Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 26 Issue 2, 2016, pp.225-233

SCREENING OF MULTIFUNCTIONAL BACTERIAL INOCULANTS WITH LIGNOCELLULOSE DEGRADATION ABILITY FOR AGRICULTURAL APPLICATIONS

Gyöngyvér Mara[1]*, Annamária Becze[1,2], Orsolya Varga[1], Éva Laslo[1], Florin Oancea[3]
[1]Sapientia Hungarian University of Transylvania, Faculty of Economics, Socio-Human Sciences and Engineering, Department of Bioengineering, Miercurea Ciuc 530104, Libertăţii Sq, No. 1, Romania.
[2]Politehnica University of Bucharest, Faculty of Applied Chemistry and Material Sciences, Department of Analytical Chemistry and Environmental Engineering, 1-7 Polizu Street, R4, Bucharest 011061, Romania.
[3]The National Institute for Research & Development in Chemistry and Petrochemistry, Bioresources Department, 202 Spl. Independentei, Bucharest 060021, Romania

ABSTRACT: We developed a flexible screening procedure for the selection of multifunctional bacterial agro-inoculant strains, with lignocellulose material degradation ability. Initial screening was done to select the bacteria isolates from ensilaged grass / legumes, able to growth on minimal media, with (carboxymethyl)cellulose, xylan or phytate as carbon source. On these isolates we tested the antagonism toward plant pathogens, ability to produce siderophores and compatibility with lactic acid bacteria. We selected a strain, SZE102A, able to growth on plant residues, with a highly antagonistic activity toward plant pathogens and compatible with lactic acid bacteria. We identified this strain as being *Bacillus licheniformis*, by a polyphasic taxonomic approach.

Keywords: Bacterial inoculants, high residue farming, bale silage, plant pathogens, lactic acid bacteria

INTRODUCTION

Bacterial inoculants are used in agriculture mainly for plant growth promoting applications (Bashan, 1998, Berg, 2009, Laslo et al., 2012) and for an improved fermentation and aerobic stability of silage grass and/or legumes. (Filya et al., 2000, Weinberg & Muck, 1996, Wilkinson & Davies, 2013). Bacterial inoculants strains, able to colonize and degrade lignocellulose material, could be useful for both applications, as treatment of plant residues, i.e. on conservation farming systems, and as inoculation of cutted grass and /or legumes, baled at high moisture.

Such applications are of particular interest for sustainable agriculture practices on wetland areas. High residues / conservation agriculture systems are highly recommended for wetland area (Faulkner et al., 2011, Hobbs et al., 2008, Scherr & McNeely, 2008), mainly due to reduced erosion and nutrient leakage, resulted from low-tillage interventions. However, such high residues systems present several disadvantages. High residues covering the soil promote survival of soil born plant pathogens (Bockus & Shroyer, 1998), including mycotoxigenic fungi (Beyer et al., 2006, Dill-Macky & Jones, 2000, Pereyra & Dill-Macky, 2008). Soil coverage decreases soil temperature (Turmel et al., 2015, Page et al., 2013, Baker et al., 2007). Higher carbon inputs into soil reduce nitrogen availability (Geisseler et al., 2010, Turmel et al., 2015). Both soil decreased temperature and reduced nitrogen availability delay the development of the cultivated plants on the early stages (Kravchenko & Thelen, 2007). Plant residues treatment, with bacterial inoculants able to colonize and accelerated the decomposition of lignocellulose material, antagonist against plant pathogens and with plant growth promoting characteristics, are one of the solutions for counteracting the negative effects of high residues covering the soil, on conservation farming (Raut et al., 2015, Sicuia et al., 2012a).

Nutritive values of grass and/or legumes from wetland are increased by frequent cutting (Cop et al., 2009). However, in such wet area, sun drying grass and/or legumes is more difficult, silage on plastic wrapped bale being an attractive alternative. For such approach of producing bale silage, bacterial inoculants, able to initiate the lignocellulose material decomposition, for an enhanced fermentation, and displaying both anaerobic / aerobic metabolism, are needed.

Our aim on this work was to develop and to use a flexible screening procedure for the selection of bacterial inoculants strains, able to growth on lignocellulose material and useful for both mentioned agricultural applications, treatment of plant residues coverage on conservation farming systems and inoculation of wrapped silage bale. We will further detail this procedure, presenting and discussing the results obtained through its application.

MATERIALS AND METHODS:

Microorganism and culture media. We used different type of silage (corn silage, grass silage, alfalfa silage) as source of bacteria with lignocellulose degradation ability. Isolation was done on minimal media supplemented with components usually found on plant cell wall / lignocellulose material – cellulose, xylan, phytate. We used M63 minimal medium, containing the following ingredients in 1 liters: K_2HPO_4 61.5 mM; KH_2PO_4 38.5 mM; $(NH_4)_2SO_4$ 15.1 mM, oligo-elements (0.5 ml of 1 mg/ml $FeSO_4$ in 0.01 M HCl; 1 ml of 1M $MgSO_4$ solution), 1 mL of 1 mg/ml thiamine solution, and 5 mL of SPV-4 trace elements

Correspondence*: *Gyöngyvér Mara, Sapientia Hungarian University of Transylvania, Faculty of Economics, Socio-Human Sciences and Engineering, Department of Bioengineering, Miercurea Ciuc 530104, Libertăţii Sq, Nr. 1, Romania, Tel.: +40 266 314 657, Fax: +40 266 372 099, email: maragyongyver@csik.sapientia.ro*

solution (Kung et al., 1991). All the used reagents were provided by Sigma-Aldrich (St. Louis, MO, USA). Into this minimal media we introduced, as carbon source and energy, 0.5 g/l of one of the followings: (*i*) microcrystalline cellulose (d50 Merck Millipore, Darmstadt, Germany, 10-30 μm particle size), (*ii*) carboxymethlycellulose sodium salt (Sigma-Aldrich, 50-200 cP viscosity; 90 kDa), (*iii*) xylan, (Sigma-Aldrich, from beechwood, >90% xylose residues) and phytate, inositol hexakisphosphate disodium salt hydrate (Sigma-Aldrich, from rice). Prior autoclaving, we adjusted the pH to 7.0 using 5 M NaOH. We added 18 g of agar (Merck-Millipore) per liter to prepare the agar plates, which we used for isolation of pure culture. We used non-inoculated agar plates as control.

Taxonomical identification of bacterial strains. We made an identification of bacterial isolates able to use components usually found on plant cell wall / lignocellulose material (cellulose, xylan, phytate) as sole source of carbon and energy using a polyphasic approach (Vandamme et al., 1996, Tilak et al., 2005). We tested the ability to used various substrates by using Api® strip (BioMeriueux, Marcy-l'Étoile, France). We confirm the initial taxonomical identification with 16S rRNA gene-based sequence analysis. We used BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) for determination of the nucleotide sequence of obtained PCR products. Sequences were analyzed with ABI 310 Genetic Analyzer (Applied Biosystems, USA). We compared the resulted sequences with those existing on NCBI Gene Bank by using BLAST (Basic Local Alignment Search Tool) Programme.

Assay of the antagonistic activity toward plant pathogens. We used the confrontation assay method, on complex agar medium, containing peptone 10 g, D-dextrose 40 g, yeast extract 10 g, agar 18 g, for determination of the antagonistic activity (Laslo et al., 2012). The plant pathogens strains which we used were: *Botrytis aclada / B. allii* DSM876, *Rhizoctonia solanii* DSM 22845, *Tiarosporella phaseolina / Macrophomina phaseolina* DSM 62744, *Fusarium graminearum* DSM4527, *Verticillium dahliae* DSM 63083, *Phytophthora sojae* ATCC 16708, *Pythium ultimum* DSM 62987, *Sclerotinia sclerotiorum* DSM 1946. We maintained these fungal plant pathogens on Czapek-Dox agar, containing sucrose 30 g, $NaNO_3$ 3 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, agar 15 g, in 1000 ml distilled water (Atlas, 2010). Tested bacterial isolated were grown overnight at 28°C in King's B broth. We spread 0.1 ml of each isolate liquid culture on complex agar plates, using a Drigalski inoculation loop. We axenically placed in the middle of agar plates, streaked with different bacterial isolates, agar disc of each tested fungal plant pathogens, with a diameter of 7 mm. We incubated the agar plates at 28°C and we measured the diameter change of the fungal mycelium after 7 days. We made control plate, wherein each fungal plant pathogen was grown on complex media agar plate without spread bacterial isolates. We calculated the inhibitory effect (IR%) of the bacterial isolates using the following formula:

$$IR\% = 100\frac{C-B}{B}$$

where C is the diameter of the control fungal plant pathogen mycelium, grown in absence of tested bacterial isolates and B the diameter of the fungal plant pathogen mycelium grown in the presence of the bacterial isolates.

Determination of the siderophores production. We used a variant of the Chrome Azurol S agar method (Oldal et al., 2002), based on the competition for ferric (Fe^{3+}) ions complexation between the indicator dye, Chrome Azurol S (CAS), and siderophore produced by the tested bacterial isolate. Briefly the composition of the used CAS agar, per liter, was: 6 g piperazine, 0.6 g NaOH, 15 g proteose-peptone, 15 g $MgSO_4.7\ H_2O$, 15 g K2HPO4, 10 ml glycerol, 20 g agar, 900 ml pure water, 60.5 mg CAS dissolved in 50 ml pure water, 10 mg $FeCl_3.6\ H_2O$ and 72.9 mg hexadecyl-trimethyl-ammonium bromide (HDTMA), dissolved in 50 ml pure water. Bacterial suspensions were prepared in saline (NaCl 9 g/L), and turbidity was set to 0.3 (OD_{600}). Bacterial suspensions were injected on the middle of CAS agar plate, using 5 µl of bacterial suspensions. We worked on three repetitions for each isolate. The plates were incubated at 28°C for 48 hours. We measured the diameter of the yellow zone, resulted from formation of siderophores - ferric complexes.

Determination of the compatibility with lactic acid bacteria. We used the dual confrontation assay. We grew bacterial isolates and lactic acid bacteria on de Man, Rogosa and Sharpe (MRS) agar (Oxoid), on anaerobic conditions. We grew the bacterial isolated overnight at 28°C in nutrient broth and lactic acid bacteria on MRS broth. We inoculated a streak of 0.1 ml of each isolate liquid culture on MRS agar plates, on 1/3 from the middle of the agar plate and on the opposite site we inoculated the tested lactic acid bacteria. We incubated anaerobically the plates on 30°C for 2 days and then we evaluated the growth of lactic acid bacteria confronted with the tested bacterial isolates.

Evaluation of the growth of lactic acid bacterial strain on plant material. We tested the growth of bacterial strain which was proved to be the most compatible with lactic acid bacteria on plant material – grass (*Lolium perene*) and alfalfa (*Medicago sativa*). We used BHM liquid minimal media, with the following composition: 1 g/l NH_4NO_3, 0.02 g/l KH_2PO_4, 0.2 g/l $MgSO_4{\cdot}7H_2O$, 0.2 g/l $CaCl_2$, 0.05 g $FeCl_3{\cdot}6H_2O$ (Singh et al., 2013). To this minimal medium we added plant biomass (grass or alfalfa), in an amount of 23.5 g/l. The tested bacterial strain was grown overnight on Nutrient broth, at 28°C for 24 hours. We used 0.4 ml bacterial suspension (OD_{600}= 1) for inoculation of 20 ml BHM medium with plant biomass, aseptically distributed into a 100 ml conical. We incubated the inoculated media at 28°C for 48 hours, on an incubated rotary shaker, at 145 rpm. We sampled 1 ml of medium, after 12, 24, 36 and 48 hours, and wherein this samples we determined the number of colony-forming units, after a series of decimal dilutions, inoculation on Nutrient agar, incubation at

28°C for 24 hours, and enumeration of the resulted colonies.

Statistical analysis. We performed all the experiments in triplicate. Colony forming units, cfu, per ml, were log-transformed, with the calculation of standard errors. Statistical relevance was established by ANOVA and linear mixed model (Bolker et al., 2009). The Excel software (Office 365 - Excel 2016, Microsoft, Redmont, WA, USA) was used to make calculations and to draw figures.

RESULTS AND DISCUSSION:

We selected several bacterial isolates able to growth on minimal media wherein the sole carbon and energy sources is represented by the components usually found on plant cell wall / lignocellulose material – cellulose, xylan, phytate. We characterized them, including in term of purity. In Table 1 we present these strains and their taxonomical identity. The majority of these strains are Gram-positive spore forming bacteria, with ability to growth both aerobically and anaerobically, by nitrate / nitrite respiration or by fermentation (Nakano & Zuber, 1998). One strain was identified as belonging to *Weissella* genera (*W. paramesenteroides*), a Gram-positive, catalase-negative, non-endospore forming bacteria, with interesting biotechnological application as probiotics, recently reviewed (Fusco et al., 2015).

Table 1.

Bacterial strains able to grown on minimal media wherein the sole carbon and energy sources is represented by the components usually presented in lignocellulose material

Bacterial strain	Origin	Used carbon sources
Bacillus aryabhattai (KF101*)	Corn silage	Phytate
Bacillus subtilis subsp. *subtilis* (SZX102)	Grass silage	Xylan
Bacillus simplex (LE101B)	Alfalfa silage	Cellulose
Bacillus subtilis subsp. *inaquosorum* (SZF101B2)	Grass silage	Phytate
Bacillus subtilis subsp. *inaquosorum* (SZX102A*)	Grass silage	Xylan
Bacillus subtilis subsp. *inaquosorum* (SZE102B*)	Grass silage	Cellulose
Bacillus subtilis subsp. *inaquosorum* (SZC102B*)	Grass silage	Carboxymethlycellulose
Bacillus licheniformis (SZF102)	Grass silage	Phytate
Bacillus licheniformis (SZX101B)	Grass silage	Xylan
Bacillus licheniformis (SZE102A*)	Grass silage	Cellulose
Bacillus licheniformis (SZC101A)	Grass silage	Carboxymethylcellulose
Paenibacillus pabuli (KX101*)	Corn silage	Xylan
Paenibacillus amylolyticus (KC102)	Corn silage	Carboxymethylcellulose
Weissella paramesenteroides (LC101B*)	Alfalfa silage	Carboxymethylcellulose

On these strains we determined their antagonistic activity towards 8 fungal plant pathogens and their capacity to produce siderophores – Figure 1.

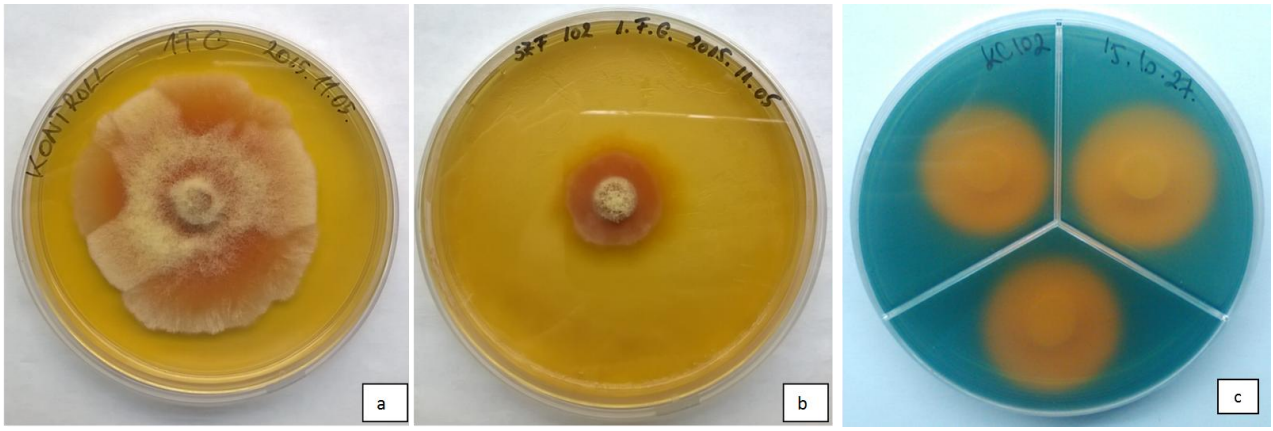

Fig. 1. Examples of determinations of the antagonistic activity against fungal plant pathogens and of the capacity to produce siderophores on the selected strains. a – control, development of *F. graminearum* DSM4527 on complex media; c - development of *F. graminearum* DSM4527 on media wherein strain *Bacillus licheniformis* SZF102 was spread; c – siderophores production by *Paenibacillus amylolyticus* KC102.

We made the antagonism tests against fungal plant pathogens of the bacterial strains in triplicates. We calculated the mean values of the inhibitory effect as average of the values obtained on each repetition. The resulted mean values of inhibitory effect values are represented in Figure 2.

Bacterial strain *B. aryabhattai KF101** shown an inhibitory effect on tested fungal pathogens, with the lower recorded value for *T. phaseolina* (13.97%), while the maximum value has been reached in the case of oomycete species *P. sojae* (100%). Till now strains belonging to *B. aryabhattai* species were demonstrated to promote plant growth into a microcosmos soil (Lee et al., 2012) or to enhance zinc bioavailability for plant (Ramesh et al., 2014). According to our information, this is the first signalization of antagonistic properties for a strain from this *Bacillus* species.

Strains of *B. licheniformis* presented an inhibitory effect of 100% in the case of species of fungal plant pathogens B. *dahliae, B. aclada (B. alli)* and *P. sojae*, showing values of inhibitory effects between 55.46% - 95.99% for the other five other species of fungal plant pathogens. Antagonism of *B. licheniformis* species against fungal plant pathogens is well known (Lee et al., 2006, Tendulkar et al., 2007, Slimene et al., 2015). The lower inhibitory effect, of 7.69%, was registered for the strain of *B. simplex* LE101B, against *S. sclerotinum*. Although this LE101B bacterial strain showed antagonistic effects on all species of fungi tested, the values of the inhibitory effect were lower than for the others tested bacterial strains. Plant beneficial *B. simplex* strains, including due to their antagonism against plant pathogens, were demonstrated to produce mainly volatile compounds (Gutiérrez-Luna et al., 2010, Santoyo et al., 2012, Campos et al., 2010). Our experimental conditions, of confrontation on complex media, do not address well the active volatile producing fungi. For such strains, the double sandwich confrontation method (Raut et al., 2014) is more suitable.

Bacterial Strains of *B. subtilis* subsp. *inaquosorum* proved to be the most effective, form all 14 bacterial strains tested for the antagonistic activity. The strain *B. subtilis* subsp. *inaquosorum SZE102B** presented the inhibitory effect of 100% for 6 fungal plant pathogens. *B. subtilis* subsp. *inaquosorum* SZC102B* and SZF101B2 demonstrated a 100% inhibitory effects for 5 fungal plant pathogen and *B. subtilis* subsp. *inaquosorum* SZX102A* strain totally inhibited 4 fungi. All tested strains of *B. subtilis* subsp. *inaquosorum* proved to be 100% effective against *T. phaseolina, V. dahliae B. aclada (B. alli)* and *P. sojae*. The strain *B. subtilis* subsp. *subtilis* SZX102 present 100% inhibitory effect for 5 fungal plant pathogens, *T. phaseolina, V. dahliae, B. aclada (B. alli), S. sclerotiorum, P. sojae*, 95.08% inhibitory effect for *R. solani*, 81.55% for *F. graminearum* and 78.2% for *P. ultimum*. Strains from *Bacillus subtilis* group are well known to be very actives as plant pathogens antagonist (Morikawa, 2006, Nagorska et al., 2007, Ongena & Jacques, 2008, Sicuia et al., 2015). However, our results underline the complexity of this *B. subtilis* group, wherein strains of different subspecies shown different biological activities.

Strains included into *Paenibacillus* genera have been shown to be weaker competitors than strain which was included into *B. subtilis* group, especially of those included into subsp. *inaquosorum.* Strain *P. amylolyticus KC102* proved to be effective at 100% for 3 of the fungal plant pathogens: *V. dahliae*, *B. aclada (B. alli)* and *S. sclerotiorum.* In the case of the other five plant pathogen strains, the inhibitory effect ranged between 60.42% - 77.99%. Strain *P. pabuli KX101** proved to be 100% effective on 4 plant pathogen strains: *V. dahliae, B. aclada (B. alli), S. sclerotiorum* and *P. sojae.* In the case of the other 4 plant pathogen strains the inhibitory effect ranged between 55.25%-87.92%. Strains from *Paenibacillus* genera are well known for their beneficial effects on cultivated plants – especially those from *P. polymyxa* (Mousa & Raizada, 2015). *P. amylolyticus* strains were proved to be active against *Fusarium oxysporum* f. sp. *radicis-lycopersici* in hydroponics (stonewool) substrate (Validov et al., 2007). *P. pabuli* strains were reported to be active against several plant pathogens (Kobayashi et al., 2015), including *Phytophthora parasitica*, in an *in vivo* assay (Wang et al., 2012). Strains included into *P. pabuli* species were reported to produce several extracellular enzymes active on polysaccharides, including cellulases (Juarez-Jimenez et al., 2008, de Castro et al., 2011, Archna et al., 2015).

Interesting, we found that a strain from a specie included into lactic acid bacteria group, *W. paramesenteroides LC101B**, proved significant inhibitory effect against plant pathogen. It exhibited an inhibitory effect of 100% against 3 plant pathogens: *V. dahliae, B. aclada (B. alli)* and *S. sclerotiorum*. In the case of the other tested five plant pathogens the inhibitory effects ranged between 72.09-93.14%. *W. paramesenteroides* was isolated from fermented sausages and differentiated from *Leuconostoc* genera more than 20 years ago (Collins et al., 1993). Presence of bacterial populations taxonomically classified a *W. paramesenteroides* was described on various plant materials (Sade et al., 2016, Chen et al., 2012, Fusco et al., 2015). Despite the fact that *W. paramesenteroides* was reported to produce a bacteriocin, weissellin A (Papagianni & Papamichael, 2011), antagonism toward plant pathogen of strains from such a lactic acid bacteria species was not yet described, according to our knowledge.

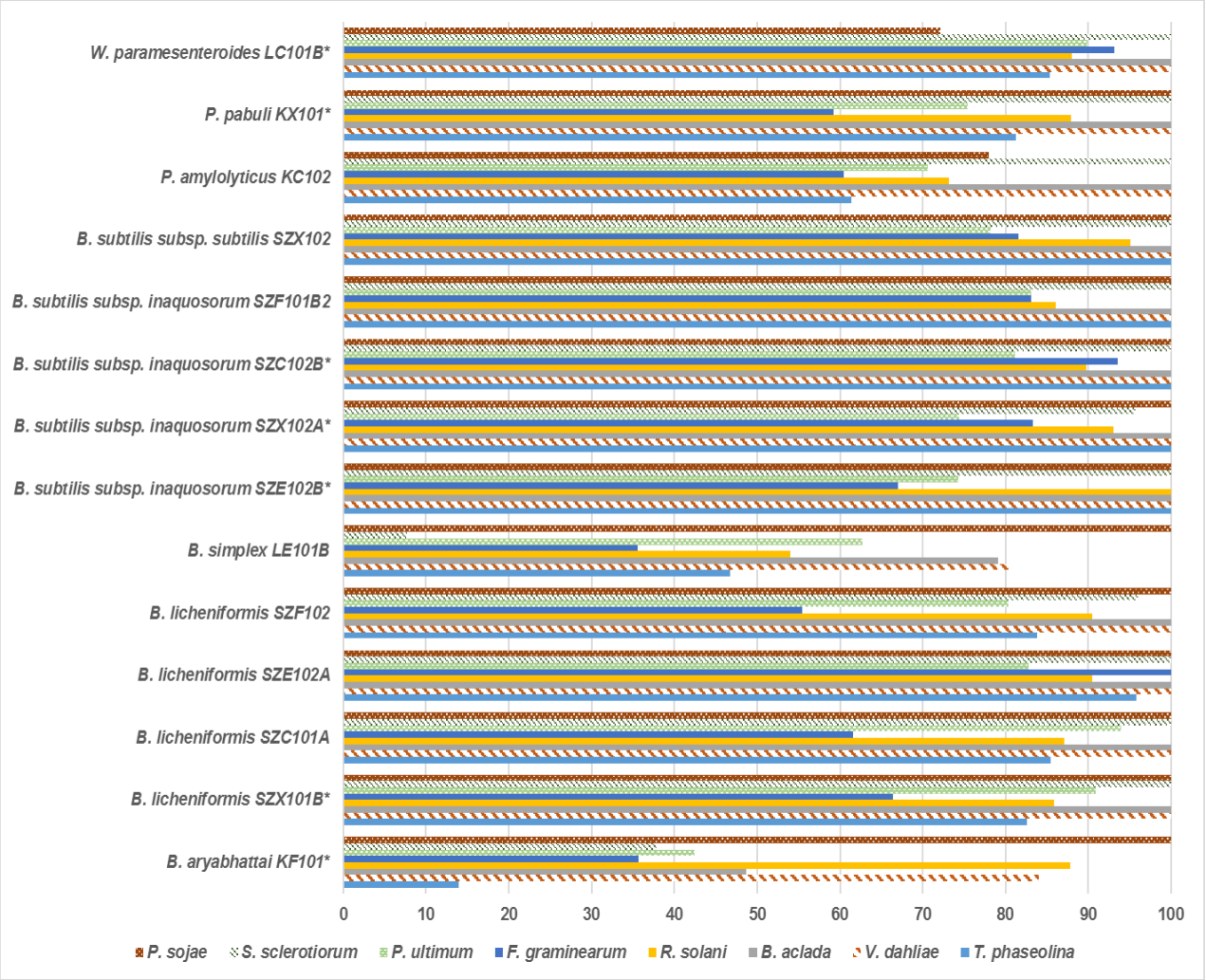

Fig. 2. The antagonistic activity toward fungal plant pathogens of the bacterial strains with lignocellulose material degradation ability

All the tested strains, initially isolated on minimal media with lignocellulose components, cellulose, xylan, phytate, as sole carbon and energy sources, shown a significant antagonism toward tested fungal plant pathogens. This is not unusual, a direct relationship being already demonstrated between the (hemi)cellulolytic activity and antagonism toward plant pathogens (Budi et al., 2000, Krechel et al., 2002, Pastor et al., 2012).

Such characteristics, lignocellulose degradation and antagonism toward plant pathogens, were demonstrated to be beneficial to cultivated plants. Wheat straw and cellulolytic microorganism application are enhancing nodulation efficiency and growth of fenugreek (Abd-Alla & Omar, 1998). Cellulolytic activity induced into soil by inoculating microorganisms was directly correlated with the suppression of seedling blight of barley caused by *F. culmorum* (Rasmussen et al., 2002). Compost inoculation with such (ligno)cellulolytic and antagonistic microorganisms improves its suppressiveness toward soil born disease (Hadar & Papadopoulou, 2012, Kausar et al., 2014).

We evaluated an additional characteristic beneficial to plant, production of siderophores. Siderophores are low molecular weight compounds, secreted by microorganism for solubilization, transport and intake of ferric ions from soil (Loper & Buyer, 1991). Bacterial strains producing siderophores are both competitors towards others microorganisms, due to specific sequestration of a limited resource, soluble ferric ions (Buysens et al., 1996) and biostimulants of plant growth, due to promotion of iron acquisition (Colombo et al., 2013). From our tested bacterial isolates, six strains (*B. aryabhattai* KF101*, *B. subtilis* subsp. *inaquosorum* SZF101B2, *B. subtilis* subsp. *inaquosorum* SZX102A*, *B. subtilis* subsp. *inaquosorum* SZX102A*, *B. licheniformis* SZE102A, *W. paramesenteroides* LC101B*) were able to produce siderophores. The average values of yellow spot diameter produced on CAS agar are illustrated in figure 3.

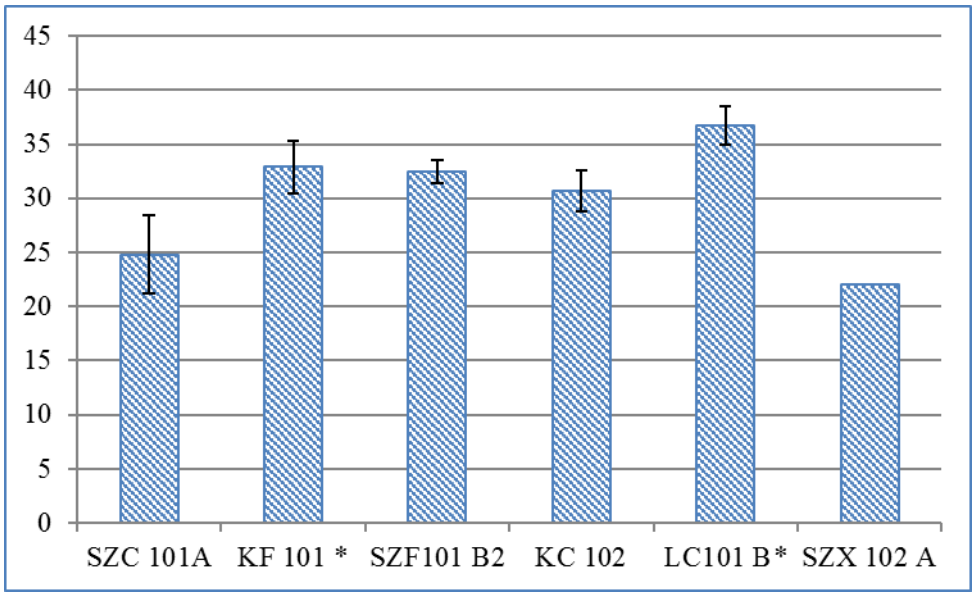

Fig. 3. The quantity of siderophores produced by the tested bacteria strains

We tested the compatibility with lactic acid bacteria. One of the tested strain, *B. licheniformis* SZE102 A proved to be compatible with 14 of the lactic acid bacteria strain tested – Table 2.

Table 2.

Compatibility of B. *licheniformis* SZE 102A strain with lactic acid bacteria strain

Lactic acid bacteria strains	***B. licheniformis SZE 102A***
Lactobacillus pentosus C11	+
Lactobacillus plantarum subsp. A5	+
Lactobacillus pentosus A7	+
Enterococcus fecalis B3	+
Lactobacillus pentosus C10	+
Lactobacillus pentosus C2	+
Lactobacillus plantarum A1	-
Lactobacillus pentosus C15	-
Weissella paramesenteroides Luc 2	+
Pediococcus pentosaceus Luc 1	-
Enterococcus faecalis Szen 1	+
Leuconostoc lactis N19	-
Lactobacillus plantarum C5	-
Enterococcus faecalis N21	+
Lactobacillus paracases N16	-
Lactobacillus pentosus N3	-
Lactobacillus plantarum C6	+
Lactobacillus acidophilus H9	+
Pediococcus parvulus H17	-
Lactobacillus buhneri H1	-
Lactobacillus brevis H15	-
Weissella paramesenteroides Szen ana 2	+
Pediococcus pentosaceus Luc ana2	+
Lactobacillus plantarum subsp. Szen 1 ana	-
Pediococcus pentosaceus Luc ana 1	+

This bacterial strain *B. licheniformis* SZE102

A was also the most active against *Fusarium graminearum*. These mycotoxigenic fungi represent the major health hazard on both agricultural applications envisaged by our bacterial inoculant selection, treatment of plant residues covering the soil on conservative farming systems and grass bale silage. Overwintering and perithecia development / formation of *F. graminearum* / *Gibberella zeae* is promoted by plant residues (Dill-Macky & Jones, 2000, Beyer et al., 2006, Pereyra & Dill-Macky, 2008) and plant residues treatments with antagonists could reduce perithecia formation and spore release (Sicuia et al., 2012b). Also, *F. graminearum* mycotoxins represent a major hazard for grass silage, especially when aerobic conditions occur (Eckard et al., 2011, Alonso et al., 2013). An antagonistic strain, able to survive on anaerobic conditions and very competitive on aerobic conditions, like *B. licheniformis* SZE102 A could prevent the development of mycotoxigenic fusaria and mycotoxins production. Being considered a strain with

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 26 Issue 2, 2016, pp.225-233

potential, *B. licheniformis* SZE102 A was deposited for patent purposes and patented. This strain demonstrates ability to grown well on liquid minimal media with biomass as sole source of carbon and energy. On the liquid BHM media containing grass, after 48 hours, reached a reaches a value of 6.0×10^7 cfu/ml, and in the case of liquid BHM medium containing alfalfa reached a value of 8.06×10^7 cfu/ml after 48 hours.

CONCLUSIONS:

Several bacterial isolates able to growth on minimal media wherein the sole carbon and energy sources is represented by the components usually found on plant cell wall / lignocellulose material, cellulose, xylan, phytate, were selected.

These bacterial isolated were identified by polyphasic taxonomy and tested for their *in vitro* antagonism toward fungal plant pathogen. The strain KF101*, belonging to *Bacillus aryabhattai* species, bacteria known mainly for their plant growth promoting activity, was demonstrated to be antagonist also toward plant pathogens, with an inhibitory effect of 100% to *Phytophthora sojae*.

A lactic acid bacteria strain, LC101B*, identified as *Weissella paramesenteroides*, shown an inhibitory effect of 100% toward *Verticillium dahliae*, *Botrytis aclada* (*B. alli*) and *Sclerotinia sclerotiorum*.

On CAS agar six of the tested strains were proved to produce siderophores, lactic acid bacteria strain, *W. paramesenteroides* LC101B*, being the most active.

The strain SZE102 A, identified as B. *licheniformis*, is a powerful antagonist toward *Fusarium graminearum*, a mycotoxigenic fungal pathogen, which generate health hazard on both conservation farming systems and wrapped bale silage. This strain is compatible with lactic acid bacteria and have potential for application as inoculant of plant residues covering the soil on conservation agricultural system and for grass silage.

ACKNOWLEDGMENT:

This research work was carried out with the support of MECS-UEFISCDI, Programme PN2 Partnerships, project PN-II-PT-PCCA-2013-4-0846-159/2014 CERES and MFE-AI / ANCS-OI, POS.CEE O2.1.1, project P-1734/2012 SILOPREP: Microbial biopreparates for increasing the quality of silages.

REFERENCES:

Abd-Alla HM, Omar AS, 1998. Wheat straw and cellulolytic fungi application increases nodulation, nodule efficiency and growth of fenugreek (Trigonella foenum-graceum L.) grown in saline soil. *Biology and Fertility of Soils* **26**, 58-65.

Alonso VA, Pereyra CM, Keller LaM, *et al.*, 2013. Fungi and mycotoxins in silage: an overview. *Journal of Applied Microbiology* **115**, 637-43.

Archna S, Priyank V, Nath YA, Kumar SA, 2015. Bioprospecting for extracellular hydrolytic enzymes from culturable thermotolerant bacteria isolated from Manikaran thermal springs. *Research Journal of Biotechnology* **10**, 33-42.

Atlas RM, 2010. *Handbook of microbiological media*. CRC press.

Baker JM, Ochsner TE, Venterea RT, Griffis TJ, 2007. Tillage and soil carbon sequestration—What do we really know? *Agriculture, Ecosystems & Environment* **118**, 1-5.

Bashan Y, 1998. Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnology Advances* **16**, 729-70.

Berg G, 2009. Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology* **84**, 11-8.

Beyer M, Klix MB, Klink H, Verreet JA, 2006. Quantifying the effects of previous crop, tillage, cultivar and triazole fungicides on the deoxynivalenol content of wheat grain - a review. *Journal of Plant Diseases and Protection* **113**, 241-6.

Bockus WW, Shroyer JP, 1998. The impact of reduced tillage on soilborne plant pathogens. *Annual Review of Phytopathology* **36**, 485-500.

Bolker BM, Brooks ME, Clark CJ, *et al.*, 2009. Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in ecology & evolution* **24**, 127-35.

Budi SW, Van Tuinen D, Arnould C, Dumas-Gaudot E, Gianinazzi-Pearson V, Gianinazzi S, 2000. Hydrolytic enzyme activity of Paenibacillus sp. strain B2 and effects of the antagonistic bacterium on cell integrity of two soil-borne pathogenic fungi. *Applied Soil Ecology* **15**, 191-9.

Buysens S, Huengens K, Poppe J, Hofte M, 1996. Involvement of pyochelin and pyoverdin in suppression of Pythium-induced damping-off of tomato by Pseudomonas aeruginosa 7NSK2. *Applied and Environmental Microbiology* **62**, 865-71.

Campos VP, Pinho RSCD, Freire ES, 2010. Volatiles produced by interacting microorganisms potentially useful for the control of plant pathogens. *Ciência e Agrotecnologia* **34**, 525-35.

Chen YS, Wu HC, Lo HY, *et al.*, 2012. Isolation and characterisation of lactic acid bacteria from jiang-gua (fermented cucumbers), a traditional fermented food in Taiwan. *Journal of the Science of Food and Agriculture* **92**, 2069-75.

Collins MD, Samelis J, Metaxopoulos J, Wallbanks S, 1993. Taxonomic studies on some Leuconostoc-like organisms from fermented sausages - description of a new genus Weissella for the Leuconostoc paramesenteroides group of species. *Journal of Applied Bacteriology* **75**, 595-603.

Colombo C, Palumbo G, He J-Z, Pinton R, Cesco S, 2013. Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. *Journal of Soils and Sediments* **14**, 538-48.

Cop J, Vidrih M, Hacin J, 2009. Influence of cutting regime and fertilizer application on the botanical composition, yield and nutritive value of herbage of wet grasslands in Central Europe. *Grass and Forage Science* **64**, 454-65.

De Castro ALM, Vollu RE, Peixoto RS, *et al.*, 2011. Cellulolytic potential of a novel strain of paenibacillus sp isolated from the armored catfish parotocinclus maculicauda gut. *Brazilian Journal of Microbiology* **42**, 1608-15.

Dill-Macky R, Jones RK, 2000. The effect of previous crop residues and tillage on Fusarium head blight of wheat. *Plant Disease* **84**, 71-6.

Eckard S, Wettstein FE, Forrer H-R, Vogelgsang S, 2011. Incidence of Fusarium species and mycotoxins in silage maize. *Toxins* **3**, 949-67.

Faulkner S, Barrow W, Keeland B, Walls S, Telesco D, 2011. Effects of conservation practices on wetland ecosystem services in the Mississippi Alluvial Valley. *Ecological Applications* **21**, S31-S48.

Filya I, Ashbell G, Hen Y, Weinberg ZG, 2000. The effect of bacterial inoculants on the fermentation and aerobic stability of whole crop wheat silage. *Animal Feed Science and Technology* **88**, 39-46.

Fusco V, Quero GM, Cho G-S, *et al.*, 2015. The genus Weissella: taxonomy, ecology and biotechnological potential. *Frontiers in Microbiology* **6**, 155.

Geisseler D, Horwath WR, Joergensen RG, Ludwig B, 2010. Pathways of nitrogen utilization by soil microorganisms - A review. *Soil Biology & Biochemistry* **42**, 2058-67.

Gutiérrez-Luna FM, López-Bucio J, Altamirano-Hernández J, Valencia-Cantero E, Cruz HR, Macías-Rodríguez L, 2010. Plant growth-promoting rhizobacteria modulate root-system architecture in Arabidopsis thaliana through volatile organic compound emission. *Symbiosis* **51**, 75-83.

Hadar Y, Papadopoulou KK, 2012. Suppressive Composts: Microbial Ecology Links Between Abiotic Environments and Healthy Plants. *Annual Review of Phytopathology* **50**, 133-53.

Hobbs PR, Sayre K, Gupta R, 2008. The role of conservation agriculture in sustainable agriculture. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **363**, 543-55.

Juarez-Jimenez B, Rodelas B, Martinez-Toledo MV, *et al.*, 2008. Production of chitinolytic enzymes by a strain (BM17) of Paenibacillus pabuli isolated from crab shells samples collected in the east sector of central Tyrrhenian Sea. *International Journal of Biological Macromolecules* **43**, 27-31.

Kausar H, Razi Ismail M, Saud HM, Othman R, Habib S, Siddiqui Y, 2014. Bio-efficacy of microbial infused rice straw compost on plant growth promotion and induction of disease resistance in chili. *Compost Science & Utilization* **22**, 1-10.

Kobayashi A, Kobayashi YO, Someya N, Ikeda S, 2015. Community Analysis of Root- and Tuber-Associated Bacteria in Field-Grown Potato Plants Harboring Different Resistance Levels against Common Scab. *Microbes and Environments* **30**, 301-9.

Kravchenko AG, Thelen KD, 2007. Effect of winter wheat crop residue on no-till corn growth and development. *Agronomy Journal* **99**, 549-55.

Krechel A, Faupel A, Hallmann J, Ulrich A, Berg G, 2002. Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode Meloidogyne incognita (Kofoid & White) Chitwood. *Canadian Journal of Microbiology* **48**, 772-86.

Kung L, Tung RS, Maciorowski KG, Buffum K, Knutsen K, Aimutis WR, 1991. Effects of plant cell-wall-degrading enzymes and lactic-acid bacteria on silage fermentation and composition. *Journal of Dairy Science* **74**, 4284-96.

Laslo É, György É, Mara G, Tamás É, Ábrahám B, Lányi S, 2012. Screening of plant growth promoting rhizobacteria as potential microbial inoculants. *Crop Protection* **40**, 43-8.

Lee JP, Lee S-W, Kim CS, *et al.*, 2006. Evaluation of formulations of Bacillus licheniformis for the biological control of tomato gray mold caused by Botrytis cinerea. *Biological Control* **37**, 329-37.

Lee S, Ka J-O, Song H-G, 2012. Growth promotion of Xanthium italicum by application of rhizobacterial isolates of Bacillus aryabhattai in microcosm soil. *The Journal of Microbiology* **50**, 45-9.

Loper JE, Buyer JS, 1991. Siderophores in microbial interactions on plant surfaces. *Mol. Plant-Microbe Interact* **4**, 5-13.

Morikawa M, 2006. Beneficial biofilm formation by industrial bacteria Bacillus subtilis and related species. *Journal of Bioscience and Bioengineering* **101**, 1-8.

Mousa WK, Raizada MN, 2015. Biodiversity of genes encoding anti-microbial traits within plant associated microbes. *Frontiers in Plant Science* **6**, 25.

Nagorska K, Bikowski M, Obuchowskji M, 2007. Multicellular behaviour and production of a wide variety of toxic substances support usage of Bacillus subtilis as a powerful biocontrol agent. *Acta Biochimica Polonica* **54**, 495-508.

Nakano MM, Zuber P, 1998. Anaerobic growth of a "strict aerobe"(Bacillus subtilis). *Annual Reviews in Microbiology* **52**, 165-90.

Oldal B, Jevcsák I, Kecskés M, 2002. A sziderofortermel} o képesség szerepe Pseudomonas-törzsek növénypatogénantagonista hatásának biológiai vizsgálatában. *Biokémia* **26**, 57-63.

Ongena M, Jacques P, 2008. Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology* **16**, 115-25.

Page K, Dang Y, Dalal R, 2013. Impacts of conservation tillage on soil quality, including soil-borne crop diseases, with a focus on semi-arid grain cropping systems. *Australasian Plant Pathology* **42**, 363-77.

Papagianni M, Papamichael EM, 2011. Purification, amino acid sequence and characterization of the class IIa bacteriocin weissellin A, produced by Weissella paramesenteroides DX. *Bioresource Technology* **102**, 6730-4.

Pastor N, Carlier E, Andres J, Rosas SB, Rovera M, 2012. Characterization of rhizosphere bacteria for control of phytopathogenic fungi of tomato. *Journal of Environmental Management* **95**, S332-S7.

Pereyra SA, Dill-Macky R, 2008. Colonization of the residues of diverse plant species by Gibberella zeae and their contribution to Fusarium head blight inoculum. *Plant Disease* **92**, 800-7.

Ramesh A, Sharma SK, Sharma MP, Yadav N, Joshi OP, 2014. Inoculation of zinc solubilizing Bacillus aryabhattai strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India. *Applied Soil Ecology* **73**, 87-96.

Rasmussen PH, Knudsen IMB, Elmholt S, Jensen DF, 2002. Relationship between soil cellulolytic activity and suppression of seedling blight of barley in arable soils. *Applied Soil Ecology* **19**, 91-6.

Raut I, Badea-Doni M, Calin M, *et al.*, 2014. Effect of volatile and non-volatile metabolites from Trichoderma spp. against important phytopathogens. *Revista de Chimie* **65**, 1285-8.

Raut I, Oancea F, Şesan TE, *et al.*, 2015. Trichoderma asperellum Td36–Versatile strain for treatment of high residue agricultural systems and nutraceutical crops. *Journal of Biotechnology* **208**, S62.

Sade E, Lassila E, Bjorkroth J, 2016. Lactic acid bacteria in dried vegetables and spices. *Food Microbiology* **53**, 110-4.

Santoyo G, Orozco-Mosqueda MDC, Govindappa M, 2012. Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of Bacillus and Pseudomonas: a review. *Biocontrol Science and Technology* **22**, 855-72.

Scherr SJ, Mcneely JA, 2008. Biodiversity conservation and agricultural sustainability: towards a new paradigm of 'ecoagriculture'landscapes. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* **363**, 477-94.

Sicuia O-A, Oancea F, Constantinescu F, Dinu S, Cornea CP, 2012a. Bacillus strains useful in improving vegetal mulch technology trough bio-activation. *Romanian Biotechnological Letters* **17**, 7610-9.

Sicuia O-A, Oancea F, Cornea C-P, 2012b. New screening methods for evaluation of Fusarium sporulation inhibition by Bacillus biocontrol strains. *Scientific Papers, UASVM Bucharest, Series F Biotechnologies* **16**, 67-72.

Sicuia OA, Constantinscu F, Cornea CC, 2015. Biodiversity of Bacillus subtilis group and beneficial traits of Bacillus species useful in plant protection. *Romanian Biotechnological Letters* **20**, 10737-50.

Singh S, Moholkar VS, Goyal A, 2013. Isolation, Identification, and Characterization of a Cellulolytic Bacillus amyloliquefaciens Strain SS35 from Rhinoceros Dung. *ISRN Microbiology* **2013**, 728134.

Slimene IB, Tabbene O, Gharbi D, *et al.*, 2015. Isolation of a Chitinolytic Bacillus licheniformis S213 Strain Exerting a Biological Control Against Phoma medicaginis Infection. *Applied Biochemistry and Biotechnology* **175**, 3494-506.

Tendulkar SR, Saikumari YK, Patel V, *et al.*, 2007. Isolation, purification and characterization of an antifungal molecule produced by Bacillus licheniformis BC98, and its effect on phytopathogen Magnaporthe grisea. *Journal of Applied Microbiology* **103**, 2331-9.

Tilak K, Ranganayaki N, Pal K, *et al.*, 2005. Diversity of plant growth and soil health supporting bacteria. *Current science* **89**, 136-50.

Turmel MS, Speratti A, Baudron F, Verhulst N, Govaerts B, 2015. Crop residue management and soil health: A systems analysis. *Agricultural Systems* **134**, 6-16.

Validov S, Kamilova F, Qi S, *et al.*, 2007. Selection of bacteria able to control Fusarium oxysporum f. sp radicis-lycopersici in stonewool substrate. *Journal of Applied Microbiology* **102**, 461-71.

Vandamme P, Pot B, Gillis M, De Vos P, Kersters K, Swings J, 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiological reviews* **60**, 407-38.

Wang HC, Li WH, Chen QY, *et al.*, 2012. A Rapid Microbioassay for Discovery of Antagonistic Bacteria for Phytophthora parasitica var. nicotianae. *Phytopathology* **102**, 267-71.

Weinberg ZG, Muck RE, 1996. New trends and opportunities in the development and use of inoculants for silage. *Fems Microbiology Reviews* **19**, 53-68.

Wilkinson JM, Davies DR, 2013. The aerobic stability of silage: key findings and recent developments. *Grass and Forage Science* **68**, 1-19.