# Evaluation of biofungicide activity of endophytic bacteria isolated from blackberry bush (*Rubus ulmifolius* Schott) against two phytopathogenic fungi

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# INTRODUCTION

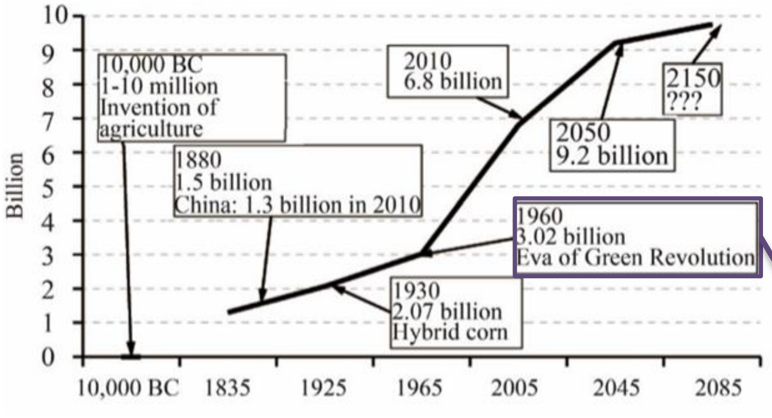



**Figure 1. World population growth [1].**

Popp and Lakner (2013)

*Average Annual Varietal Releases by Crop and Region*

| Crop | Average Annual Releases | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1965-70 | 1971-75 | 1976-80 | 1981-85 | 1986-90 | 1991-95 | 1996-2000 |
| Wheat | 40.8 | 54.2 | 58.0 | 75.6 | 81.2 | 79.3 | 85.0 |
| Rice | 19.2 | 35.2 | 43.8 | 50.8 | 57.8 | 54.8 | 58.5 |
| Maize | 13.4 | 16.6 | 21.6 | 43.4 | 52.7 | 108.3 | 71.3 |
| Sorghum | 6.9 | 7.2 | 9.6 | 10.6 | 12.2 | 17.6 | 14.3 |
| Millet | .8 | .4 | 1.8 | 5.0 | 4.8 | 6.0 | 9.7 |
| Barley | 0.0 | 0.0 | 0.0 | 2.8 | 8.2 | 5.6 | 7.3 |
| Lentils | 0.0 | 0.0 | 0.0 | 1.8 | 1.8 | 3.9 | 4.0 |
| Beans | 4.0 | 7.0 | 12.0 | 18.5 | 18.0 | 43.0 | 45.0 |
| Cassava | 0.0 | 1.0 | 2.0 | 15.8 | 9.8 | 13.6 | 15.0 |
| Potatoes | 2.0 | 10.4 | 13.0 | 15.9 | 18.9 | 19.6 | 20.0 |
| All Crops | | | | | | | |
| Latin America | 37.8 | 55.9 | 65.9 | 92.5 | 116.2 | 177.3 | 140.0 |
| Asia | 27.2 | 59.6 | 66.8 | 86.3 | 76.7 | 81.2 | 80.0 |
| Middle East and North Africa | 4.4 | 8.0 | 10.2 | 12.2 | 28.4 | 30.5 | 85.0 |
| Sub-Saharan Africa | 17.7 | 18.0 | 23.0 | 43.2 | 46.2 | 50.1 | 55.0 |
| All Regions | 87.1 | 132.0 | 161.8 | 240.2 | 265.8 | 351.7 | 360.0 |

Evenson *et al*. (2005)

HIGH YIELD CROP VARIETIES

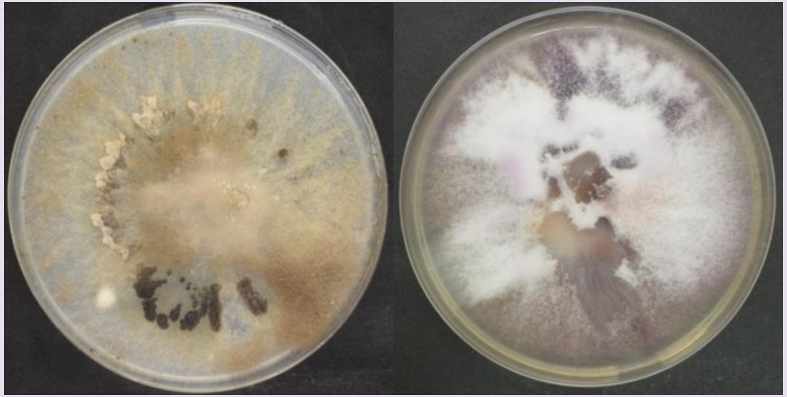
IRRIGATION

MACHINERY

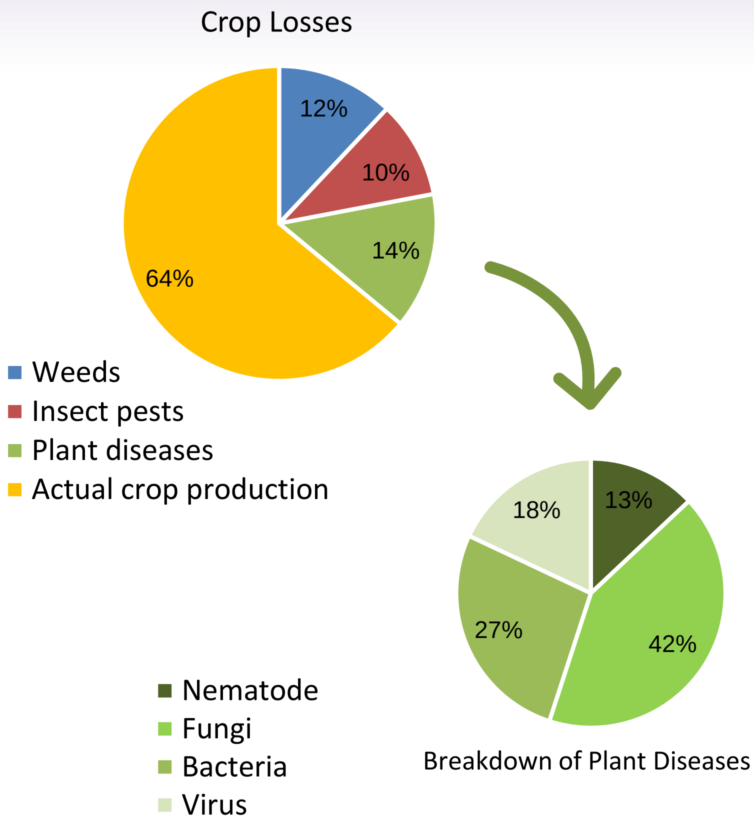
FERTILIZER/PESTICIDES

# INTRODUCTION

Main Fungal Diseases



*Botrytis cinerea* *Fusarium oxysporum*




(Khan and Anwer, 2011)

# INTRODUCTION

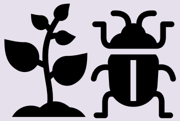
Traditionally, **chemical products** have been used to control plant diseases

Consequences

$CO_2$ 


Resistances increase


Environmental impact

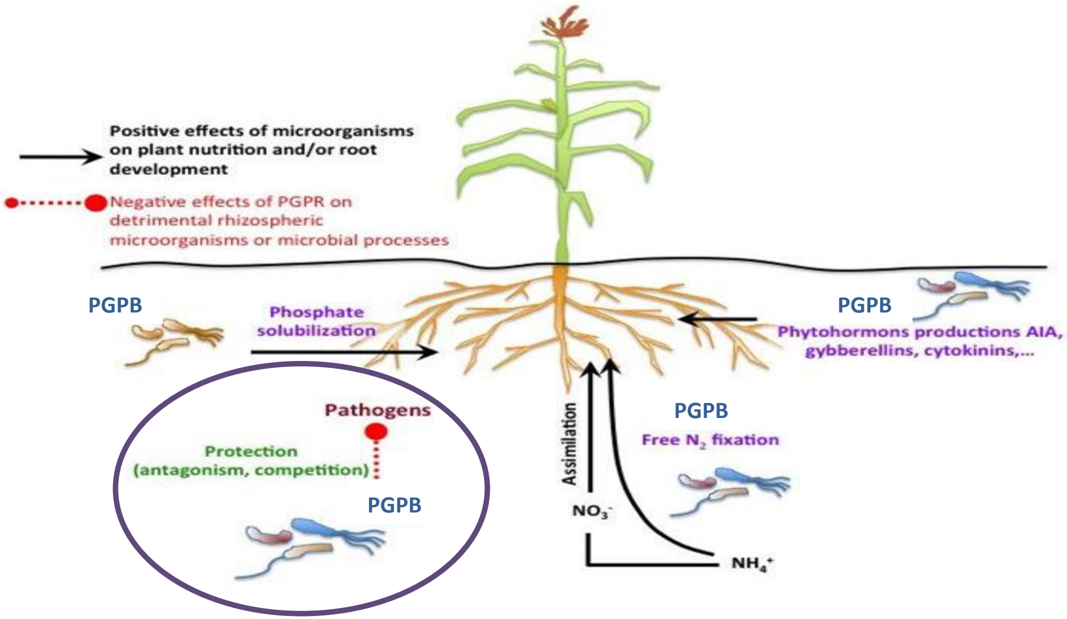

Unspecific

Long-term unsustainable system

# INTRODUCTION

**ALTERNATIVE:**

PGPB: **Plant Growth Promoting Bacteria**. Microorganism which increases both plant growth and resistance against pathogens.




Comte *et al.* (2008)

# OBJECTIVES

## OBJECTIVES

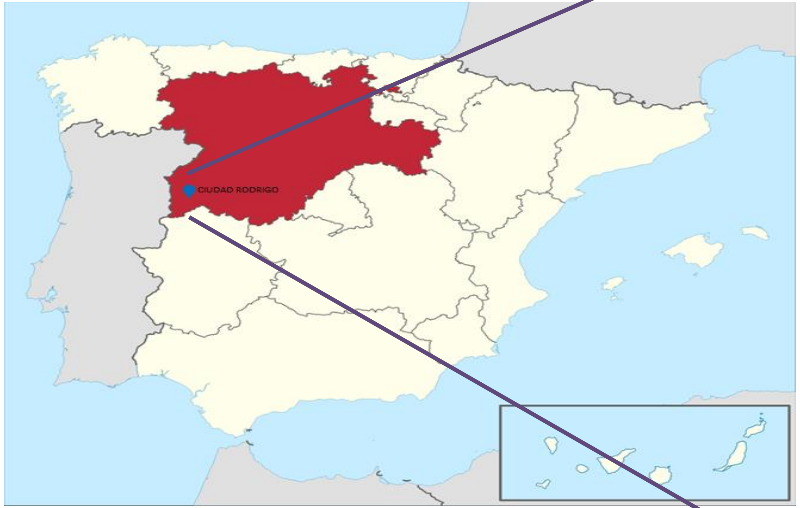
The aim of the present work is to **characterize endophytic bacteria from blackberry bush (*Rubus ulmifolius* Schott) and the evaluation of antifungal activities against *Botrytis cinerea* and *Fusarium sp*.**

- Isolation and characterization of endophytic bacteria of blackberry bush (*Rubus ulmifolius* Schott).
- Analysis of biodiversity of the isolates.
- Genotypic characterization (specific and intraspecific).
- Phenotypic characterization of antifungal activity *in vitro*
- Phenotypic characterization of antifungal activity *in silico*
- Selection of those strains with positive results

# RESULTS AND DISCUSSION

# RESULTS AND DISCUSSION: bacterial isolation

*Rubus ulmifolius* Schott




CULTUROMIC ANALYSIS

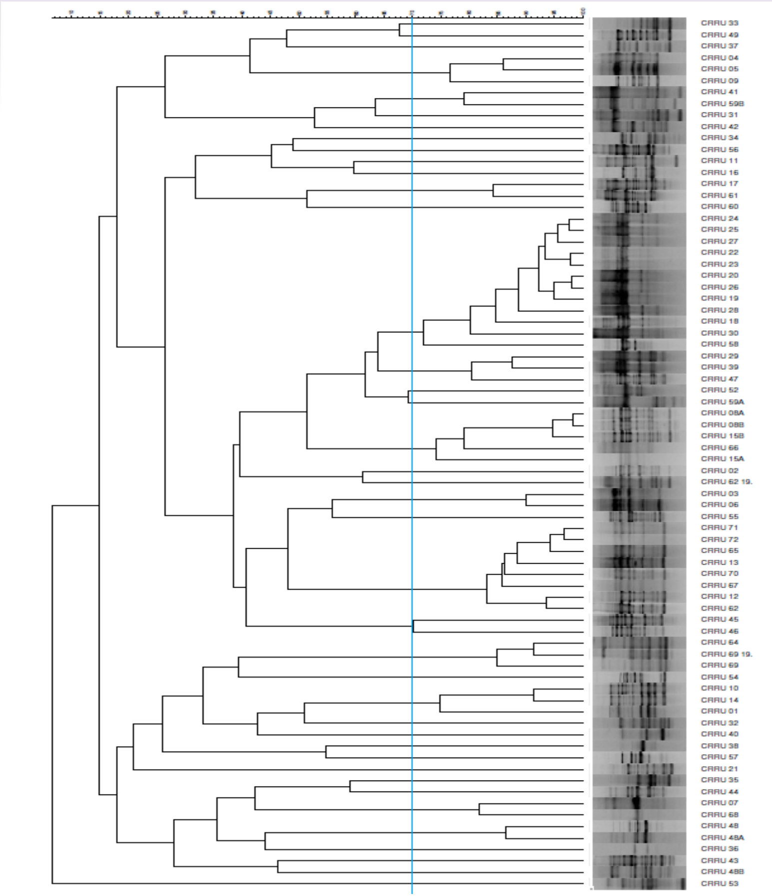
METAGENOMIC ANALYSIS

# RESULTS AND DISCUSSION: culturomic analysis

70 isolates

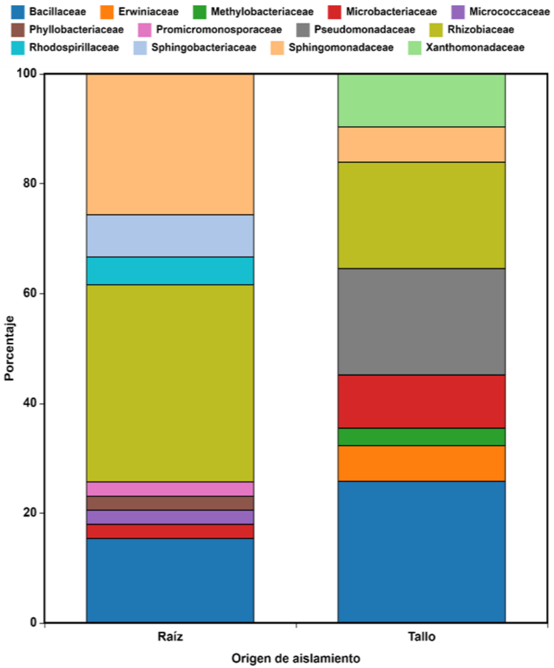
**RAPD technic**: Random Amplification of Polymorphic DNA

- Pearson correlation coefficient.
- Strains are grouped according band number and position
- Percentage of similarity = 75 %
- 70 strains → 39 groups



# RESULTS AND DISCUSSION: genotypic characterization

## 16S Sequencing

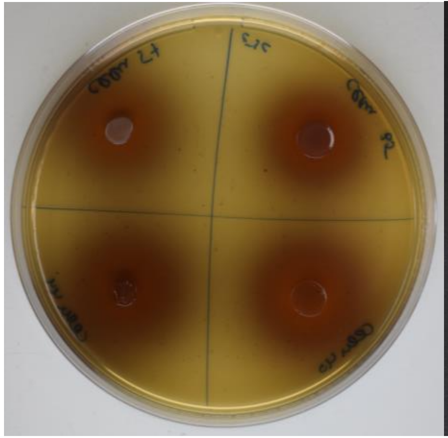



| GROUP | STRAIN | IDENTIFICATION | % IDENTITY |
|---|---|---|---|
| 1 | CRRU 33 | *Agrobacterium rubi* ATCC 23308 | 100 |
| 1 | CRRU 49 | - | - |
| 2 | CRRU 37 | *Curtobacterium albidum* JCM 1344 | 96.7 |
| 3 | CRRU 04 | - | - |
| 3 | CRRU 05 | *Sphingobium aromaticiconvertens* RW16 | 98.8 |
| 3 | CRRU 09 | - | - |
| 4 | CRRU 41 | *Xanthomonas campestris pv. campestris* HTX 16S | 99.3 |
| 5 | CRRU 59 B | *Bacillus drentensis* NBRC 102427 | 99.8 |
| 6 | CRRU 31 | *Xanthomonas vesicatoria* ATCC 35937 | 99.9 |
| 7 | CRRU 42 | *Pantoea septica* X122 | 98.9 |
| 8 | CRRU 34 | *Curtobacterium herbarum* P 420/07 | 99.6 |
| 9 | CRRU 56 | *Pseudomonas syringae pv. syringae* NCPPB 281 | 99.0 |
| 10 | CRRU 11 | *Neorhizobium galegae* NBRC 14965 | 99.6 |
| 11 | CRRU 16 | *Neorhizobium huautlense* SO2 | 99.2 |
| 12 | CRRU 17 | *Neorhizobium galegae* NBRC 14965 | 99.6 |
| 12 | CRRU 61 | - | - |
| 13 | CRRU 60 | *Mesorhizobium cantuariense* ICMP 19515 | 100 |
| 14 | CRRU 18 | - | - |
| 14 | CRRU 19 | *Bacillus subtilis subsp. subtilis* str. 168 | 100 |
| 14 | CRRU 20 A | - | - |
| 14 | CRRU 20 B | - | - |
| 14 | CRRU 22 | - | - |
| 14 | CRRU 23 | - | - |
| 14 | CRRU 24 | - | - |
| 14 | CRRU 25 | - | - |
| 14 | CRRU 26 | - | - |
| 14 | CRRU 27 | - | - |
| 14 | CRRU 28 | - | - |
| 14 | CRRU 30 | - | - |
| 14 | CRRU 58 | - | - |
| 15 | CRRU 29 | *Pseudomonas syringae pv. coryli* NCPPB 4273 | 99.9 |
| 15 | CRRU 39 | - | - |
| 15 | CRRU 47 | - | - |
| 16 | CRRU 52 | *Inquilinus ginsengisoli* Gsoil 080 | 99.7 |
| 17 | CRRU 59 A | *Cellulosimicrobium funkei* W6122 | 99.8 |

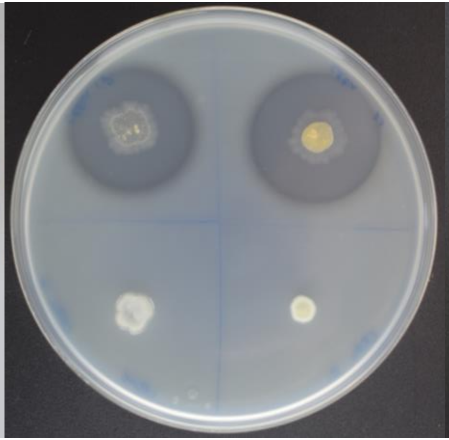
| GROUP | STRAIN | IDENTIFICATION | % IDENTITY |
|---|---|---|---|
| 18 | CRRU 08 | - | - |
| 18 | CRRU 15 | *Mucilaginibacter rubeus* EF23 | 98.6 |
| 18 | CRRU 66 | - | - |
| 19 | CRRU 02 | *Sphingobium aromaticiconvertens* RW16 | 97.6 |
| 20 | CRRU 03 | *Rhizobium soli* DS-42 | 100 |
| 20 | CRRU 06 | - | - |
| 21 | CRRU 55 | *Novosphingobium sp*. A1K012 | 100 |
| 22 | CRRU 12 | - | - |
| 22 | CRRU 13 | - | - |
| 22 | CRRU 62 | - | - |
| 22 | CRRU 65 | - | - |
| 22 | CRRU 67 | - | - |
| 22 | CRRU 70 | *Rhizobium sophorae* strain LMG 27901 | 100 |
| 22 | CRRU 71 | - | - |
| 22 | CRRU 72 | - | - |
| 23 | CRRU 45 | *Rhizobium soli* DS-42 | 98.5 |
| 24 | CRRU 46 | *Rhizobium soli* DS-42 | 99.8 |
| 25 | CRRU 64 | *Sphingobium aromaticiconvertens* RW16 | 98.1 |
| 25 | CRRU 69 | - | - |
| 26 | CRRU 54 | *Curtobacterium herbarum* P 420/07 | 100 |
| 27 | CRRU 01 | - | - |
| 27 | CRRU 10 | *Novosphingobium sp.* MN2-9 | 99.4 |
| 27 | CRRU 14 | - | - |
| 28 | CRRU 32 | *Rhizobium skierniewicense* Ch11 | 98.7 |
| 29 | CRRU 40 | *Pseudomonas lutea* OK2 | 100 |
| 30 | CRRU 38 | *Pantoea anthophila* LMG 2558 | 97.5 |
| 31 | CRRU 57 | *Inquilinus ginsengisoli* Gsoil 080 | 100 |
| 32 | CRRU 21 | *Arthrobacter crystallopoietes* DSM 20117 | 98.6 |
| 33 | CRRU 35 | *Pseudomonas silesiensis* A3 | 99.9 |
| 34 | CRRU 44 | *Rhizobium leguminosarum* LMG 14904 | 95.5 |
| 35 | CRRU 07 | *Sphingobium aromaticiconvertens* DSM 12677T | 98.4 |
| 36 | CRRU 68 | *Sphingobium aromaticiconvertens* RW16 | 98.6 |
| 37 | CRRU 48 | *Methylobacterium bullatum* F3.2 | 100 |
| 38 | CRRU 36 | *Xanthomonas campestris pv. campestris* XCC-C7 | 99.6 |
| 39 | CRRU 43 | *Sphingomonas mali* NBRC 15500 | 98.4 |

# RESULTS AND DISCUSSION: antifungal activity *in vitro*

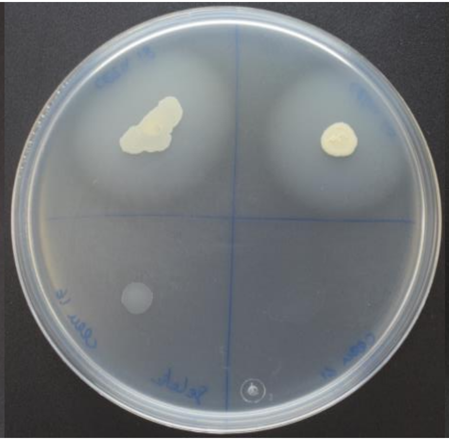
## Lytic enzymes synthesis



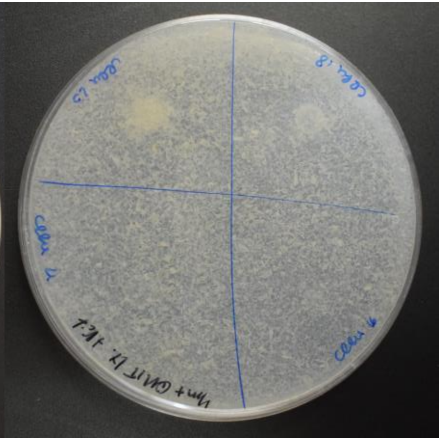
**Glucanases**: CRRU 35, CRRU 40, CRRU 44 and CRRU 57 strains growing in esculin medium. Four strains have shown positive results.



**Milk proteases** : CRRU 16, CRRU 18, CRRU 20 and CRRU 21 strains growing in powder milk medium. CRRU 18 and 20 strains have shown positive results.



**Gelatin Proteases** : CRRU 16, CRRU 18, CRRU 20 and CRRU 21 strains growing in gelatin medium. CRRU 18 and 20 strains have shown positive results.



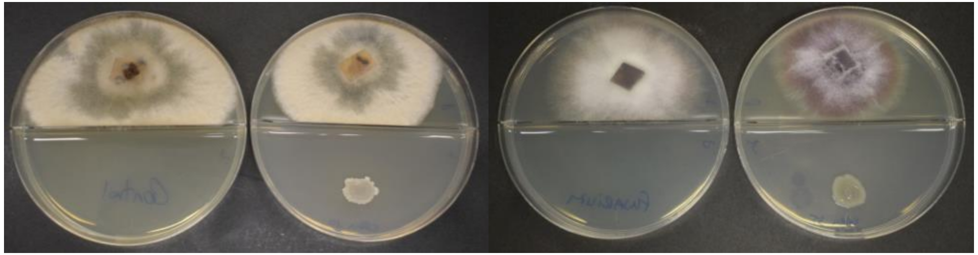
**Chitinases**: CRRU 16, CRRU 18, CRRU 20 and CRRU 21 strains growing in chitin medium. None of the strains have shown positive results.

# RESULTS AND DISCUSSION: antifungal activity *in vitro*

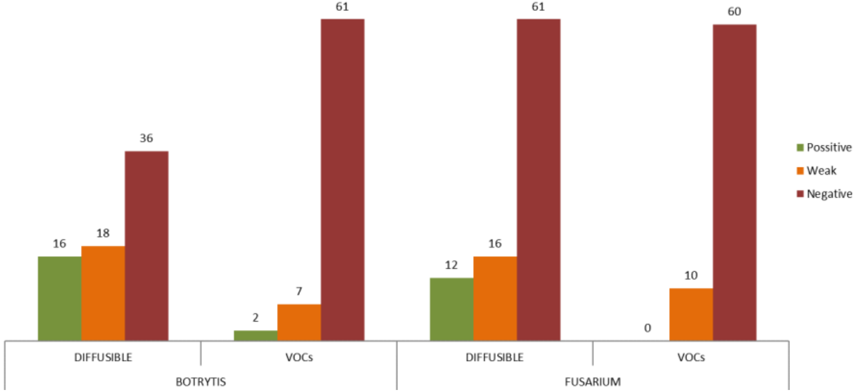
## Diffusible compounds

Antifungal activity mediated by diffusible compounds against *Botrytis* (up) and against *Fusarium* (down).

## Volatile Organic Compounds (VOC)



Antifungal activity mediated by VOCs against *Botrytis* (left) and against *Fusarium* (right).



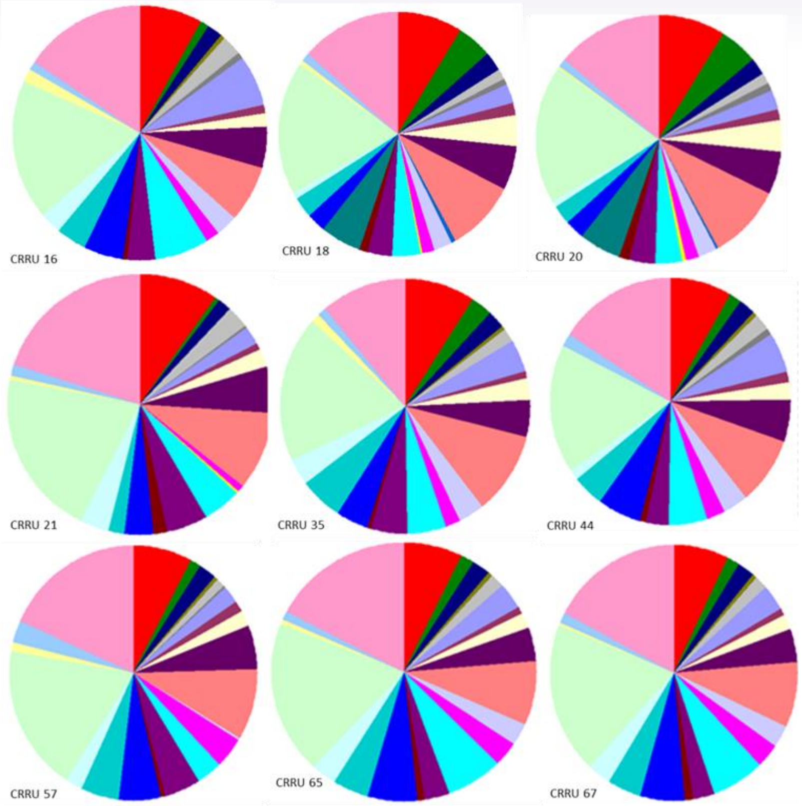
# RESULTS AND DISCUSSION: strain selection

| Strain | | Diffusible agaisnt *B. cinerea* | Diffusible agaisnt *F. oxysporum* | VOCs agaisnt B. cinerea | VOCs agaisnt F. oxysporum |
|---|---|---|---|---|---|
| CRRU 16 | *Neorhizobium huautlense* CRRU 16 | + | - | + | - |
| CRRU 18 | *Bacillus subtilis subsp. subtilis* CRRU 18 | + | + | + | - |
| CRRU 20 | *Bacillus amyloliquefaciens* CRRU 20 | - | + | - | - |
| CRRU 21 | *Arthrobacter crystallopoietes* CRRU 21 | - | + | - | - |
| CRRU 35 | *Pseudomonas silesiensis* CRRU 35 | + | + | + | - |
| CRRU 44 | CRRU44 | + | - | - | - |
| CRRU 57 | *Inquilinus ginsengisoli* CRRU 57 | + | - | + | + |
| CRRU 65 | *Rhizobium sophorae* CRRU 65 | + | - | - | - |
| CRRU 67 | *Rhizobium sophorae* CRRU 67 | - | - | - | + |

# RESULTS AND DISCUSSION : genomes studies

| Strain | Size (nt) | G+C Content (%) | Nº of contigs | Nº of CDSs | rRNAs |
|---|---|---|---|---|---|
| CRRU 16 | 8,111,545 | 61.1 | 106 | 8,236 | 48 |
| CRRU 18 | 3,976,137 | 46.5 | 53 | 4,078 | 95 |
| CRRU 20 | 3,969,796 | 46.5 | 46 | 4,067 | 95 |
| CRRU 21 | 4,671,503 | 67.9 | 132 | 4,423 | 57 |
| CRRU 35 | 6,272,183 | 57.1 | 137 | 5,995 | 63 |
| CRRU 44 | 5,482,309 | 62.8 | 59 | 5,385 | 54 |
| CRRU 57 | 7,960,204 | 69 | 357 | 7,712 | 49 |
| CRRU 65 | 7,539,570 | 60.7 | 87 | 7,743 | 49 |
| CRRU 67 | 7,537,940 | 60.7 | 72 | 7,738 | 51 |

# RESULTS AND DISCUSSION : genomes studies




| SYSTEMS | CRRU 16 | CRRU 18 | CRRU 20 | CRRU 21 | CRRU 35 | CRRU 44 | CRRU 57 | CRRU 65 | CRRU 67 |
|---|---|---|---|---|---|---|---|---|---|
| Cofactors, Vitamins, Prosthetic Groups, Pigments | 219 | 147 | 147 | 175 | 186 | 144 | 181 | 178 | 178 |
| Cell Wall and Capsule | 34 | 80 | 81 | 12 | 54 | 31 | 35 | 40 | 40 |
| Virulence, Disease and Defense | 50 | 39 | 39 | 27 | 46 | 37 | 53 | 53 | 57 |
| Potassium metabolism | 11 | 3 | 3 | 3 | 9 | 11 | 11 | 13 | 13 |
| Photosynthesis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Miscellaneous | 73 | 24 | 24 | 49 | 38 | 38 | 23 | 40 | 40 |
| Phages, Prophages, Transposable elements, Plasmids | 19 | 15 | 15 | 5 | 1 | 17 | 9 | 4 | 4 |
| Membrane Transport | 170 | 41 | 41 | 42 | 81 | 89 | 48 | 81 | 81 |
| Iron acquisition and metabolism | 30 | 23 | 23 | 13 | 19 | 25 | 33 | 22 | 22 |
| RNA Metabolism | 44 | 70 | 69 | 40 | 53 | 39 | 45 | 46 | 46 |
| Nucleosides and Nucleotides | 146 | 95 | 94 | 105 | 97 | 93 | 128 | 102 | 102 |
| Protein Metabolism | 190 | 157 | 157 | 176 | 209 | 156 | 215 | 197 | 201 |
| Cell Division and Cell Cycle | 0 | 6 | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| Motility and Chemotaxis | 77 | 42 | 42 | 0 | 69 | 54 | 7 | 71 | 71 |
| Regulation and Cell signaling | 45 | 26 | 26 | 12 | 38 | 43 | 86 | 74 | 74 |
| Secondary Metabolism | 5 | 7 | 7 | 8 | 4 | 4 | 4 | 6 | 6 |
| DNA Metabolism | 182 | 62 | 62 | 81 | 99 | 91 | 81 | 167 | 167 |
| Fatty Acids, Lipids, and Isoprenoids | 101 | 55 | 55 | 90 | 93 | 56 | 107 | 72 | 72 |
| Nitrogen Metabolism | 14 | 19 | 19 | 28 | 13 | 11 | 12 | 23 | 23 |
| Dormancy and Sporulation | 2 | 92 | 91 | 1 | 3 | 1 | 1 | 1 | 1 |
| Respiration | 131 | 41 | 41 | 67 | 86 | 103 | 129 | 144 | 143 |
| Stress Response | 105 | 46 | 45 | 34 | 110 | 71 | 118 | 108 | 109 |
| Metabolism of Aromatic Compounds | 75 | 12 | 12 | 65 | 64 | 26 | 52 | 76 | 76 |
| Amino Acids and Derivatives | 483 | 294 | 294 | 372 | 374 | 293 | 428 | 466 | 465 |
| Sulfur Metabolism | 44 | 7 | 8 | 9 | 20 | 3 | 28 | 11 | 11 |
| Phosphorus Metabolism | 30 | 16 | 16 | 23 | 25 | 29 | 62 | 31 | 33 |
| Carbohydrates | 404 | 220 | 223 | 351 | 211 | 261 | 416 | 403 | 403 |

# RESULTS AND DISCUSSION : genomes studies

| Strains | Lytic enzymes synthesis | Diffusible antifungal molecules | Antifungal VOCs |
|---|---|---|---|
| CRRU 16 | Endo-β-1,3-1,4 glucanase (licheninase) (EC 3.2.1.73)<br>β-glucosidase (EC 3.2.1.21)<br>α-amylase (EC 3.2.1.1) | NRPS | Terpenes<br>Phenilpropanoid |
| CRRU 18 | Endo-β-1,3-1,4 glucanase (licheninase) (EC 3.2.1.73)<br>β-1,4-glucanase (cellulose) (EC 3.2.1.4)<br>α-amylase (EC 3.2.1.1) | Endo-β-1,3-1,4 glucanase (licheninase) (EC 3.2.1.73)<br>Endoglucanase H (EC 3.2.1.4)<br>NRPS | Terpenes<br>Phenilpropanoid |
| CRRU 20 | β-1,4-glucanase (cellulose) (EC 3.2.1.4)<br>β-glucosidase (EC 3.2.1.21)<br>α-amylase (EC 3.2.1.1) | Endo-β-1,3-1,4 glucanase (licheninase) (EC 3.2.1.73)<br>Endoglucanase H (EC 3.2.1.4)<br>NRPS | Terpenes<br>Phenilpropanoid |
| CRRU 21 | β-glucosidase (EC 3.2.1.21)<br>α-amylase (EC 3.2.1.1) | NRPS | Terpenes<br>Phenilpropanoid |
| CRRU 35 | β-glucosidase (EC 3.2.1.21)<br>α-amylase (EC 3.2.1.1) | NRPS | Phenilpropanoid |
| CRRU 44 | β-1,4-glucanase (cellulose) (EC 3.2.1.4)<br>β-glucosidase (EC 3.2.1.21)<br>α-amylase (EC 3.2.1.1)<br>Endo-1,4-β-xylanase (EC 3.2.1.8) | NRPS | Terpenes<br>Phenilpropanoid |
| CRRU 57 | β-glucosidase (EC 3.2.1.21)<br>α-amylase (EC 3.2.1.1)<br>1,4-β-xylosidase (EC 3.2.1.37) | NRPS | Terpenes<br>Phenilpropanoid |
| CRRU 65 | β-glucosidase (EC 3.2.1.21)<br>α-amylase (EC 3.2.1.1)<br>Endo-1,4-β-xylanase (EC 3.2.1.8) | Endo-β-1,3-1,4 glucanase (licheninase) (EC 3.2.1.73)<br>Endoglucanase H (EC 3.2.1.4) | Terpenes<br>Phenilpropanoid |
| CRRU 67 | β-glucosidase (EC 3.2.1.21)<br>α-amylase (EC 3.2.1.1)<br>Endo-1,4-β-xylanase (EC 3.2.1.8) | Endo-β-1,3-1,4 glucanase (licheninase) (EC 3.2.1.73)<br>Endoglucanase H (EC 3.2.1.4) | Terpenes<br>Phenilpropanoid |

# CONCLUSIONS

## CONCLUSIONS

Results obtained in this work have allowed us to stablish the following conclusions:

- Evaluation of endophytic bacteria isolated from blackberry plants (*Rubus ulmifolius* Schott) has shown an efficient strategy in the selection of new bacteria with antifungal activity.
- Selected strains isolated from blackberry bush may represent a strong ally against some of the worst pathogens that agriculture faces, such as *Botrytis cinerea* and *Fusarium oxysporum*.

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THANK YOU VERY MUCH!!