International Journal of Science and Research (IJSR)
ISSN: 2319-7064
ResearchGate Impact Factor (2018): 0.28 | SJIF (2018): 7.426

# Cultivated Strawberry (*Fragaria x ananassa*) and Wild Strawberry (*Duchesnea indica*) Rhizosphere associated Microbes as Inoculants to Promote Early Vegetative Growth of Strawberry

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**Abstract:** ***The effect of cultivated (Fragaria x ananassa) and wild strawberry (Duchesnea indica) rhizosphere microbes on the growth of strawberry (var. chandler) was investigated in a glasshouse experiment. Four bacterial (B1-B4) and three fungal (F1-F3) strains isolated from the cultivated strawberry rhizosphere and two bacterial (B5 and B6) strains isolated from the wild strawberry rhizosphere were used as inoculants. The recommended dosage of chemical fertilizer (CF) was used as the reference treatment while the control was maintained without adding microbes or CF. The pH of the growth media and nitrogenase activity of strains were measured. Microbes were morphologically and biochemically characterized. According to the results, all strains showed acidic pHs. Only the strain, B2 showed nitrogenase activity. Total plant and shoot biomass of strawberry were significantly ($p \leq 0.05$) improved by B1, B2, B4 and F2, while root biomass was improved ($p \leq 0.05$) by strains, B2 and B4. Petiole length was significantly increased ($p \leq 0.05$) with B1, F1 and F2. All fungal isolates were belonged to the genus Aspergillus. Bacterial strains B1, B2 and B4 were identified as Bacillus sp., Enterobactor sp. and Pseudomonas sp., respectively. Other bacterial strains were not identified. The findings show the ability of rhizosphere isolates to improve early vegetative growth of strawberry, highlighting their significance as potential biofertilizers.***

**Keywords:** biofertilizer, fungal and bacterial strains, rhizosphere, strawberry, vegetative growth

## 1. Introduction

Plant growth promoting microbes (PGPM) are a heterogeneous group of organisms [1] associated with roots showing the ability to enhance plant growth [2,3]. Bacterial species such as *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to enhance plant growth in many crops [2,4,5,6]. Mycorrhizal fungi [7] and *Penicillium*[8,9] have also been recorded as plant growth promoters. The plant growth promotion by microbes entails either by providing the plant with growth promoting substances synthesized by them [10,11,12] or facilitating the uptake of nutrients [13,14]. On the other hand, they can compete with phytopathogens in the soil, thus act as biocontrol agents [1,11]. Over the years, scientists have attempted to develop biofertilizers for strawberries [15,16,17,18,19,20], as the strawberry cultivation is heavily depend on agrochemicals. High doses of agrochemicals diminish soil quality and fertility due to their negative impacts on soil fauna and microbes [21,22], eventually causing many environmental issues. Farmers at present have been compelled to use more fertilizers and pesticides in order to maintain a reasonable crop yield. While identifying the negative impacts of these agrochemicals, the use of environmentally-friendly biofertilizers had become the focal point among agriculturists. Previous studies show the potential use of microbial growth promoters as biofertilizers in strawberry [1,23,24,25,26]. The use of biofertilizers can markedly reduce the use of chemical fertilizers [27]. Therefore, this study focused on isolation of suitable rhizosphere microbial strains from cultivated and wild strawberry, and to test their potential to use as biofertilizers to promote the early growth of strawberry (*Fragaria x ananassa*).

## 2. Research Methodology

### Isolation and screening of rhizosphere associated bacteria and fungi

Rhizosphere-associated bacteria and fungi were isolated from the rhizosphere of cultivated (*Fragaria x ananassa*) and wild strawberry (*Duchesnea indica*) using Nutrient Agar (NA) and Potato Dextrose Agar (PDA), respectively [10,12]. Cultivated strawberry plants were obtained from the Agricultural Research Station at SeethaEliya, Sri Lanka while the wild strawberry plants were collected from an Organic Tea Plantation in Hatton (Figure 1). The isolated bacterial strains were symbolized by the letter B while fungal strains by the letter F, followed by a strain number.

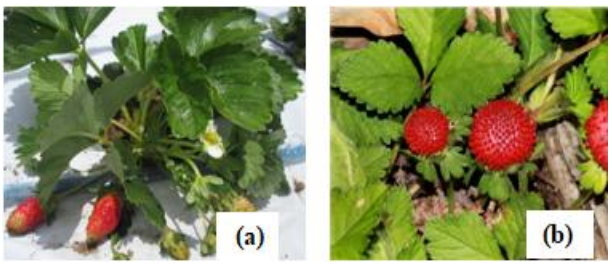


**Figure 1:** Growth habits and fruits of (a) cultivated strawberry (*Fragaria x ananassa* Duch. variety chandler), and (b) wild relative of strawberry (*Duchesnia indica*).

All bacterial isolates were cultured in nitrogen-free Combined Carbon Medium (CCM)[28,29], which is used to identify presumptive diazotrophs. Nitrogenase activity of the presumptive diazotrophs was subsequently evaluated by the Acetylene Reduction Assay (ARA) [10,30,31,32]. Growth pH of bacterial and fungal isolates were measured [33]. Out of 24 bacterial isolates, six bacterial strains (four isolated from cultivated strawberry rhizosphere, B1 – B4 and two isolated from wild strawberry rhizosphere, B5 – B6) and three fungal isolates (isolated from cultivated strawberry rhizosphere, F1 – F3) were used as inoculants in the pot experiment. The selection of isolates for the plant assay was based on their ability to survive in N free medium, nitrogenase activity and acidic pHs.

**Characterization of fungal and bacterial isolates**

Fungal isolates used in the assay were characterized by observing their morphological features. The slide culture technique [34] was used to observe the fine structures of the fungal isolates. The bacterial strains were identified through biochemical tests including gram stain, catalase, oxidase, oxidase-fermentation, citrate, Methyl-Red (MR), Voges-Proskauer (VP), urease and glucose fermentation. As an additional test, nitrate test was conducted on Gram +ve bacteria. All tests were carried out by following the standard protocols of American Society for Microbiology (ASM) Microbe Library (http://www.microbelibrary.org/home).

**Plant assay**

A glasshouse pot experiment was conducted for a period of two months during June/July, 2012 at the Agricultural Research Station at SeethaEliya, Sri Lanka (6º93' N - 80º81' E, elevation 1868 m, 20 ºC day and 11.6 ºC night temperature) to evaluate the potential of isolates to promote early growth of cultivated strawberry plants. One week old tissue-cultured strawberry (var. chandler) plantlets were grown in pots (height 15 cm, top diameter 12 cm, bottom diameter 10 cm) containing a potting mixture of sand, coir dust and forest soil in 3:2:1 ratio (by volume and as recommended by the Department of Agriculture, Sri Lanka). After two weeks, the potting mixtures were inoculated with the selected bacterial and fungal isolates, separately. Inoculants (106 bacterial cells or fungal spores /mL) were prepared and diluted 10 times, and 20.0 mL of the diluents were used in the inoculants. Chemical fertilizers (CFs) were not added for inoculated plantlets. The recommended dosage of CFs (Albert solution) was used in the reference treatment. The control (CONT) was maintained without adding microbes or CFs. Five replicates for each treatment were arranged according to Completely Randomized Design (CRD). Plants were harvested destructively after two months. The number of leaves, petiole lengths in mother plants and number of runners were recorded and the dry weights of roots, shoots and runners were taken after oven drying at 60 ºC to a constant weight. The normal distribution of the data were checked by Anderson Darling normality test. Parametric data were analyzed using Analysis of Variance (ANOVA) and means were separated by Tukey's simultaneous mean separation test. Non parametric data were analyzed using Kruskal Wallis test. Statistical analyses were performed using Minitab Statistical package (Minitab® 16.2.1, 2010).

## 3. Results and Discussion

**Rhizosphere associated bacteria and fungi isolated from rhizosphere**

In total, 19 and 5 bacterial isolates were isolated from cultivated and wild strawberry rhizospheres, respectively, out of which only six isolates (B1 - B4 from cultivated strawberry and B5 - B6 from wild strawberry) demonstrated growth in CCM. The strain, B2 changed the color of CCM into blue, whereas the rest of the isolates changed the color to yellow confirming their ability to produce acids. The strain, B2 showed nitrogenase activity in ARA (4.04 nmoles/h/tube). All other isolates did not show nitrogenase activity, possibly due to their low fixation levels. Nitrogen is one of the limiting nutrients for plant growth, and hence the ability of rhizosphere microbes to fix nitrogen is important for their plant growth promoting activity. Nitrogenase activity of bacteria isolated from rhizosphere of sesame, maize, wheat, lettuce, pepper and rice has been reported in previous studies [10].

The fungal isolates, F1, F2 and F3 converted the culture medium pH into 5.5, 6.1 and 6.4, respectively, whereas bacterial isolates B1- B6 were in the pH range of 4.4 - 6.3. Thus, all isolates preferred acidic conditions. The acid-producing rhizosphere microorganisms have the ability to promote plant growth [11,35], as acids produced by these microbes are directly or indirectly involved in promoting the growth of plants [8,14,36]. Microbes produce growth hormones such as Indole Acetic Acid (IAA) [11], and Gibberelic acid (GA)[11,37], which are known plant growth promoting hormones. In addition, most naturally occurring bio-controlling agents preferred acidic growth pHs [38]. Thus, the present results indirectly suggest that both fungal (F1-F3) and bacterial (B1–B6) strains have the ability to act as plant growth promoters.

**Characterization of microbial isolates**

According to microscopic observations, all fungal filaments were hyaline and septate. Collumella were blackish in colour in all three strains, with F1 and F3 showing globular-shape whereas F2 being rod-shape. In all three strains, hyaline conidia were linked with each other forming chains. All three strains were identified as *Aspergillus*-like species [39,40].

Based on biochemical test results given in table 1, the three bacterial isolates (B1, B2 and B4) were identified as *Bacillus* sp. [41], *Enterobactor* sp. [42] and *Pseudomonas* sp. [43], respectively. The bacterial strains, B3, B5 and B6 were not identified.

**Table 1:** Biochemical characterization of bacterial isolates, B1- B6 isolated from strawberry rhizosphere

| Biochemical test | Bacterial isolate | | | | | |
|---|---|---|---|---|---|---|
| | B1 | B2 | B3 | B4 | B5 | B6 |
| Gram stain | + | - | + | - | + | + |
| Shape | rod | rod | rod | cocci | rod | rod |
| Catalase test | - | + | - | + | + | + |
| Oxidase test | - | + | - | +/- | + | + |
| Oxidative – Fermentation test (after 48 of incubation) | + | + | - | +/- | + | + |
| Citrate test | + | + | - | + | - | - |
| MR | - | - | - | - | + | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| VP | + | + | - | - | + | + |
| Nitrate test | + | nc | - | nc | - | + |
| Urease (after 18 hours of incubation) | + | + | - | - | + | + |
| Urea broth | nc | - | nc | + | nc | nc |
| Glucose fermentation | + | + | - | - | + | + |

+, positive results: -, negative results: +/-, confusing results: nc, not conducted

**Growth performance of strawberry**

The growth assay demonstrated the ability of rhizosphere isolates to promote growth. Total plant and shoot biomass were significantly improved ($p \leq 0.05$) by B1, B2, B4 and F2 isolates in comparison to the reference treatment, where plants were given only the recommended dosage of CF (Figure 2). The root biomass was significantly ($p \leq 0.05$) higher in plants grown with B2 and B4 strains than in the control (Figure 2). Plants provided with CF showed the least growth during the experiment.

The isolates, B1, F1 and F2, significantly ($p \leq 0.05$) extended the petiole length in comparison to the control (Figure 3). Root weight ratio (RWR) showed no significant differences among treatments. However, in all treatments the relative contribution of shoot to the total plant biomass was higher than that of root (Figure 4).

According to the Kruskal-Wallis test, the number of leaves per mother plant was not significantly different between isolates ($p \geq 0.05$). However, the highest mean rank was recorded in F3 over all other isolates with a positive Z value (1.27). Runner counts ($p \leq 0.1$) and their dry biomasses ($p \leq 0.05$) were significantly different among treatments with the best Z values in B6 (1.46) and B4 (1.45) for runner production. The highest biomass of runners was recorded in B4 (Z = 1.72) (Table 2).

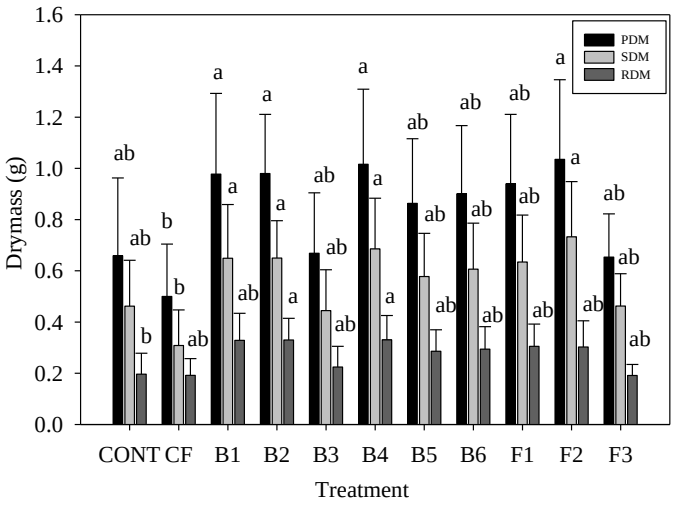


**Figure 2:** Shoot (SDM), root (RDM) and total plant (PDM) biomass (dry) of strawberry plants treated with different fungal (F1-F3) and bacterial (B1-B6) isolates in comparison to control (CONT, with no chemical or biofertilizers) and chemical fertilizers (CF, recommended dosage of CF). Different letters indicate significant differences at 5% probability level. Vertical bars show standard errors of means (SEM).

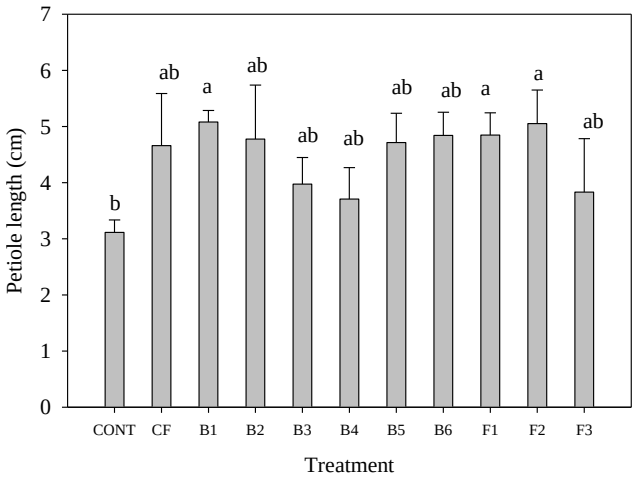


**Figure 3:** Petiole length (cm) of strawberry under different treatments. Different letters on the columns show significant differences at 5% probability level. Vertical bars on the columns show standard errors of means (SEM).

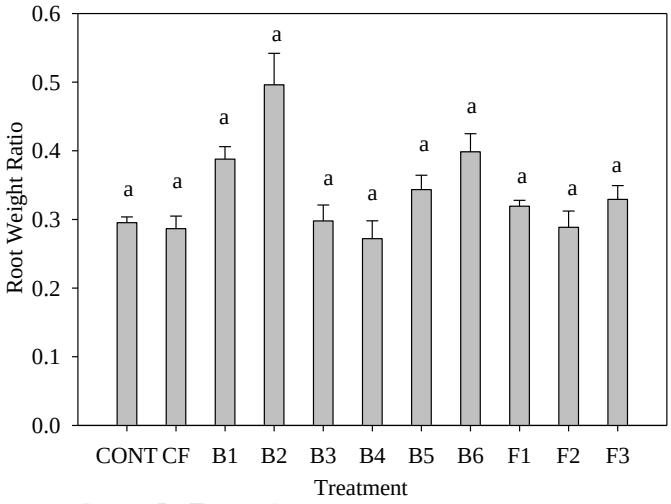


**Figure 4:** Root Weight Ratio (RWR) of strawberry plants under different fungal (F1-F3) and bacterial (B1-B6) isolates, control (CONT, with no chemical or biofertilizers) and chemical fertilizers (CF, recommended dosage of CF). RWR values were not significantly different among treatments at 5% probability level. Vertical bars are the standard errors of means (SEM)

**Table 2:** Number of runners/ mother plant and their dry masses

| Treatment | Mean number of runners/mother plant | Mean dry mass of runners (g)/mother plant |
|---|---|---|
| CONT | 0.840 | 0.103 |
| CF | 0.300 | 0.046 |
| B1 | 1.333 | 0.266 |
| B2 | 1.429 | 0.375 |
| B3 | 0.833 | 0.093 |
| B4 | 1.364 | 0.330 |
| B5 | 1.286 | 0.249 |
| B6 | 1.400 | 0.195 |
| F1 | 1.000 | 0.204 |
| F2 | 1.278 | 0.303 |
| F3 | 0.500 | 0.087 |

According to the growth assay, six bacterial isolates (B1 – B6) and fungal isolates (F1 and F2), have shown the potential to improve the early vegetative growth of strawberry. Previous studies [18,19,44] have also shown growth improvements in strawberry when applied with rhizosphere-originated inoculants. The rhizosphere-isolated *Pseudomonas putida* showed growth improvements in strawberry when combined with arbuscular mycorrhizal fungi [44]. The mixed cultures of *Pseudomonas* and *Bacillus* are also used as inoculants to improve the vegetative growth of strawberry [17]. *Pseudomonas fluorescens* C7r12 and arbuscular mycorrhizal fungi showed growth improvements in strawberry [19]. In favour, the present study showed early growth improvements in strawberry when rhizosphere-originated bacterial strains were used as single-strain inoculants. Mycorrhizal inoculation is a common practice in strawberry to improve growth and yield. However, use of non mycorrhizal fungal inoculants in strawberry has not been recorded in literature.

Therefore, the present study showed some promising results on the use of non mycorrhizal fungal inoculants as potential biofertilizers in strawberry for the first time. Fungal isolates F1 and F2 showed some promising growth improvements in comparison to the control showing their prospects as biofertilizers in strawberry cultivation. Surprisingly, strawberry recorded the least growth with the application of CFs. Early studies revealed that the inputs of agrochemicals diminish soil quality, fertility and microbial diversity in soils [45,46]. Thus, microbial treatments are more effective than that of CFs at least in their early growth promotion.

Strawberry plants are propagated by planting cuttings of stolons. It is also reported that the vegetative growth and runner production in strawberry varieties are solely light [47,48,49] and/or temperature dependent [47]. However, present study revealed that the microbial inoculation to mother plants plays a facilitative role in the growth of daughter-plants, as the daughter-plants originated from the inoculated mother plants enhance their chances of re-colonization by the inoculants [50].

## 4. Conclusion

An initial growth improvement in strawberry can be achieved with the application of some rhizosphere isolates from cultivated and wild strawberries. Isolates B1 (*Bacillus* sp.), B2 (*Enterobacter* sp.), B4 (*Pseudomonas* sp.) and F2 (*Aspergillus* sp.) were the best among all strains tested. Further studies are needed to evaluate the flowering and fruit production under field conditions in order to validate the potential use of these microbial strains as biofertilizers in strawberry.

## 5. Acknowledgement

The work has been partially funded by a research grant provided by the University Grant Commission, Sri Lanka (UGC/ICD/RG/02/2012/10). The authors wish to express gratitude to Dr. Mangalika Nugaliyaddha, Officer-In-Charge of Agricultural Research Station SeethaEliya for providing glasshouse and other facilities to carry out the study.

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