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Original article

Study of Some Biocontrol Bacterial Isolates and Evaluation of Their Antifungal and Insecticidal Effects [1]

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Abstract

The biological control of pathogenic diseases and other pests by the introduction of microorganisms beneficial has been proposed as an alternative to chemical control.

The objective of our work is to determine the elements on which this interaction between antagonistic bacteria and bioagressors of honey bee such as secondary metabolites, is based. The present work involves the identification of a collection of strains belonging to the *Bacillus* genus with the demonstration of the synthesis of secondary metabolites such as enzymes with hydrolytic effect and the characterization of their bioactive molecules as well as the study of their antagonist effect against *Aspergillus* species, known to infect honey bee (*Apis mellifera*) brood, causing stonebrood disease over all larval stages and insecticidal effect against *Galleria mellonella*. These isolats are from the rhizosphere soil of three cultivated plants (Nèfle, Barley and potato) in the region of Boumerdes (North Algeria).

The macroscopic and microscopic identification tests allowed us to select strains with characteristics identical to those of *Bacillus* sp.

The study of the different enzymes (cellulase, chitinase and phosphatases) show for the majority of strains a good production, which explains their efficiency against *Aspergillus sp*. In fact, the biological control tests carried out in vitro by these bacterial strains have shown that it is possible to limit the mycelium growth of *Aspergillus* sp. and that the use of this biological treatment makes it possible to maintain the disease at an acceptable threshold.

Concerning the insecticidal effect of bacteria on the larvae of the wax moth *Galleria mellonella,* very significant results are achieved with 100% mortality recorded 8 days after treatment at the high dose (1.10^7 ufc/ml).

It appears that these isolates could find their place in biotechnological applications aimed at improving yields and preserving the environment for sustainable development.

Field surveys and laboratory experiments were conducted during the season 2009/10 and 2010/11 in witchweed (*Striga hermonthica* [Del.] Benth.) endemic areas in Sudan to investigate the host specificity of witchweed populations collected from different locations with respect seed germination and haustorium initiation in response to sorghum root exudates and extracts. Field surveys were conducted to collect seeds from witchweed plants growing under their respective hosts; sorghum and millet. A total of fifteen witchweed populations were collected. Tow *in vitro* experiments were conducted at the Phytopathology Center and

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Biology Laboratory, Faculty of Agricultural Sciences, University of Gezira, Sudan to study the effects of root exudates and root extracts of sorghum cv. Abu-70, cv. Wad Ahmed and cv. Hakika on percentage of seed germination and haustorium initiation. Treatments (fifteen witchweed populations and three sorghum cultivars) were arranged in a factorial completely randomized design with three replicates. Data were collected and subjected to analysis of variance. Means were separated for significant using Duncan's Multiple Range Test (at $p \leq 0.5$). The results showed that, there were significant differences in seed germination and haustorium initiation of witchweed in response to root exudates and root extracts among sorghum cultivars and among the witchweed populations. However, the highest seed germination and haustorium initiation percentages attained by each of the witchweed population were on their respective hosts. This study suggests two levels of physiological specialization in witchweed in Sudan: intercrop specialization and intra-crop specialization. Moreover, two strains of witchweed are suggested, one specific to sorghum and the other, to millet. The existence of variability and host specificity within witchweed populations are suggested to be based almost entirely on differential response of *Striga* isolates to root exudates and root extracts from host.

Keywords: Antagonism, *Bacillus* Sp., Bioactive Molecule, *Galleria Mellonella*, Rhizosphere, *Aspergillus*.

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INTRODUCTION

The microbial flora of the soil is very varied; it has various effects influencing the development of plants. These microorganisms can also improve their competitiveness and responses to external stress (Kloepper 1992; Glick 1995). Some soils have the ability to suppress disease expression by involving frequently non-pathogenic bacteria (Cook and Rovira, 1997). Among the bacteria that colonize the rhizosphere, Plant Growth Promoting Rhizobacteria (PGPR) are used in the stimulation of plant growth and as biological control agents.

Most of these bacterial strains have antagonistic properties against pathogenic fungi as well as against certain harmful insects. They are a promising alternative to chemical fertilizers and chemical pesticides. Alternative control measures such as the use of antagonists are necessary and need to be explored (Chérif et al., 2002; Silva et al., 2004).

Among the antagonists that prevail in soil saturated with microflora, we almost always meet species of the genus *Pseudomonas* and *Bacillus*, these microorganisms have a great capacity to produce antimicrobial substances and to enter into space and nutritional competition with pathogenic agents. Biological Control of pathogens and pest is more beneficial to the environment compared to chemical control (Nautiyal, 2001) and natural insecticides have several advantages over synthetic compounds because of their rapid biodegradation and the reduction of environmental risks (Plettner et al., 2017).

Material and methods

Bacterial isolates

For the realization of the experiments, we used 24 bacterial strains of the *Bacillus* genera, isolated from the rhizosphere of some plants cultivated in the region of Boumerdes which is a coastal region situated at the North Algerian.

The insect

The insect chosen for this study is the wax moth. *Galleria mellonella*, raired at the laboratory. It was placed in an oven set at 32 ° C, the larvae are fed to a powder of pollen mixed with honey. After emergence, the butterflies are collected and placed in glass jars for mating and laying eggs.

Fungal strains

We used two fungal strains of *Aspergillus*, A1 and A2 isolated from an apiary in the region of Ain Sefra (wilaya of Naâma) situated at the south Algeria.

Phenotypic characterization

The identification of *Bacillus* sp. is performed by different methods based on morphological, biochemical and physiological criteria.

Indeed, after purification, the colonies obtained on solid medium are observed with the naked eye. The macroscopic study is based on the identification elements given by Joffin and Leyral (2006) such as shape, size, relief, etc.

The microscopic study was based on Gram staining (Prevot, 1977) and spore staining (Singleton, 2005), for biochemical characterization we used about twenty tests such as test of catalase, nitrate reductase, mannitol-mobility, Voges Proskauer and hydrolysis of starch, gelatin and casein (Meyer et al., 2004).

Enzyme production

Enzymes searched in our study are the starch hydrolyzing enzyme using nutrient agar medium supplemented with 1% soluble starch, chitinase using an agar medium supplemented with 10g of Chitin, Cellulases on M9 agar supplemented with 10g of cellulose and 1.2g of yeast extract (Benzina et al, 2016). The phosphatases are investigated on PVK medium by streaking in central streaks on petri dishes, incubated at 30 ° C. for 72 hours (Nautiyal, 2001).

Study of the antagonistic activity of isolated bacterial strains against Aspergillus

The selection of antagonist strains is carried out according to Backer and Cook (1988), on the PDA medium. This method consists of depositing a 5 mm diameter mycelial disk of the cryptogamic isolate in the center of a petri dish and incubating for 24 hours at 25 ° C.

After incubation, we set a bacterial suspension on the periphery of the dish in an opposite way, after which the dish is incubated at 25 ° C. The control is prepared in the same way except that the Petri dish contains the mycelium only without the bacterial strain.

The results are read in comparison with the control, after 7 to 15 days of incubation at 25 ° C., depending on the size of the inhibition zones.

Only the bacterial strains having shown an antagonistic activity in the previous selection are retained for a second confrontation with the fungal isolates on the culture medium mentioned above, in order to confirm their antagonistic activities and to measure the percentages of inhibition.

The percent inhibition was calculated according to: I (%) = (1-Dn / D0) X 100

Dn: diameter in the presence of the bacterial strain.

D0: diameter in absence of the bacterial strain (control).

Evaluation of insecticidal activity

One strain of *Bacillus* sp. have been tested on larvae of wax moth *Galleria mellonella*. In effect, 4 doses were used, the treatment took place by ingestion. For the assessment of *G. mellonella*'s rate of mortality, daily counts of mortality were made, and corrected mortality was determined.

RESULTS and DISCUSSION

Phenotypic characterization

After incubation of the 25 bacterial strains isolated on nutrient agar medium at 30 ° C. for 24 hours, we observed the appearance of distinct colonies visible to the naked eye, having the specific morphological criteria of the genus Bacillus.

Strains are characterized by rapid growth on this medium, giving cream to whitish colonies. Microscopic observation reveals strictly straight bacilli, the cells appear isolated or grouped together. After performing the Gram stain, the bacterial wall of the strains tested appears purplish blue in the form of straight bacilli, so they are gram-positive bacilli.

Staining of bacterial spore with Malachite green revealed the presence of spores in some strains of Bacillus sp, for some we even observed the spore after Gram stain.

On the other hand, the appearance of the deep violet color on the oxidase disk in the strains 1, 14, 13 shows a positive response, indicating that the phenylene diamine reactant has been oxidized to form a colored compound. in purple (Singleton, 2005). In addition, the absence of purple staining in strains 2 and 15 indicates that there was no oxidation of the reagent. Thus, the appearance of gaseous bubbles (effervescence) on the slide in strains 1, 13, 15, 16, 17 and 24 show a positive response, indicates a

decomposition of hydrogen peroxide, the absence of effervescence in strain 25, indicates that there was no decomposition.

Moreover, isolates have shown positive results for the test of catalase, nitrate reductase, mannitol-mobility and gelatinase. In contrast, strains expressed negative hydrolysis test against TRP, GLU. The results of the ADH, URE, ARA, ADI appeared variables.

Plant growth-promoting rhizobacteria or PGPRs form a diverse group of microorganisms with two main characteristics, the ability to colonize the rhizosphere on the one hand and, on the other hand, a positive influence on the growth of the plant with which they are associated (Loper and Gross, 2007).

Production of enzymes

From the results obtained, we found that our bacterial strains belonging to the genus *Bacillus* isolated from the soil showed a degradation of several substrates such as proteins and complex sugars (starch, cellulose) by the production of a very diverse range of enzymes such as proteases, amylases, chitinase, phosphatase and cellulases. These strains can colonize rhizospheres quickly and efficiently (Lemanceau, 1992).

Indeed, it turns out from the results obtained that 100% of the strains tested produce amylase, 75 % produces chitinase, 50% produce cellulase and finally 17 % produce phosphatase (Figure 1).

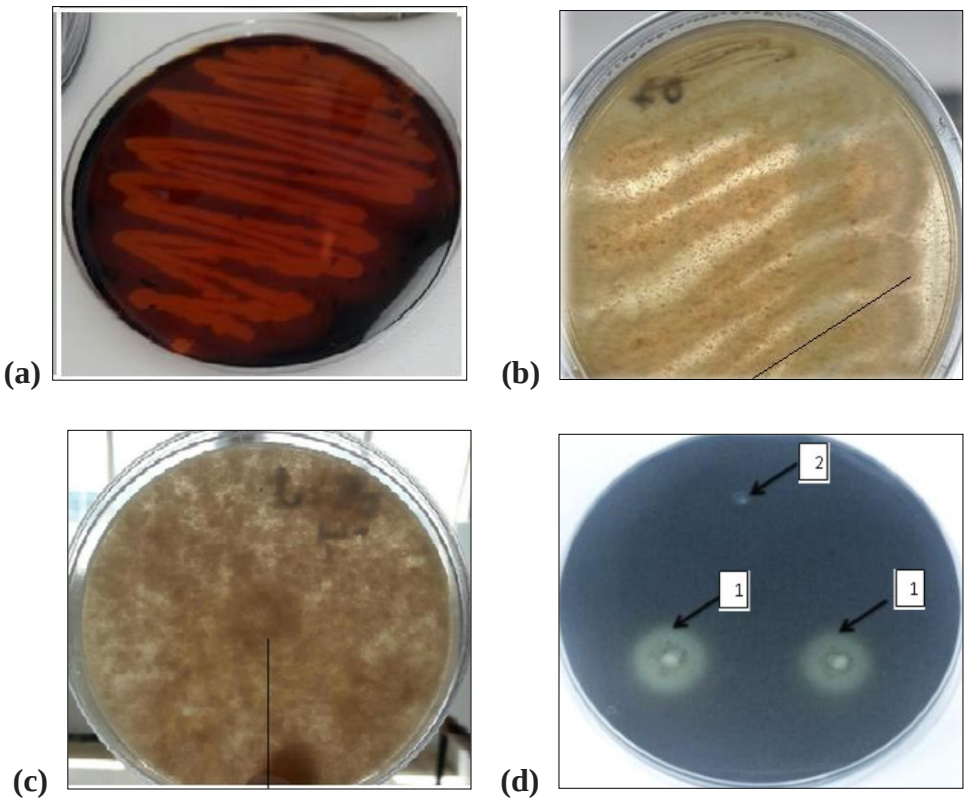

Figure 1. Enzymes production tests; amylase (a), chitinase (b), cellulase (c) and phosphatase (d) (For the phosphatase, the arrows ; 1: shows a clear halo around the colony, indicating the production of phosphatase. 2: a negative control (non solubilization of phosphorus)

Bacillus species are still the major industrial microorganisms in applied microbiology, and are good candidates for the production of large quantities of extracellular enzymes of industrial interest. They are characterized by the strong capacity of their secretory system; they produce various extracellular hydrolytic enzymes (Devine, 1995).

Cellulases are important enzymes that are involved in the control of fungi and oomycetes through their ability to degrade cell wall compounds (Larito et al., 1996). Moreover, Chitin is found in the exoskeleton of insects, fungi, yeast, and algae, and in the internal structures of other vertebrates. chitinolytic enzymes are becoming important in their biotechnological applications, especially the chitinases exploited in agricultural fields to control pathogens and insects. The enzymes chitinases are involved in human healthcare, especially in human diseases like asthma (Simoes Nunes and Philipps-Wiemann, 2018).

Recent studies have established high specificity of phosphatases in substrate recognition and important roles in plant signaling pathways, such as pathogen defense and stress regulation, light and hormonal signaling, cell cycle and differentiation, metabolism, and plant growth Schweighofer and Meskiene (2015).

Results of antagonistic activity of bacterial strains in vitro

The antagonistic potency of bacterial isolates of *Bacillus* sp. was tested by inhibition of mycelial growth of two isolates of Aspergillus A1 and A2.

The diameter of the mycelial colonies was greatly reduced in the presence of Bacillus antagonist strains compared to the uninoculated control.

The intensity of inhibition varies according to the fungal strain tested and the bacterial strain used (Table 1).

Table 1. Inhibition of mycelial growth of *Asergillus sp.* isolates by the most active isolates of *Bacillus* sp. tested on PDA medium.

Strains	*Aspergillus* (A1)	*Aspergillus* (A2)
S1	49.41%	82.35%
S2	52.94%	82.35%
S3	35.29%	90.58%
S4	32.94%	90.58%
S5	50.58%	76.48%
S6	41.17%	76.47%
S7	0%	68.23%
S8	0%	68.23%
S9	47.05%	68.23%
S10	47.05%	68.23%
S11	0%	64.70%
S12	0%	64.70%
S13	35.29%	70.58%
S14	41.17%	70.58%
S15	0%	64.70%
S16	0%	64.70%

We noticed that the 16 strains of *Bacillus* tested have an antagonistic effect on the A2 strain of *Aspergillus* sp with percentages of inhibition which exceed 60%.

On the other hand, these same strains showed less activity against the A1 strain of *Aspergillus* sp. which we have found that the inhibition percentages do not exceed 50%. While 6 strains (S7, S8, S11, S12, S15 and S16) have no antagonistic effect.

The antagonistic activity tests on isolated fungi indicate that these strains show significant antagonistic activity with respect to both strains of *Aspergillus* sp.

Secondary metabolites produced by Bacillus and Pseudomonas species exhibit antimicrobial and antifungal activity against various phytopathogenic agents (Ongena et al., 2008)

Bacillus are known for their important antifungal activity, they produce a variety of potent metabolites and hydrolytic enzymes (Rahman et al., 2007) that leads to the formation of irreversible spores in the phospholipid double layer of the pathogen; These antifungal peptides therefore inhibit the growth of a large number of fungi, *Aspergillus*, *Penicillium*, *Fusarium*, bacteria and oomycetes (Munimbazi and Bullerman, 1998).

The antagonistic activity tests on isolated fungi indicate that these strains show significant antagonistic activity with respect to both strains of *Aspergillus* sp.

The use of beneficial microorganisms for the management of soil-related diseases has attracted the attention of scientists in recent years. Antagonistic bacteria use different mechanisms of antagonism, namely antibiotics, siderophore production, secretions of various enzymes, hormone synthesis and resistance induction in plants (Thomashow and Weller 1990; Pierson and Weller, 1994; Amer and Utkhede, 2000; Manjula et al., 2000; Collins and Jacobson, 2000).

Evaluation of the insecticidal activity of an isolate of Bacillus

The corrected daily mortality rate obtained after treatment of Galleria mellonella L5 larvae by isolates S1 shows a mortality in the individuals treated with the high dose from the first day with almost 7%, this mortality evolves in the time according to the concentration tested to reach 100% after treatment with the concentrations C1, C2, C3 and C4 respectively the 8th, 9th, 11th and 13th day.

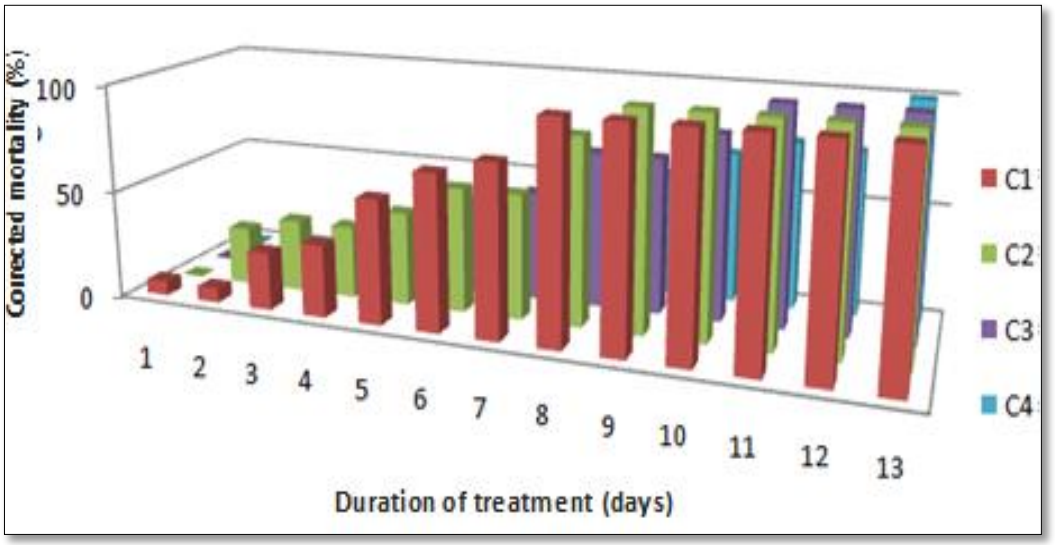

Figure 2. Corrected mortality rate L5 larvae of *G. mellonella* treated with *Bacillus* sp. strain-S1 at concentrations: C_1 =3. 10^4 ufc/ml, C_2 =2. 10^5 ufc/ml, C_3=7.10^6 ufc/ml, C_4=1.10^7 ufc/ml. (The ANOVA test reveals a significant difference at the 5% threshold between concentrations ($p<0.013674$), as well as for time ($p<0.003700$)).

In fact, Bacillus thurigiensis, Bacillus popillia, Bacillus alevi, Bacillus larvae, Bacillus lentimorbus and Bacillus sphaericus have a particular capacity to induce mortality in certain insects (Lacoursiere and Boisvert, 2004). B. thuringensis, which is the most commonly used entomopathogenic bacterium in biological control, acts by release of toxins (Joung and Cote, 2001). The interaction of Bacillus toxins with digestive system epithelial cell receptors causes insect death following disruption of the osmotic regulation of these cells. (Lacoursiere and Boisvert, 2004). The effect of strain Bacillus sp. (HF911367) isolated from south of Algeria, on the 5th instar nymphs L. migratoria were studied. Results obtained one week after treatment indicated that treated nymphs were very highly susceptible to bacterial suspensions, with rates of 65 % (Oulebsir-Mohandkaci et al., 2015). However, Injection of strain S4 of bacterium from the genus Bacillus isolated from larvae of an lepidopteran insect, for individuals of Galleria mellonella resulted mortality of 83.33% (Benzina et al., 2017).

Conclusion

Our work focuses on the identification of *Bacillus* sp. strains, their characterization and the demonstration of the synthesis of secondary metabolites as well as the study of their activity on a phytopathogenic fungus.

The tests carried out in vitro allowed us to demonstrate the antagonistic effects of some bacterial strains. These non-negligible antagonistic effects could be added to other control methods. In parallel, the study of the insecticidal activity of some isolated strains against *Galleria mellonella* has demonstrated a remarkable entomopathogenic power.

The tests we have developed can serve as a basis for the demonstration of antibiotic and anzymatic properties of *Bacillus* and the development of other tests will reveal other aspects of the antagonistic activity of bacterial strains and search for more effective species for use in the control of soil diseases as well as pests.

In fact, these isolates could find their place in biotechnological applications aimed at improving yields and preserving the environment for sustainable development. Many studies have revealed model strains, but stability and reproducibility must be guaranteed. beneficial effects of these rhizobacteria on the scale of field agronomic practices.

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