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 enzimes 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.

MATERIALS AND METHODS

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.

Motility

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.

Plant growth promotion activity

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.

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.

Ability to mineralize the lignocelullosic material

In detection of the ability to mineralize lignocelullosic material we used Strathkelvin Strathtox respirometer.

The vegetal substrate consisted of wheat straw, that was subjected to some grinding steps, using manual fragmentation at 1-2 cm length and grinding at 15000rpm using a simple blender (Macapá P-102) and afterwords 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, IFIN-HH. We employed grinded wheat straw as vegetal substrate for this test because it is a lignocellulosic material that can release nutrients only after an enzymatic digestion. To create a favorable environment for biological activity, substrate was moistured with a sterile suspending solution, concerning of saline phosphate buffer (PBS), that contains: 8g NaCl; 0,2 g KCl; 1,44 g Na_2HPO_4; 0,24g KH_2PO_4 in 1L distilled water. As a buffer solution it can keep pH at nearly constant value, does not submit the microorganisms to osmotic stress, and does not influence 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, Romania, and Usa2 *Bacillus subtilis* strain was isolated from garlic rhizosphere, Dolj County, Romania.

The bacterial strains were refreshed on LBA medium using exhaustion loop technique for obtaining isolated colonies. And the overnight cultures were used to prepare the inoculum. Bacterial inoculum was prepared in glass tubes with screwed lids containing 20ml sterile PBS. Bacterial biomass was taken from the isolated colonies with sterile cotton applicators (Böttger, 94249 Bodenmais / Germany), and suspended in the PBS inoculation fluid from the tube.

When the bacterial suspension reached the optical density of 0.3 A at 600nm, the inoculum concentration was considered optimal for use. Samples were prepared in Erlenmeyer flasks with 100ml volume of tailings, in which were added 0,25 g of sterile cellulosic substrate (made from ground wheat straw) and 24ml sterile PBS 1ml bacterial inoculum.

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

$$\frac{vegetal_material_quantity\langle g\rangle \times 100}{inoculation_fluid_quantity\langle ml\rangle + inoculum_quantity\langle ml\rangle} = \frac{0,25 \times 100}{24+1} = 1\%$$

Those microorganisms that can metabolize the lignocellulosic substrate could rich an exponential multiplication phase, leading to increased oxygen consumption. This respiratory activity of the samples was detected using Strathkelvin Strathtox respirometer.

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

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

The positive control provide information on the development of the tested bacterial strain, by growing it on usual culture medium. This allows the microorganism to multiply in proper feeding but in the incubation conditions provided during the course of the test.

The type of samples prepared for analyze in 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. This laboratory respirometer allows making tests under controlled temperature conditions. The device needs to be calibrated once a day before any test at the sated temperature parameter. By calibration are established the maximum and minimum points of a captured signal. 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 process the samples at 24°C temperature. We calibrate 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.

Samples were analysed with the system for minimum 3 hours measurements were registered every second but we collect 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 grater 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. There were grown in LB broth medium at 28ºC within 150 rpm shaking. Two days old cultures were pelleted and resuspended in 10mM $MgSO_4$ supplemented with 1,2 mg/ml ampicillin. This antibiotic is used to prevent infections with bacteria naturally present on the larvae surface. The bacterial inoculum concentration was indirectly determined by reading his optical density as absorbance at 600nm with CECIL CE 2021 spectrophotometer, knowing that an OD at 600nm of 1 corresponds to 10^8cfu/ml (see table 3).

G. mellonella larvae were reared on Haydak medium at 30ºC environment. To prepare one ration of Haydak medium were necessary 400g of cornflour, 200g flour, and 200g wheat bran, which were freeze for 7-10 days and then sterilized at 60-70°C for 2 hours. To the sterile composition were added 100g dryed yeast and milk powder 200g. At this mixture, 350ml glycerin, and 350 ml honey and beeswax were added. 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, via the hindmost left proleg. Ten larvae were injected at each dilution in tree replicates. Control larvae were injected with 5µl of only 10mM $MgSO_4$ plus 1,2 mg/ml ampicillin in order to measure any potentially lethal effects of the physical injection process. Injected larvae were stored on Haydak medium at 30ºC and darkness, and scored as dead or alive at 24, 48 and 72h post infection. Larvae were considered dead if they 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, fig. 1).
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 et al., 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 et al., 2007).

The results showed that the the strain of *B. subtilis* Usa2 produced cellulase but no cellulase was produced by the *B. laterosporus* 56.1s (fig. 2).

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

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 (fig. 5).

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, fig. 6).

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, fig. 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 (table 6).

The inocuity test revealed an intense pathogen reaction of Usa2 *B.subtilis* strains towards *G. mellonella* (fig. 8). The insect toxic reaction to this bacterial strain reveals a similar response within the mammalians.

The mortality among the *G.mellonella* larvae when injected with 56.1s *Brevibacillus laterosporus*, does not reach 50% not even at the higher concentration of the inoculum (fig. 9).

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.*

Both 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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REFERENCES

ALIPPI A. M., PERELLO A. E., SISTERNA M.N.; GRECO N.M.; CORDO C.A., 2000. Potential of spore-forming bacteria as biocontrol agents of wheat foliar diseases under laboratory and greenhouse conditions, *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* ISSN 0340-8159 , 107(2): 155-169.

BAILEY K.L., LAZAROVITS G., 2003. Suppressing soil-borne diseases with residue management and organic amendments. *Soil & Tillage Research* 72: 169–180

BAOYU T., YANG J., ZHANG K.Q., 2007. Bacteria used in the biological control of plant-parasitic nematodes: populations, mechanisms of action and future prospects. *FEMS Microbiology Ecology.* 61: 197–213.

BLANCO-CANQUI H. and LAL r., 2008. No-Tillage and Soil-Profile Carbon Sequestration: An On-Farm Assessment. *Soil Science Society of America Journal* 72**:** 693-701.

BOCKUS W.W., BOWDEN R.L., CLAASSEN M.M., GORDON W.B., HEER W.F., SHORYER J.P., 1997. Time of application and winter wheat genotype affect production of large seed after fungicide application. *Canadian Journal of Plant Science* 77: 567-72.

DAVID W.A and DONALD C. R., 2009. Economic Performance of Alternative Tillage Systems in the Northern Corn Belt. Agronomy Journal *101: 296-304*

DRURY C.F., TAN C.-S., WELACKY T.W., OLOYA T.O., HAMILL A.S., WEAVER S.E., 1999. Red clover and tillage influence on soil temperature, water content, and corn emergence. *Agronomy Journal* 91:101-108.

FAGERIA N. K., BALIGAR V.C., and BAILEY B.A., 2005. Role of Cover Crops in improving Soil and Row Crop Productivity. *Communications in Soil Science & Plant Analysis*. 36:2733-2757.

KUO S., SAINJU U.M., and JELLUM E.J., 1997a. Winter cover crop effects on soil organic carbon and carbohydrate in soil. *Soil Science Society of America Journal.* 61:145–152.

KUO S., SAINJU U.M., and JELLUM E.J., 1997b. Winter cover cropping influence on nitrogen in soil. *Soil Science Society of America Journal.* 61:1392–1399.

LIU A., MA B.L., BOMKE A.A., 2005. Effects of cover crop on soil aggregate stability, total carbon, and polysaccharides. *Soil Science Society of America Journal.* 69*(6):*2041-2048.

PARICHI M., STĂNILĂ ANCA-LUIZA, CRUCERU N., 2006. Solurile principalelor unităţi de relief din România. *Analele Universităţii Spiru Haret Seria Geografie,* 9.

SAINJU U.M., WHITEHEAD W.F., and SINGH B.R., 2003. Cover crops and nitrogen fertilization effects on soil aggregation and carbon and nitrogen pools. *Canadian Journal Soil Science*. 83:155–165.

TRIPLETT, G. B. Jr. and WARREN A. Dick, 2008. No-Tillage Crop Production: A Revolution in Agriculture! *Agronomy Journal.* 100:S-153–S-165.

VETCH J.A., and RANADALL G.W., 2000. Enhancing no-tillage systems for corn with starter fertilizers, row cleaners, and nitrogen placement methods. *Agronomy Journal.* 92:309–315.

VILLAMIL M.B., BOLLERO G.A., DARMODY R.G., SIMMONS F.W., BULLOCK D.G., 2006. No-till corn/soybean systems including winter cover crops: Effects on soil properties. *Soil Science Society of America Journal.* 70*(*4*):*1936-1944.

WUEST S.B., 2007. Surface versus incorporated residue effects on water-stable aggregates. *Soil & Tillage Research* 96:124-130.

TABLES

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	R.D.I.P.P./ efficient in biocontrol of the soil borne fungi complex which produce seedlings "damping off"; plant growth promotion
FL 400/ *Paenibacillus graminis*	R.D.I.P.P./ plant growth promotion
Ps 33/ *Serratia plymuthica*	R.D.I.P.P./ biofilm forming strain
56.1s/ *Brevibacillus laterosporus*	R.D.I.P.P./ antagonistic activity *in vitro* against several soil borne fungi
Hm s1/*	R.D.I.P.P./ antagonistic activity *in vitro* against several soil borne fungi
Cn s2/*	R.D.I.P.P./antagonistic activity *in vitro* against several soil borne fungi
Sp s2/*	R.D.I.P.P./ antagonistic activity *in vitro* against several soil borne fungi
Tm s2/*	R.D.I.P.P./ antagonistic activity *in vitro* against several soil borne fungi
Mz b1/*	R.D.I.P.P./ antagonistic activity *in vitro* against several soil borne fungi
Cp b5/*	R.D.I.P.P./ antagonistic activity *in vitro* against several soil borne fungi

* = identification in progress

Table 2

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

Table 3

Optical density measurements of bacterial inoculums used in the inocuity test

OD 600nm	Non diluted inoculum (A)	Dilution 1 (A)	Dilution 2 (A)	Dilution 3 (A)	Dilution 4 (A)	Dilution 5 (A)	Dilution 6 (A)
B. subtilis Usa2	1,435	0,312	0,034	0,007	0,004	0,003	0,001
B. laterosporus 56.1s	1.900	0.252	0.100	0.070	0.020	0.010	0.001

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*

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

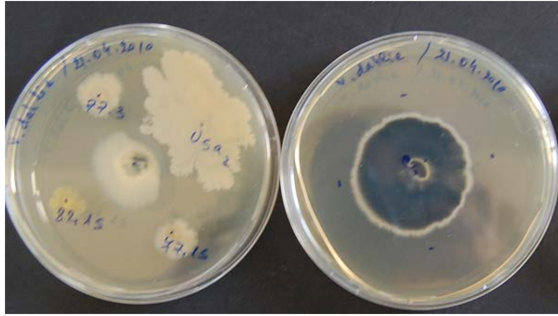
Table 6

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

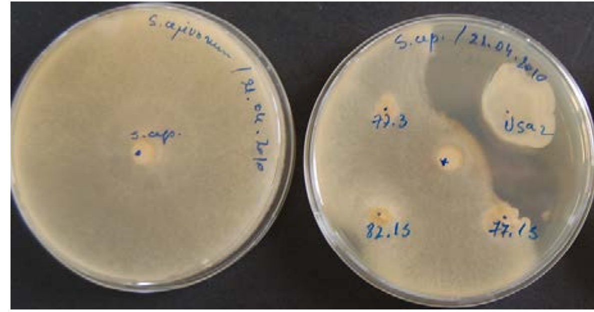
Reading time (minutes)	*Brevibacillus laterosporus* 56.1s ($mg\ O_2/L$)			*Bacillus subtilis* Usa2 ($mg\ O_2/L$)		
	Nagative control	56.1s (tree repetition average)	Positive control	Nagative control	Usa2 (tree repetition average)	Positive control
0	8,80	7,55	1,49	7,87	7,17	5,10
10	8,80	7,60	1,48	8,17	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

Strain Usa2 - *Bacillus subtilis*

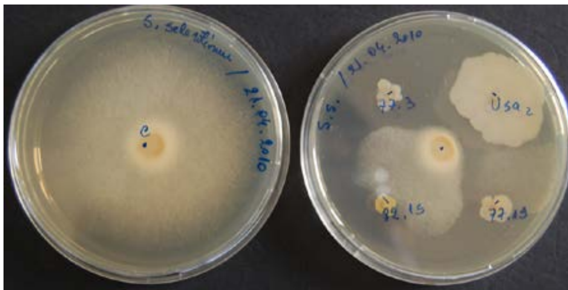
Versus Verticilium dahlie



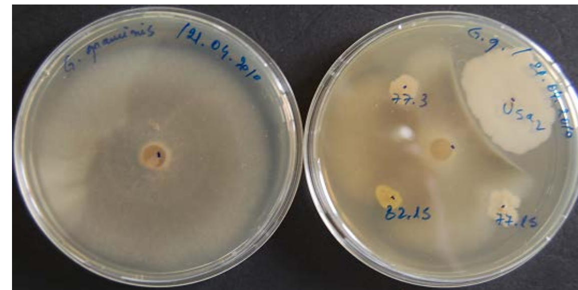
Versus Sclerotium cepivorum



Versus Sclerotinia sclerotiorum



Versus Gaemanomices graminis

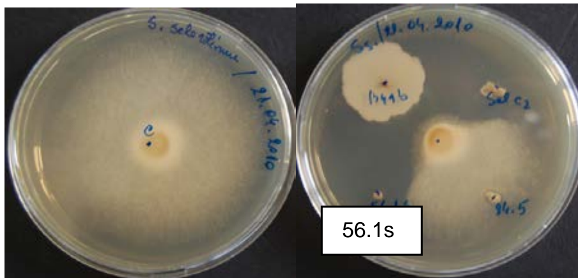


Versus Rhizoctonia solani

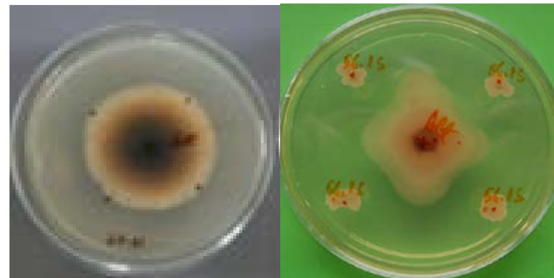
Versus Fusarium graminearum

Strain 56.1s- *Brevibacillus laterosporus*

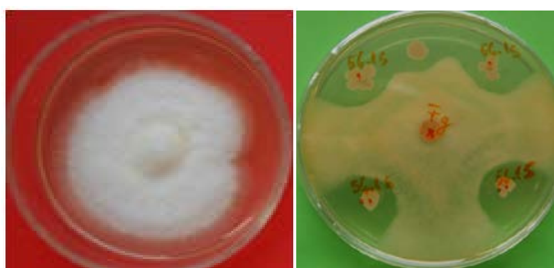
Versus Sclerotinia sclerotiorum



Versus *Alternaria spp.*



Versus Fusarium graminearum



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

Fig. 1 *In vitro* test of the bacterial isolates indicating antagonistic activity against phytopathogenic fungi (72 h incubation at 28°C).

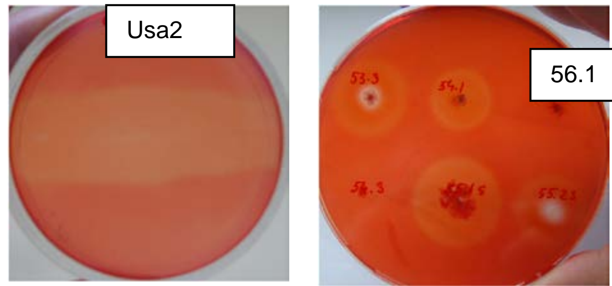
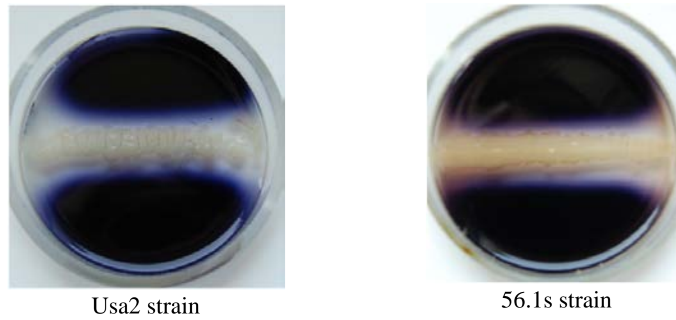


Fig. 2 Cellulase test of the strains Usa2 and 56.1s.



Usa2 strain 56.1s strain

Fig. 3 Amylase production resulting by starch degradation

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

Fig. 4 C6-HHL degradation by Usa2 and 56.1s

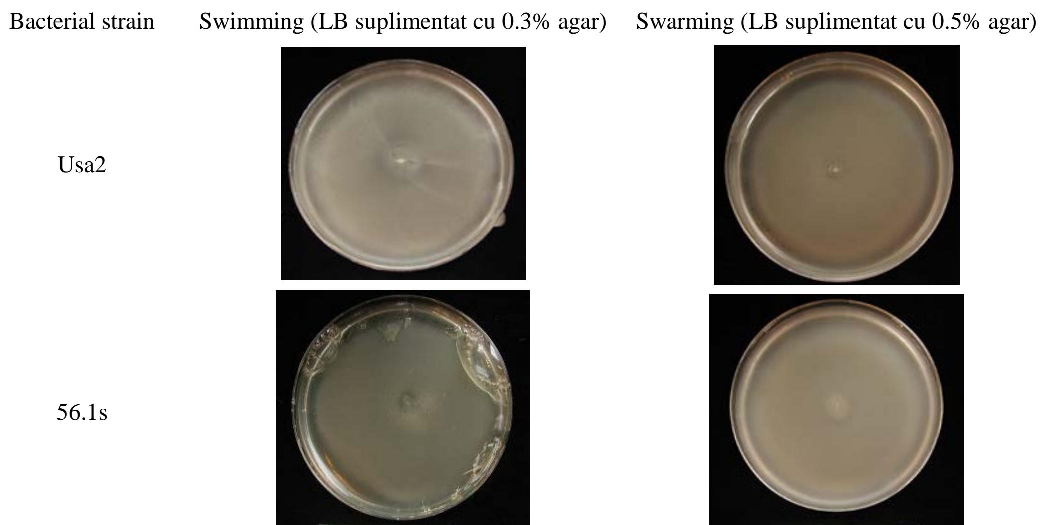


Fig. 5 The swimming and swarming mobility of the strains Usa2 and 56.1s on LB media suplimented with 0,3% agar and respectively with 0,5% agar. Photos at 18 hours incubation at 28°C

56.1s *B. laterosporus* Uninoculated control

Fig. 6 Tomato plants seed inoculated with the strain 56.1s vs. Uninoculated control (3 growth weeks)

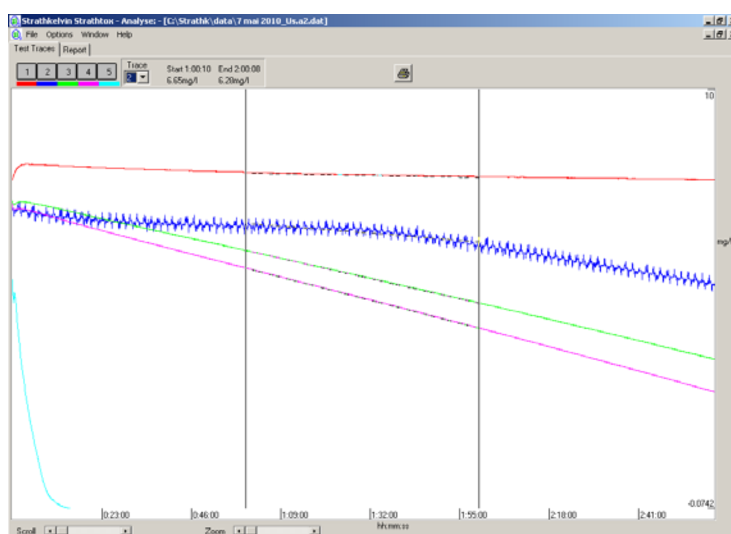


Fig. 7 Graphic representation of the respiratory rates from Usa2 test report of Strathkelvin Strathtox respirometry analyze for detection of the ability to mineralize lignocellulosic material. On flask number one is the negative control represented in red color within the graphic. Flask number 2 (blue), 3 (green) and 4 (magenta) contains our testing samples of Usa2. The chart presents the evolution of the mineralizing activity over the 3 hours of analyze. Fifth flask contains the positive control, presented in graphic with pale blue color.

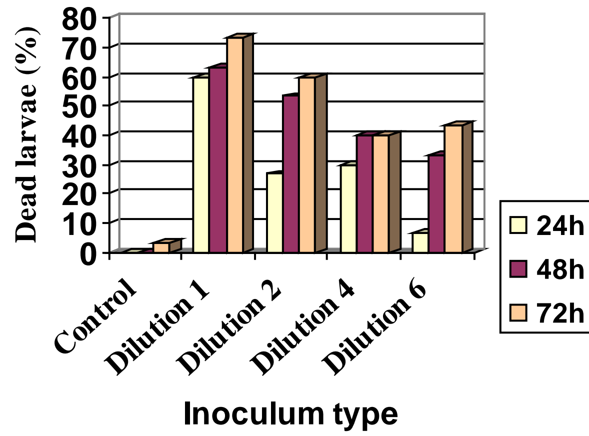


Fig. 8 Toxic effect of Usa2 *Bacillus subtilis* inoculum on *Galleria mellonella* larvae

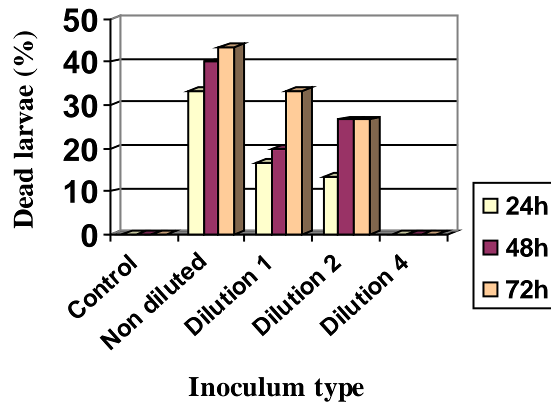


Fig. 9 Wax moth mortality when injected with 56.1s *Brevibacillus laterosporus* strain