf surfaces during daylight hours under field conditions (11). Thus, our discovery that epiphytic rhamnolipid-producing bacteria are also normal inhabitants of leaf surfaces, coupled with the knowledge of the mechanism of antagonism to the target pest, con-

stitute a cautious but optimistic view that biological control of zoosporic plant pathogens can be achieved by management of the physical and chemical environment, which would enhance the activities of introduced or naturally occurring biosurfactant-producing bacteria.

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