Developing biopesticides for control of citrus fruit pathogens of importance in global trade

By

Joseph Obagwu

Promoter: Prof. Lise Korsten
Co-promoter: Dr. Thierry Regnier
Department: Microbiology and Plant Pathology
Faculty: Natural and Agricultural Sciences
Degree: Ph.D. (Plant Pathology)

RESUMÈ

Bacillus isolates originally isolated from Valencia and Shawmut oranges were screened for inhibitory activity against citrus pathogens; *Guignardia citricarpa* cause of citrus black spot, *Penicillium digitatum* and *P. italicum* the cause of green- and blue mold respectively. The potential antagonists were tested both *in vitro* and *in vivo*, and isolates were tested either on their own or in combination with sodium bicarbonate (SB). Plant extracts, which included aqueous garlic clove and *Coprosma repens* were also evaluated for antifungal activity against the fungal pathogens. In addition, *C. repens* extracts were also evaluated for their antibacterial properties against *Escherichia coli* 0157-H7 *Salmonella typhimurium*, *Staphylococcus aureus* and *Vibrio cholerae,* known food-borne pathogens of importance in food safety of fresh fruits. The study revealed the following:

1. Fifty percent of the *Bacillus* isolates showed *in vitro* inhibitory activities against *P. digitatum* and *P. italicum.* However, only three isolates: F1, L2 and L2-5 provided effective and consistent control of both pathogens in both *in vitro* and *in vivo* tests. Isolates were not effective in controlling latent infections of *G. citricarpa*.

2. The combination of isolates with SB (1% w/v) resulted in a synergistic reaction. Subsequently, a remarkable improvement in the biocontrol activity of the three

isolates was recorded particularly with F1. The combination of F1 with SB was as effective as the commercial fungicide treatment giving complete control of both diseases. Consistency of product performance was shown throughout the season.

3. Effective *in vivo* control of both *P. digitatum* and *P. italicum* was achieved with aqueous garlic clove extracts. Extracts alone (1 000 - 10 000 ppm) was not as effective as the commercial fungicide treatment. Combining extracts with vegetable oil improved biological activity. A combination of extracts (1 000 ppm) with oil (0.1% v/v) was as effective as the fungicide treatment which gave complete control of both diseases.

4. Ethanol extracts of *C. repens* were effective in preventing the development of black spot lesions, and in controlling both green- and blue mold. More effective control of CBS was achieved compared to green- and blue mold. The active compound in the plant extracts falls within the family of the hydroxycinamics.

5. In addition to its antifungal activities, extracts of *C. repens* possessed antibacterial activities and were effective in controlling both the *in vitro* and *in vivo* establishment of *E. coli* 0157-H7, *S. typhimurium*, *S. aureus* and *V. cholerae*.

Appendix 1. Identity of *Bacillus* species screened and status of evaluation

Code	Name	Status [a]	
		In vitro	*In vivo*
268	*Bacillus subtilis*	x	-
F1	*Bacillus subtilis*	x	x
L2-5	*Bacillus subtilis*	x	x
OPF1	*Bacillus subtilis*	x	-
OP2-5	*Bacillus subtilis*	x	-
L3	*Bacillus subtilis*	x	-
719 C	*Bacillus subtilis*	x	-
OPL2A	*Bacillus subtilis*	x	-
143	*Bacillus amyloliquefasciens*	x	-
T1	*Bacillus subtilis*	x	-
L2	*Bacillus subtilis*	x	x
565	*Bacillus subtilis*	x	-
T2	*Bacillus subtilis*	x	-
L2-2	*Bacillus subtilis*	x	-
LIA	*Bacillus subtilis*	x	-
80	*Bacillus subtilis*	x	-
2	*Bacillus subtilis*	x	-
814	*Bacillus licheniformis*	x	-
642	*Bacillus subtilis*	x	-
341	*Bacillus subtilis*	x	-

[a] "x" means applicable, "-" means not applicable

Appendix 2. Common poisonous plants growing in gardens and in the wild

American Ivy
Apple
Apricot
Azalea
Bird-of-Paradise
Bittersweet
Black locust
Bunch Berry
Caladium
Calla Lily
Castor Bean
Cherry
Choke Cherry
Colocasia
Convallaria Majalis
Cowbane
Crowfoot
Daffodil
Delphinium
Daphne
Dieffenbachia
Digitalis
English Ivy
Foxglove
Hedera Helix
Hemlock
Hens and Chickens
Holly
Hyscinth
Hydrangea
Iris
Jack-in-the-Pulpit
Jessamine
Jimsonweed
Jonquil
Lantana
Larkspur
Ligustrum
Lily-of-the-Valley
Locoweed
Locust
Mayapple
Mistletoe
Monstera
Morning Glory
Narcissus
Nightshade
Oleander
Philodendron
Plum
Poinciana
Poinsettia
Poison Oak
Pokeweed
Prickly Poppy
Privet
Rhubarb
Rhododendron
Ricinus Communis
Skunk Cabbage
Strawberry Bush
Sweet Pea
Thorn Apple
Wisteria
Yew

Reference: www.webhome.idirect.com/bom2luv/plants.htm.

Appendix 3. Chemical composition of the RSM-CAS medium (Leong, 1986)

Treatment	Molecular weight	Weight of chemical	Volume of water	Concentration
$FeCl_3.6H_2O$	27.0	23.0	27.0	1M
$KH_2.PO_4$	126.09	13.61	0.1 L	1 M
Sucrose	302.31	30.81	0.3 L	30%
$ZnSO_4.7H_2O$	287.56	0.2013	1 L	0.0007 M
$MnSO_4.4H_2O$	169.01	0.1521	1 L	0.0009 M
Thiamine. HCl	337.27	0.02	1 L	20mg L^{-1}
Casamino acids	-	30	0.3 L	10%
Biotin	233.31	0.001	1 L	1mg L^{-1}
HCl	-	1ml	1 L	10nM

Appendix 4. Vibrio Diagnostic Agar, a medium used for the isolation of *Vibrio cholerae*

Ingredient	Quantity (g)
Sodium Chloride	10.0
Sucrose	20.0
Sodium Citrate	10.0
Sodium Thiosulphate	10.0
Special Peptone	10.0
Ox Bile	5.0
Yeast Extract	5.0
Sodium Taurocholate	3.0
Ferric Citrate	1.0
Bromothymol Blue	0.04
Thymol Blue	0.04
Agar	15.0

Appendix 5. Standard 1 Nutrient Agar, a medium used for the enumeration, isolation and enrichment of bacteria

Ingredient	Quantity (g)
Special Peptone	15.6
Yeast Extract	2.8
Sodium Chloride	5.6
D (+) Glucose	1.0
Agar	12.0

Appendix 6. Violet-Red-Bile-MUG Agar, a selective medium for the simultaneous detection and enumeration of *Escherichia coli*

Ingredient	Quantity (g)
Brain Heart Infusion	7.0
Peptone	4.0
Lactose	9.0
Bile Salt No.3	1.5
Neutral Red	0.03
Crystal Violet	0.002
MUG	0.1
Sodium Chloride	4.5
di-Sodium Phosphate	1.0
Agar	13.0

Appendix 7. MacConkey Agar, a selective agar for the isolation of *Salmonella*

Ingredient	Quantity (g)
Peptone	20.0
Lactose	10.0
Bile Salt No.3	1.5
Sodium Chloride	5.0
Neutral Red	0.003
Crystal Violet	0.001
Agar	13.5

Appendix 8. Identity, methodology and time of dip applications of different treatments evaluated on a semi-commercial scale

Treatment	Product	Concentration	Date
Experiments done in 2000 (A)			
Control	Water	-	June & August
Avogreen powder	*Bacillus subtilis*	75g/100 L water	June & August
Avogreen liquid	*Bacillus subtilis*	250 ml/100 L water	June & August
Biocoat	*Candida saitoana*	406g product/ 15 L water	June & August
Biocure	*Candida saitoana*	406g product/ 15 L water	June & August
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	June & August
Experiments done in 2000 (B)			
Control	Water	-	September
Avogreen powder	*Bacillus subtilis*	75g/100 L water	September
Avogreen liquid	*Bacillus subtilis*	250 ml/100 L water	September
Biocoat	*Candida saitoana*	406g product/ 15 L water	September
Biocure	*Candida saitoana*	406g product/ 15 L water	September
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	September
F1	*Bacillus subtilis*	10^8 cell ml^{-1}	September
L2-2	*Bacillus subtilis*	10^8 cell ml^{-1}	September
L2	*Bacillus subtilis*	10^8 cell ml^{-1}	September
F1 + SB	-	-	September

Appendix 8 continued

Treatment	Product	Concentration	Date
L2-5 + SB	-	-	September
L2 + SB	-	-	September
SB	Sodium bicarbonate	1% w/v	September

Experiments done in 2001 (A)

Control	Water	-	June & August
Avogreen powder	*Bacillus subtilis*	75g/100 L water	June & August
Avogreen liquid	*Bacillus subtilis*	250 ml/100 L water	June & August
Biocoat	*Candida saitoana*	406g product/ 15 L water	June & August
Biocure	*Candida saitoana*	406g product/ 15 L water	June & August
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	June & August
F1	*Bacillus subtilis*	10^8 cell ml^{-1}	June & August
L2-2	*Bacillus subtilis*	10^8 cell ml^{-1}	June & August
L2	*Bacillus subtilis*	10^8 cell ml^{-1}	June & August
F1 + SB	-	-	June & August
L2-5 + SB	-	-	June & August
L2 + SB	-	-	June & August
SB	Sodium bicarbonate	1% w/v	June & August

Experiments done in 2001 (B)

Control	Water	-	September
Avogreen powder	*Bacillus subtilis*	75g/100 L water	September
Avogreen liquid	*Bacillus subtilis*	250 ml/100 L water	September

Appendix 8 continued

Treatment	Product	Concentration	Date
Biocoat	*Candida saitoana*	406g product/ 15 L water	September
Biocure	*Candida saitoana*	406g product/ 15 L water	September
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	September
F1	*Bacillus subtilis*	10^8 cell ml^{-1}	September
L2-2	*Bacillus subtilis*	10^8 cell ml^{-1}	September
L2	*Bacillus subtilis*	10^8 cell ml^{-1}	September
F1 + SB	-	-	September
L2-5 + SB	-	-	September
L2 + SB	-	-	September
SB	Sodium bicarbonate	1% w/v	September
Experiments done in 2002			
Control	Water	-	August & September
Fungicide	Imazalil + guazatine	1g +1 ml product/ L water	August & September
F1	*Bacillus subtilis*	10^8 cell ml^{-1}	August & September
L2-2	*Bacillus subtilis*	10^8 cell ml^{-1}	August & September
L2	*Bacillus subtilis*	10^8 cell ml^{-1}	August & September
F1 + SB	-	-	August & September

Appendix 8 continued

Treatment	Product	Concentration	Date
L2-5 + SB	-	-	August & September
L2 + SB	-	-	August & Sept