SELECTION OF BACTERIAL STRAINS USEFULL IN BIOACTIVE MULCH AGRICULTURAL SYSTEMS

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Abstract: This study referes to the use of "bioactive mulch" (vegetal mulch suplimented with antagonistic beneficial bacteria) as an alternative to chemical fertilization of the crops and application of pesticides. Therefore, we selected two bacterial strains with biological activity *in vitro* against soil borne fungi *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Fusarium graminearum.* The selected strains, Usa2 *Bacillus subtilis* and 56.1s *Brevibacillus laterosporus* were analized for important biological traits like enzymes production (amilase, lactonase and cellulase), plant growth promotion, ability to mineralize the lignocellulosic material and for their mobility. Finnaly, the inocuity test was performed to establish the toxic potential of the izolates against mammalians.

Key words: cover crop, mulch, beneficial bacteria, biocontrol

INTRODUCTION

Converting winter cover crop (WCC) into mulch layer on top of the soil appears to be one of the most promising conservation practices in agriculture (Drury et al., 1999; Villamil et al., 2006; Triplett and Dick, 2008). Winter cover crops can maintain or increase soil content of C and N (Kuo et al., 1997a,b; Sainju et al., 2003; Liu et al., 2005) and these effects are largely responsible for the changes in physical properties associated with their use (Fageria et al, 2005). Maintenance of a crop residue layer over the soil surface helps to protect from raindrop impact and cycles of freezing–thawing and drying–wetting (Stănilă, 2006; Wuest, 2007; Blanco-Canqui and Lal, 2008).

In the mean time large amounts of crop residue on the soil surface may particularly favor pathogens that survive between crops in the infected/infested residue (Bockus and Shoryer, 1998; Mack and Jones, 2000, Bailey and Lazarovits, 2003). Crop residues lying on the surface reduce soil temperatures. Lower soil temperatures caused by plant residues retarded seed germination and crop growth, including root system development, promote root disease and finally reduced crop yields (Kapser et al.1990; Vetsch and Randall, 2000; Archer and Reicosky, 2009).

In order to counter the action of these negative side effects for cover crop mulches we proposed: (*i*) the application on crop residues laying down the soil of an antagonist to soil born phytopathogens and (*ii*) the inoculation with diazotroph plant growth promoting rhizobacteria (PGPR) of the seed of cash crop established on treated mulches. Antagonist to plant pathogen (like fungi from genus *Trichoderma* or bacteria from genus *Bacillus*) are intended to reduce the primary inoculum for both foliar infecting and root infecting soil born phytopathogens. Diazotroph PGPR (like *Azospirillum*) aim to promote the development of cash crop seedling and to improve the nutrition of the plant with slow developing root system.

We called this system "bioactive mulch", because it includes viable microbial inoculants: diazotroph PGPR applied to cash crop seeds and antagonist (to phytopathogens) inoculated on the WCC mulch. The bacterial antagonist used for in the bioactive mulch system should have several characteristics, like: (*i*) antagonism to soil born fungi; (*ii*) capacity to colonize the crop residue; (*iii*) ability to mineralize the lignocelullosic material; (*iv*) plant growth promoting activity (*v*) inocuity for macroorganism. In this paper we present the procedure which we used for screening from local

isolated of bacterial strains useful for bioactive mulch system and a short description of the selected strains.

MATERIAL AND METHOD

Two hundred and seventy two bacterial strains were isolated from soil and vegetal samples from six different counties in the south of Romania: Constanţa, Dolj, Olt, Călăraşi, Ilfov and Dâmboviţa. These were selected based on several traits: antagonistic capacity *in vitro* against several important soil borne fungi, enzimes production (lactonase, cellulase, amylase), motility (swimming and swarming motility) and plant growth promotion.

Isolation of microorganisms consisted of distributing 1 g of soil or vegetal sample in 15 ml Nutrient Broth (NB) media in Erlenmeyer flasks follow by incubation at 28°C and 150 rpm for 24 hours. After, 50-100 μl from the culture were distributed with a Drigalsky ooze on nutrient agar (NA) or Luria Bertani agar (LBA) media and incubated at 28°C.

To identify the spores producing strains, 2 ml from each culture were placed in sterile glass tubes and treated at 100°C on water bath for 15 minutes. 100 μl were placed on NA and incubated at 28°C.

After 24 and 48 incubation hours the morfology of the grown colonies was noted and they were purified in new Petri plates with NA media.

Antagonistic activity

The antagonistic activity of the isolates was tested *in vitro* against the soil borne fungi: *Fusarium graminearum, Alternaria spp, Botrytis cinerea, Gaemanomices graminis, Pythium de barianum, Rhizoctonia solani, Sclerotinia sclerotiorum, Sclerotium cepivorum, Sclerotium bataticola* and *Verticilium dahlie.*

The fungi were refreshed on potato-dextrose-agar (PDA) media and incubated at 28°C for 5 days. The bacterial strains were grown on Luria-Bertani agar (LBA) at 28°C for 24 hours.

The test was performed on PDA media and the distance between the bacteria and the calibrated fungal plug (5 mm) was 2 cm. The plates were incubated at 28°C and analyzed at 24, 48 and 72 hours for the inhibition of the mycelia growth due to bacteria.

The test was repeated 3 times.

Enzymes production: cellulase, amylase, lactonase

Cellulase detection assay

Cellulase activity was determined by the breakdown of the substrate carboxymethylcellulose. Bacterial strains were inoculated on plates with minimal media supplemented with 1% carboxyl-methyl-cellulose (CMC). After growth for 5 days at 28°C the plates were stained for 30 min with 0.3% Congo Red. Plates were subsequently rinsed with excess tap water and the dye was fixed by incubation with a 10% acetic acid solution for 15 minutes. The presence of a clear halo indicates the presence of cellulase activity.

Amylase detection assay

The strains were striated on nutrient agar (NA) media supplemented with 0,4% soluble starch. The plates were incubated at 28°C for 48-72 hours and then treated with iodine solution. The presence of a clear halo arrownd the bacterial strains indicated the presence of amilase activity.

Lactonase detection assay

The strains were inoculated in 2 ml of LB broth containing 5 μM of C6-hexanoyl homoserine lactone (C6-HHL) and grown overnight (ON) at 28°C and 150 rpm. As a negative control the same media without bacteria was also incubated under these conditions. The next day a LC agar plate containing 50 μg/ml of kanamicin was overlaid with Cv026 by spreading 250 μl of an ON culture. Wells were punctured into the plate (5 mm in diameter) and filled with a 100 μl of bacterial culture. The plates were incubated ON at 28°C and scored for the presence or absence of purple halos. Absence of purple halos indicates that all of the C6-HHL was degraded.

<u>Motility</u>

The strains were tested for their swimming and swarming motility. All strains were refreshed on LB agar plates supplemented with 1,8% agar and grown ON at 28 °C. LB agar plates (25 ml) containing 0,3% (swimming) or 0.5% (swarming) bacto-agar were prepared fresh and were dried for 30 min in the laminar flow cabinet. Each plate was toothpick inoculated and scored for swimming and swarming motility after 18 h incubation at 28°C.

<u>Plant growth promotion activity</u>

The plant growth promotion activity was tested *in vivo* in growth chamber conditions. We used as a model the tomato plants *Lycopersicon esculentum* cv. Unibac.

The microorganisms used in this study are presented in table 1. They were grown on LB broth media at 28^0C and 150 rpm for 16 hours.

Table 1

Microorganisms used *in vivo* experiment

Strain code/ Taxonomy	Provenience/ Characteristics
WCS 365/ *Pseudomonas fluorescens* Pozitive Control	IBL, Leiden, Netherlands/ good colonizer of tomato roots; efficient in biocontrol of *Fusarium oxysporum* f. sp. *radicis lycopersici.*
B49b/ *Bacillus subtilis* Positive Control	I.C.D.P.P./ efficient in biocontrol of the soil borne fungi complex which produce seedlings "damping off"; plant growth promotion.
FL 400/ *Paenibacillus graminis*	I.C.D.P.P./ plant growth promotion.
Ps 33/ *Serratia plymuthica*	I.C.D.P.P./ biofilm forming strain.
56.1s/ *Brevibacillus laterosporus*	I.C.D.P.P./ antagonistic activity *in vitro* against several soil borne fungi.
Hm s1/*	I.C.D.P.P./ antagonistic activity *in vitro* against several soil borne fungi.
Cn s2/*	I.C.D.P.P./ antagonistic activity *in vitro* against several soil borne fungi.
Sp s2/*	I.C.D.P.P./ antagonistic activity *in vitro* against several soil borne fungi.
Tm s2/*	I.C.D.P.P./ antagonistic activity *in vitro* against several soil borne fungi.
Mz b1/*	I.C.D.P.P./ antagonistic activity *in vitro* against several soil borne fungi.
Cp b5/*	I.C.D.P.P./ antagonistic activity *in vitro* against several soil borne fungi.

* = identification in progress.

The experiment had 12 variants each with 4 repetitions. Each repetition consisted in 5 plants and each treatment had 20 plants in total.

Tomato seeds were disinfected in two steps, first with ethanol 70% for 30 seconds at 60 rpm and secondly with sodium hypochlorite 4% for 15 minutes. After the ethanol was removed the seeds were washed three times with sterile distilled water (SDS) and after the hypochlorite treatment the seeds were rinsed for 2 hours with SDS at each 25 minutes the water being refreshed.

Seeds were inoculated by immersion in 3 ml bacterial suspension at 10^7 cfu/ml and 2% carboxyl-methyl-cellulose follow by sowing in the sterile growth pouches "cyg" (Mega International). Pouches were wetted with nutritive solution Hoagland 0,25% during the next 3 weeks.

After 3 weeks the total length of the roots was assessed.

<u>Ability to mineralize the lignocelullosic material</u>

In order to detect the ability of the selected bacterial strains to mineralize lignocelullosic material we used Strathkelvin Strathtox respirometer.

As lignocelulosic substrate we used wheat straws because this type of vegetal material can release nutrients only after an enzymatic digestion. The straws were subjected to some grinding steps, like manual fragmentation at 1-2 cm length and grinding at 15000rpm using a simple blender (Macap P-102) and afterwards a mill for grain (ZM200, Retsch). The resulted powder was packed in PE bags (0,25 g/pc) and gamma sterilized with an ionizing radiation dose of 30 ± 2 kGy at IRASM, Horia Hulubei National Institute of Physics and Nuclear Engineering.

To create favorable conditions for the expression of microbiological activity, the substrate was

moistened with a sterile solution of saline phosphate buffer (PBS: 8g NaCl; 0,2 g KCl; 1,44 g Na$_2$HPO$_4$; 0,24g KH$_2$PO$_4$ in 1L distilled water). The buffer solution maintained the pH at a constant value, did not affect the microorganisms with osmotic stress and did not influenced the organic nutritional composition of the substrate.

The tested bacterial strains were *Brevibacillus laterosporus* 56.1s and *Bacillus subtilis* Usa2. The 56.1s *Brevibacillus laterosporus* strain was isolated from soil collected from south Bărăgan area, and Usa2 *Bacillus subtilis* strain was isolated from garlic rhizosphere, Dolj County, Romania.

The bacterial strains were refreshed on LBA medium in order to obtain pure isolated colonies. Overnight cultures were used to prepare the inoculum. Pure colonies were collected with sterile cotton applicators (Böttger, 94249 Bodenmais / Germany) and suspended in sterile PBS previously distributed in glass tubes with screwed lids. The bacterial concentration was established at 10^{8}ufc/ml by measuring the optical density at 600nm, when bacterial suspension reached the absorbance of 1A the inoculum concentration was considered optimal for use. Samples were prepared in 100ml Erlenmeyer flasks, in which were added 0,25g of sterile cellulosic substrate (made from ground wheat straw), 24ml sterile PBS and 1ml bacterial inoculum.

The concentration of lignocellulosic material within suspension was determined using the equation:

$$\frac{vegetal\_material\_quantity\langle g\rangle \times 100}{inoculation\_fluid\_quantity\langle ml\rangle + inoculum\_quantity\langle ml\rangle} = 1\%$$

If the lignocelullosic material is mineralized, the microorganisms that metabolized it rich an exponential multiplication phase, that leads to increased oxygen consumption. This respiratory activity of the samples was detected using Strathkelvin Strathtox respirometer.

Samples were analyzed comparing with two references, a negative control without bacterial inoculum and a positive control where the bacterial inoculum was grown on usual culture medium (Luria Bertani broth).

In the negative control the lignocellulosic substrate has not been inoculated with microorganisms and was kept under identical conditions of incubation with test samples, showing maximum amount of oxygen that could be found in samples without biodegradation activity. It provides real-time information regarding the behavior of ideal samples that do not have biological activity to degrade vegetal substrate.

In the positive control the microorganisms could multiply in favorable conditions and consumed the oxygen from the test vial.

The type of samples prepared for each test are presented in table 2. Samples were incubated at 28 - 30°C with 150rpm shaking. After two days of incubation, the samples were processed on Strethkelvin Strathtox with the respirometry program. The laboratory respirometer allows to perform the tests under controlled temperature conditions. The device needs to be calibrated once a day before any test at the sett temperature parameter. Through calibration the maximum and minimum points of a captured signal are established. For example, at a working temperature of 20°C the maximum current must be included in the range of 200-600pA, and the minimum value between 0-10 pA. The calibration of the oxygen sensor is auto-correlated within the programmed temperature selected for processing the samples. It means that for the 20°C temperature the maximum amount of oxygen in a saturated sample is 8,4 mgO$_2$/L.

In our tests we processed the samples at 24°C temperature. We calibrated the system according to the protocol described in the instruction manual. So, the high point calibration was established using distilled water saturated in oxygen, and zero point was determined in 0,5% sodium sulphite solution. After calibrating procedure samples where assigned to each active electrode. For respirometry tests were required a minimum of 20ml of sample. Since the samples had in composition abrasive particles of grinded straw that could break the oxygen membrane it was required a prior filtration through sterile filter paper.

Table2

Sample preparation for the test which detects
the capacity to mineralize vegetal substrate

Crt. no.	Sample	Repetition	Sample content			
			Vegetal substrate (potential growth source) <g>	PBS (inoculation fluid) <ml>	Bacterial inoculum <ml>	LB broth (growth medium) <ml>
1	Negative reference	-	0,25	25	-	-
2	Tested sample	R1	0,25	24	1	-
3	Tested sample	R2	0,25	24	1	-
4	Tested sample	R3	0,25	24	1	-
5	Pozitive reference	-	-	-	1	24

Samples were analyzed with the system for minimum 3 hours and measurements were registered every second but we collected only the values from 15 to 15 minutes. The number of electrodes that were fitted on the Strathtox system permitted three repetitions of the tested samples along with two references.

Galleria mellonella inocuity test

Analysis of insect responses to pathogens can provide an accurate indication of the mammalian response to pathogen microorganisms (21, 25). An inocuity test on larvae of wax moth *Galleria mellonella* have been used previously as an infection model for studying bacterial human pathogens (Seed and Dennis, 2008). It is known that the innate immune systems of insects such as *G. mellonella* share some similarities of structural and functional homology to the innate immune systems of mammals (21), therefore, *G. mellonella* shown an attractive alternative infection model.

The inocuity test that we used has been described by Seed and Dennis (2008). The bacterial strains that we tested were *Bacillus subtilis* Usa2 and *Brevibacillus laterosporus* 56.1s. These were grown in LB broth medium at 28ºC and 150 rpm shaking. Two days old cultures were pelleted and resuspended in 10mM $MgSO_4$ supplemented with 1,2 mg/ml ampicillin. This antibiotic was used to prevent infections with bacteria naturally present on the larvae surface. The bacterial inoculum concentration was indirectly evaluated by measuring the optical density with CECIL CE 2021 spectrophotometer, at 600nm (see table 3).

Table 3

Optical density measurements of bacterial inoculums used in the inocuity test

Bacterial strain		Non diluted inoculum	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5	Dilution 6
B. subtilis Usa2	OD 600nm	1,435	0,312	0,034	0,007	0,004	0,003	0,001
	CFU/ml	$6,85 \times 10^7$	$\mathbf{6,85 \times 10^6}$	$6,85 \times 10^5$	$6,85 \times 10^4$	$6,85 \times 10^3$	$6,85 \times 10^2$	$6,85 \times 10$
B. laterosporus 56.1s	OD 600nm	1.900	0.252	0.100	0.070	0.020	0.010	0.001
	CFU/ml	$9,2 \times 10^7$	$\mathbf{9,2 \times 10^6}$	$9,2 \times 10^5$	$9,2 \times 10^4$	$9,2 \times 10^3$	$9,2 \times 10^2$	$9,2 \times 10$

G. mellonella larvae were raised at 30ºC on Haydak medium (400g of corn flour, 200g flour, and 200g wheat bran, which were freeze for 7-10 days and then sterilized at 60-70°C for 2 hours, in the sterile composition were added 100g dryed yeast and milk powder 200g, 350ml glycerin and 350 ml honey and beeswax. The final composition needs to be welled homogenised an preserved at 4ºC.

To perform the inocuity test, last stage larvae were numbed about 5 minutes at 4ºC, and then injected with 5µl bacterial inoculum using a Hamilton syringe, behind the hindmost left proleg. Each inoculum concentration was tested in tree replicates using ten larvae for replicate. As control we used

10mM MgSO$_4$ suplimented with 1,2 mg/ml ampicillin, in order to analyze the effects of the injection process. Larvae were stored at 30ºC and darkness, on Haydak medium, and scored as dead or alive at 24, 48 and 72h post treatment. The larvae were considered dead if they did not move when touched with a pipette tip.

RESULTS

Performed tests led to the selection of two bacterial strains, 56.1s and Usa2. Which were identified based on their 16S rDNA and fatty acid profile. 56.1s was classified as *Brevibacillus laterosporus* species and Usa2 as *Bacillus subtilis*.

The antagonistic activity test showed that the selected strains are producing antifungal metabolites which inhibit the growth of several fungi (table 4, figure 1).
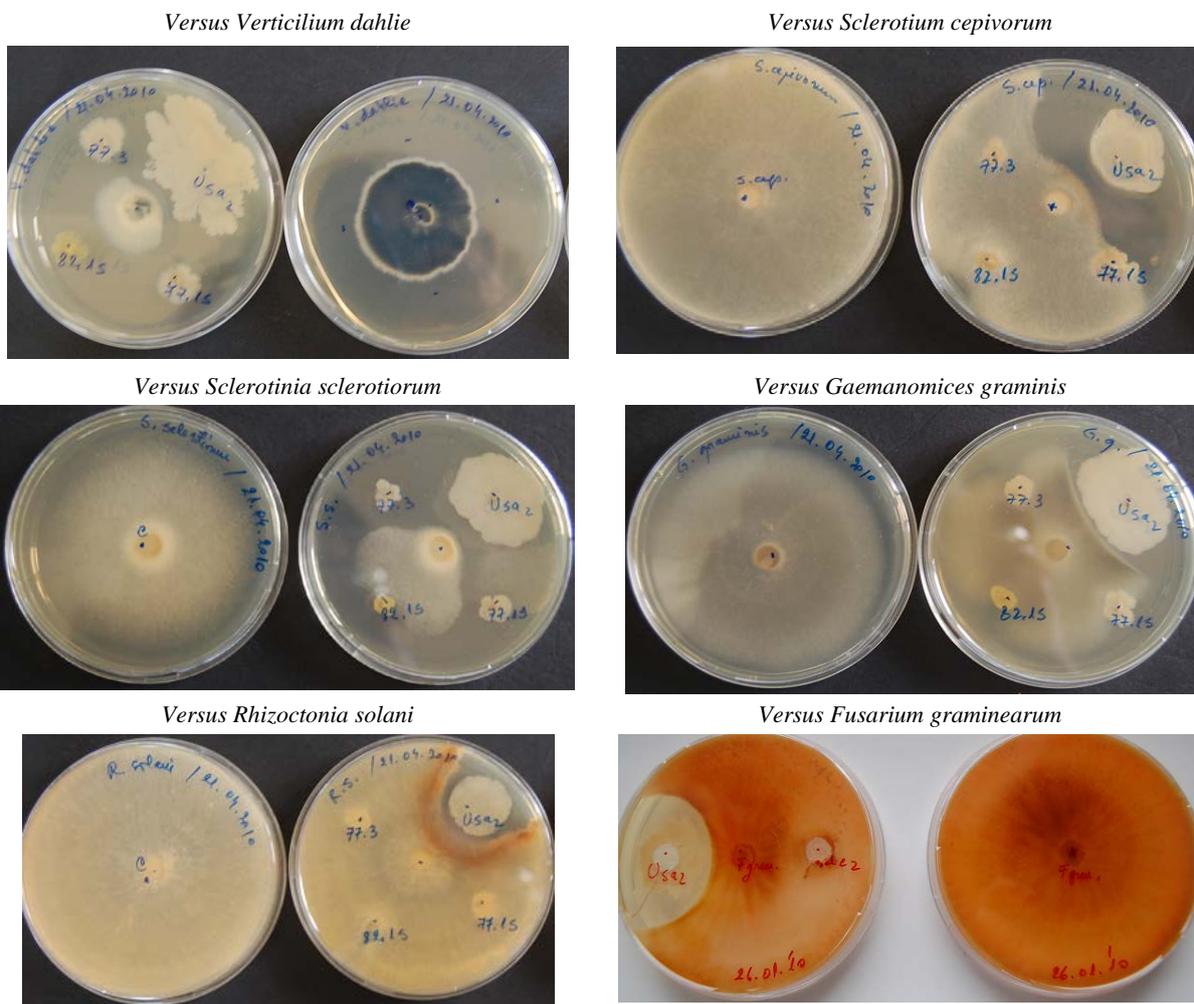
Table 4

In vitro test of the bacterial isolates antagonistic activity against phytopathogenic fungi
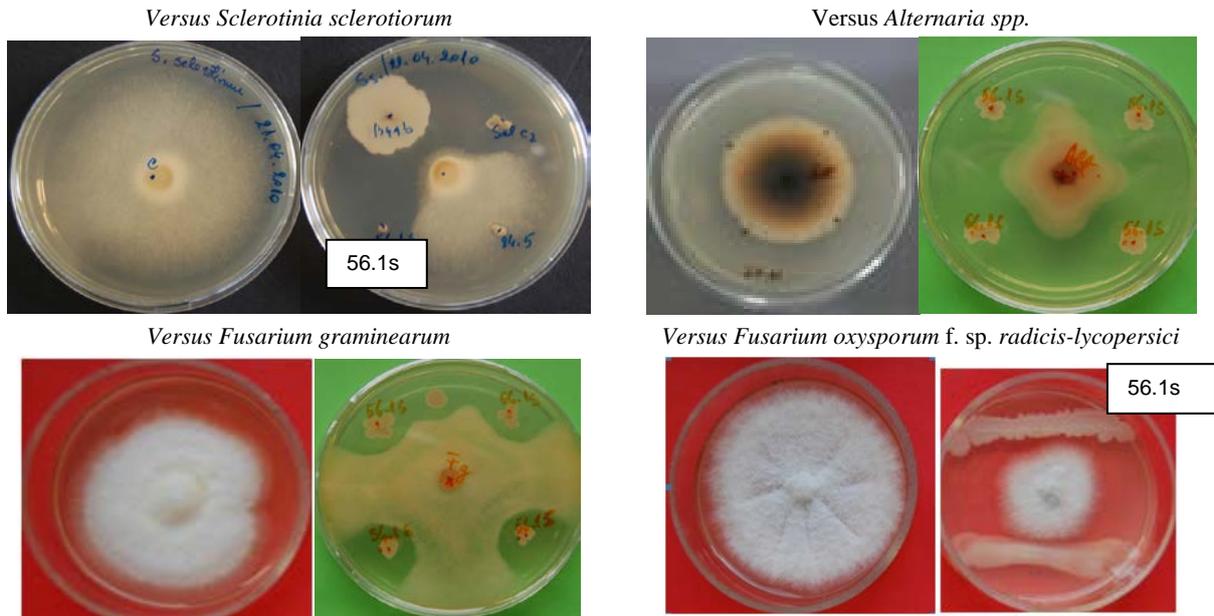(inhibition zone after 72 h of incubation at 28°C).

Phytopathogenic fungi */ Strain code- Strain taxonomy	Fg	Alt	Bc	Ss	Sc	Gg	Pdb	Vd	Rs	Forl	Sb
	Inhibition zone (mm)										
56.1s- *Brevibacillus laterosporus*	5	5	2	8	0	1	0	0	0	5	2
Usa2 – *Bacillus subtilis*	6	6	5	6	17	2	0	4	4	5	3

*Fg=Fusarium graminearum, Alt=Alternaria spp, Bc=Botrytis cinerea, Ss=Sclerotinia sclerotiorum, Sc=Sclerotium cepivorum, Gg=Gaemanomices graminis, Pdb= Pythium de barianum, Vd=Verticilium dahlie, Rs=Rhizoctonia solani, Forl =Fusarium oxysporum f. sp. radicis-lycopersici; Sb=Sclerotium bataticola

Strain Usa2 - *Bacillus subtilis*

Versus Verticilium dahlie   *Versus Sclerotium cepivorum*

Versus Sclerotinia sclerotiorum   *Versus Gaemanomices graminis*

Versus Rhizoctonia solani   *Versus Fusarium graminearum*

Strain 56.1s- *Brevibacillus laterosporus*

Versus Sclerotinia sclerotiorum

Versus Alternaria spp.


Versus Fusarium graminearum

Versus Fusarium oxysporum f. sp. *radicis-lycopersici*

Figure 1. *In vitro* test of the bacterial isolates indicating antagonistic activity against phytopathogenic fungi (72 h incubation at 28°C).
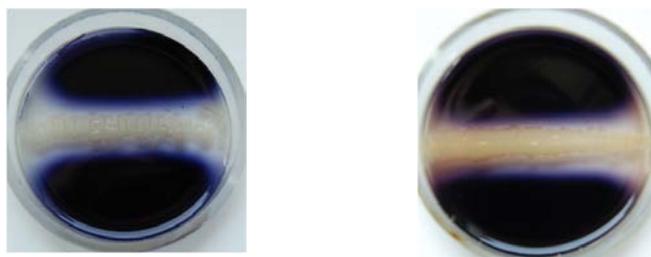
The highest inhibition zones against most of the fungi were induced by *Bacillus subtilis* Usa2. Soil borne biocontrol of phytopathogenic fungi *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Fusarium graminearum* presented by our strain of *Brevibacillus laterosporus in vitro* tests is scientific news. To our knowledge this is the first report about the ability of *B. laterosporus* to control these two fungi *in vitro*. There is one paper in which this species is mentioned to control the leaf phytopathogenic fungi *Alternaria triticimaculans* in wheat (Allipi A. M. & colab., 2000). Instead, there are several publications mentioning the use of *B. laterosporus* species in controling harmful insects from Coleoptera and Lepidoptera order and nematodes like *Heteroderaglycines*, *Trichostrongy-luscolubriformis* and *Bursaphelenchus xylophilus* (Baoyu Tian& colab., 2007).

The results showed that the Usa2 strain of *B. subtilis* degraded cellulose. No cellulase was produced by the *B. laterosporus* 56.1s (figure 2).
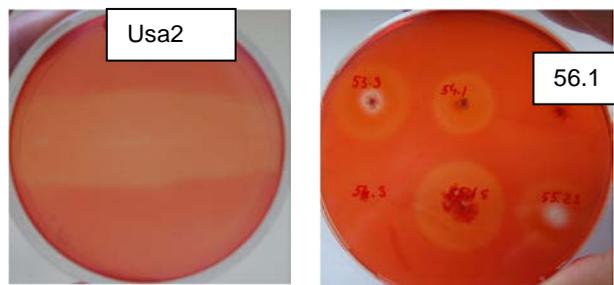

Figure 2. Cellulase test of the strains Usa2 and 56.1s.

Both strains degraded the starch from the media thus proving that they are producing amylase (figure 3). The results revealed that both strains produced lactonase (figure 4).

Usa2 strain   56.1s strain
Figure 3. Producerea de amilază prin descompunerea amidonului

Sample plate with the two tested strains comparing to undegraded lactone

Control plate with positive reaction on Ps33 strain and negative reaction on B49b strain

Figure 4: C6-HHL degradation by Usa2 and 56.1s

The selected strains were tested for their mobility by swim or swarm and the results showed that both types of mobility were present for both strains (figure 5).

Bacterial strain   Swimming (LB suplimentat cu 0.3% agar)   Swarming (LB suplimentat cu 0.5% agar)

Usa2

56.1s

Figure 5. The swimming and swarming mobility of the strains Usa2 and 56.1s on LB media suplimented with 0,3% agar and respectively with 0,5% agar. Photos at 18 hours incubation at 28°C.

Plant growth promotion test which was performed in gnotobiotic system in sterile "cyg" pouches showed that the selected strain 56.1s didn't stimulated significantly the growth of the tomatoes roots comparing with the uninoculated control (table 5, figure 6).

Table 5

Total length of the tomatoes roots at 3 weeks after sowing (mm)

Treatment	Total length of the roots (mm)
Uninoculated control	184.29
WCS 365	219.05
B 49 b	187.5
FL 400	174.54

Ps 33	173.5
56. 1s	184.75
Hm s1	200.86
Cn s2	214.38
Sp s2	181.93
Tm s2	187
Mz b1	225.79
Cp b5	199.28

56.1s *B. laterosporus*   Uninoculated control
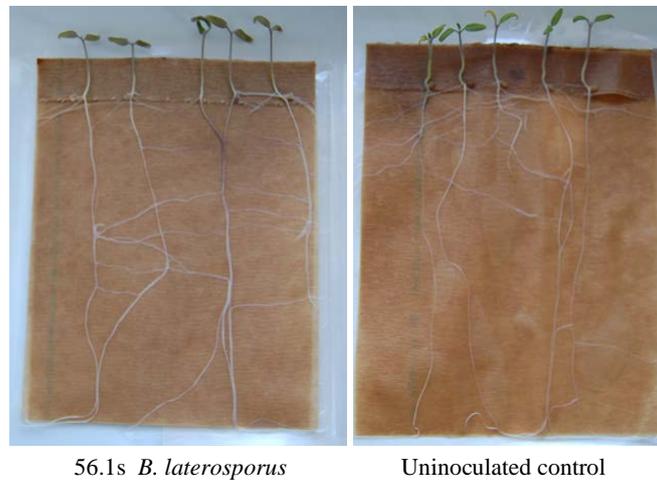
Figure 6: Tomato plants seed inoculated with the strain 56.1s vs. Uninoculated control (3 growth weeks)

From the tested strains, significant growth of the roots comparing with the untreated control were registered in the variant were the seeds were inoculated with the strain Mzb1. This experiment will be repeated and the Usa2 strain will be also tested.

The mineralization of lignocellulosic material test performed on the two bacterial strains, using Strathkelvin Strathtox respirometry system, showed that the respiration rate of Usa2 increased over the analyzing time, and after tree hours the quantity of oxygen decrease to 3,86 mg O_2/L (table 6, figure 7). This means that the bacterial strain could growth on the moistened wheat straw by mineralizing the lignocellulosic material. The 56.1s *B. laterosporus* could not decompose the lignocellulosic substrate and the level of oxygen in the sample flasks were nearly high as in the negative control (see table 6).
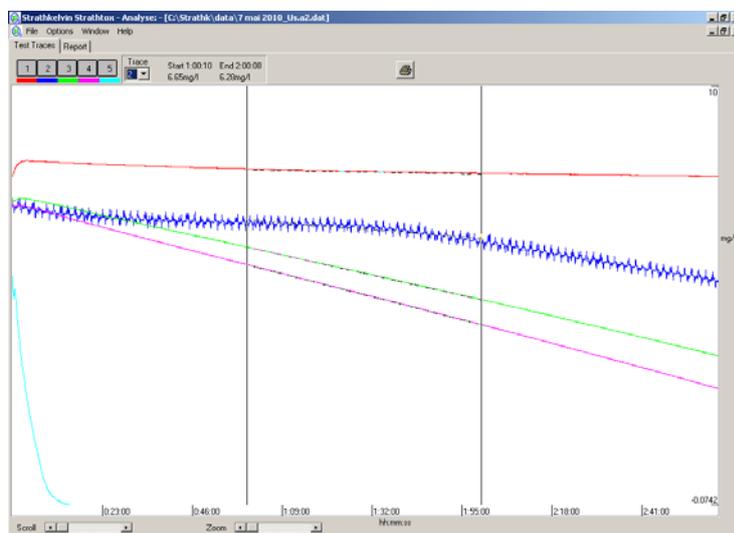
Figure 7: Graphic representation of the respiratory rates from Usa2 test report of Strathkelvin Strathtox respirometry analyze for detection of the ability to mineralize lignocellulosic material. On flask number one is the negative control represented in red color within the graphic. Flask number 2 (blue), 3 (green) and 4 (magenta) contains our testing samples of Usa2. The chart present the evolution of the mineralizing activity over the 3 ours of analyze. Fifth flask contains the positive control, presented in graphic with pale blue color.

Table 6

Respiration rates of the bacterial strains tested for the ability to mineralize the lignocellulosic material

Reading time (minutes)	*Brevibacillus laterosporus* 56.1s (mg O$_2$/L)			*Bacillus subtilis* Usa2 (mg O$_2$/L)		
	Nagative control	56.1s (tree repetition average)	Positive control	Nagative control	Usa2 (tree repetition average)	Positive control
0	8,80	7,55	1,49	7,87	7,17	5,10
10	8,80	7,60	1,48	8,17	7,02	0,17
20	8,80	7,44	1,27	8,13	6,85	0,00
30	8,80	7,35	1,13	8,09	6,71	0,00
40	8,81	7,22	0,90	8,06	6,49	0,00
50	8,81	7,38	0,88	8,03	6,34	0,00
60	8,81	7,44	0,65	8,01	6,16	0,00
70	8,81	7,40	0,56	7,98	6,03	0,00
80	8,81	7,52	0,56	7,96	5,87	0,00
90	8,81	7,54	0,54	7,95	5,70	0,00
100	8,81	7,61	0,43	7,93	5,53	0,00
110	8,82	7,36	0,31	7,92	5,33	0,00
120	8,82	7,45	0,23	7,9	5,13	0,00
130	8,82	7,42	0,17	7,89	4,92	0,00
140	8,82	7,55	0,19	7,88	4,73	0,00
150	8,83	7,34	0,11	7,86	4,53	0,00
160	8,83	7,10	0,80	7,86	4,29	0,00
170	8,84	7,45	0,60	7,84	4,08	0,00
180	8,84	7,55	0,05	7,83	3,86	0,00

The inocuity test revealed the toxic effect of the strain Usa2 *B.subtilis* towards *G. mellonella* (see figure 8). The mortality among the *G.mellonella* larvae when injected with 56.1s *Brevibacillus laterosporus*, does not reach 50% even at the maximum concentration tested (see figure 9).
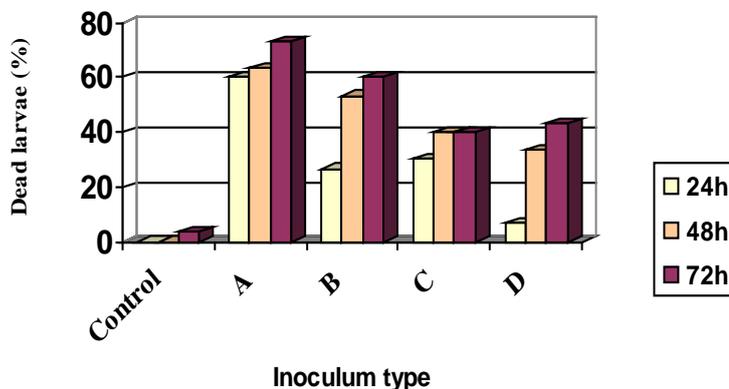
Figure 8: Toxic effect of *Bacillus subtilis* Usa2 strain on *Galleria mellonella* larvae. The larvae were injected with bacterial inoculum of A: $6,85 \times 10^6$ ufc/ml, B: $6,85 \times 10^5$ ufc/ml, C: $6,85 \times 10^3$ ufc/ml, D: $6,85 \times 10$ ufc/ml.
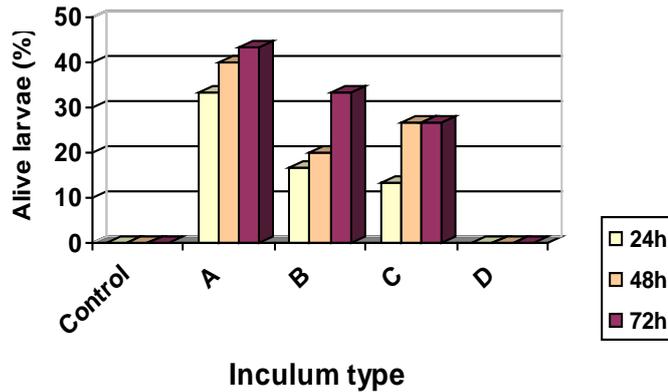
Figure 9: Toxic effect of *Brevibacillus laterosporus* 56.1s strain on *Galleria mellonella* larvae. The larvae were injected with bacterial inoculum of A: $9,2 \times 10^7$ ufc/ml, B: $9,2 \times 10^6$ ufc/ml, C: $9,2 \times 10^5$ ufc/ml, D: $9,2 \times 10^3$ ufc/ml.

CONCLUSIONS

The test performed to detect the antagonistic activity *in vitro* showed that the highest inhibition zones against most of the phytopathogenic fungi were induced by *Bacillus subtilis* Usa2.

The biocontrol of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *Fusarium graminearum* by the strain 56.1s of *Brevibacillus laterosporus in vitro* is scientific news. To our knowledge this is the first report about the ability of *B. laterosporus* to control these two fungi *in vitro.*

Selected strains, Usa2 and 56.1s produced amilase and lactonase, but just Usa2 produced cellulase.

The mobility test showed that the strains were mobile both by swimming and swarming, therefore beeing able to colonise the vegetal material represented by mulch.

Plant growth promotion test performed on tomatos plants revealed that the strain 56.1s didn't significantly promoted the growth of the roots, but this experiment will be repeated on corn, sunflower and bean plants taking along the strain Usa2.

The mineralization of lignocellulosic material test performed on the two bacterial strains, using Strathkelvin Strathtox respirometry system, revealed that Usa2 *B. subtilis* strain, unlike 56.1s B. *laterosporus*, can mineralize the wheat straw.

The infection model of *G. mellonella* larvae revealed the pathogen potential of the Usa2 *B.subtilis* strain against mammalian. These inocuity test results exposed the value of *in vivo* screening for virulence within bacterial strains.

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