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Impact of Diazotrophic Bacteria on Germination and Growth of Tomato, with Bio-control Effect, Isolated from Algerian Soil

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Abstract: 10 soils samples were collected from the west region of Bejaia-Algeria in aseptic conditions. The preliminary germination test on tomatos seeds allowed screening of 4 simples from which 40 different colonies were isolated and purified. These last were submitted to in vitro germination test, while 20 strains with an inductor effect on the germination were selected and tested for the presence of stimulator effect of growth of tomato in vivo. Biochemical (on API 50 CHB/E gallery), and phylogenetic (DNAr 16S sequencing) identification, tests researching characteristics promoting plant growth (production of IAA (Indol Acetic Acid) phyto-hormone, enzymes, siderophores, free nitrogen fixation, and an antagonistic test on five phytopathogenic fungi (*Botrytis cinerea; Phytophtora cactorum, phytophtora cinnamomi; Fusarium oxysporum and Verticillium dahliae*) were applied for the strain showing positive in vivo germination results on tomato.The results of this last test led to select four strains: S11, S6, S15, and S12 that greatly affected tomato growth, with stems length (cm) of 9.212, 9.280, 9.362 and 9.858, respectively, compared to the control (6.400). Biochemical and phylogenic identifications of these strains allowed to affiliate them as following: *Arthrobacter agilis* (S6), *Streptomyces Sp* (S11), *Bacillus sp13* (S15) and *Mycobacterium Sp* (S12). The four strains produced several enzymes, high indol acetic acid (phyto-hormone) quantity (mg/l: 38.47 (S11); 86.90 (S6); 93.96 (S12) and 190.23 (S15), siderophores, nitrogen fixing. It also showed an antagonistic activity against the phytopathogenic fungi tested with variable plant growth inhibition (PGI) %:0-78.51% for *Bacillus sp13*; 29-74%for *Streptomyces sp*; 0-72%for *Arthrobacter agilis*; and 0-69% for *Mycobacterium sp*. These results prove that the strains are compatible for tomato growth stimulation.

Keywords: Diazotrophic-Bacteria, Enzymes, Phytopathogens, Bio-control, Tomato, Algeria

1 Introduction

Soil is a natural environment in which microorganisms live, multiply and die [11], considered also as physical support, and provide mineral elements requisite for plants. In soil, rhizosphere is a biologically active zone characterized by a diversity of life. After its first definition by Lorenz Hiltner in 1904 as; ...zone in soil in which microflora is influenced by plant roots , many others were proposed that soil adhering to roots when it were rigorously shaken [33].

In the rhizosphere, these microorganisms could be beneficial by promoting plant growth and health, therefore, [2], or harmful causing diseases and plant death. Plant growth stimulating bacteria can take place by direct or indirect mechanisms. The first one implicates; atmospheric nitrogen fixation, supplying non available nutrients, production of plant growth regulators (phyto-hormones) (De Salamone et al., 2005) and ethylene synthesis inhibition [36]. Concerning the indirect mechanisms, it includes phytopathogenic agents removing by competition for space and nutrients, enzymes production, inhibition of enzymes and toxins produced by pathogens and through plant defense mechanisms induction [2]. Phytopathogens living in soil are varied and can induce serious problems for vegetable production [17]. Pesticides utilization, in spite of their effect against plant parasites in short time, but, they often act negatively with serious threats on ecosystems and

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public health [18]. For this raison, application of biological control agents based on natural processes is increased these last years, in order to repair some disadvantages of chemical treatments. Mechanisms involved in the biological control include: antibiosis, competition for space and nutrients, defense plant stimulating by synthesis of many metabolites and combination of different mechanisms [28].

In this study we present four bacterial strains isolated from Algerian rhizospheric soil (Bejaia), followed by their purification and identification, testing their capacity to stimulate the germination and growth of tomato seeds, to synthesize extracellular enzymes, other metabolic activities, at last their application in antagonistic tests against some tomato phytopathogenic fungi

2 MATERIAL AND METHODS

2.1 Soils sampling

10 rhizospheric soil samples were randomly collected on many sites of the west coast of Bejaia. (Boulimat, Saket, Beni-ksila) in January, 2011. Samples were taken with metallic carrot (20cm length) previously sterilized, stored at 4°C and transported immediately to laboratory [22].

2.2 Vegetable material

In the present work, tomato seeds (*Lycopersicum esculentum Mill*) are investigated for *in vitro* and *in vivo* inoculation tests. Seeds were provided by the Algerian society PROFERT (Production-Fertilization), originated from seeds Voltz France, with a germination rate (96%) and 99% of purity.

2.3 Experimental Design and Plant Culture

2.3.1 Preliminary germination test

The phosphate saltsolution (PBS) was prepared, sterilized and distributed in 11sterilized flasks (samples and control), in ratio of 90ml/flask. 10 g of each soilsamplewere added to each flask; the solution was shaken for 10min for homogenization, and then left to pause.

2.3.2 Tomato seeds sterilization

Tomato seeds were surface sterilized according to the protocol of (Gtz et al.2006) with some modifications. Firstly, by washing for 1 min with 70% ethanol with kind agitation, followed by 15 min treatment in sodium hypochlorite (12%), ending by six subsequent rinsing steps with sterile distilled water for at least 15 min.

2.3.3 Inoculation of seeds with the bacterial strains

The surface sterilized seeds were added to each PBS-soil solution in a ratio of 5 seeds/bottle. The mixture was left 20 min for shaking. A control containing 5 seeds suspended in 90 ml of PBS without soil was prepared in parallel [7].

2.3.4 Flasks preparing

11 glass bottles (250ml), each one containing a thin layer of PCA (Plate Count Agar) medium saddle with card cotton and aluminum paper are autoclaved (120°C/20 min). After agar solidification, the surface sterilized seeds were put on the agar layer. All the flasks were incubated in darkness, at room temperature/7 days. The germination rate was measured every day and results were obtained according to the following equation: % G= (NG/ 5) X 100, where; NG is the number of germinated seeds [7]. Serial dilution was performed up to$10^{-3}$for positives samples, and an aliquot of 1 ml of this suspension was spread on the plates containing different media: PCA, NF-b, Jensen, YMA, M2. Plates were incubated at 30°C / 24- 72h. The positive flasks will be used in the second test. So every typical bacterial colony was picked up and re-streaked in the same fresh agar plate cited before, and used for the second germination test. Every flask was treated thrice and put in darkness at room temperature; the germination rate was compared to the control. Isolates showing good results comparing to the control will be used as an inoculants of tomato seeds in pots under greenhouse.

2.4 Impact of bacterial strains on inoculated seeds under greenhouse

In order to test the effect of isolates inoculation on tomato stem length; pots (1.5L volume) were previously sterilized and filled up with agriculture sieved soil (pH H_2O=8.60, pH KCL= 7.70, humidity H%= 7.70 %). 5 sterilized tomato seeds were sown in each plastic pot in 1cm depth. 10 ml of each bacterial solution was added respectively to each lot treated. Seeds were watered every day with nearby water under natural conditions of light/darkness. After germination only one plant was kept by pot [23].

2.5 Characterization and molecular identification of strains

Bacterial strains showing a positive effect on seeds germination, growth of tomato are characterized and identified by using standard morphological, physiological, and biochemical tests completed with a phylo-genetic identification based on rRNA 16S

sequencing. The biochemical identification was tested on API50CHB/E gallery. The phylo-genetic characterization was done as described by cite31. The different steps were performed in Resbiog Laboratory, Faculty of Pharmacy, Seville University (Spain).

2.6 Plant Growth Promoting Characters

2.6.1 Enzymatic activities

Many enzymatic activities were tested: a cellulase was revealed using the method of [6]. Lipase and esterase activities were tested following Sierra (1957), modified by [6]. A positive activity was detected by apparition of transparent halo around the colonies. The protease activity of the strain was also tested according to [4]. The medium contains a milk-casein as unique carbon source, then, bacteria degrading casein shows a clear zone around the colonies. For the soluble starch decomposition, a test of amylase was carried out following to [35]. The medium was inoculated by the bacterial strain by putting an agar cylinder (5 mm ϕ) on the surface. After incubation, a lugol solution previously prepared was added at the surface. Starch hydrolyzing shows a clear zone around the cylinders. Concerning phosphatase activity the method of [27] was monitored on YED-P medium. A positive activity generates a transparent halo around the colonies. The urease activity was also tested using the protocol of Christensen (1946).

2.6.2 IAA production

To reveal the ability of the strains to produce the indol acetic acid phyto-hormone-auxins) This test was performed using the method of [26], using the NF-b minimal liquid medium (K_2HPO_4 -6g; KH_2PO_4 - 4g; $MgSO_4$,$7H_2O$- 0.2g; NaCl 0.1g $CaCl_2$- 0.02g; $FeCl_3$ - 0.01g ; Na_2MoO_4 - 0.002g ; yeast extract - 0.05g ; pH-7,1 ; glucose 10% per liter),0.5mg/ml of tryptophan solution was added.
Strains were also tested for two activities implicated in the biological control; the Chitinase activity, according to the protocol of [16], and the Siderophores production revealed by Chrome Azurol S (CAS) assay (Schwyn and Neilands, 1986).

2.7 Effect of bacterial strain and some tomatos phytopathogenic fungi

Five tomatos phytopathogenic fungi of the collection of (Resbiog Laboratory: faculty of pharmacy, university of Seville) were used: *Verticillium dahliae (Vd), Fusarium oxysporum (Fo), Phytophthora cinamomi (Pci), Phytophthora cactorum (Pca), Botrytis cinerea (Bc)*.

The effect of bacterial strains on fungi was studied on TSA medium (Tryptic Soy agar) following to [30]. In each TSA plate, three cylinders containing each one a different colony were deposed at equidistance (1cm) to the Petri side, and one fungal strain slide (4 mm diameter) was placed in the middle of the plate. Petri plates containing each one a fungal strain were prepared to serve as controls. Plates and are incubated at 25 $\pm$2°C/5-7 day. A mycelium growth rate was noted. The apparition of inhibition zone was mentioned every day comparing to the control. Each test was repeated thrice, and the growth percentage inhibition (PGI %) was calculated according to the following formula: PGI%= KR-R_1/KR_x 100: KR: is the distance in mm covered by the fungi in the control plate, and R_1: the distance in mm between the fungi and bacterial colony in the plates.

2.8 Statistical analysis

Seeds germination rate data, stem length of tomato plants and PGI% recorded, were statistically analyzed by ANOVA method, using Fischers test (LSD) at interval of 95% ($p \leq 0.05$).

3 RESULTS

3.1 Preliminary germination test

Four soil samples among the 10 tested: E_3, E_9, E_{10}, E_{11} showed a stimulator effect on tomato germination seeds comparing to the control. This result could be explained by a probable presence of bacteria possessing a PGPR effect in these four samples. After successive dilutions followed by inoculation on different agar media, forty pure colonies were been distinguished (coded S1 to S40).

3.2 Second in vitro germination test

After seven days of incubation in darkness at room temperature, the percentage of germination was calculated for each flask, the results obtained after statistical analysis ($P \leq 0.05$), showed that 20 strains among the 40 tested had a percentage of germination exceeding 40% (value recorded to the control) (Fig 1).

3.3 Growth of inoculated seeds under greenhouse

Results showed that among the 20 strains, there are 4 strains with the best effect on tomato stem length ($p \leq 0.05$), comparing to the control not inoculated. The strains are: S11 S06 - S15 and S12, with respective length values of: 9.212 cm$\pm$ 1.706, 9.280 cm$\pm$ 2.709, 9.362 cm$\pm$ 1.618 and9, 858 cm$\pm$1,369, comparing to 6.400 cm$\pm$ 0.894 for the control (Fig.2).

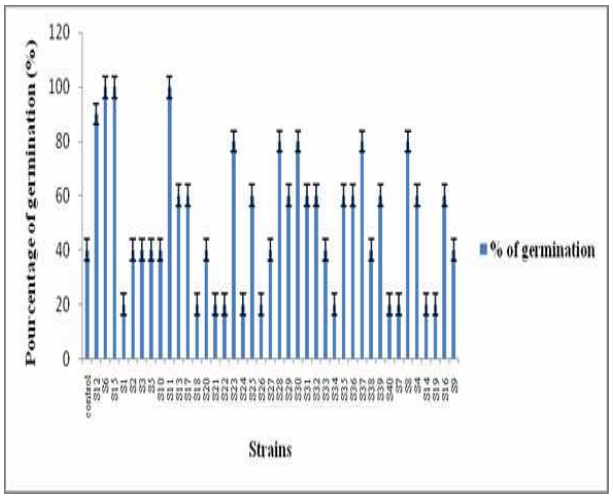

Fig. 1: Positive effect of the 20 strains on germination percentages of tomato seeds

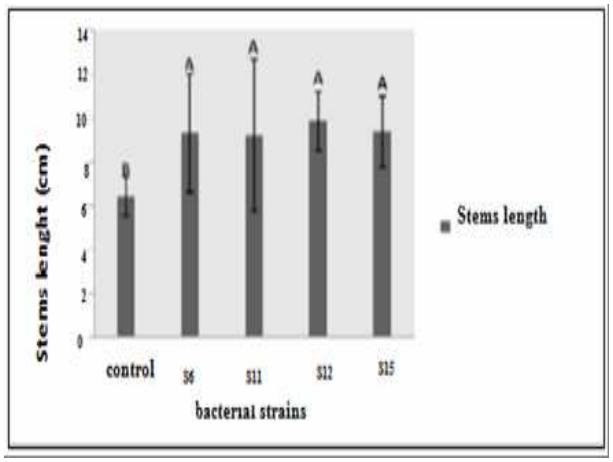

Fig. 2: Stems length of the control and inoculated tomato under greenhouse.

Letters (A, B) correspond to the homogenous groups obtained by Fisher LSD test and ANOVA, ($p \leq 0, 05$) analysis.

3.4 Strain identification

3.4.1 Biochemical and phylo-genetic identifications

Results of rRNA 16S sequenced and analyzed revealed that the strain S12 is very close to the strain *Mycobacterium Sp*. with a similarity of 98%. For S15, its very similar to the strain *Bacillus Sp* 13 (97.9%). Concerning, S06, it shows a high similarity with the strain *Arthrobacter agilis* (98.9%), finally, S11 presents a high resemblance with *Streptomyces Sp*, (99%). These results corroborate with the biochemical characters obtained on gallery API 50 CHB/E

3.4.2 Beneficial enzymatic activities

All the results are summarized in Table 1. It is noted that each strain among the four tested have at least five positive activities, but it appears clearly that the strain S12 (*Mycobacterium Sp*) is very rich with enzymatic equipment.

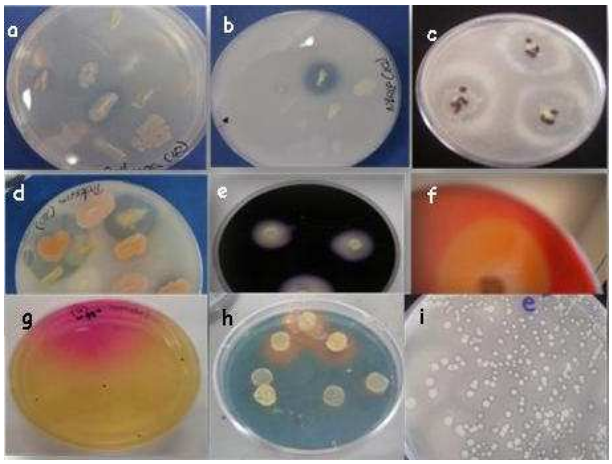

Fig. 3: Enzymatic activities

a:chitinase; b: phosphatase; c:lipase ; d:protease ; e:anylase ; f:cellulose ; g:urease ; h:siderophores ; i:esterase

3.4.3 IAA production

Results showed that the strain S15 produced the highest IAA concentration (190.235mg/l), followed by S12 (93.960mg/l), then S06 (86.901 mg/ml), finally, S11 with 38.470 mg/l. These concentrations could be the major plants growth promoting factors.

3.4.4 Antagonism

The effect of the strains on the fungal growth inhibition is presented on (Fig.5). *Streptomyces Sp* inhibited all the pathogens with percentages inhibition (PGI %) included between 29.62% and 74.81%, with an average of 55.99%. The *Mycobacterium Sp*, *Bacillus Sp* 13 and *Arthrobacter agilis* inhibited four pathogens among the five tested with PGI% values going from 0-69.62%; 0-78.51%; 0-72.60% with averages of 43.43%; 54.66%; and 37.33%, respectively (Fig4-a).

Very attractive inhibition zones were obtained (Fig5); showing that the selected strain have an antagonistic effect against the phytopathogenic fungi, which could be explained by the presence several enzymes (Table 1).

Table 1: Results of the different applied activities on the four strains.

Strains	Phosphatase	Cellulase	Esterase	Lipase	Protease	Chitinase	Amylase	Urease	Siderophore	NF-b
S06	+	-	+	-	-	+	+	+	+	+++
S12	+	-	+	+	+	+	+	+	+	+++
S15	+	+	+	+	+	+	+	-	+	+++
S11	+	-	+	-	+	-	+	+	-	+++

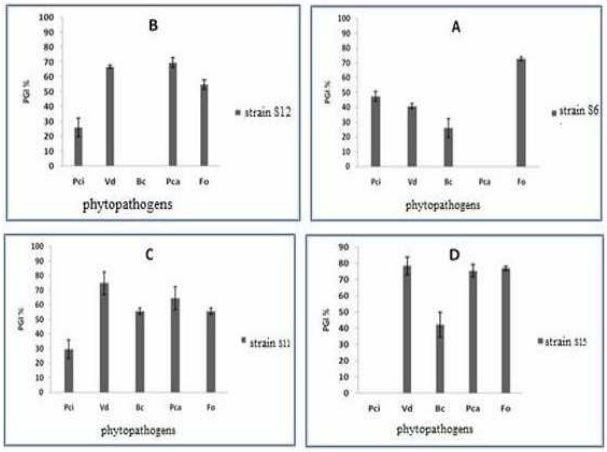

Fig. 4: Mycelium growth inhibition rates of phytopathogens in presence of S06, S12, S11 and S15strains.

A: S06; B: S11; C: S12; D: S15; F: strains together. Letters (A, B, C) correspond to homogeneous groups obtained by ANOVA analysis with fisher LSD test ($p \leq 0{,}05$). *Verticillium dahliae (Vd), Fusarium oxysporum (Fo), Phytophthora cinamomi (Pci), Phytophthora cactorum (Pca), Botrytis cinerea (Bc)*

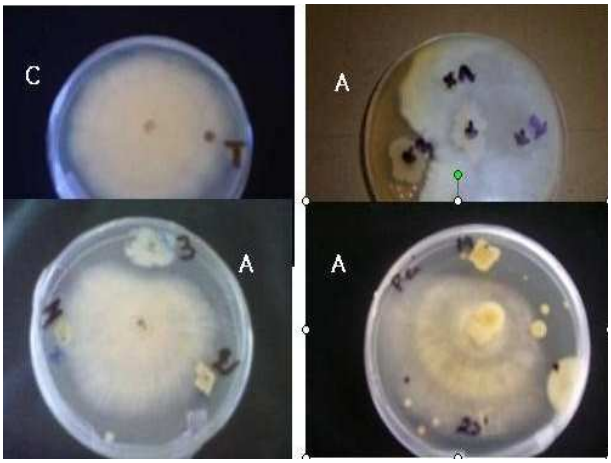

Fig. 5: Positives antagonism results showing clearly the inhibition zones

C : Control: *Verticillium dahliae* without bacteria. A: presence of *Mycobaterium Sp+ Verticillium dahliae*

4 DISCUSSION

The results of the work led to select 4 strains with good effect on tomato stem growth, these strains belonged either to Actinomycetes group or *Bacillus* genus. So, our results corroborate with literature stipulating that *Bacillus* and Actinomycetes are very abundant in rhizosphere of many plants [10].

Auxin production and growth stimulation are concomitant [34], but many studies reported that bacteria have also the aptitude to degrade this phyto-hormone [21]. In spite of the high stem length values recorded by *Mycobacterium Sp*, this strain produces less than Bacillus *Sp13* (table 1). [15] explained this decreasing in IAA concentration by synthesis of enzymes like; IAA oxidase, and IAA peroxidase.

In addition to antibiotic synthesis, actinomycetes are produced various enzymes like: protease, chitinase, cellulase, and amylase involved in complex organic matter degradation in soil. ([25,32].
In table 1, among the 3 actinomycetes, *Mycobacterium Sp* shows seven 7 positive enzymatic activities, in addition to siderophores production and nitrogen fixating (good growth on NF-b medium). [13] demonstrated that the strain *Mycobacterium flavum* 301 fix nitrogen. This could explain the best stem length obtained in presence of *Mycobacterium Sp* comparing to others strains.
Phosphate solubilization is widely involved in plant growth stimulation. [3]. Also, protease activity could have more advanced effect, it influences the auxin synthesis by releasing of amino-acids such as tryptophane which is the precursor of IAA synthesis and other related substances [19]. Urease is produced by the three strains of actinomycetes, this enzyme was considered as soil quality indicator, because of its relationship with organic matter content [20].
Its important to specify that a good bio-control agent must include a good persistence degree and aggressiveness, and, in parallel, not pathogen for the host plant.
The antagonistic effect of bacteria can be attributed to lipase, protease, and chitinase activities which can act synergistically [29]. Besides, the inhibitory effect of actinomycetes against phytopathogenic fungi *Fusarium sp.*, *Phytophtora sp.*,and*Verticillium sp.*, had been patented [9]. This antagonistic activity can be also attributed to siderophores synthesis in iron restricted

conditions by the bio-control agents against phytopathogenic fungi of tomato (Tri Wahyudiet al., 2011).

Many studies confirmed the antagonistic effect of actinomycetes chitinase positive, which can explain the effect of the two strains; *Arthrobacter agilis* and *Mycobacterium Sp.* Microbial chitinases are more efficient when combined with other mechanisms [14]. Thus, our results concord with these work, because all the strains possessed many activities in parallel. [21] showed that plant growth stimulation by bacteria is not only depended on IAA quantity produced, but roots colonization must be taken place in the same time. This can explain the tomato growth stimulation by *Mycobacterium Sp*, an endophytic bacterium isolated from the roots vascular tissues (Deljou et al., 2010).

The application of the two genera will be interesting for the inhibition of tomatos phytopathogens. Because of it rich in different enzymes benefic for soil fertilization, and stimulation of germination and growth of tomato, may be du to IAA production, phosphate solubilization and iron fixation. In addition, Nitrogen fixing which is the main parameter in plant growth promoting bacteria. Therefore, the four Algerians bacterial strains appear more compatible with tomato growth, showing a highest stem length values.

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