Antagonistic activity of bacterial endophytes against major soilborne pathogens of soybean

Brunda KS, Shamarao Jahagirdar and Kambrekar DN

Abstract
The antagonistic effect of 30 bacterial endophytes of soybean collected from northern Karnataka and parts of Maharashtra against Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium oxysporum were assayed in vitro through dual culture plate technique. The bacterial endophytes RB-KK-6 (40.78%), SB-BS-6 (50.08%) and LB-BU-1 (47.02%) were found effective against S. rolfsii and the isolates SB-DG-11 (47.41%), LB-BiN-8 (41.22%) were effective against R. bataticola. The effective bacterial endophytes against F. oxysporum were RB-HS-1 (41.99%), SB-BiJ-9 (40.07%), LB-BU-1 (54.20%) and LB-BV-2 (51.64%). Based on molecular characterization the effective bacterial endophytes were identified as Acinetobacter sp. (RB-HS-1), Alcaligenes faecalis (RB-KK-6), Sienotrophomonas sp. (SB-BiJ-9), Bacillus pumilus (SB-DG-11 & LB-BiN-8), Paenalcaligenes sp. (LB-BU-1), Bacillus cereus (SB-BS-6) and Brevibacillus sp. (LB-BV-2).

Keywords: Soybean, Bacterial endophyte, Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium oxysporum

1. Introduction
Soybean (Glycine max (L.) Merril) also known as ‘golden bean’ or ‘miracle bean’, is one of the premier agricultural crops in India. Soybean, with over 40 per cent proteins and 20 per cent oil has now been recognized as a potential supplementary source of edible oil and nutritional food. It is gaining popularity on account of its unique characteristics and adaptability to varied agro-climatic conditions. The unique chemical composition of soybean seed, which includes a number of nutraceutical compounds such as isoflavones, tocopherol and lecithin has made it one of the most valuable agronomic crops in the world. Despite having made rapid stride for both coverage and total production, soybean still suffers on the productivity front. Soil borne plant pathogens can significantly reduce the yield and quality of soybean. Some of these pathogens are particularly challenging because they often survive in soil for many years and attack the crop irrespective of its crop growth stage. Biological control in agriculture is gaining much attention when environment and soil health are taken into account. Endophytes might interact more closely with the host plant and therefore, could be more effective biocontrol agents in sustainable crop production and offer unique opportunity for crop protection and biological control.

Endophyte refers to fungi or bacteria which, during entire or part of their life cycle, invades the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of the disease [1]. Endophytes benefit the plant by promoting plant growth, improving resistance to multiple stress and offering protection from diseases and insects. Endophytic fungi are of biotechnological interest due to their ability to produce antibacterial, antiviral, antitumor, antidiabetic and immunosuppressive compounds that also act as biocontrol agents [2]. Inspite of the increased number of reports about the beneficial traits of endophytic microbes towards the crop plants protecting their host against pathogens and promotion of plant growth, there is a dearth of information regarding the use of different endophytic microorganisms for the management of soil borne fungal pathogens and growth promotion of soybean in India. India being a tropical country with great biodiversity offer more chances to chance the endophytes that are suitable to produce bioactive compounds. Hence, the present investigation was undertaken to tap the endophytic bacterial diversity of soybean and to screen them for the antagonistic activity against major soil borne pathogens.
2. Material and methods

Isolation of bacterial endophytes

Healthy soybean plants collected from major soybean growing areas of northern Karnataka viz., Belagavi, Bidar, Dharwad, Haveri and parts of Maharashtra viz., Kolhapur and Sangli were used for isolation as per the procedure suggested [3]. Roots, stems and leaves were washed to remove dirt and cut into sections. Surface sterilized sections were rinsed with 0.02 M potassium phosphate buffer three times (0.1 ml aliquot from the last wash was taken and transferred to Petri plate which served as sterility check). One gram of plant parts was macerated with nine ml of potassium phosphate buffer in pestle and mortar. Further serial dilution was made up to 10^6 dilution and were plated using the streak plate method. The plates were incubated for 48-72 h for the observation of the colonies and isolated colonies were picked up and streaked again on fresh nutrient agar plates for further purification.

**In vitro evaluation of bacterial endophytes**

Soil borne fungal pathogens viz., Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium oxysporum isolated from infected soybean plant samples were used for screening of the endophytes. Dual culture plate technique was adopted for testing the antagonistic activity of the endophytes against the soil borne pathogens on PDA plates. The fungal pathogen was inoculated at one side of Petri plate and bacterial endophyte was streaked on the opposite side of the plate. For this, actively growing cultures of both endophyte and pathogens were used. Petriplate inoculated only with the pathogen served as control. Per cent inhibition over control was worked out according to the formula [4]

\[ \text{I} = \frac{C - T}{C} \times 100 \]

Where,
- I = Per cent inhibition
- C = mycelial growth in control (mm)
- T = mycelial growth in treatment (mm)

Molecular characterization of the effective endophytes: The total genomic DNA from pure culture of the different isolates of bacteria were extracted by the CTAB (Cetyl Trimethyl Ammonium Bromide) method [5] with some modifications. PCR amplification of rDNA sequences were conducted by using the universal bacterial primers. Finally, the amplified products of the representative samples were sent for sequencing at Chromos Biotech Ltd., Bengaluru. The obtained sequence results were analyzed using Basic Local Alignment Search Tool (BLAST) algorithm available at http://www.ncbi.nlm.nih.gov.

3. Results and Discussion

A total of 30 bacterial endophytes (6 from root, 13 from stem and 11 from leaf) were obtained from 25 different locations of northern Karnataka and parts of Maharashtra. They were subjected for in vitro screening against major soil borne pathogens of soybean viz., Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium oxysporum for their antagonistic activity. A total of six bacterial root endophytes were evaluated against the three pathogens by dual culture technique and results are presented in Table 1. Efficacy of root endophytes against three pathogens ranged from 0.14 to 41.99 per cent mycelial inhibition. Among the six endophytes evaluated against S. rolfsii, the isolate RB-KK-6 showed the maximum mycelial inhibition of 40.78 per cent. The root isolate RB-HS-1 showed the maximum mycelial inhibition of 41.99 per cent against F. oxysporum. None of the isolates showed any inhibition above 40 per cent against R. bataticola.

### Table 1: In vitro evaluation of soybean bacterial root endophytes against Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium oxysporum

<table>
<thead>
<tr>
<th>Bacterial endophyte</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. rolfsii</td>
</tr>
<tr>
<td>RB-HS-1</td>
<td>16.15(23.68)*</td>
</tr>
<tr>
<td>RB-HB-2</td>
<td>10.10(18.51)</td>
</tr>
<tr>
<td>RB-HY-3</td>
<td>8.74(17.18)</td>
</tr>
<tr>
<td>RB-DM-4</td>
<td>0.14(1.64)</td>
</tr>
<tr>
<td>RB-DK-5</td>
<td>5.15(13.09)</td>
</tr>
<tr>
<td>RB-KK-6</td>
<td>40.78(39.67)</td>
</tr>
<tr>
<td>S.Em. ±</td>
<td>0.37</td>
</tr>
<tr>
<td>C. D. @ 1%</td>
<td>1.46</td>
</tr>
</tbody>
</table>

* Arc sine values

Results clearly indicated that all 13 bacterial stem endophytes significantly inhibited the mycelial growth of all the three pathogens. Efficacy of stem endophytes against three pathogens ranged from 5.21 to 50.08 per cent mycelial inhibition. Among the 13 bacterial stem endophytes evaluated against S. rolfsii, the isolate SB-BS-6 showed the maximum mycelial inhibition of 50.08 per cent. The isolate SB-DG-11 showed the maximum inhibition of 47.41 per cent against R. bataticola. The stem isolate SB-BJ-9 showed the maximum mycelial inhibition of 40.07 per cent against F. oxysporum Table 2.

### Table 2: In vitro evaluation of soybean bacterial stem endophytes against Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium oxysporum

<table>
<thead>
<tr>
<th>Bacterial endophyte</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. rolfsii</td>
</tr>
<tr>
<td>SB-BJ-1</td>
<td>28.10(31.99)*</td>
</tr>
<tr>
<td>SB-BJ-2</td>
<td>32.55(34.77)</td>
</tr>
<tr>
<td>SB-BU-3</td>
<td>5.21(13.17)</td>
</tr>
<tr>
<td>SB-BU-4</td>
<td>40.07(39.26)</td>
</tr>
<tr>
<td>SB-BK-5</td>
<td>34.08(35.70)</td>
</tr>
<tr>
<td>SB-BS-5</td>
<td>50.08(45.03)</td>
</tr>
<tr>
<td>SB-BKh-7</td>
<td>40.04(39.24)</td>
</tr>
<tr>
<td>SB-BV-8</td>
<td>35.13(36.34)</td>
</tr>
<tr>
<td>SB-Bij-9</td>
<td>18.40(25.39)</td>
</tr>
<tr>
<td>SB-DM-10</td>
<td>13.32(21.39)</td>
</tr>
<tr>
<td>SB-DG-11</td>
<td>15.78(23.39)</td>
</tr>
<tr>
<td>SB-DK-12</td>
<td>18.19(25.23)</td>
</tr>
<tr>
<td>S.Em. ±</td>
<td>0.26</td>
</tr>
<tr>
<td>C. D. @ 1%</td>
<td>1.04</td>
</tr>
</tbody>
</table>

* Arc sine values

A total of 11 bacterial leaf endophytes were evaluated against the three pathogens. Efficacy of leaf endophytes against three pathogens ranged from 0.22 to 54.20 per cent mycelial inhibition. Among the 11 bacterial leaf endophytes evaluated against S. rolfsii, the isolate LB-BU-1 showed the maximum mycelial inhibition of 47.02 per cent The isolate LB-Bin-8 showed the maximum inhibition of 41.22 per cent against R. bataticola. The leaf isolates LB-BU-1 showed the maximum mycelial inhibition of 54.20 per cent followed by the isolate LB-BV-2 with an inhibition of 51.64 per cent against F. oxysporum Table 3.
Effective endophytes excrete extra cellular lytic enzymes that of HCN, hydrolytic enzymes, siderophore and antibiotics towards Rhizoctonia bataticola. The effective bacterial isolates were selected. The bacterial endophytes RB were effective against KK bacterial isolates were selected. The bacterial endophytes RB

Table 3: In vitro evaluation of soybean bacterial leaf endophytes against Sclerotium rolfsii, Rhizoctonia bataticola and Fusarium oxysporum

<table>
<thead>
<tr>
<th>Bacterial endophyte</th>
<th>S. rolfsii</th>
<th>R. bataticola</th>
<th>F. oxysporum</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-BU-1</td>
<td>47.02 (43.27)</td>
<td>17.91 (20.03)</td>
<td>54.20 (47.39)</td>
</tr>
<tr>
<td>LB-BV-2</td>
<td>30.95 (33.79)</td>
<td>18.79 (23.68)</td>
<td>51.64 (45.92)</td>
</tr>
<tr>
<td>LB-BV-3</td>
<td>37.98 (38.03)</td>
<td>35.77 (36.72)</td>
<td>29.80 (33.07)</td>
</tr>
<tr>
<td>LB-HA-4</td>
<td>3.69 (11.02)</td>
<td>12.03 (20.29)</td>
<td>23.48 (28.97)</td>
</tr>
<tr>
<td>LB-BiB-5</td>
<td>16.78 (24.17)</td>
<td>0.22 (1.56)</td>
<td>19.52 (26.20)</td>
</tr>
<tr>
<td>LB-BiI-6</td>
<td>15.28 (23.00)</td>
<td>0.31 (2.52)</td>
<td>12.44 (20.60)</td>
</tr>
<tr>
<td>LB-BiI-7</td>
<td>18.35 (25.35)</td>
<td>41.22 (39.92)</td>
<td>30.58 (33.55)</td>
</tr>
<tr>
<td>LB-DN-9</td>
<td>17.29 (24.56)</td>
<td>30.81 (33.70)</td>
<td>32.87 (34.96)</td>
</tr>
<tr>
<td>LB-KK-10</td>
<td>27.69 (31.73)</td>
<td>0.96 (4.41)</td>
<td>17.64 (24.82)</td>
</tr>
<tr>
<td>LB-SK-11</td>
<td>21.95 (27.92)</td>
<td>35.23 (36.39)</td>
<td>31.60 (34.19)</td>
</tr>
<tr>
<td>S. Em. ±</td>
<td>0.33</td>
<td>0.98</td>
<td>0.37</td>
</tr>
<tr>
<td>C. D. @ 1%</td>
<td>1.30</td>
<td>3.93</td>
<td>1.49</td>
</tr>
</tbody>
</table>

* Arc sine values

Based on the dual culture method of screening, eight effective bacterial isolates were selected. The bacterial endophytes RB-KK-6 (40.78%), SB-BS-6 (50.08%) and LB-BU-1 (47.02%) were effective against S. rolfsii and the isolates SB-DG-11 (47.41%), LB-BiI-8 (41.22%) were effective against R. bataticola. The effective bacterial endophytes against F. oxysporum were RB-HS-1 (41.99%), SB-BJ-9 (40.07%), LB-BU-1 (54.20%) and LB-BV-2 (51.64%). Endophytic bacteria use different mechanisms like production of HCN, hydrolytic enzymes, siderophore and antibiotics to out compete the fungal phytopathogens. Further, some effective endophytes excrete extra cellular lytic enzymes that are responsible for their antagonistic abilities. The results are in agreement with the earlier works.[6,8]

Molecular characterization of the effective bacterial endophytes: Eight effective bacterial endophytes of soybean were characterized based on cultural, morphological and molecular methods and results revealed that the isolates RB-HS-1 was similar to Acinetobacter sp., the root isolate RB-KK-6 has shown similarity to Alcaligenes faealis, the isolate SB-BJ-9 was identified as Stenotrophomonas sp., the isolates SB-DG-11 and LB-BiI-8 had shown similarity to Bacillus pumilus, the isolate LB-BU-1 was similar to Paenalcaligenes sp., the isolate SB-BS-6 was similar to Bacillus cereus and the isolate LB-BV-2 has shown similarity to Brevibacillus sp. The bacterial endophytes were identified by 16S rDNA sequence analysis. Similar endophyte identification procedure was followed [9] where tomato bacterial endophytes were identified as Lateimonas aestuarii, Pseudomonas lini, Bacillus pumilus and Bacillus sp. by using16S rDNA sequence analysis. Kulkinsky-Sobral [10] isolated Stenotrophomonas maltophilia from root samples of soybean. Yuliar et al. [11] isolated the endophytic bacteria and identified them by 16S rDNA sequence analysis as Acinetobacter, Bacillus sp., B. pumilus, B. cereus, Alcaligenes sp. and Stenotrophomonas sp. and evaluated them for plant growth promotion and antagonistic activity against R. solani. Nhu and Diep [12] isolated and screened the endophytic bacteria for plant growth promotion and the effective endophytes were identified as Bacillus sp., Bacillus subtilis and Acinetobacter sp.

Table 4: Comparative analysis of sequence similarity of association of native bacterial endophytes

<table>
<thead>
<tr>
<th>Source</th>
<th>Endophyte</th>
<th>Comparison with already reported endophytes/ bioagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem</td>
<td>SB-BS-6</td>
<td>Bacillus cereus strain AIMST1.Cit.6(Citrus sp., leaf, Malaysia)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus cereus strain SS, SNC06 (Mangrove plants, root, India)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Paenalcaligenes suwonensis strain ABC02-12 (Spent mushroom compost, Korea)</td>
</tr>
<tr>
<td>Leaf</td>
<td>LB-BU-1</td>
<td>Bacillus cereus strain LB8 (Capillaris sp., China)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus cereus strain KK2 (River water, India)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus sp.strain KCN26 (Banana rhizosphere, India)</td>
</tr>
<tr>
<td></td>
<td>(100% similarity)</td>
<td>(99% similarity)</td>
</tr>
<tr>
<td>Leaf</td>
<td>LB-BV-2</td>
<td>Brevibacillus laterosporus strain DY23 (Nicotiana tabacum, stem, China)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brevibacillus laterosporus strain LAM0313 (Rice rhizosphere, China)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brevibacillus laterosporus strain ZQ2 (Apple rhizosphere, China)</td>
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<tr>
<td></td>
<td></td>
<td>Brevibacillus laterosporus strain HB 2667 (Bamboo rhizosphere, India)</td>
</tr>
<tr>
<td></td>
<td>(100% similarity)</td>
<td>(99% similarity)</td>
</tr>
<tr>
<td>Root</td>
<td>RB-HS-1</td>
<td>Acinetobacter sp. LCE1 (Water, Argentina)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acinetobacter sp. KT46 (Mine soil, China)</td>
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<td></td>
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<td>Acinetobacter johnsonii strain 018S (Thalassia hemprichii, China)</td>
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<td></td>
<td></td>
<td>Acinetobacter johnsonii strain CIP 64.6 (Toona sinensis, China)</td>
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<tr>
<td></td>
<td></td>
<td>Acinetobacter oryzae strain B23 (Oryza sativa, China)</td>
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<tr>
<td></td>
<td>(100% similarity)</td>
<td>(99% similarity)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Source</th>
<th>Endophyte</th>
<th>Comparison with already reported endophytes/ bioagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem</td>
<td>SB-BiI-9</td>
<td>Stenotrophomonas sp. EnB-bsy2 (Black soybean seeds, China)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stenotrophomonas maltophilia strain FLA3(Rice rhizosphere, India)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stenotrophomonas sp. strain HPC107 (Soil, India)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stenotrophomonas sp. strain SCT-8 (Bursaphelenchus xylophillus,China)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stenotrophomonas sp. strain A18 (Microcystis aeruginosa, China)</td>
</tr>
<tr>
<td></td>
<td>(99% similarity)</td>
<td>(99% similarity)</td>
</tr>
<tr>
<td>Leaf</td>
<td>Stem</td>
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<tr>
<td>Stem</td>
<td>Stem</td>
<td>Root</td>
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</tbody>
</table>

4. Conclusions
Diversified culturable endophytic bacteria with an addition advantage of antagonism against soilborne pathogens were identified and characterized. Further investigation on their bioefficacy, plant growth promotion activity under field condition and mechanism of action against the pathogens can be carried out which helps in complete understanding and usage of endophytes as effective bioagents in plant diseases management.

5. References