ANTAGONISTIC EFFECT OF SOME RHIZOBACTERIA ON THE BIOLOGICAL ACTIVITYOF RALESTONIA SOLANISEARUM

By NERHAN ABD ELSALAM EID ABD ELAAL

B.Sc. Agric. Sc. (Plant Pathology), Ain Shams Univ., 2005

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In (Agricultural Science)

Department of Microbiology Faculty of Agriculture Ain Shams University

Approval Sheet

ANTAGONISTIC EFFECT OF SOME RHIZOBACTERIA ON THE BIOLOGICAL ACTIVITYOF RALESTONIA SOLANISEARUM

By NERHAN ABD ELSALAM EID ABD ELAAL

B.Sc. Agric. Sc. (Plant Pathology), Ain Shams Univ., 2005

••

Date of Examination: 19 / 10 / 2015

ANTAGONISTIC EFFECT OF SOME RHIZOBACTERIA ON THE BIOLOGICAL ACTIVITYOF RALESTONIA SOLANISEARUM

By NERHAN ABD ELSALAM EID ABD ELAAL

B.Sc. Agric. Sc. (Plant Pathology), Ain Shams Univ., 2005

Under the supervision of:

Dr. Elshahat Mohamed Ramadan

Prof. of Agric. Microbiology, Dep. Microbiology, Faculty of Agriculture, Ain Shams University (Principal Supervisor)

Dr. Enas Abd-Eltawab Hassan

Associate Prof. of Agric. Microbiology Dep., Faculty of Agriculture, Ain Shams University.

Dr. Abeer El-Morsy Ahmed El-Hadidy

Associate researcher Professor of Plant Pathology Unit, Desert Research Center.

ABSTRACT

Nerhan Abd El Salam Eid Abd El Aal. Antagonistic Effect of some Rhizobacteria on the Biological Activity of *Ralestonia solanisearum*". Unpublished M.Sc. Thesis. Department of Microbiology, Faculty of Agriculture, Ain Shams University, 2015.

Bacterial wilt caused by Ralstonia solanacearum phylotypeII sequevarI (race 3 biovar 2), is considered one of the most destructive bacterial diseases of potato plant. The aim of this study was to evaluate the potential of bacterial antagonists to suppress the causative bacteria. In this regard, 420 isolates of *R. solanacearum* were recovered from different habitats at different Egyptian districts. The variation between isolates was assessed on the basis of pathogenic potentials to tomato seedlings in the greenhouse. The evaluation indicated that, the highest potential, as shown by the wilt severity was obtained by 11 out of 420 isolates. The most virulent R. solanacearum isolates were identified by special techniques. To lay down a biological control protocol, a collection of 318 isolates of rhizobacteria from rhizosphere of different plants, were tested for antibiosis against R. solanacearum selected strains. In vitro, 14 isolates were more effective in suppressing the pathogen at different levels of relative power of antibiosis. These isolates were categorized into three groups according to their Gram-staining reaction, cell morphology and cultural characteristics. Further evaluation, under the greenhouse condition, has shown that six of the isolates were effective in suppressing disease development, as expressed by the Area Under Disease Progress Curve (AUDPC). The assessment of cyanide and siderophore production for selected antagonistis revealed that M3 and M5 isolates gave the highest values of HCN wheaeas maximum siderophore production was recorded by M4, M5 isolates. The selected isolates were identified as: Streptomyces toxytricini, Stenotrophomonas maltophilia strain IAM 12423 , Stenotrophomonas maltophilia strain ATCC 19861 , Bacillus pseudomycoides and Brevibacillus brevis. The recovered of antimicrobial compounds from supernatants of these strains were conducted using chlorophorm, petroleum ether and ethylacetat individually to find that chlorophorm phase at 100 ppm for Bacillus pseudomycoides M3, Streptomyces toxitricini C5 and Stenotrophomonas maltophilia M5 gave antagonistic effect spectrum of against R. solanacearum strains wheras petroleum ether phase at 50 ppm inhibited the most selected strains of R. solanacearum in vitro. The efficient organic phase of each strain was separated to many fractionated bands by Thin Layer Chromatography(TLC). The antagonistic bacteria promoted the potato growth and inhibited the bacterial pathogens under semi field conditions. The total count of *R. solanacearum* in the rhizosphere decreased gradually during three months and the count of antagonistic bacteria didn't change. The growth parameters were improved by the tested rhizobacteria.

Keywords: Microbial antagonists, biological control, *B. pseudomycoides*, *Brevibacillus brevis*, *Ralstonia solanacearum*, *Streptomyces toxytricini* and *Stenotrophomonas maltophilia*, PCR, 16S rDNA

ACKNOWLEDGMENT

Praise and thanks be to ALLAH, the most merciful for assisting and directing me to the right way.

Great acknowledgement goes in particular to **Prof. Dr. El shahat Mohamed Ramadan**, Prof. of Agric. Microbiology, Faculty of Agriculture, Ain Shams University and **Dr. Enas Abd El Tawab Hassan**, Associate Professor of Agric. Microbiology, and Fac. of Agric., Ain Shams University for their supervision, scientific advice and for their sincer co-operation in revising this manuscript.

Gratitude is due to **Dr. Naglaa Moussa Balabel** Associate professor, Head of plant bacterial disease department, Plant Pathology, Res. Inst. ARC, for valuable endless advises, guidance and sincere help.

Thanks are also due to **Dr. Abeer El Morsy Ahmed El Hadidy,** First researcher, Head of Plant Disease unit, Plant Protection Department Desert Research Center.

Sincere thanks are also due to **Dr. Mohamed Abd El Aziz Balah** for encouragement, support and faithful assistance throughout the study. Many thanks are due to **Dr. Ahmed Ismaeil**, researcher in Plant Protection Department Desert Research Center. For his support and faithful assistance throughout of this study

I am grateful to **Dr. Ahmed Soliman Mohamed** researcher, and all colleagues in Plant Protection Department, Desert Research Center,

Full respect to my lovely mother who gave me love and encouragement all the time. Thanks to my husband for his encouragement and full support all the time, and thanks to my lovely little kids.

CONTENTS

	Page
LIST OF TABLES	
LIST OF FIGURES	
LIST OF ACRONYMS	
1. INTRIDUCTION	1
2. REVIEW OF LITRATURE	3
2.1. Ralstonia solanacearum, a destructive bacterial plant	4
pathogen	
2.1.1 Distribution of <i>Ralstonia solanacearum</i>	4
a. In Egypt	4
b. In other countries	4
2.1.2 The Disease Symptoms	6
21.3 Occurrence and Host range of <i>Ralstonia</i>	7
solanacearum	
2.1.4 The Spread and survival of Ralstonia solanacerum	8
2.2 The brown rot disease and the problem of exports to the	9
European Union	
2.3 Ralstonia solanacearum races and biovars	10
2.3.1 Morphological and cultural characteristics	10
2.3.2 Detection of Ralstonia solanacearum:	11
 a. Detection by tomato bioassay test 	11
(pathogenicity test)	
b. Detection on selective media	11
c. Detection by Immunofluorescence Antibody	12
Stain (IFAS)	
2.4 Biocontrol agents for controlling potato brown rot disease	12
2.4.1 Plant growth promoting rhizobacteria for biocontrol	13
of plant diseases	
a. Florescent pseudomonads bacteria	14
b. Bacillary bacteria	16
c. Actinomycetes	17
2.4.2 The mode of action of biocontrol agents	18
2.4.2.1 Antibiosis	20
2.4.2.2 Siderphores production and competition for iron	21
iron	

		Page
,	2.4.2.3 Volatile compounded (HCN)	23
,	2.4.2.4 Competition for infection sites and nutrients	24
,	2.4.2.5. Induced systemic resistance	26
2.4	.3 Biocontrol potential of plant growth promoting	28
	bacteria against bacterial wilt	
3. MATEI	RIALS AND METHODS	29
3.1 Materia	al	29
3.1.1 Soi	l and collected samples	29
3.1.1.1	Source of collected samples	29
3.1.1.2	Samples of antagonistic rhizobacteria	29
3.1.1.3	Cultivated soil	29
	ant materials	30
3.1.3 Cl	nemical fertilizers	30
3.1.4 M	aterials used for formulation of dry state inoculant.	31
	rowth media, buffers and assay solutions	31
3.1.5.1	Media used	31
a.	King's medium B. (KB medium) (King et al., 1954)	31
b.	Nutrient agar (Jacobs and Gerstein, 1960)	31
c.	Oxidation/Fermentation (OF) medium (Fahy and	32
	Persley, 1983)	
d.	Gelatin medium (Collins and Patricia, 1984)	32
e.	Starch agar (Collins and Patricia, 1984)	32
f.	Thornley's medium 2A Agrinine medium (Lelliot	33
	and Stead, 1987)	
g.	(SMSA) Semi Selective Medium of South Africa	33
8.	(Elphinstone et al., 1996)	
h.	Nutrient Glucose agar medium (Dowson, 1957)	34
i.	_	34
	Trypticase soy broth agar (TSBA) (Rao, 1977)	
J.	Glycerol-nitrate agar medium (Waksman, 1961)	34
3.1.5.2		35
	Modified CAS (Chrome Azurol S) assay solution	35
	(Alexander and Zuberer, 1991)	
3.1.5.3.	Buffers used	36
	Buffers used for Immunofluorescence Antibody	36
	Stain (IFAS) test (Lelliot and Stead, 1987)	
a.	Phosphate buffer (0.05 M)	36
b.	Pellet Phosphate buffer (0.01 M)	36

	Page
c. Phosphate buffer saline (0.01 M)	36
d. Phosphate glycerine buffer (0.1 M)	36
3.2. Methods	37
3.2.1.Isolation and characterization of <i>Ralstonia</i> solanacearum as a causal of potato brown rot disease from different habitats	37
a. potato tuber	37
b. potato stems	37
c. irrigation water	38
d. Soil	38
e. Weeds associated with potato fields	38
3.2.2 Pathogenic potential of Ralstonia solanacearum	38
isolates 3.2.3. Diagnosis and Identification of the pathogenic	39
bacteria	3)
3.2.3.1 Physiological and biochemical characteristics	39
3.2.3.2 Plating on the SMSA medium	39
3.2.3.3 Immunofluorescence Antibody Stain (IFAS)	40
3.2.3.4 Biovar determination of the R. solanacearum	40
3.2.3.5. Real-Time PCR(Taq-Man)assay	41
3.2.3.6. Phylotype analysis of <i>R.solanacearum</i> by Multiplex- PCR	41
3.2.4 Rhizobacteria for potato brown rot control	42
3.2.4.1 Isolation of antagonistic rhizobacteria	42
3.2.4.2 Preliminary screening for antagonistic activity in	43
vitro	
3.2.4.3 Evaluation of biocontrol potential <i>in vivo</i>	44
A. Inoculum preparation of antagonistic rhizobacteria	44
B. Inoculum preparation of pathogenic bacteria:	44
C. Pot experiment under greenhouse conditions:	44
3.2.4.4 Evaluation antagonistic interaction among selected strains	45
3.2.5 Identification of selected antagonist :	45
3.2.5.1 Sequence analysis for 16S rRNA gene	45
3.2.6 Assay the antibacterial activity of antagonist	46
(Elucidation of biocontrol mechanisms of antagonist)	••
3.2.6.1. HCN production (Wei <i>et al.</i> , 1991)	46
3.2.6.2. Quantative assessment of siderophore production	47

	Page
1-Preparation of standard curve of deferoxamine mesylate	47
2- Preparation of culture for examination	47
3- Quantative method for determination siderophores	48
3.2.6.3 Production of extracellular bioactive	48
metabolite(s).	
A. Culture preparation:	48
B. Extraction of antibacterial substances	48
C. Determination of antibacterial activity of crude	49
extract	
d. Fractionation of crude extract	49
e. Characterization of the partially purified antibacterial compounds	50
3.2.7 Preparation of different formulation of biocontrol agent.	50
A- Preparation of carrier material	50
B- Preparation of bacterial antagonists	51
C- Preparation of different formula of solid state	51
inoculant.	
D- Survival of antagonistis on carrier materials	51
formulation	
3.2.8 Evaluation of biocontrol agent against potato brown rot	51
pathogen under greenhouse condition	
3.2.8.1 Preparation of biocontrol agent	52
3.2.8.2 Preparation of bacterial pathogen	52
3.2.8.3 Experimental design.	52
3.2.8.4 Parameters determined	53
3.2.8.4.1 Vegetative parameters	53
3.2.8.4.2 Assay of enzymes activity:	53
a. Peroxidase activity	53
b. Polyphenol oxidase activity	53
3.2.8.4.3 Microbiological parameters:	54
a. Bacterial load	54
b. Streptomyces load	54
c. Ralstsonia solanacearum load	54
3.2.9 Statistical analysis	54
4. RESULTS AND DISCUTIONS	55
4.1 Isolation and characterization of <i>Ralstonia solanacearum</i>	55
isolates:	
4.2 Identification of the pathogenic bacteria.	58

	Page
4.2.1 Physiological and biochemical characteristics	58
4.2.2 Biovar determination of the pathogen	59
4.2.3 Real-Time PCR assay:	59
4.2.4 Phylotype analysis of <i>R.solanacearum</i> by Multiplex-PCR	60
4.3 Isolation of rhizobacteria	60
4.4 Screening of antagonistic microorganisms	62
4.5 Evaluation of biocontrol potential	63
4.6 Assay of antagonistic activities	67
4.6.1 Cyanide production	67
4.6.2 Siderophore mediated biocontrol	69
4.7 <i>In vitro</i> test for cross interaction between the selected antagonistis	70
4.8 Identification of antagonistic bacteria by molecular analysis	70
4.9 Production of antimicrobial compounds	73
4.9.1 Antibacterial activity of crude extract	73
4.10 Evaluation of antibacterial activity of purified fractions <i>in vitr</i> o	76
4.11 Partial purification of the possible antibacterial compounds using High Pressure Liquid Chromatography Mass Spectroscopy (HPLC-MS) profile	82
4.12 Survival of tested bacteria on different carriers during the storage period	97
4.13 <i>In vivo</i> evaluation of potential bio-control agent	99
4.13.1 Counts of some bacterial groups in the rhizosphere during growth period	100
4.13.2 Counts of <i>R. solanacearum</i> in the rhizosphere during growth period	102
4.13.3 Growth characteristic of plant	105
3.13.4. Enzyme activity in plant	106
5. SUMMARY	109
6. REFERENCE	116
ARABC SUMMARY	