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**ORIGINAL RESEARCH PAPER**

# TESTING SOME WILD *Bacillus* spp. STRAINS AS POTENTIAL BIOCONTROL AGENTS

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The present investigation follows the study of twenty-four *Bacillus* spp. strains isolated from different samples (compost, municipal waste, crops waste, silage, forest soil, rhizosphere soil, poultry litter, cattle dung and rotting wood) collected from different areas from Galați region, Romania and the assessment of their potential as biocontrol agents against molds. The obtained results demonstrated that the tested indicator fungal strains, *Penicillium expansum* MIUG M11, *Geotrichum candidum* MIUG M63, *Aspergillus glaucus* MIUG M61 and *Fusarium graminearum* MIUG M59 were sensitive to varying degrees towards the *Bacillus* spp. action, with growth inhibition degrees ranging from 56.75% to 100%. Four *Bacillus* spp. isolates possess the ability to produce extracellular chitinase, which could be also used for the control of fungi and insects. By using the radial diffusion method, a hydrolysis zone of colloidal chitin between 27.0-34.5 mm was highlighted. The selected *Bacillus* spp. strains can be promising antifungal agents for pest control in agriculture and food industry.

**Keywords:** *Bacillus* spp., antifungal activity, chitinase activity, bio-fungicides

## Introduction

Using agrochemicals to protect crops against various fungal plant pathogens is a widespread action in agriculture. Nevertheless, this aspect should be limited, because of the toxic effects of these chemicals on the environment and on human health (Martinez-Absalon *et al*., 2012). It is well-known that large quantities of these chemicals are applied to crops, but only a very small percentage (0.1%) reaches the target pathogen (Gajbhiye *et al*., 2010).

Soil borne plant pathogens are a major problem in many open field and greenhouse crops. Pathogens are often able to survive for several years in the soil as a dormant form with environmentally persistent resting structures, until a susceptible crop is cultivated (Gajbhiye *et al*., 2010). *Fusarium oxysporum* is an utterly ubiquitous phytopathogen that causes root rot, vascular wilt and damping off in many plant species (tomato) (Hibar *et a*l., 2006; Gajbhiye *et al*., 2010). *Aspergillus* spp. can invade important seed crops such as soybean, peanut, corn and cotton seed (Nishanth Kumar *et al*., 2013). Biological control is a very promising alternative instead of an extended use of pesticides, which are often expensive and accumulate in plants or soil, having adverse effects on humans (Gajbhiye *et al*., 2010). Microbial products such as enzymes, inhibitors, antibiotics and toxins are very promising as biopesticides against plant pests and pathogens (Ghormade *et al*., 2011). *Bacillus* spp. have been shown to have characteristics which make them useful as biocontrol agents against plant pathogens (Haggag, 2008; Martinez-Absalon *et al*., 2012) being able to produce biological active substances that are capable of disintegrating the fungal cell walls, including chitinase (Islam *et al*., 2012; Youcef-Ali *et al*., 2014). Mycobacillins, surfactins, mycosubtilins and fungistatins, produced by the *Bacillus* species, are some of the antifungal peptides that are widely studied (Islam *et al*., 2012). The species *that belong to the Bacillus* genus that can produce antibiotics are as follows: *B. subtilis*, *B. polymyxa*, *B. brevis*, *B. licheniformis*, *B. circulans*, *B. pumilus*, and *B. cereus* (Hibar *et al*., 2006; Haggag, 2008; Chen *et al*., 2013). The *Bacillus* species present a wide range of antimicrobial activities since they are used as antifungal agents, antiviral agents, antiameobocytic agents and antimycoplasma agents (Yilmaz *et al*., 2006). *Bacillus subtilis* is the most characterized member of the *Bacillus* genus, and it has become a paradigm organism of Gram-positive bacteria. *B. subtilis* has many characteristics as excellent biocontrol agent, including the production of structurally diverse antibiotics, formation of viable spores, promotion of plant growth, and a completely ubiquitous presence in soil (Gajbhiye *et al*., 2010).

The enzymatic lysis of fungal cell walls through extracellular chitinases has been highlighted as a mechanism of biocontrol by bacterial agents. This is mostly due to the antifungal property of chitinases. Microbial production of chitinase has received a worldwide attention of both industrial and scientific environments, not only because of its wide spectrum of applications but also due to the lacuna of an effective production method (Zarei *et al*., 2011). Chitinases are constituents of several bacterial species such as: *Aeromonas* spp., *Serratia* spp., *Vibrio* spp., *Streptomyces* spp. and *Bacillus* spp. (Kuddus and Ahmad, 2013). The presence of chitin in soils or foliage increased the number of chitinolitic microorganisms, thus providing pathogen control (Bressan and Figueiredo, 2010; Velusamy and Das, 2014).

The main aim of this research was to assess the efficiency of some *Bacillus* spp. isolates as biocontrol agents based on their ability to inhibit moulds based on their potential to produce the secondary metabolites and extracellular chitinase.

## Materials and methods

### *Isolation of bacterial strains*

Different samples, such as compost, municipal waste, crops waste, silage, forest soil, rhizosphere soil, poultry litter, cattle dung and rotting wood were taken from different areas of Galați, Romania. One gram of each sample was suspended in 9 mL sterile physiological salt (0.9% NaCl) and shaken vigorously for 2 min. (Yilmaz *et al*., 2006). The samples were heat-shocked at 80°C for 10 min in a water bath (Albert *et al*., 2005; Gajbhiye *et al*., 2010). This treatment was undertaken to ensure that all vegetative forms are destroyed and that only the bacterial spores remain viable. Then, 10 µL of samples were spread onto PCA (Plate Count Agar) medium and incubated at 37°C, for 48 h (Albert *et al*., 2005). The cultures were purified by subculturing. The test that characterizes the *Bacillus* ssp. genus including the endospore forming rods, the microscopic aspects and Gram staining, was carried out according to Bergey's Manual of Systematic Bacteriology (De Vos *et al*., 2009). The pure cultures (AGR1-24 coded) were preserved in 40% (v/v) glycerol, at the temperature of -70ºC.

### *Indicator fungi*

The fungal strains, *Penicilium expansum* MIUG M11, *Alternaria alternata* MIUG M60, *Geotrichum candidum* MIUG M63, *Cladosporium herbarum* MIUG M62, *Aspergillus glaucus* MIUG M61 and *Fusarium graminearum* MIUG M59 used for the present investigation were provided by the Collection of Microorganisms of Bioaliment Research Platform (acronym MIUG), belonging to Dunărea de Jos University of Galati, Romania. The fungal strains were individually grown on PDA (Potato Dextrose Agar) slants at 28°C, for 5 days, until complete sporulation (Hibar *et al*., 2006). Afterwards, the spores were collected from the slants using sterile saline solution (0.9 % NaCl). The counting of the spore suspension was done by using a Thoma cytometer. In order to assess the antifungal activity, the spore suspension was diluted as to reach the indicated spore concentration.

### *Antifungal activity assay*

The determination of the inhibitory effect of the bacterial isolates upon the indicator fungi was carried out according to the spot inoculation method and diffusion method (Kumar *et al*., 2009). The PDA medium was cooled down at 42°C and then poured into Petri dishes and mixed with 1 mL of spores suspensions ($10^6$ spores/mL) of the indicator fungal strains. Afterwards, with standardized sterile loops (1 µL) only a single colony of *Bacillus* spp. isolates was picked up and introduced into the PDA agar plate and incubated at 28°C, for 6 days. After the incubation, the inhibition zone of the fungal growth was measured and expressed as mm.

Furthermore, based on the inhibition growth rate upon the fungal strains, only two *Bacillus* spp. strains were studied. The antifungal compounds production was performed in a PD broth medium, that was inoculated with 2% (v/v) bacterial

inoculums and had a 0.5 OD at 610 nm, being after incubated on a SI 300-R rotary shaker (Lab Companion, USA), at 37°C, for 72 h, 180 rpm (Zarei *et al*., 2011; Das et al., 2012). The cultures were centrifuged at 8000 rpm for 15 min, at 4°C, and the cell free supernatants were used for *in vitro* antifungal assay as reported by Cortes-Zavaleta *et al*. (2014). Briefly, the cell free supernatants were mixed with PDA (pH 4.0) to achieve a final concentration of 10% (v/v) and poured into Petri dishes. The resulting media was inoculated centrally with 5 µL of fungi spore suspension ($10^4$ spores/mL) and incubated at 28°C, for 6 days. The control plates containing PDA media mixed with sterile deionised water, in the same proportions as mentioned above, were also prepared and inoculated. After the incubation period, the colonies diameters of both treated (AT) and control (AC) plates were determined from the mean of perpendicular diameter measurements, assuming it was a circular growth. The percentage of growth inhibition (I) was calculated as:

$$I = 100 \times \frac{Ac-At}{Ac}, \% \quad (1)$$

***Screening of chitinase producing bacteria***

Colloidal chitin was prepared using chitin (Carl Roth) after the modified method of Hsu and Lockwood (1975). The chitin powder (40 g) was slowly added in 600 mL of concentrated HCl and kept for 60 min at 30°C by with vigorous stirring. The chitin was precipitated as a colloidal suspension by slowly adding 2 L of MilliQ water at 4–10°C. The suspension was collected by vacuum filtration on a coarse filter paper and washed by suspending it in about 5 L of MilliQ water. The wash was repeated 3 times until the pH of the suspension was 3.5. After the above treatment, the loose colloidal chitin was used as substrate (Kuddus and Ahmad, 2013).

For the screening of chitinase producing bacteria, basal agar medium supplemented with colloidal chitin was used. The basal medium consisted of (g/L): $KNO_3$ 10; $K_2HPO_4 \cdot 3H_2O$, 10; $MgSO_4 \cdot 7H_2O$, 5; NaCl, 5; agar, 20 and colloidal chitin 20 (w/v), pH 6.0. The bacteria were inoculated in point on the surface of the solid medium in Petri dishes and incubated for 8 days, at 37°C (Kuddus and Ahmad, 2013). The colonies, that displayed clearance zones on an opaque background, were considered as chitinase-producing bacteria (Lamine *at al*., 2012). A hydrolysis index was calculated as a ratio between the hydrolysis zone diameter and the colony diameter.

Further, the active strains were cultivated in a liquid basal medium supplemented with 2% colloidal chitin. The media was inoculated with 2% (v/v) bacterial inoculum (with 0.5 OD at 610 nm) and incubated at 37°C, for 72 h, at 180 rpm, on a SI 300-R rotary shaker (Lab Companion, USA) (Zarei *et al*., 2011; Das *et al*., 2012). The bacterial cultures were centrifuged at 8000 rpm, for 15 min, at 4°C and the cell free supernatants were used for the chitinase activity assay by radial diffusion method. Hence, 10 mL of sterile colloidal chitin agar medium, cooled down to 42°C were poured in Petri dishes. After solidification, in the medium several wells were made (10 mm diameter) and then, in each well, 200 µL of crude enzyme extracts were placed. The Petri dishes were

incubated at 37°C for 8 days. During the incubation, the active chitinase extracts presented a clear zone around the wells. The hydrolysis zone was measured and expressed as mm.

## Results and discussion

### *Evaluation of the antagonism against fungal indicator strains*

Twenty four *Bacillus* spp. isolates, as well as their secondary metabolites, were tested in order to determine if they act as antifungal products against some indicator fungal strains. These strains that were isolated from the biological samples showed a potential antifungal activity, both for the antagonistic strains and also for their crude fermentation products that significantly inhibited the growth of *F. graminearum* MIUG M59, *C. herbarum* MIUG M62 and *A. alternata* MIUG M60 (Table 1).

**Table 1.** Antifungal activity of *Bacillus* spp. isolates against the indicator fungal strains

| Isolates code | Inhibition zone (mm)* | | | |
|---|---|---|---|---|
| | Indicator fungal strains | | | |
| | *F. graminearum* MIUG M59 | *C. herbarum* MIUG M62 | *P. expansum MIUG* M11 | *A. alternata* MIUG M60 |
| AGR1 | -** | 1.04±0.20 | - | - |
| AGR2 | - | 1.27±0.23 | - | - |
| AGR3 | 1.57±0.21 | 1.72±0.01 | - | - |
| AGR4 | 1.45±0.07 | 1.50±0.20 | 2.92±0.21 | 1.66±0.14 |
| AGR5 | 2.11±0.17 | 1.73±0.01 | - | 1.50±0.20 |
| AGR6 | 2.23±0.21 | 1.52±0.31 | - | 4.50±0.02 |
| AGR7 | - | 1.73±0.02 | - | 2.00±0.04 |
| AGR8 | - | 1.52±0.03 | - | 2.14±0.01 |
| AGR9 | 1.72±0.15 | 1.64±0.01 | - | - |
| AGR10 | - | 1.65±0.31 | - | 3.00±0.05 |
| AGR11 | 2.40±0.01 | 2.35±0.05 | - | 3.16±0.01 |
| AGR12 | 2.53±0.02 | 1.52±0.14 | 2.28±0.01 | 3.33±0.02 |
| AGR13 | 2.21±0.02 | 1.92±0.21 | - | 1.29±0.02 |
| AGR14 | - | 2.17±0.02 | - | 2.37±0.22 |
| AGR15 | - | 1.86±0.04 | - | - |
| AGR16 | - | 1.84±0.18 | - | - |
| AGR17 | 1.57±0.14 | 1.40±0.25 | 2.71±0.02 | 1.41±0.25 |
| AGR18 | 1.52±0.01 | 1.62±0.02 | - | 3.16±0.01 |
| AGR19 | - | - | - | - |
| AGR20 | - | - | - | - |
| AGR21 | - | 1.80±0.01 | - | 2.57±0.18 |
| AGR22 | - | 2.25±0.02 | - | 1.40±0.02 |
| AGR23 | 1.40±0.23 | 1.69±0.03 | 1.83±0.01 | 2.30±0.01 |
| AGR24 | - | - | - | - |

* The value represents the mean of the triplicate analysis; -** lack of inhibition zone

The data shown in Table 1 indicate that *C. herbarum* MIUG M62, *A. alternata* MIUG M60 and *F. graminearum* MIUG M59 strains were sensitive to the antifungal compounds that were produced by the tested bacterial isolates. Instead, the *G. candidum* MIUG M63, *A. glaucus* MIUG M61 and *P. expansum* MIUG M11 indicator strains displayed resistance towards the antifungal metabolites produced by *Bacillus* isolates.

The literature stipulates that several species of Bacillus spp. are capable of producing biologically active molecules, including antifungal substances (Youcef-Ali *et al*., 2014). The obtained results are in accordance with those reported by Munimbazi and Bullerman (1998), who stated that *Bacillus pumilus* produced extracellular antifungal metabolites which inhibited the mycelia growth of many species of *Aspergillus spp., Penicillium spp.* and *Fusarium spp*. Cell free supernatants of the two most potent *Bacillus spp.* isolates (AGR11 and AGR12) were tested for their *in vitro* antifungal activity. The results demonstrated that the moulds (*P. expansum* MIUG M11, *G. candidum* MIUG M63, *A. glaucus* MIUG M61 and *F. graminearum* MIUG M59) were sensitive to various volumes of the cell free supernatants (CFS) obtained by submerged cultivation with selected *Bacillus spp.* strains, with inhibition rates ranging from 56.75% to 100%. Cell free extracts obtained with *Bacillus spp.* selected strains, coded AGR11 and AGR12, showed no competitive activity against *A. alternata* MIUG M60 and *C. herbarum* MIUG M62 strains (Table 2 and Fig. 1). As it can be observed in Fig. 1, the colonies of Bacillus AGR11 appeared on the PDA medium although only cell free supernatant was added, which can be explained by the presence in the supernatant of the bacterial spores, after centrifugation.

**Table 2.** Growth inhibition percentage (I) of selected mould strains by *Bacillus* spp. in the presence of cell free supernatants, after 6 days of cultivation on PDA, at 28°C

| Indicator fungal strains | Isolates code | Growth inhibition (I), (%) |
|---|---|---|
| *F. graminearum* MIUG M59 | AGR11 | 56.75 |
| | AGR12 | 62.16 |
| *A. alternata* MIUG M60 | AGR11 | 0.00 |
| | AGR12 | 0.00 |
| *C. herbarum* MIUG M62 | AGR11 | 0.00 |
| | AGR12 | 0.00 |
| *P. expansum* MIUG M11 | AGR11 | 70.58 |
| | AGR12 | 100.00 |
| *G. candidum* MIUG M63 | AGR11 | 58.53 |
| | AGR12 | 65.85 |
| *A. glaucus* MIUG M61 | AGR11 | 70.27 |
| | AGR12 | 66.66 |

Youcef-Ali *et al*. (2014), demonstrated that *B. subtilis* and *B. mojavensis* have a strong antifungal activity especially against *Candida albicans* due to the fact that these strains produce lipopeptides (surfactin, iturin and fengycin) and cell-wall degrading enzymes. Moreover, *B. subtilis* SE14 represents a promising biocontrol agent which produces persistent antifungal compounds against *Rhizoctonia solani* (Elkahoui *et al*., 2012). Cyclo (L-Pro-D-Leu), an antifungal compound identified as diketopiperazine, produced by *Bacillus cereus* subsp. *thuringienis,* strongly inhibited the mycelia growth of *Aspergillus flavus* MTCC 277 and *Aspergillus niger* MTCC282 (Nishanth Kumar *et al*., 2013).

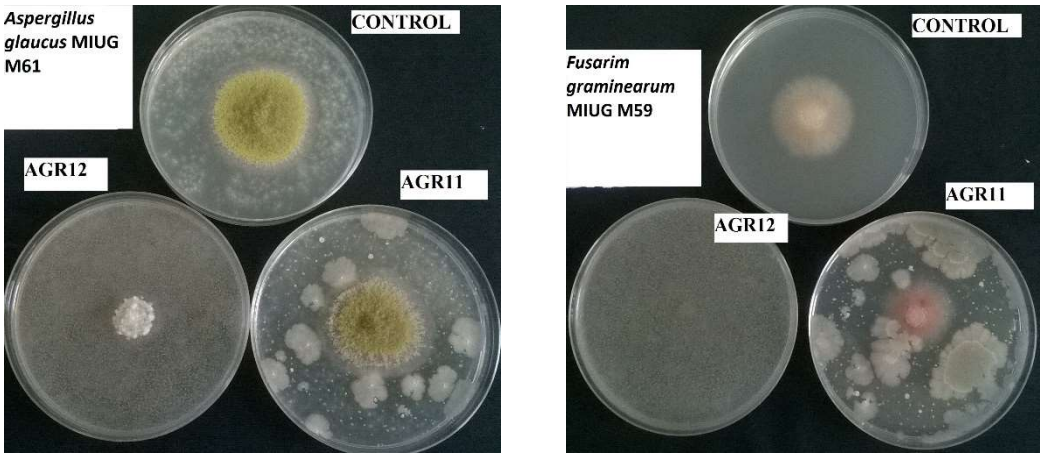


**Figure 1.** Growth of *A. glaucus* MIUG M61 and *F. graminearum* MIUG M59 on PDA, in the presence of cell free extracts of *Bacillus* spp. isolates (AGR11 isolate – right and AGR12 - left), after 6 days, at 28°C

### *Testing the chitinase production ability*

A fast screening of chitinolytic bacteria was performed by spotting the inocula of *Bacillus* spp. isolates on plates containing basal media supplemented with colloidal chitin as the only source of carbon.

The chitin degrading strains formed colonies that ranged between 15.0-22.5 mm in diameter, being surrounded by a clear zone, thus indicating the extracellular chitinase biosynthesis. Only four *Bacillus* spp. isolates produced a clear zone between 27.0-34.5 mm by hydrolyzing the colloidal chitin. The value of hydrolyses index ranged between 1.33 and 2.10 (Fig. 2). Lamine at al., 2012 reported that *Serratia marcescens* DSM $30121^T$ produced a clear zone of more than 0.5 cm by hydrolyzing the colloidal chitin from a minimal salt medium.

Furthermore, based on the chitinase production potential, the crude enzyme extracts of two potential isolates AGR2 and AGR19, were selected. Both of the tested strains indicated a hydrolysis zone of the colloidal chitin of 33.0 mm (for AGR2 isolate) and 34.0 mm (for AGR19 isolate) (Fig. 3).

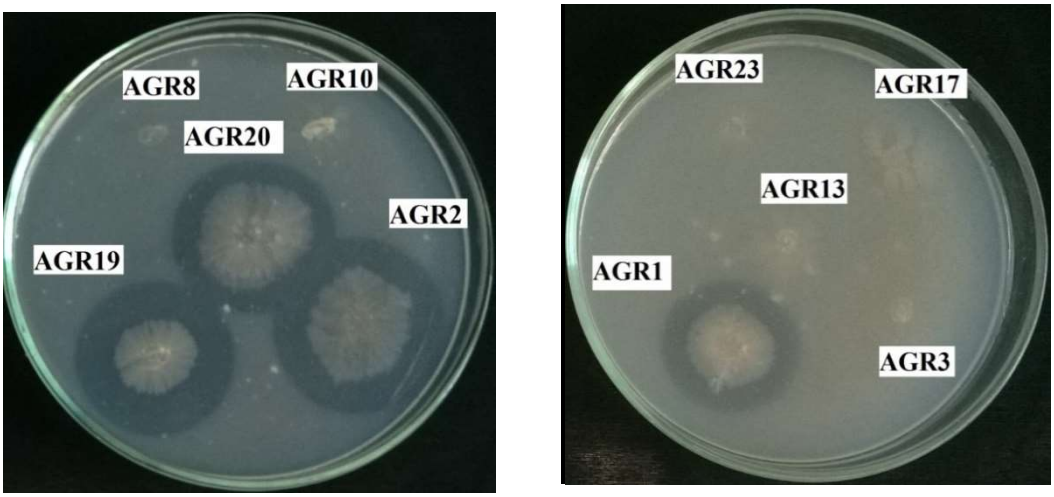


**Figure 2.** Plate assay showing the extracellular chitinase production of *Bacillus* spp. isolates in the basal medium supplemented with 2% colloidal chitin, after 8 days of cultivation, at 37°C

Kuddus and Ahmad (2013) studied a total of 58 morphologically different chitinolytic bacteria isolated from soil samples collected from different habitats of Lucknow, India. Based on the colloidal chitin degradation and the diameter of the clearance zone (>0.2 cm) on CCA plate, only six colonies were selected for secondary screening in broth media and tested for enzyme activity.

Kuddus and Ahmad (2013) stated that the maximum chitinase production was obtained at 37°C and pH 8.0, after 24–48 h of incubation of *Aeromonas hydrophila* HS4 (93.27 U/mL) and at 37°C and pH 7.0, after 48 h incubation of *Aeromonas punctata* HS6 (73.43 U/mL). Instead, a high level of chitinase activity (33.5 U/mL) was observed in the nutrient broth with 0.5% colloidal chitin at pH 7.5 and at 35°C after 3 days of incubation of *Bacillus amyloliquefaciens* SM3 (Das *et al*., 2012).

By comparing these results with those achieved regarding the antifungal activity, it can be concluded that the bacterial isolates that exhibited chitinolytic activity (AGR1, AGR2, AGR19 and AGR20) do not have the capacity to produce antifungal compounds so that to inhibit the growth of the fungal strains. Thus, the antifungal activity was assured by another type of compounds other than the chitinolytic

enzymes. The results are in accordance with Zarei *et al*. (2011), who mentioned that not all chitinases had antifungal activity on a native chitinase and that there are some of them that have partial antifungal activity against a wide range of phytopathogens isolated from *Serratia marcescens* B4A.

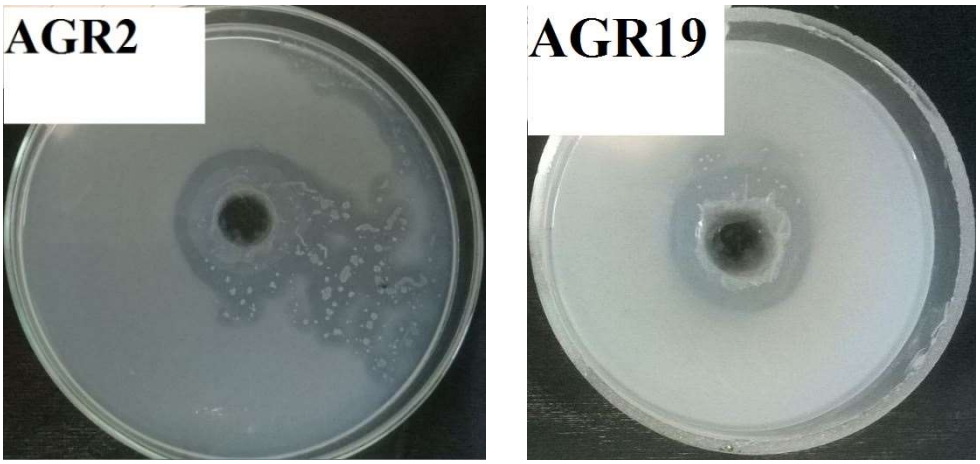


**Figure 3.** Hydrolysis zone of the colloidal chitin (2%) from the crude extracts of AGR2 (left) and AGR19 (right) strains, after 8 days of incubation at 37°C

## Conclusions

Based on the antagonistic activity of the wild *Bacillus* spp. isolates, *in vitro* tests showed that most of the strains had antifungal activity against *F. graminearum* MIUG M59, *C. herbarum* MIUG M62 and *A. alternata* MIUG M60. This matter is very important because these mould strains are the main contaminants of plants. The obtained data revealed that the antifungal activity was not caused by enzymes such as chitinase, mainly due to the fact that the chitinase activity is exhibited by different bacterial isolates (AGR2 and AGR19) than those responsible for the antifungal activity (AGR11 and AGR12). Furthermore, the antifungal activity of these bacterial isolates offers good perspectives for the control of raw materials contaminants (moulds or insects) in regards to agriculture, home-made food or food industry.

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