

Testing of Bacterial Endophytes from Non-Host Sources as Potential Antagonistic Agents against Tomato Wilt Pathogen *Ralstonia solanacearum*

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Abstract

The study was taken up with the objective of testing whether the endophytic organisms isolated from crops that are normally non-hosts to the bacterial wilt pathogen *Ralstonia solanacearum* possessed pathogen-antagonistic activity and to evaluate the selected isolates for the alleviation of wilt disease in the target tomato crop through horizontal movement of promising organisms. Sixteen endophytic bacteria (EB) isolated from the micropropagated cultures of grape, watermelon and papaya were tested for potential antagonistic effects against *R. solanacearum* tomato isolate “NH-01” through agar-well diffusion assay. *Enterobacter cloacae* from papaya (EB-11) displayed the maximum antagonistic effect followed by *Bacillus subtilis* (EB-06) and *B. flexus* (EB-07) from watermelon and *B. pumilus* (EB-02) from grape. Testing the above organisms for crop protection through seed fortification of susceptible tomato cv. Arka Vikas at sowing in *R. solanacearum* inoculated (*Ral+*) organic cocopeat showed EB-02 and EB-11 promising (33% and 32% survival, respectively, four weeks after sowing against 15% in *Ral+* control). A second trial showed 37%, 28%, 21% and 55% seedling survival 6 weeks after sowing for EB-02, EB-06, EB-07 and EB-11 respectively, compared to 2.5% in non-treated control. Assessing the four endophytes for crop protection in *Ral+* sick-soil through seedling fortification at transplanting indicated less disease incidence in treated sets (40%, 40%, 20% and 20% survival, respectively, six weeks after transplanting) over non-fortified control (5%). Endophytic fortification of seedlings through hypocotyl inoculation showed some systemic resistance induction upon seedling transplanting to sick soil but not with petiole fortification. Seedling growth was enhanced by the isolates EB-06 and EB-07. The study thus identifies four endophytic organisms from crops unrelated to tomato possessing potential antagonistic activity against the wilt pathogen and prospects for exploitation as biocontrol agents coupled with seedling growth promotion effects.

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Keywords

Bacterial Wilt, Biocontrol, Endophytic Bacteria, Growth Promotion, *Solanum lycopersicum*

1. Introduction

Bacterial wilt caused by *Ralstonia solanacearum* (formerly called *Pseudomonas solanacearum*) is one of the major limiting factors in the cultivation of tomato and other *solanaceous* crops in the tropics and subtropics worldwide [1] [2]. The wide host range of this pathogen, soil- and water-borne nature and the adaptability to survive in soil in the absence of host plants for long periods make the control of this disease very challenging [1]-[4]. The common management strategies include the adoption of resistant varieties, crop rotation and the use of resistant root stocks [1]-[6].

Biocontrol approach exploiting microorganisms with antagonistic activity against the pathogen assumes significance as an alternate strategy for the management of plant diseases [7]-[9]. In the case of *R. solanacearum*, this includes the usage of rhizospheric colonizers [10]-[12] and the endophytic organisms [9] [13]-[15]. Competitive rhizospheric colonization, niche exclusion, substrate competition and production of secondary metabolites including antibiotics and siderophores are attributed in the control of pathogens by the antagonistic bacteria [7] [8] [16]. Endophytic microorganisms colonize plants internally and they share an integral and inseparable association with the host [7] [16] [17]. They are known to colonize the vascular system which forms the niche for the wilt pathogen too [15] [16] [18] [19]. Extensive colonization of plant tissue by the endophytes create a barrier-effect whereby they compete and prevent pathogenic organisms from establishing themselves [7] [16]. Endophytes are generally considered to be predominantly root colonizers [8] [16] [18] [20]. Extensive colonization of shoot tissue by bacterial endophytes including intracellular cytoplasmic and periplasmic niches has been documented recently [21].

At this laboratory, a number of endophytic bacteria were isolated as tissue culture-associated organisms from different crop species such as grape [22] [23], watermelon [24] and papaya [25]. Several of these organisms have been hardy spore-formers which offer the advantage of developing durable spore-based formulations. Endophytic organisms associated with other plant species if they possess antagonistic activity against the pathogen, they could be considered as potential biocontrol agents in the target susceptible crop through their lateral introduction. This is on the assumption that the susceptible target genotype may not naturally possess such antagonistic organisms or the specific isolates. This advantage applies particularly to the organisms isolated from crops that are unrelated to the target crop or are normally non-hosts to the pathogen. Among the above crops, papaya may be affected [10] [26] but is not a very common known host, while others are not known as normal hosts. The present study was taken up with the objective of screening a select group of endophytic organisms isolated from crops such as grape, watermelon and papaya for potential antagonistic activity against this pathogen and to test the utility of the selected organisms in alleviating the bacterial wilt disease in tomato.

2. Materials and Methods

2.1. Endophytic Organisms

Sixteen endophytic bacterial isolates (EB) from crop sources which are normally non-hosts to the pathogen, namely, grape, watermelon and papaya (coded as EB01 - EB16) were taken up for screening towards any antagonistic activity against the bacterial wilt pathogen. The EB isolates included *Bacillus pumilus* and *Brevibacillus* sp. isolated from *in vitro* grown grape as alcohol-resistant spore-formers [22] [23], *B. megaterium*, *B. fusiformis*, *B. pumilus*, *B. subtilis* and *B. flexus* from triploid watermelon culture again as alcohol-tolerant organisms [24], and *Microbacterium esteraromaticum*, *Pantoea ananatis*, *Enterobacter cloacae*, *Brevundimonas aurantiaca*, *Bacillus benzoevorans*, *Sphingomonas* sp., *Methylobacterium rhodesianum* and *Agrobacterium tumefaciens* from papaya shoot-tip cultures [25] (Table 1). The isolates were maintained as glycerol (20%) stocks at -80°C and revived by spotting the cultures on nutrient agar (NA) one or two days prior to their use in different trials.

Table 1. List of endophytic bacteria isolated from micropropagated stocks of different plant species employed in *Ralstonia solanacearum* antagonism trials.

| EB No. | Identity of the organism | Isolate | Crop source | NCBI 16S rRNA gene seq. acc no. | Reference | Description |
|--------|--------------------------------------|------------|-------------|---------------------------------|-----------|---|
| EB-01 | <i>Brevibacillus</i> sp. | ARBG1 | Grape | DQ 116777 | [23] | Gram – ve spore former |
| EB-02 | <i>Bacillus pumilus</i> | ARBG2 | Grape | AY496869 | [22] | Gram + ve spore former |
| EB-03 | <i>Bacillus megaterium</i> | 3xWMARB-1 | Watermelon | NA | [24] | Gram + ve spore former |
| EB-04 | <i>Bacillus fusiformis</i> | 3xWMARB-2 | Watermelon | DQ306025 | [24] | Gram + ve spore former |
| EB-05 | <i>Bacillus pumilus</i> | 3xWMARB-3 | Watermelon | DQ306026 | [24] | Gram + ve spore former |
| EB-06 | <i>Bacillus subtilis</i> | 3xWMARB-4 | Watermelon | DQ306027 | [24] | Gram + ve spore former |
| EB-07 | <i>Bacillus flexus</i> | 3xWMARB-5 | Watermelon | DQ306024 | [24] | Gram + ve spore former |
| EB-08 | <i>Bacillus pumilus</i> | 3xWMARB-6 | Watermelon | NA | - | Gram + ve spore former |
| EB-09 | <i>Microbacterium esteraromatium</i> | Pap.ViBa-1 | Papaya | DQ346728 | [25] | Gram + ve <i>Actinobacterium</i> , |
| EB-10 | <i>Pantoea ananatis</i> | Pap.ViBa-2 | Papaya | EF088342 | [25] | Gram – ve γ - <i>proteobacterium</i> |
| EB-11 | <i>Enterobacter cloacae</i> | Pap.ViBa-3 | Papaya | EF088345 | [25] | Gram – ve γ - <i>proteobacterium</i> |
| EB-12 | <i>Brevundimonas aurantiaca</i> | Pap.ViBa-4 | Papaya | DQ346729 | [25] | Gram – ve α - <i>proteobacterium</i> |
| EB-13 | <i>Bacillus benzoovorans</i> | Pap.ViBa-5 | Papaya | DQ346730 | [25] | Gram + ve spore former |
| EB-14 | <i>Sphingomonas</i> sp. | Pap.ViBa-6 | Papaya | DQ346734 | [25] | Gram – ve α - <i>proteobacterium</i> |
| EB-15 | <i>Methylobacterium rhodesianum</i> | Pap.ViBa-7 | Papaya | DQ346736 | [25] | Gram – ve α - <i>proteobacterium</i> |
| EB-16 | <i>Agrobacterium tumefaciens</i> | Pap.ViBa-8 | Papaya | DQ346737 | [25] | Gram – ve α - <i>proteobacterium</i> |

NA, not available.

2.2. Culture of Pathogen

Ralstonia solanacearum strain “NH01” (NCBI Acc. no. KJ399970) isolated from the bacterial ooze of a wilted tomato plant [27] constituted the pathogen unless mentioned differently. The culture was stored as glycerol stocks at -80°C and revived on Kelman [28] medium fortified with 2, 3, 5 triphenyl tetrazolium chloride (KM-TTC) as described elsewhere [29]. An additional isolate of *R. solanacearum* “IH-01” was used for confirmation of antagonistic assay results *in vitro*.

2.3. Media and Culture Conditions

KM-TTC medium was used for raising the culture of pathogen while NA was employed for growing the EB isolates. Pre-sterilized single-use 9 cm plates were used for raising the cultures of both the pathogen and the endophytes whereas 12×12 cm square plates were employed in antagonism trials. Both the above were sourced from Hi Media Biosciences, Mumbai.

2.4. Agar-Well Diffusion Assay to Test Pathogen-Antagonistic Activity by Endophytic Organisms

The lawn of pathogen was prepared in square plates (60 - 70 ml KM-TTC medium per 12×12 cm plate) by spreading 200 μl of 0.1 OD (600 nm) *R. solanacearum* “NH-01” culture derived from 2-day old KM-TTC source plates. Prior to the introduction of challenge organisms, *R. solanacearum* was allowed to establish on KM-TTC medium for 3 - 4 h at 30°C . Wells of 7 - 8 mm diameter (approx.) were made with the distal end of a sterile 200 μl disposable pipette-tip and 50 μl of 0.2 OD (600 nm) suspension of EB isolates (01 - 16) prepared

from 1 - 2 day-old NA plate cultures was added in marked wells. After incubation at 30°C for 2 - 4 days, the plates were scored for the extent of zone of inhibition. The experiment was repeated twice for confirmation including the additional isolate “IH-01”.

2.5. Testing the Promising Endophytes for Crop Protection through Seed Fortification

Based on the outcome from agar-well diffusion assay, four isolates namely EB-02 (*Bacillus pumilus*), EB-06 (*B. subtilis*), EB-07 (*B. flexus*) and EB-11 (*Enterobacter cloacae*) were taken up for further trials to check the ability of the organisms to protect the tomato crop. Cultivar Arka Vikas, which is highly susceptible to the pathogen [27] was employed as the target plant. Seeds were fortified with single endophytes by soaking in bacterial inoculum prepared in sterile water (0.1 OD at 600 nm) for 1 h and they were sown in organic cocopeat (CP) [27] in 50 cavity protrays at the rate of 4 seeds per cavity (approx. 5 g CP) after removing the excess liquid inoculum. The CP was inoculated by applying 5 ml of 0.1 OD suspension of *R. solanacearum* prepared in autoclaved water from 2-day KM-TTC plate cultures [27]. There were two control treatments, namely endophyte non-fortified seeds sown in *Ralstonia* inoculated or non-inoculated CP (*Ral+* and *Ral-* control, respectively). Ten replications were employed per treatment with five cavities forming one replication. Separate trays were employed for different treatments to avoid the cross spreading of inoculum across different treatments. Trays were incubated in a glasshouse under identical conditions and seed germination was recorded after 7 and 10 days. The disease incidence (%) was recorded after 2, 3 or 4 weeks based on the extent of irreversible wilting or seedling mortality [27]. The experiment was repeated thrice, twice as above and the next time employing CP that was inoculated with pathogen a week prior to the date of sowing.

2.6. Assessing Selected Endophytes for Crop Protection Capability at Transplanting

Seedlings of Arka Vikas were grown in fresh CP medium in 98 cavity protrays and were transplanted at 4 weeks stage with the intact ball of CP to *Ralstonia* sick soil in 50 cavity protrays as per Thomas and Upreti [29]. Two days prior to transplanting, the seedlings were fortified by drenching with 2 ml of 0.1 OD suspensions of the isolates EB-02, EB-06, EB-07 or EB-11. Following transplanting, the root zone was again drenched with 5 ml endophyte inoculum. The seedlings were incubated under glasshouse conditions and the disease incidence was recorded weekly for six weeks [29]. There were five replications per treatment with four plants constituting one replication. The experiment was repeated once.

2.7. Testing the Endophytic Isolates for Any Direct Adverse Effects on Tomato

Considering that the endophytes employed in this study were derived from other plant species, they were tested for any pathogenic effect on tomato by petiole-excision inoculation of seedlings [27]. The seedlings were grown in 5” plastic pots and at 4 week stage, the lower most green leaf was excised and a 10 µl droplet of 0.1 OD inoculum prepared in sterile water was deposited. There were two control treatments, namely, seedlings applied with sterile water or with *R. solanacearum* inoculum.

2.8. Testing the Capability of Organisms to Induce Systemic Resistance

This was done on 4 week old seedlings two days prior to their transplanting to *Ralstonia* sick soil through petiole-excision inoculation as well as hypocotyl inoculation. In the first trial, the petiole was excised and a 10 µl droplet of 0.1 OD inoculum was applied as mentioned above. In the latter, the seedlings were given a hypodermic injection with approx. 10 µl EB inoculum at the lower part of the hypocotyl. The control sets were injected with sterile water. There were 20 seedlings per treatment and the seedlings were transplanted to *Ralstonia* sick soil in 50 cavity protrays [29].

2.9. Testing the Selected Organisms for Growth Promotion Effects on Tomato Seedlings

The four selected EB isolates were tested for growth promotion effects on “Arka Vikas” at seedling stage. The seeds were soaked in 0.1 OD bacterial suspension for 1 h and then sown in CP in 98 cavity protrays. Germination was recorded after 10 days and the seedling growth was assessed based on shoot height and the fresh weight of root, shoot and the whole seedling through destructive sampling 4 weeks after sowing [29].

2.10. Statistical Methods

The trials were conducted in completely randomized design under glasshouse conditions. The experimental conditions and data analysis were as described earlier [27] [29].

3. Results

3.1. Agar-Well Diffusion Assay Employing Endophytic Organisms

Four endophytic isolates, namely EB-02 (*Bacillus pumilus*) from grape, EB-06 (*B. subtilis*) and EB-07 (*B. flexus*) from watermelon and EB-11 (*Enterobacter cloacae*) from papaya displayed clear inhibition zone development against *R. solanacearum* while four other isolates (EB-08, EB-09, EB-10, EB-13) displayed slight zone of inhibition (**Figure 1**). The results with the first four isolates appeared consistent in two repeat trials which also held well with the second isolate, “IH-01” (**Figure 1**). EB-15 (*Methylobacterium* sp.) was not included in this trial as it was a very slow grower. Based on the extent of clear zone development, the organisms were categorized into four groups constituting EB-11 in group-1 displaying notable inhibition zone development, EB-02, EB-06 and EB-07 in group-2 showing obvious inhibition zone, EB-08, EB-09, EB-10 and EB-13 in group-3 with negligible zone of inhibition (EB-08 showed some inconsistency), and the rest in group-4 (**Table 2**). It was significant to note that although there were three *B. pumilus* isolates (EB-02, EB-05 and EB-08), only one isolate (EB-02 from grape) showed clear and consistent inhibition-zone development.

3.2. Testing the Promising Endophytes for Crop Protection through Seed Fortification

In the trial where the seeds were sown in freshly pathogen-inoculated CP after fortification with EB-02, EB-06, EB-07 or EB-11, germination was unaffected except in EB-11 where it showed a notable reduction (**Table 3**). The control seeds (EB non-fortified set) did not show notable differences in percent germination in *Ral+* and

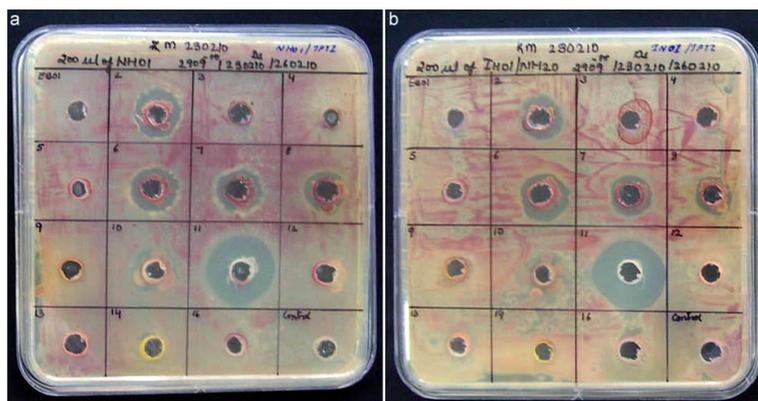


Figure 1. Agar-well diffusion assay to test pathogen-antagonistic activity against *Ralstonia solanacearum* by fifteen endophytic organisms marked 1 - 16 (excluding EB-15 which showed very slow growth) plus distilled water control; Prominent clear-zone development by the endophytic bacterial isolate EB-11 followed by EB-02, EB-06 and EB-07 in the trial employing the isolate “NH-01” (a) or the isolate “IH-01” (b).

Table 2. Classification of endophytic organisms based on the extent of clearing/antagonistic effect against *Ralstonia solanacearum* in agar-well diffusion assay.

| No. | Antagonistic effect | Clear zone diameter | Isolates |
|-----|-------------------------------------|---------------------|--|
| 1 | Notable inhibition zone development | >20 mm | EB-11 |
| 2 | Obvious inhibition zone development | 15 - 20 mm | EB-02, EB-06, EB-07 |
| 3 | Slight inhibition zone development | 10 - 15 mm | EB-08, EB-09, EB-10, EB-13 |
| 4 | No inhibition zone development | None | EB-01, EB-03, EB-04, EB-05, EB-12, EB-14, EB-15, EB-16 |

Table 3. Extent of seed germination and the incidence of seedling mortality in tomato “Arka Vikas” after sowing the endophyte-fortified seeds in freshly pathogen inoculated cocopeat (a), or inoculated one week prior to the sowing (b).

| Aspect | Treatment | | | | | |
|--|--------------------------|--------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| | Control: <i>Ral</i> - CP | Control: <i>Ral</i> + CP | EB02/ <i>Ral</i> + CP | EB06/ <i>Ral</i> + CP | EB07/ <i>Ral</i> + CP | EB11/ <i>Ral</i> + CP |
| Freshly pathogen inoculated cocopeat | | | | | | |
| Seed germination (%) | | | | | | |
| Day 7 | 85 ^a | 80 ^{a,b} | 77.5 ^{a,b} | 85 ^a | 85 ^a | 67.5 ^b |
| Day 10 | 92.5 ^a | 82.5 ^{a,b} | 85 ^{a,b} | 87.5 ^a | 90 ^a | 72.5 ^b |
| Seedling mortality (%) | | | | | | |
| 2 week | 0 | 40.8 ^a | 28.3 ^a | 33.3 ^a | 15.8 ^b | 40.8 ^a |
| 4 week | 0 | 51.6 ^a | 49.1 ^a | 46.6 ^a | 47.4 ^a | 55.8 ^a |
| 6 week | 0 | 85.0 ^a | 66.6 ^a | 81.6 ^a | 81.6 ^a | 68.3 ^a |
| Cocopeat inoculated with pathogen one week prior to sowing | | | | | | |
| Seed germination (%) | | | | | | |
| Day 7 | 82.5 ^a | 75 ^a | 82.5 ^a | 82.5 ^a | 77.5 ^a | 82.5 ^a |
| Day 10 | 92.5 ^a | 80 ^b | 87.5 ^{a,b} | 87.5 ^{a,b} | 82.5 ^b | 97.5 ^a |
| Seedling mortality (%) | | | | | | |
| 2 week | 0 | 16.6 ^a | 0 ^b | 5.8 ^b | 8.3 ^{a,b} | 7.5 ^{a,b} |
| 4 week | 0 | 60.1 ^a | 59.9 ^a | 45.8 ^{a,b} | 62.4 ^a | 38.1 ^b |
| 6 week | 0 | 97.5 ^a | 63.3 ^{b,c} | 72.5 ^b | 79.1 ^{a,b} | 45.0 ^c |

Seeds were fortified by soaking for 1 h in endophytic bacterial suspension of 0.1 OD prepared in water. *Ral*- CP and *Ral*+ CP: *Ralstonia* non-inoculated and inoculated cocopeat, respectively. EB 02, *Bacillus pumilus*; EB06, *B. subtilis*; EB07, *B. flexus*; EB 11, *Enterobacter cloacae*. Values followed by the same letter in a row are not significantly different; *Ral*- CP control treatment was not included in the statistical analysis for seedling mortality.

Ral- CP. The disease symptoms appeared by two weeks in control sick (*Ral*+) CP medium as wilting of seedlings (41%) and the extent of mortality increased with time registering 52% by four weeks and 85% by six weeks. The four EB-treated sets showed relatively less disease incidence at 2 weeks but appeared almost on par with *Ral*+ control by 4 weeks. By 6 weeks, the EB-02 and EB-11 fortified sets offered some protection (33% and 32% survival, respectively) compared with *Ral*+ control (15%). A second trial where the seed sowing was done on CP one week after the inoculation with the pathogen, relatively less disease incidence was observed with EB-seed fortified treatments (Table 3). On the other hand, a third trial employing freshly inoculated CP showed high disease incidence in the presence of endophytes on par with the *Ral*+ control while a fourth trial failed to show disease incidence indicating a loss in the virulence of the pathogen (data not shown).

3.3. Assessing Selected Endophytes for Crop Protection at Transplanting

The results from these trials indicated that the endophytes were able to offer some protection when the transplanting was undertaken to the sick soil as per the observations over six weeks (Figure 2(a)). The results were confirmed in a repeat trial where the seedlings were monitored for four weeks which indicated that while the EB isolates offered some protection, they were not effective in safeguarding the crop entirely from the pathogen (Figure 2(b)).

3.4. Testing the Organisms for Any Direct Adverse Effects on Tomato Seedlings

No obvious adverse effects were observed following petiole-excision inoculation of “Arka Vikas” seedlings with the endophytic isolates (Figure 3). The seedlings that were given inoculation with *R. solanacearum* showed

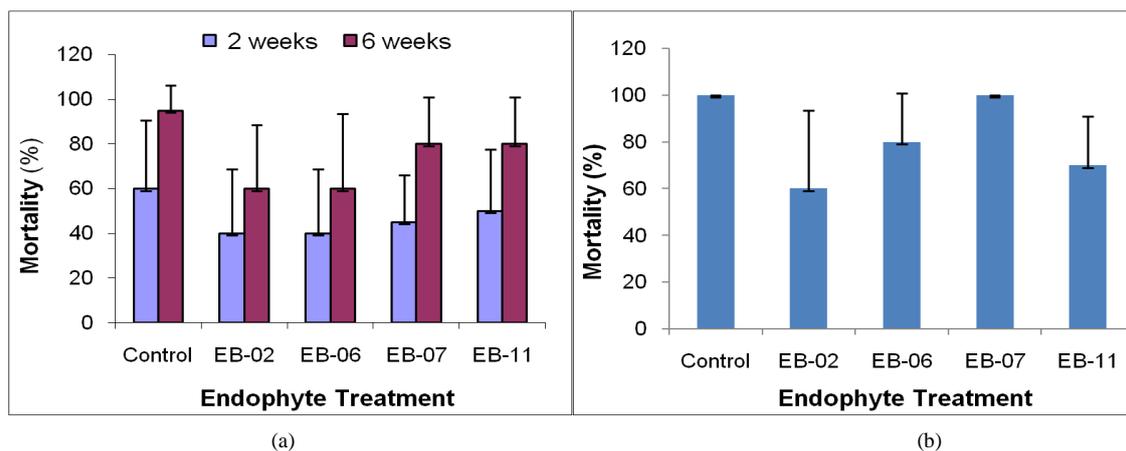


Figure 2. Assessing selected endophytic bacterial isolates for the ability to protect tomato crop by transplanting to *Ralstonia* freshly inoculated sick soil in pro trays (a) or in soil inoculated with the pathogen one week prior to transplanting (b); Vertical bars indicate SD.



Figure 3. Testing the endophytic isolates for any direct adverse effects on tomato through petiole-excision inoculation; *Ral+*, *Ralstonia* inoculated set; EB-02, EB-06, EB-07 and EB-11, endophytic bacteria inoculated sets; DW, distilled water control.

quick disease development and high mortality.

3.5. Testing the Organisms for Systemic Resistance Induction

The seedlings that were transplanted to *Ral+* sick soil following EB fortification through petiole-excision inoculation did not offer any advantage over control in terms of reducing the disease incidence; on the other hand, the extent of disease incidence appeared more than that in the control set (Figure 4(a)). The seedlings fortified through hypocotyl inoculation, however, offered some reduction in the extent of disease incidence during the next six weeks of monitoring, particularly with EB-02 and EB-06 (Figure 4(b)).

3.6. Testing the Selected Organisms for Growth Promotion Effects on Tomato Seedlings

Seed germination was unaffected except in EB-11 fortified treatment where a notable reduction in germination was observed (Table 4). Root growth in seedlings, which is a major indicator of the ability of the plants to perform in the field, was significantly more for the isolates EB-06, EB-07 and EB-11. The former two isolates also proved superior in terms of seedling shoot height, shoot weight and gross seedling weight over control although the differences were not significant in all the instances. Overall, the seedling growth was enhanced with EB-06 and EB-07 in comparison with the other treatments.

4. Discussion

The study identifies endophytic organisms from three unrelated crops of tomato, namely grape, papaya and watermelon, with antagonistic activity against the tomato wilt pathogen *R. solanacearum*. The above plant species

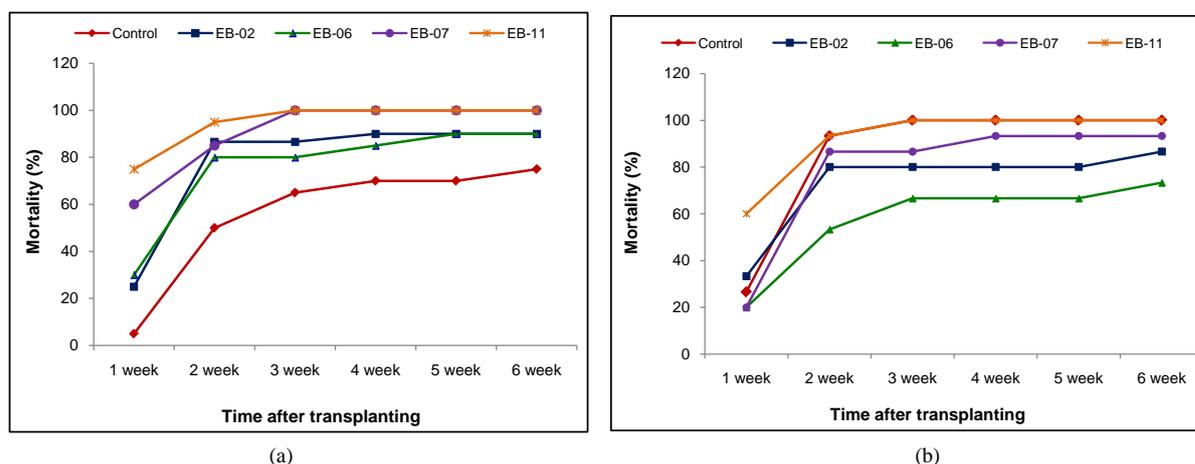


Figure 4. Testing the capability of endophytic organisms to induce systemic resistance to *Ralstonia solanacearum* through petiole excision inoculation (a) or through hypocotyl inoculation (b) before seedling transplanting to *Ralstonia* sick soil.

Table 4. Extent of seed germination and the growth characteristics of seedlings derived after seed fortification with the endophytic bacterial isolates by pre-sowing soaking inoculation.

| Endophytic isolate | Germination (%) | Seedling height (cm) | Shoot wt (mg/seedling) | Root wt (mg/seedling) | Seedling wt (mg/seedling) |
|------------------------|-------------------|----------------------|------------------------|-----------------------|---------------------------|
| EB02 | 85.0 ^a | 23.3 ^a | 1109 ^a | 106 ^a | 1216 ^a |
| EB06 | 87.5 ^a | 29.5 ^b | 1249 ^a | 183 ^b | 1432 ^{a,b} |
| EB 07 | 90.0 ^a | 29.9 ^b | 1367 ^a | 179 ^b | 1546 ^{a,b} |
| EB 11 | 72.5 ^b | 27.9 ^b | 1084 ^a | 156 ^b | 1240 ^a |
| Non-inoculated Control | 92.5 ^a | 27.5 ^b | 1184 ^a | 107 ^a | 1314 ^{a,b} |
| Significance | * | ** | NS | ** | NS |
| P | 0.0261 | 0.0053 | 0.710 | 0.0042 | 0.238 |
| CD (P = 0.05) | 10.57 | 3.02 | 370.3 | 42.45 | 274.6 |

*, **; significant at P = 0.05 and P = 0.01, respectively, NS, not significant. Values followed by the same letter in a column are not significantly different.

are not normally known to be the host to this pathogen for the exception of an isolated report on papaya [26]. As per the *in vitro* agar-diffusion assays, *E. cloacae* appeared the most promising among the four organisms with clear antagonistic activity. *E. cloacae* is a proteobacterium (γ -subclass) similar to *R. solanacearum* (β -subclass), and has been reported as an antagonist to *R. solanacearum* [11] [15] [30]. The other potentially useful organisms included *B. pumilus*, *B. subtilis* and *B. flexus*. Of these, *B. subtilis* has been reported as an antagonist and efficient biocontrol agent against *R. solanacearum* [31] [32]. *B. pumilus* is not normally known as a biocontrol agent against this pathogen. It is pertinent to note that only one of the three *B. pumilus* isolates (EB-02 from grape) showed clear inhibition-zone development while the other two isolates from watermelon did not show such consistent antagonistic effects. This suggests the strain specificity as an influencing factor during biocontrol approaches.

A genotype or crop species which is naturally susceptible to a pathogen may or may not be bearing the antagonistic organisms against the pathogen in question. This aspect is under our detailed investigations. As per Feng *et al.* [33], *Ralstonia* resistant tomato genotype possessed more cultivable and antagonistic endophytic bacteria than the susceptible check. Identification of antagonistic endophytes associated with crops that are genetically unrelated to tomato and not normally susceptible to the pathogen offers the scope for trans-crop introduction or horizontal transfer of organisms across plant species. It is likely that the same organisms may also form natural endophytes of tomato, but the strains in that event could be different. It is now envisaged to explore the resistant genotypes of tomato for the antagonistic organisms. In this respect, the observation by Feng *et al.*

[33] that the resistant tomato genotypes possessed more cultivable and antagonistic endophytic bacteria than the susceptible check assumes significance.

Testing the different organisms for their ability to alleviate the disease incidence by introducing them through seed fortification just before sowing in sick soil offered some biocontrol effect particularly when the cocopeat medium was inoculated a week prior to the sowing rather than freshly inoculated sowing medium which is more akin to field sick soil. However, it was not possible to prevent the disease incidence entirely or save the crop wholly as is the case with other studies too where a significant reduction in disease incidence is often reported [9]-[15], [30]-[33]. The same was the impression from the transplanting trials to the sick soil at 4 week stage. In the normal course, it is possible to raise the seedlings under pathogen-free conditions. The seedlings normally come in contact with the pathogen at the time of field transplanting. A reduction in disease incidence would be advantageous as otherwise no harvestable crop is obtained from the wilt inflicted plants.

Although *E. cloacae* appeared the most effective organism as per the agar-diffusion assays, it did not prove so in comparison with the other organisms. *E. cloacae* being a proteobacterium, may be sensitive to the soil conditions and thus may not be a good rhizosphere competitor. On the other hand, members of *Bacillus* genus have the advantage of being hardy spore-formers which may help in preparing stable bioformulations and they would form better candidates towards bio-control [10] [11] [31]. The spores may remain dormant during storage. However, they possess the ability to sense the environmental conditions and respond with germination with the return of favorable conditions [34]. In this respect, the three isolates EB-02, EB-06 and EB-07 proved advantageous. The isolates EB-06 and EB-07 also showed enhancement in root and gross seedling growths.

Although a number of organisms could be identified as potential antagonistic agents in *in vitro* screenings, the effectiveness may vary under soil conditions. Lack of consistency appeared to be a limitation with the use of antagonistic organisms in the glasshouse trials. The ability of the organism to survive in the bio-formulations, in rhizospheric soil or to colonize the plants may vary. This may be particularly so with the endophytes. Bio-control applications in the field have been limited by inconsistent disease control effects [9] [33]. It is generally observed that while a number of beneficial organisms are identified in the laboratory, a lower number are successful in glasshouse trials and still fewer function effectively under practical conditions [9] [35].

5. Conclusion

In conclusion, this study identifies four endophytic organisms from crops unrelated to tomato possessing potential antagonistic activity against the wilt pathogen with prospects for exploitation as biocontrol agents coupled with seedling growth promotion effects. It would now warrant more translational research to see how far these organisms are able to protect the tomato crop in the field.

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