**Bacillus amyloliquefaciens** as a Biocontrol Agent Improves the Management of Charcoal Root Rot in Melon

E. Bakhshi¹, N. Safaie¹*, and M. Shams-Bakhsh¹

**ABSTRACT**

Seventy five bacterial strains were isolated from cucurbits rhizosphere and examined for antagonistic activities against *Macrophomina phaseolina* isolate 44, the causal agent of melon charcoal root rot disease. The results of screening strains including B2, B11, B12 and BKN showed high potential of antagonistic activities against the pathogen in laboratory experiments. Inhibition of mycelial growth varied from 54.3 to 62.22%, 39.43 to 54.82%, 78.52 to 100%, and 64.45 to 88.89% in dual culture, volatile metabolite, antibiotic production, and cell free culture tests, respectively. In greenhouse experiment, seed treatment with strain B2 significantly (P= 0.01) controlled the disease by increasing plant growth indices including height, shoot and root fresh weight, shoot and root dry weight by 37.98, 36.27, 32.97, 34.44, and 30.39%, respectively, as compared to the control. The biochemical and physiological tests as well as gyrA sequence confirmed these four strains as *Bacillus amyloliquefaciens*. Results indicated that strain B2 could be an important new biological control agent for charcoal root rot disease of melon.

**Keywords:** Antagonistic activity, Biological control, Cucurbits *Macrophomina phaseolina*, Rhizosphere.

**INTRODUCTION**

Melon (*Cucumis melo* L.) is one of the most popular cucurbits in the world. It is the third most important vegetable crop next to tomato and onion (Ghebretinsae *et al*., 2007). This crop is susceptible to a large number of disease agents such as seedling and root pathogens that cause serious damages to this plant. One of these soilborne pathogens is *Macrophomina phaseolina* (Tassi) Goid, the causal agent of charcoal root rot. This polyphagous pathogen attacks and infects more than 500 plant species in 100 families of monocots and dicots (Jana *et al*., 2003). It is a major disease in dry regions of Asia, Africa, the Americas, and Australia. Germination of sclerotia of *M. phaseolina* might be triggered by root exudates from melon seedlings (Smith, 1969). The germinated sclerotia can infect the primary root and cause seedling blight (Livingston, 1945). If infection occurs before the emergence of secondary roots, the plants die. Less severely infected seedlings, however, survive and establish secondary roots and grow to mature plants. *M. phaseolina* infection leads to rotting of roots followed by rotting of stalks, resulting in lodging of the plant at later stages (Partridge *et al*., 1984).

Protection of root from infection of the fungus with microbial inoculants is targeted for eco-safe management of this disease (Spadaro and Gullino, 2004; Droby *et al*., 2009). The use of microorganisms or their secondary metabolites to prevent plant diseases are environment friendly, safe, and may provide long-term protection to the crop (San Lang *et al*., 2002; Fernando *et al*., 2003).
Many bacteria such as *Bacillus amyloliquefaciens* (Arguelles-Arias et al., 2009; Arrebola et al., 2010), *B. subtilis* (Obagwu and Korsten, 2003; Romero et al., 2007), *Pantoea agglomerans* (Usall et al., 2008; Yu et al., 2010), *Pseudomonas fluorescens* (Mikani et al., 2008) and *Serratia plymuthica* (Meziane et al., 2006) have been reported as effective biological agents against root rot pathogens of cucurbits. Among these, *Bacillus* spp. have been studied as one of the excellent biocontrol agents against plant pathogens. They produce a wide range of antimicrobial cyclic lipopeptides (iturins, fengycins and surfactins), enzymes (chitinase and β-1,3-glucanase), and antifungal Volatile Organic Compounds (VOCs), which inhibit growth of plant pathogens (Arrebola et al., 2010).

In this study, we aimed to isolate rhizobacterial isolates belonging to genus *Bacillus* from cucurbits fields with the objective of obtaining the efficient strains representing high disease control performance against charcoal root rot in vitro and greenhouse conditions.

**MATERIALS AND METHODS**

**Fungal Pathogen and Antagonistic Bacteria**

*Macrophomina phaseolina* isolate 44 (Mph44) was obtained from Plant Pathology Department of Tarbiat Modares University, Tehran, Iran. This isolate had been proved to be highly virulent in previous pathogenicity test.

All the bacteria isolated from the rhizosphere of cucurbit fields from Tehran province (Moradabad and Palaein villages) in 2013. Serial dilutions from collected soil samples were prepared and spread on Nutrient Agar (NA) medium. Petri dishes were incubated at 27°C for 48 hours. All bacterial colonies were purified and maintained at 4°C.

**Investigation of Antagonistic Mechanisms In Vitro**

For selection of bacterial antagonists, antagonistic activity of bacterial strains was tested against Mph44 using preliminary dual culture procedure (Dennis and Webster, 1971). Four bacterial strains (10⁹ CFU mL⁻¹) were placed on Petri dishes (9 cm diameter) at equal distances from each other and around the periphery of the mycelial plug (5 mm diameter). In control plates, only the pathogenic fungus was grown. When control plates were completely covered by the fungus, the experiment was stopped and the bacterial antagonists were selected based on their ability to produce inhibition zone against *M. phaseolina* using the Equation (1):

\[ IG = \frac{(C-T)}{C} \times 100 \]

Where, *IG*: Percent of Inhibition Growth; *C*: Growth in Control (radial growth of fungus in control); *T*: Growth in Treatment (radial growth in treatment or dual culture).

After that, volatile metabolites production test for antagonistic bacteria was performed following Fernando et al. (2005) procedure. One hundred-microliter suspension of each antagonistic bacterial strain (10⁹ CFU mL⁻¹) was smeared on fresh Nutrient Agar (NA). Sterile distilled water was used as a control. A 3-day-old PDA plug (5 mm diameter) of *M. phaseolina* with mycelium and microsclerotia was placed in the center of PDA medium. Dishes containing fungal pathogen were placed inverted on NA medium dishes containing bacterial strains and were sealed with parafilm. Petri dishes were maintained in dark at 28°C for three days. Inhibition percent of fungal mycelial growth was calculated using the Equation (1).

Production of antibiotics by bacterial antagonists was determined using Kraus and Lopper (1990) method. One hundred-microliter suspension of each antagonistic bacterial strain (10⁹ CFU mL⁻¹) was cultured on fresh PDA. The Petri dishes were incubated in dark at 26°C for 72 hours. After
three days of bacterial growth, the colonies were wiped off by sterile cotton swap and the plates were exposed to Chloroform vapor and UV light for two hours and air flow under sterile condition for 30 minutes, in sequence. Sterile distilled water was used as a control. Then, a 3-day-old PDA plug (5 mm diameter) of *M. phaseolina* with mycelium and microsclerotia was placed in the center of Petri dishes. The Petri dishes were incubated at 28°C for three days. The percentage of pathogen growth inhibition was calculated by Equation (1).

Bacterial cell-free supernatant assay (Singh and Deverall, 1984) was done to select the best effective antagonistic bacteria. To study the effects of the cell-free supernatant on radial growth of fungus, the cell-free supernatant was added to autoclaved PDA medium to a final concentration of 25% (v/v), just before pouring the plate. After the medium was set, a 5 mm diameter disc from an actively growing fungus was inoculated at the center of the plate. In the control plate, the cell-free supernatant was substituted with 25% (v/v) sterile Nutrient Broth (NB). The plates were incubated at 28°C. Radius of the fungal colony was measured after 5 days.

All the experiments were performed with three treatments each with three replications in a Completely Randomized Design (CRD).

### Identification of Bacterial Antagonists

The isolates were characterized based on their morphological, physiological, and biochemical characteristics (Schaad *et al.*, 2001). The four best bacterial isolates were identified in detail through *gyrA* analysis (Roberts *et al.*, 1994).

The *gyrA* sequences of four isolates were used to elucidate the phylogenetic position of the strains by comparing these sequences with published sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed with MEGA software (v. 4.0).

### In Vivo Assays

#### Seed Coating Formulation

Bacterial suspensions were prepared from cells grown in shaking incubator at 27°C and 160 rpm in Luria Bertani (LB) broth. The bacterial cells were harvested by centrifugation at 5,000 rpm for 5 minutes. The pellet was re-suspended in sterile Distilled Water (DW) to adjust the bacterial suspension to 10⁹ cells mL⁻¹ (Kim *et al.*, 1997) and mixed with equal volume of 1% CarboxyMethylCellulose (CMC) and added to disinfested seed. Then, bacterized seeds were transferred to dry sterilized filter paper and allowed to dry in laminar flow for 8 hours. The initial population of bacterial cells on the seed was about 10⁷-10¹⁰ colony per seed. Shadegan cultivar of melon was used in this experiment. Carboxin thiram was used as effective fungicide for chemical control of charcoal root rot by seed treatment at rate of 0.15% (w/w).

#### Greenhouse Experiments

In greenhouse examinations, the effectiveness of bacterial antagonists and carboxin thiram fungicide was evaluated on inhibition of pathogen and promotion of melon growth indices. Plastic pots were filled with pasteurized field soils, perlite, and peat moss (1:1:1 ratio). Field soil was sterilized by autoclaving two times. Inoculums of fungal pathogen were prepared by growing them on millet grains in 100 mL flasks as follow: the grains were soaked in distilled water, autoclaved twice (121°C for 45 minutes) and inoculated with three agar discs (5 mm diameter) of 5-day-old fungal culture. Flasks were incubated at 28°C in dark for 21 days. The inoculums were then mixed with soil (10 g kg⁻¹ soil) (Etebarian *et al.*, 2000). Four treated melon seeds were
planted to each pot and the pots were kept at 25-33°C and allowed to grow for 60 days. Pots were watered every other day until seedling emergence and daily thereafter. There were three groups of treatments with three replications for each treatment, arranged in a completely randomized design. Treatments included: (1) Pathogen (as control), (2) *B. amyloliquefaciens*+pathogen, (3) Carboxinthiram+pathogen. The experiment was carried out in a glasshouse at day/night temperatures of 32/25°C with natural day light without supplementary lighting from September to December 2013, in Tehran. The effect of tested antagonist on plant growth was measured in terms of plant height. Thereafter, at the end of experiment, the above ground parts of plants were cut into small pieces and shoot fresh weight was recorded, then, those parts were dried at 60°C for three days and dry weight of shoot pieces was recorded. Moreover, Growth Promotion Efficacy (GPE) was calculated to clarify the relative effect of tested antagonist on plant biomass according to the following Equation (2):

\[
\text{GPE} \, (\%) = \frac{G_T - G_C}{G_C} \times 100
\]  

(2)

Where, GPE refers to Growth Promotion Efficacy, \(G_T\) refers to Growth parameter in antagonist-Treated group, and \(G_C\) refers to Growth parameter in the Control group.

Antagonist Population Dynamics in Melon Rhizosphere

*Bacillus* rif1, the spontaneous mutant of *B. amyloliquefaciens* B2, was obtained by plating the cell suspension (1×10⁹ CFU mL⁻¹) on NA medium containing different concentrations of rifampicin (1, 5, 10, 20, 35, 50, 100 and 150 µg mL⁻¹). The plates were incubated at 28°C for 72 hours and mutant colonies were picked up. The mutants were evaluated for stability of resistance by sub-culturing 10 times on NA+rifampicin (100 µg mL⁻¹). Population dynamics of B2 strain was examined on roots at 7, 14, 21, 28, 35, 42, 49 and 56 days after sowing. Root sampling was done by uprooting seedlings, and each root piece was suspended in water. The suspension was incubated at 30°C for 1 hour, on a rotary shaker at 150 rpm. After serial dilution, the suspension was plated on NA medium with rifampicin (100 µg mL⁻¹). Inoculated plates were incubated at 28°C for 48 hours. The observed colonies were counted and expressed as log₁₀ CFU cm⁻¹ root. All experiments were performed in three replications. Plants treated by sterile water were used as control.

Statistical Analysis

For statistical analysis, data were subjected to Analysis Of Variances (ANOVA) and the means were compared by Duncan’s Multiple Range Test (DMRT) using SPSS statistical software (version 16.0). Level of significance in different treatments was determined at 1% probability (Little and Hills, 1978).

RESULTS

In Vitro Assays

Seventy five bacterial strains, isolated from soil samples, were purified. Among them, four isolates (B2, B11, B12 and BKN) showed more than 50% growth inhibition (54.3 to 62.22%) in dual culture test. The VOCs from the antagonists produced 39.43 to 54.82% inhibition of mycelial growth of *Mph44*. The inhibition rates of mycelial growth were 78.52 to 100% in antibiotic production test. The cell-free supernatant at 25% dilution inhibited mycelial growth of *Mph44* from 64.45 to 88.89% (Figure 1, Table 1).

Identification of Bacterial Antagonists

The strains which were isolated from the rhizosphere soil of cucurbits were finally selected as promising candidates and
identifying as *Bacillus* sp. by results of morphological, physiological, and biochemical characteristics. They were gram positive, rod shaped, motile, aerobic growth and able to form spores. All strains gave positive reaction for catalase test and starch hydrolysis. Moreover, they were positive for utilization of lactic acid and D-sorbitol. The results of biochemical and physiological test were therefore guidable to discriminate the isolates from each other.

Partial gyrA nucleotide sequences of the test strains were deposited in Genbank database under accession numbers KJ136127, KJ829646, KJ829647, and KJ829648. The phylogenetic tree based on the Maximum Likelihood analysis of the gyrA sequences is given in Figure 2. Based on gyrA analysis, these strains (*Bacillus* B2, B11, B12 and BKN) were identified as *B. amyloliquefaciens* (Figure 2).

**In Vivo Assays**

**Greenhouse Experiments**

Analysis of greenhouse results showed that there was significant difference (at 1% level in Duncan test) among treatments in increasing melon growth indices and inhibition of charcoal root rot causal agent. In presence of pathogen, *B. amyloliquefaciens* B2 was the most effective antagonist against Mph44 and increased melon shoot and root fresh weights by 36.27 and 32.97%, shoot and root dry weight by 34.44 and 30.39%, respectively, in comparison with the control i.e. only

**Table 1.** Results of *in vitro* assays.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Region</th>
<th>Dual culture (%)</th>
<th>VOCs (%)</th>
<th>Production of antibiotics (%)</th>
<th>Supernatant assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B2</td>
<td>Tehran province</td>
<td>62.22</td>
<td>54.82</td>
<td>100</td>
<td>88.89</td>
</tr>
<tr>
<td>2. B11</td>
<td>Tehran province</td>
<td>58.89</td>
<td>39.43</td>
<td>100</td>
<td>79.26</td>
</tr>
<tr>
<td>3. B12</td>
<td>Tehran province</td>
<td>54.3</td>
<td>50.52</td>
<td>100</td>
<td>82.22</td>
</tr>
<tr>
<td>4. BKN</td>
<td>Tehran province</td>
<td>56.15</td>
<td>40</td>
<td>78.52</td>
<td>64.45</td>
</tr>
</tbody>
</table>

**Figure 1.** Dual culture (a), volatile compound (b), antibiotic production (c) and extra cellular liquid compound tests. In each picture, the left plate is control (*Macrophomina phaseolina* isolate 44) and the right plate is treatment (Mph44+*Bacillus amyloliquefaciens* B2).
pathogen was added. Carboxin thiram controlled the disease by increasing melon shoot and root fresh weights 89.55 and 91.26%, shoot and root dry weight 87.78 and 90.48%, respectively, in comparison with the control i.e. only pathogen was added (Table 2).

**Antagonist Population Dynamics in Melon Rhizosphere**

Survival of introduced biocontrol agents in the rhizosphere is of primary importance for biological control of plant diseases. Evaluation of the population dynamic of the *B. amyloliquefaciens* strain B2 using the rifampicin antibiotic resistance marker revealed that all the three isolates could proliferate successfully in the rhizosphere of melon, both at 30 and 60 days after seeding. The population of *Bacillus* had increasing growth from 7 to 56 days after inoculation (Figure 3).

**DISCUSSION**

Soil-borne plant pathogens such as bacteria, fungi, and nematodes annually cause major economic losses on melon plants. *Macrophomina* charcoal root rot on melon is one of the most important diseases. The lack of chemical fungicides against *M. phaseolina* and the environmental contaminations by chemicals has encouraged researchers around the world to have an increasing interest in biocontrol agents. Rhizosphere microorganisms provide primary defense line against
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Table 2. Effects of Bacillus amyloliquefaciens strains and carboxin thiram fungicide in combination with Macrophomina phaseolina isolate 44 on melon growth indices in pasteurized soil greenhouse 60 days after planting.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H (cm)</th>
<th>SFW (g)</th>
<th>RFW (g)</th>
<th>SDW (g)</th>
<th>RDW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(B2)+Seed+CMC+P</td>
<td>59.33 b</td>
<td>13.43 b</td>
<td>2.43 b</td>
<td>4.03 b</td>
<td>1.91 b</td>
</tr>
<tr>
<td>A(B11)+Seed+CMC+P</td>
<td>51.66 d</td>
<td>11.37 e</td>
<td>2.1 bc</td>
<td>3.45 d</td>
<td>1.66 e</td>
</tr>
<tr>
<td>A(B12)+Seed+CMC+P</td>
<td>55.33 c</td>
<td>12.33 c</td>
<td>2.28 b</td>
<td>3.67 c</td>
<td>1.80 c</td>
</tr>
<tr>
<td>A(BKN)+Seed+CMC+P</td>
<td>53.5 cd</td>
<td>11.88 d</td>
<td>2.15 b</td>
<td>3.60 cd</td>
<td>1.72 d</td>
</tr>
<tr>
<td>P+Seed+CMC (As control)</td>
<td>43 e</td>
<td>9.86 f</td>
<td>1.83 d</td>
<td>2.99 e</td>
<td>1.46 e</td>
</tr>
<tr>
<td>P+Seed</td>
<td>42.33 e</td>
<td>9.82 f</td>
<td>1.89 d</td>
<td>3.01 e</td>
<td>1.49 e</td>
</tr>
<tr>
<td>P+Fungicide*</td>
<td>79.33 a</td>
<td>18.69 a</td>
<td>4.30 a</td>
<td>6.63 a</td>
<td>3.80 a</td>
</tr>
</tbody>
</table>

* Means with different letters in each column are significantly different in Duncan test (P= 0.01). Data are the means of three replicates. H: Height; SFW: Shoot Fresh Weight; RFW: Root Fresh Weight; SDW: Shoot Dry Weight; RDW: Root Dry Weight; A: Antagonist; P: Pathogen; CMC: CarboxyMethylCellulose. 

Results showed that 0.15% concentration of carboxin thiram fungicide inhibited the growth of M. phaseolina by 100% in vitro, so, this concentration of carboxinthiram was used in greenhouse experiment.

Figure 3. Increasing growth of Bacillus amyloliquefaciens B2<sup>vir</sup> in melon rhizosphere.

pathogen attacks (Weller, 1988). The predominantly reported biocontrol agents for managing M. phaseolina include mycoparasitic fungi and bacteria such as Trichoderma spp., Bacillus spp., and Pseudomonas spp. (Gupta et al., 2002; Singh et al., 2008). In this study, we evaluated antagonistic effects of cucurbits rhizobacteria against Macrophomina charcoal rot on melon. High reduction of pathogen growth in in vitro tests was observed by all antagonists. We selected four potential biocontrol agents of B. amyloliquefaciens strains, namely, B2, B11, B12, and BKN to suppress the disease in greenhouse condition. Dual culture (Dennis and Webster, 1971), antibiotic production (Kraus and Loper, 1992), cell free culture (Weller et al., 1988) and volatile metabolite test (Fiddaman and Rossall, 1993) were used to observe the effect of Bacillus isolates on Macrophomina phaseolina isolate 44. These results were similar with previous studies reporting the antagonistic activity of B. subtilis (Pinchuk et al., 2002) and B. amyloliquefaciens (Liu et al., 2008; Yoshida...
et al., 2001) against plant pathogenic bacteria and fungi. The inhibitory effects of antagonists’ metabolites have been reported in various studies (Pal et al., 2001; Monte and Liobell, 2003). B. amyloliquefaciens has been reported to be effective in controlling plant pathogens by the production of antibiotics (Alvindia and Natsuaki, 2009; Chen et al., 2009) and VOCs (Koumoutsi et al., 2004; Romero et al., 2007; Arguelles-Arias et al., 2009). Arrebola et al. (2010) identified eight different VOCs from B. amyloliquefaciens PPCB004 and indicated that the in vitro radial mycelial growth of Penicillium crustosum, P. digitatum and P. italicum was inhibited by over 50, 30, and 25%, respectively, in the presence of VOCs of B. amyloliquefaciens PPCB004. Knowledge on the mechanisms of action involved in the biocontrol process could permit establishment of optimum conditions for the interaction between the pathogen and the biocontrol agent and is important for developing additional means and procedures in order to obtain better results from known antagonists. In addition, understanding the mechanism of biocontrol will help in selecting more effective and desirable strains of antagonists (Sharma et al., 2009).

In in vivo assays, heating of soils by autoclaving usually allows the pathogen to proliferate (Burgess et al., 1988). In greenhouse experiments, B. amyloliquefaciens B2 was the most effective antagonist in inhibition of Macrophomina charcoal rot and increased plant growth indices. Our greenhouse experiments showed that, in the presence of pathogen, all antagonists controlled the melon charcoal rot disease. It was determined that direct interaction of antagonist B. amyloliquefaciens isolate B2 with melon seeds increased height, shoot and root fresh weight, and shoot and root dry weight indices by 37.98, 36.27, 32.97, 34.44, and 30.39%, respectively. Ability of the four tested isolates to inhibit the growth of soil borne fungi means these isolates have several mechanisms to suppress plant pathogenic microorganisms, and have a high chance to keep their activity for protection of host plants in greenhouse. Hao et al. (2011) reported that at 1x10⁸ CFU mL⁻¹ of B. amyloliquefaciens HF-01, the incidence of green and blue mold and sour rot were 16.32, 23.64 and 31.65%, respectively, while the control fruit had more than 90% decay.

In conclusion, the results of this study indicated that use of Bacillus isolates as complementary biocontrol method could be integrated with other means of control e.g. fungicides. B. amyloliquefaciens B2 reduced disease severity in glasshouse condition, but further fundamental, multicomponent research into the interaction between host, pathogen, antagonist, and environment is required. Further studies are, however, needed to check the isolates for their ability to suppress the growth of other soil-borne plant pathogenic fungi to increase their chance by offering sufficient protection to the host plant against wide spectrum of soil-borne pathogens and, also, to investigate the mode of action of these strains in terms of inducing systemic resistance and enhancing their antibiosis activity against plant pathogens, as well as confirming the antagonistic ability of these strains in field trials at different locations.

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به عنوان عامل بیوتولیکی، بیماری Bacillus amyloliquefaciens
پوسیدگی دمازده خشکه

چکیده

به منظور بررسی کنترل بیولوژیک بیماری بوسیدگی دمازده خشکه، ناشی از قارچ Macrophomina phaseolina، اثر آنتی‌گونیستی ۱۵ استرین باکتری ناحیه ریزوسفر، بررسی گردید. در شرایط آزمایشگاهی، گیاهان خشکه روز چندانی از جدایی ۴۴ نارنجی بیماری بوسیدگی M. phaseolina، اثر بیماری Bacillus BKN، با کشت در بناهای B2، B11 و B12 بیشتر به علت میزان پوسیدگی، خشکی و بافت خشکی جزئی به‌طور ۹۷/۳۹، ۲۷/۳۰، ۹۷/۳۷ و ۴۴/۳۴ درصد منجر به بود. در آزمایش‌های گیاه‌خانه‌ای، بیمار بذری استرین B2 نشانه‌هایی از خشکی و به‌طور ارتقاء، وزن نشان آنتی‌گونیستی اردک، وزن خشکی اندام هوا و ریشه و ریشه را به ترتیب ۹۷/۳۷، ۳۵/۳۰، ۹۷/۳۷ و ۴۴/۳۴ درصد در مقایسه با شاهد کنترل افزایش داد. آزمایش‌های بیوشیمیایی، فیزیولوژیکی و مولکولری با تأکید ناحیه gyrA تأیید نمودند که تمامی این ۴ استرین به‌طور گونه Bacillus amyloliquefaciens مهم جدیدی علیه قارچ عامل پوسیدگی دمازده خشکه در نظر گرفته شود.

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یک بیماری استرینی

Bacillus amyloliquefaciens

9.607