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INSTITUTE OF AGRICULTURE AND NATURAL RESOURCES
DEPARTMENT OF PLANT PATHOLOGY

Picotechnology 340pm / 600pm Evaluation of Soysoap Formula S-101 and S-102 For Activity Against Gram-Positive Plant Pathogenic Bacteria

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SUMMARY

Biobased USA Picotechnology 340pm / 600pm Evaluation Soysoap Formula S-100 was effective in vitro at multiple concentrations in killing multiple isolates of agriculturally important Gram-positive plant pathogens. The bacteria tested in these assays were Clavibacter michiganensis subsp. nebraskensis (causal agent of Goss's wilt and blight of maize), Cl. mich. subsp. michiganensis (causal agent of bacterial canker of tomato), Cl. mich. subsp. insidiosus (causal agent of bacterial wilt of alfalfa), and Curtobacterium flaccumfaciens pv. flaccumfaciens (causal agent of bacterial wilt of dry bean). The three subspecies of Clavibacter michiganensis tested had indistinguishable sensitivities to Soysoap Formula S-100 after 22 hours treatment (2^{-14} =1/16384, or 61 ppm), while Curtobacterium flaccumfaciens pv. flaccumfaciens was much less sensitive (2^{-8} = 1/256, or 3.9 ppt). One replicate of a subset of strains was tested after 2 hours treatment; this shorter exposure time was nearly as effective as 22 hours.

OBJECTIVE

Soysoap Formula S-100 was tested in vitro at multiple concentrations to assess its potential efficacy as a protection agent against important Gram-positive plant pathogenic bacteria in greenhouse and field grown crops.

MATERIALS AND METHODS

Test Organisms

Clavibacter michiganensis subsp. nebraskensis (Cmn) Disease:
Goss' wilt and blight of maize
Isolates tested: 2579 NCPPB (Pawnee Co. Nebraska 1971)
20037 (Dawson Co. Nebraska, 2003)
200800460 (Antelope Co. Nebraska, 2008)

Clavibacter michiganensis subsp. michiganensis (Cmm) Disease:
bacterial canker of tomato
Isolates tested: JD83-1 (Jim Vick, Canadian Canners)
CF-2 (Frontier Co. Nebraska)

Clavibacter michiganensis subsp. insidiosus (Cmi)
Disease: bacterial wilt of alfalfa
Isolates tested: 239 P2

Curtobacterium flaccumfaciens pv. flaccumfaciens (Cff)
Disease: bacterial wilt of dry bean
Isolates tested: 1446 NCPPB (Hungary, 1982)
Small Red (Scotts Bluff Co. Nebraska, 2005)

Bacterial cultures had been maintained as lyophilized cultures or in Microbank vials (PRO-LAB Diagnostics, Canada) at -70°C. Culture suspensions, made from colonies grown on Tryptic Soy Agar, were grown for two to three hours in 10 ml Tryptic Soy Broth (Difco, Sparks, MD) at 27°C, sessile. The optical density of each culture was determined spectrophotometrically at 640.

Agents Tested

Tested in this assay was Soysoap Formula S-100 non-toxic surfactant
an amber, viscous solution, .

Method

A modified Minimal Inhibitory Concentration (MIC) microbiological assay was used to determine levels of resistance of plant pathogens to

this agent. Briefly, this method involves serial dilutions (1:2) of the test agent in TSB, a liquid growth medium. After the dilutions were made, an aliquot of bacterial suspensions was added to each tube, except for an uninoculated control. The tubes were incubated for 22 hours, shaking, at 27 C. Three 10 µL aliquots from each dilution were placed on the surface of a TSA plate, and the plates were incubated at 27 C for
96 hours.

For the first assay (Table 1), the test agent was diluted in ten replicates: One milliliter of Soysoap Formula S-100 concentrate was added to 1 ml of the first tube of the series and mixed; 1 ml of this tube was transferred to the second tube of 1 ml and mixed. The dilution process was repeated in subsequent tubes resulting in a final series which included the undiluted agent, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128,
1:256, 1:512, and 1:1024 dilutions.

For the second assay (Tables 2 & 3), the test agent was serially diluted as a single replicate, but in sufficient volume to dispense one milliliter aliquots for each of the two replicates of each strain tested. This dilution series extended from the undiluted agent out to 1:131,072, in two-fold dilutions.

For each of the assays, control tubes were included: broth only and bacteria + broth (no agent). An additional control was included for the second assay: the dilution series with no bacteria added was checked for the absence of contaminating bacteria. Each bacterial isolate was tested in two replicate dilutions of the treatment.

The Minimum Inhibitory Concentration of an agent is the highest dilution or lowest concentration which prevents the growth of a bacterial culture. In a standard MIC assay, bacterial growth is assessed after incubation by comparing each tube of the dilution series visually or turbidimetrically against the control tube which contains the bacterial suspension with no test agent. We modified the MIC protocol by plating triplicate 10 µL aliquots of each test dilution and controls on TSA II™ Trypticase Soy Agar (BBL, Cockeysville, MD) medium to validate and quantitate bacterial survival. The assay plates were incubated at 27 °C, examined every 24 hours, and bacterial growth was recorded for controls and each dilution 96 hours after inoculation. The assay plates were kept for an additional 10-12 days at 27 °C to determine whether there were any "escapes" or additional surviving cells.

RESULTS

The results of independent assays are presented in table format and attached. Growth of bacteria was recorded as positive (+) or negative (-) based on visible growth in the areas of inoculum (triplicate spots) on the agar plates. The minimum inhibitory concentration of agents is indicated on the tables by a yellow shading of cells; growth of bacteria by green shading.

The three subspecies of Clavibacter michiganensis tested had indistinguishable sensitivities to Soysoap Formula S-100, not surprising given their extensively confirmed relatedness. Curtobacterium flaccumfaciens pv. flaccumfaciens was much less sensitive, a surprising result because this pathogen is closely related to the other three.

DISCUSSION

There are limited bacterial control agents currently registered for crop protection in the U.S. and worldwide. Screening for cost effective bactericides including bio-based, synthetic and inorganic compounds frequently begins in the laboratory with in vitro assays to evaluate the activity of the agent against target organisms in a non-plant system under standardized conditions. In vitro activity of an agent against a

target organism suggests potential but does not predict efficacy in plant disease control.

The assays of Soysoap Formula S-100 demonstrated its in vitro activity at 22 hours against multiple isolates of four important bacterial pathogens of important crops. Even at very low concentrations Soysoap Formula S-100 was highly active against Cmn, the causal agent of Goss's wilt and blight of maize, Cmm, the causal agent of bacterial canker of tomato and Cmi, the causal agent of bacterial wilt of alfalfa (Tables 2a & 2b). Except for one of the two strains of Cmi (which may be an atypical strain), these pathogens did not survive at a concentration of 61 ppm (MIC = 1:16384), which is impressive. Previously, Cmn was tested against Soysoap S-102, and in those tests the pathogen did not survive a concentration of 980 ppm (or 0.098%) (MIC = 1:1024).

Soysoap Formula S-100 demonstrated a reduced activity at 22 hours against Cff, the agent of bacterial wilt of bean (MIC = 1:64 for one isolate and MIC = 1:128 for the other). These results for Cff were similar to the results for Soysoap S-102 vs. Cff.

The activity of Soysoap Formula S-101 at two hours was tested for one of the two replicates for two strains of Cmn, one strain of Cmm, one strain of Cmi and the Cff strain (Table 3). The results at two hours, limited as they were by assaying only one replicate of fewer strains, were similar to the results at 22 hours, but the additional incubation time did appear to allow lower concentrations of Soysoap Formula S-100 to be more effective relative to the two hour incubation.

Based on these promising results, areas for further study might include testing activity of Soysoap Formula S-101 or Soysoap S-102 (or both) at several concentrations in greenhouse and field grown plants to evaluate efficacy and phytotoxicity in planta (in plants), selection of delivery method of agent and pathogen, and time sequence studies to assess death rate of bacterial pathogens when the agent is applied prior to or after plant inoculation.

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Table 1. Initial screen of pathogens vs. dilutions of *Soysoap Formula S-101*. Results at 96 hours after treatment for 22 hours.

concentration	strain	Cff 1446		Cff small red		Cmn 2579		Cmn20037		Cmn20080	
of Formula 101	replicate	A	B	A	B	A	B	A	B	A	B
100%	undiluted	—	—	—	—	—	—	—	—	—	—
50%	1/2	—	—	—	—	—	—	—	—	—	—
25%	1/4	—	—	—	—	—	—	—	—	—	—
12.5%	1/8	—	—	—	—	—	—	—	—	—	—
6.25%	1/16	—	—	—	—	—	—	—	—	—	—
31.3 ppt	1/32	—	—	—	—	—	—	—	—	—	—
15.6 ppt	1/64	+/–	+/–	—	—	—	—	—	—	—	—
7.81 ppt	1/128	+/–	+/–	+/–	+/–	—	—	—	—	—	—
3.91 ppt	1/256	+++	+++	+++	+++	—	cont.	—	—	—	—
1.95 ppt	1/512	+++	+++	+++	+++	—	cont.	—	—	—	—
977 ppm	1/1024	+++	+++	+++	+++	—	cont.	—	—	—	—
488 ppm	1/2048	+++	+++	+++	+++	—	cont.	—	—	—	—
244 ppm	1/4096	+++	+++	+++	+++	—	cont.	—	—	—	—
122 ppm	1/8192	+++	+++	+++	+++	—	cont.	—	—	—	—
	untrted	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	sterile	—	—	—	—	—	—	—	—	—	—

Key: —, no growth (pink)(yellow for MIC); +/–, only a few colonies (green); +++, confluent growth (green); cont., contaminated

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Table 2a. Second screen of pathogens vs. dilutions of Soysoap Formula S-101. Results at 96 hours after treatment for 22 hours.

concentration	strain	Cff 1446		Cmn 2579		Cmn20037		Cmn20080	
of Formula 101	replicate	A	B	A	B	A	B	A	B
100%	undiluted	NT	NT	NT	NT	NT	NT	NT	NT
50%	1/2	NT	NT	NT	NT	NT	NT	NT	NT
25%	1/4	NT	NT	NT	NT	NT	NT	NT	NT
12.5%	1/8	NT	NT	NT	NT	NT	NT	NT	NT
6.25%	1/16	NT	NT	NT	NT	NT	NT	NT	NT
31.3 ppt	1/32	NT	NT	NT	NT	NT	NT	NT	NT
15.6 ppt	1/64	NT	NT	NT	NT	NT	NT	NT	NT
7.81 ppt	1/128	NT	NT	NT	NT	NT	NT	NT	NT
3.91 ppt	1/256	+/–	+/–	NT	NT	NT	NT	NT	NT
1.95 ppt	1/512	+/–	+/–	NT	NT	NT	NT	NT	NT
977 ppm	1/1024	+++	+++	NT	NT	NT	NT	NT	NT
488 ppm	1/2048	+++	+++	NT	NT	NT	NT	NT	NT
244 ppm	1/4096	NT	NT	—	—	—	—	—	—
122 ppm	1/8192	NT	NT	—	—	—	—	—	—
61.0 ppm	1/16384	NT	NT	—	—	—	—	—	—
30.5 ppm	1/32768	NT	NT	+++	+++	+/–	+/–	+/–	+/–
15.3 ppm	1/65536	NT	NT	+++	+++	+++	+++	+++	+++
7.63 ppm	1/131072	NT	NT	+++	+++	+++	+++	+++	+++
	no trtmnt	+++	+++	+++	+++	+++	+++	+++	+++
	diluent	—	—	—	—	—	—	—	—

Key: —, no growth (pink)(yellow for MIC); +/–, only a few colonies (green); +++, confluent growth (green); NT, not tested (blue)

Table 2b. Second screen of pathogens vs. dilutions of Soysoap Formula S-101. Results at 96 hours after treatment for 22 hours.

concentration	strain	CmmJD83-1		CmmCF-2		CmiP2		Cmi239		Dilution
of Formula 101	replicate	A	B	A	B	A	B	A	B	Only
100%	undiluted	NT	NT	NT	NT	NT	NT	NT	NT	NT
50%	1/2	NT	NT	NT	NT	NT	NT	NT	NT	NT
25%	1/4	NT	NT	NT	NT	NT	NT	NT	NT	—
12.5%	1/8	NT	NT	NT	NT	NT	NT	NT	NT	—
6.25%	1/16	NT	NT	NT	NT	NT	NT	NT	NT	—
31.3 ppt	1/32	NT	NT	NT	NT	NT	NT	NT	NT	—
15.6 ppt	1/64	NT	NT	NT	NT	NT	NT	NT	NT	—
7.81 ppt	1/128	NT	NT	NT	NT	NT	NT	NT	NT	—
3.91 ppt	1/256	—	—	—	—	—	—	+++	+++	—
1.95 ppt	1/512	—	—	—	—	—	—	+++	+++	—
977 ppm	1/1024	—	—	—	—	—	—	+++	+++	—
488 ppm	1/2048	—	—	—	—	—	—	+++	+++	—
244 ppm	1/4096	—	—	—	—	—	—	+++	+++	—
122 ppm	1/8192	—	—	—	—	—	—	+++	+++	—
61.0 ppm	1/16384	—	—	—	+/–	—	—	+++	+++	—
30.5 ppm	1/32768	+++	+++	+++	+++	+++	+++	+++	+++	—
15.3 ppm	1/65536	+++	+++	+++	+++	+++	+++	+++	+++	—
7.63 ppm	1/131072	+++	+++	+++	+++	+++	+++	+++	+++	—
	no trtmnt	+++	+++	+++	+++	+++	+++	+++	+++	NT
	diluent	—	—	—	—	—	—	—	—	NT

Key: —, no growth (pink)(yellow for MIC); +/–, only a few colonies (green); +++, confluent growth (green); NT, not tested (blue)

Table 3. Second screen of a subset of pathogens vs. dilutions of Soysoap Formula S-101. Results at 96 hours after treatment for either 2 or 22 hours.

Concentration	strain	Cff 1446 A		Cmn 2579 A		Cmn20037 A		CmmJD83-1		CmiP2	
of Formula 101	time	2 h	22 h	2 h	22 h	2 h	22 h	2 h	22 h	2 h	22 h
6.25%	1/16	—	—	NT	NT	NT	NT	NT	NT	NT	NT
31.3 ppt	1/32	—	—	NT	NT	NT	NT	NT	NT	NT	NT
15.6 ppt	1/64	+/–	—	NT	NT	NT	NT	NT	NT	NT	NT
7.81 ppt	1/128	+/–	+/–	NT	NT	NT	NT	NT	NT	NT	NT
3.91 ppt	1/256	+/–	+/–	NT	NT	NT	NT	—	—	—	—
1.95 ppt	1/512	+++	+/–	NT	NT	NT	NT	—	—	—	—
977 ppm	1/1024	+++	+++	NT	NT	NT	NT	—	—	—	—
488 ppm	1/2048	+++	+++	NT	NT	NT	NT	+/–	—	+/–	—
244 ppm	1/4096	NT	NT	—	—	—	—	+/–	—	+/–	—
122 ppm	1/8192	NT	NT	+/–	—	+/–	—	+++	—	+++	—
61.0 ppm	1/16384	NT	NT	+++	—	+/–	—	+++	—	+++	—
30.5 ppm	1/32768	NT	NT	+++	+++	+++	+/–	+++	+++	+++	+++
15.3 ppm	1/65536	NT	NT	+++	+++	+++	+++	+++	+++	+++	+++
7.63 ppm	1/131072	NT	NT	+++	+++	+++	+++	+++	+++	+++	+++
	no trtmnt	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	diluent	—	—	—	—	—	—	—	—	—	—

Key: —, no growth (pink)(yellow for MIC); +/–, only a few colonies (green); +++, confluent growth (green); NT, not tested (blue)

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