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# Synergistic inoculation of *Azotobacter vinelandii* and *Serendipita indica* augmented rice growth

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## Abstract

In the rhizosphere, microbes act as crucial constituent for plant growth and development. The root-endophytic plant growth-promoting fungus-*Serendipita indica* and nitrogen fixing bacterium *Azotobacter vinelandii* are well known for plant growth promotion and stress alleviation. Individual inoculation of *S. indica* or *A. vinelandii* strain *SRIAz3* has already been reported to augment plant growth. Therefore, it would be interesting to investigate the mutual interaction among these two microbes along with their symbiotic influence on growth of rice plant. The present study explores the interaction of *S. indica* and *A. vinelandii* strain *SRIAz3* under in vitro condition and perceive their cumulative effect on rice growth. Differential growth response of *S. indica* was observed after inoculation of *A. vinelandii* strain *SRIAz3* on different days. An enhanced *S. indica* growth in terms of hyphal radius and dry cell weight was found when *SRIAz3* was inoculated 7 days after fungal inoculation. Moreover, confocal microscopy analysis revealed that *A. vinelandii* strain *SRIAz3* cell-free supernatant increased *S. indica* spore germination. The sequential inoculation of *A. vinelandii* strain *SRIAz3* on seventh day after *S. indica* inoculation in rice variety IR64 demonstrates better growth in terms of root-shoot biomass and chlorophyll content as compared to the non-inoculated and to singly inoculated plants. These findings recommend the sequential inoculation of *S. indica* followed by *A. vinelandii* strain *SRIAz3* for the enhancement of rice growth.

**Keywords** *Serendipita indica* · *Azotobacter vinelandii* strain *SRIAz3* · Microbial interactions · Confocal microscopy · Rice

## 1 Introduction

Resource depletion and rising human population are in urgent need of crop yield enhancement.

Researchers have been utilizing many resources and employing different strategies diligently to increase the crop yield. In the rhizosphere zone, a complex relationship exists between microbes and roots of plants, wherein beneficial interactions promote plant growth and sustainable agriculture (Bandyopadhyay et al. 2016a; Finkel et al. 2017). The utilization of microbial biostimulants can be a powerful approach to revolutionize the agricultural crop yield. However, a relatively limited number of favorable plant-microbe interactions are well described and exploited (Farrar et al. 2014). Notably, *Serendipita indica* (formerly known as *Piriformospora indica*) and *Azotobacter* sp. have been documented as beneficial microbes to promote plant growth and stress alleviation. Particularly, *S. indica*, a root-colonizing plant growth-promoting fungus belongs to the order Sebacinales and can be grown axenically. In addition, *S. indica* has been reported to enhance plant growth and stress tolerance (Waller et al.

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2005; Varma et al. 2012; Jogawat et al. 2013; Gill et al. 2016; Zhang et al. 2017). Obligatorily association of *S. indica* was reported with an endofungal bacterium-*Rhizobium radiobacter* (Sharma et al. 2008). Like *S. indica*, this bacterium has similar stimulating priming and plant growth promoting effects on barley and wheat plants (Glaeser et al. 2015; Glaeser et al. 2017; Guo et al. 2017). Furthermore, *Azotobacter* is free-living aerobic nitrogen-fixing plant growth promoting rhizobacterium (Sethi and Adhikary 2012). Recently, a novel strain *SRIAz3* of *Azotobacter vinelandii* has been reported as a potential biofertilizer which improve the yield and alleviated salt stress in rice (Sahoo et al. 2014a; Dehury et al. 2018).

In recent studies, an effort has been implied to perceive the mutual microbial association viz. fungus and bacteria (Tarkka et al. 2009), wherein bacterial counterpart was reported to enhance fungal spore germination (Lumini et al. 2007; Bandyopadhyay et al. 2016b). Particularly, cell-free supernatant of *Azotobacter chrococcum WR5* increased *S. indica* spore germination (Bandyopadhyay et al. 2016b). Notably, *S. indica* showed either stimulatory, inhibitory, or neutral interaction with other microbes (Varma et al. 2012). For example, confrontation assays revealed *Pseudomonas putida IsoF*, *Azospirilium brasilense* and *Bradyrhizobium* spp. stimulated the growth of *S. indica* (Malla and Pokhare 2008; Varma et al. 2012; Ansari et al. 2014) while, *Pseudomonas fluorescens* strains *WS5* and *SS101* inhibited the growth of *S. indica*. On the contrary, *Herbaspirillum frisingense GSF30$^T$, H. lusitanum P6-12$^T$, Bacillus coagulans NCC235, Bacillus subtilis NCC09* exhibited neutral behavior with the fungus (Varma et al. 2012). Notably, in vitro interaction analysis indicated that *A. chrococcum WR5* and *A. chrococcum M4* influenced *S. indica* growth positively and negatively, respectively (Bhuyan et al. 2015). However, various reports indicated that dual inoculation of fungal-bacterium consortium enhances plant growth viz., inoculation of *S. indica* with *P. fluorescens* increased the growth of *Chlorophytum* (safed musli) plants (Gosal et al. 2010). Co-inoculation of *S. indica* with *A. chrococcum* also improved the growth and yield of *Stevia rebaudiana* (Kilam et al. 2015) and *Artemisia annua* L. plant (Arora et al. 2016).

Though individual inoculation of *S. indica* and *A. vinelandii* strain *SRIAz3* were reported to improve plant growth, further investigation is still required to explore the interaction between these microbes and their cumulative effect on the plant. Therefore, the present study was executed to examine the potential of mutual interaction of *A. vinelandii* strain *SRIAz3* with *S. indica* and to further evaluate its impact on rice growth. The following objectives were set (a): synergistic effect of *A. vinelandii* strain *SRIAz3* on *S. indica* growth, (b) effect of bacterial cell-free supernatant on germination of fungal spores and (c) impact of dual inoculation of *S. indica* and *SRIAz3* on rice plant (variety IR64) growth with the intent of future prospects of these two microbes as biofertilizer for sustainable cultivation of rice.

## 2 Materials and methods

### 2.1 *S. indica* and *Azotobacter vinelandii* growth culture and conditions

The Hill and Kaefer medium (Hill and Kafer 2001) and Jensen medium (Jensen 1942) were used to maintain the cultures of *S. indica* and *Azotobacter vinelandii* strain *SRIAz3*, respectively. Fungal discs of approximately 4 mm were inoculated in 90 mm petri plates with Hill and Kaefer agar medium followed by incubation for 7–8 days at 30 °C, whereas strain *SRIAz3* culture was maintained in Jensen's medium at 28 °C. Further, fungal-bacterial interaction was studied in Hill and Kaefer medium.

### 2.2 Effect of *SRIAz3* on *S. indica* growth

The methodology for the interaction analysis of *S. indica* and *SRIAz3* was adopted from Bhuyan et al. (2015) with slight modifications. In this study, the interaction between *S. indica* and *SRIAz3* was observed in terms of circularly extending fungal mycelia in Hill and Kaefer agar plates. Initially, the agar plates were inoculated with fungal discs measuring up to 4 mm which was obtained from a freshly grown *S. indica* culture. The experiment was performed in two sets; in the first set fungus was allowed to grow by incubating the plates in dark at 28 ± 2 °C for 3 days. Secondary culture of *SRIAz3* (O.D. 0.4 at 600 nm) was streaked towards the periphery on 3 days after fungus inoculation (dafi). The second set of experiments was conducted in a similar way except inoculation of bacterium suspension on 7 dafi. Further, growth of *S. indica* was assessed by measuring the radius of the radially growing hyphae. A control plate was maintained with *S. indica* inoculation only. On plates with *SRIAz3* inoculation on 3 dafi, hyphal radius was measured on 4th, 6th, 8th and 10th dafi. When *SRIAz3* inoculation was performed on 7 dafi, hyphal radii were measured on 8th, 9th, 10th and 11th dafi. For analysis of the interaction in broth, bacterial inoculation was performed on 7 dafi. In brief, 250 ml erlenmeyer flasks were filled up with 50 ml autoclaved Hill and Kaefer broth and were inoculated with a 4 mm disc of *S. indica*. Further, secondary culture of *SRIAz3* (O.D. 0.4 at 600 nm) was sequentially added on 7 dafi along with respective control. Both cultures were harvested after 11 days of fungal growth and filtered through Whatman No.1 filter paper. Further, the fungal biomass was washed three times with 1xPBS buffer and centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the pellet was kept in an oven at 60 °C for 48 h for dry cell weight determination (Kumar et al. 2011).

### 2.3 Impact of *SRIAz3* cell-free supernatant on *S. indica* spore germination

The methodology for isolation of bacterial cell-free supernatant was adopted from Bandyopadhyay et al. (2016b). To investigate the effect of *SRIAz3* cell-free supernatant on *S. indica* spore germination, the bacterial strain was primarily maintained on Jensen's medium and then grown on Hill and Kaefer minimal medium for 3 days. To isolate cell-free supernatant, bacterial culture was subjected to centrifugation (Labogene, Denmark) followed by supernatant filtration through 0.22 μm filter membrane (Millipore). The extracted cell-free supernatant was then added to *S. indica* spores ($10^3$ spores/ml) followed by 12 h incubation. Germinating spores were visualized using Nikon's Confocal microscope A1 at 60× magnification. In order to analyze, the effect of bacterial cell-free supernatant on fungal spore germination the procedure adopted from the Benslim et al. (2016) and following formula was used: %GS = GS/GS + NGS × 100 (% GS: percentage of germinated spores; GS: number of germinated spores; NGS: number of non-germinated spores).

### 2.4 Histochemical analysis of *S. indica–SRIAz3* interaction

Spores were isolated from dual culture plate assays of *S. indica* and *SRIAz3* on 3 and 7 dafi along with control (*S. indica* alone). The methodology for spore isolation was adopted from Hilbert et al. (2013) with slight modifications. To isolate the spores, 1 ml of autoclaved 0.02% Tween 20 solution was added to fresh *S. indica* culture plate and collected in 50 ml falcon tubes (Tarsons, India). Collected suspensions were vortexed and centrifuged for 10–15 min at 5000 g. The supernatant was discarded and pellet was mixed with 10 ml autoclaved distilled water. The spore count was determined by hemocytometer and final concentration was adjusted to $5 \times 10^5$ spores/ml with distilled water. To visualize *S. indica* spores, WGA-Alexa 488 conjugated dye (Invitrogen) was used as per the instructions described by Deshmukh et al. (2006) with slight modifications. Five microlitre of the dye from the stock solution (10 μg/ml) was used to stain the 500 μl spore sample. A drop of the stained sample was placed in the middle of the microscopic glass slide followed by gently placing the cover slip to avoid bubble formation. The images were captured using confocal microscope (Model: Nikon A1) under 60× magnification at Amity Institute of Microbial Technology, Amity University, India. The confocal settings for WGA-Alexa 488 conjugated dye is as follows; excitation-488 nm, emission- 515. Three dimensional image of the spore was constructed using confocal microscope. Further, length, breadth, and height were estimated using NIS Elements software (Nikon, Japan).

### 2.5 Rice growth conditions

Seeds of rice variety, IR64 were sterilized for 10 min in 10% sodium hypochlorite and washed five times with sterile water. Seeds were then placed on sterile germination sheets and incubated at 28 °C for 3–4 days in dark (Bagheri et al. 2013). Autoclaved distilled water was added regularly in Petri plates to maintain the moisture. The soil used in this study was sandy loam, with the following characteristics: pH 7.2; EC 0.37 $dSm^{-1}$; organic C 0.2 g $kg^{-1}$; total N 0.032%; available P 6.2 kg $ha^{-1}$ and K 73 kg $ha^{-1}$. Further, soil, sand and vermicompost were taken in ratio of 3:3:1 and autoclaved at 121 °C with the pressure of 15 psi for 2 h to prepare the mixture (Arora et al. 2016). The germination tray was filled with autoclaved mixture followed by the sowing of pre-germinated seeds. After 14 days of germination, seedlings were transferred in pots filled with 270 g of autoclaved mixture approximately (pot dimension- 11 cm height and 9 cm diameter). The seedlings were supplemented with 40 ml of half-strength Hoagland's solution twice a week (Hoagland and Arnon 1950). The experiment was carried out in controlled environmental chamber where the photoperiod of 16 h was set along with photon flux density of 700 μmol $m^{-2}$ $s^{-1}$. The day and night temperature were maintained at 32 °C and 23 °C, respectively and relative humidity was maintained at 60–80%.

### 2.6 Inoculation of *S. indica* and *SRIAz3* in rice

Spores of *S. indica* were isolated, as explained in section 2.4. Similarly, a fresh culture of *SRIAz3* was harvested by centrifugation at 10000 g for 10 min and adjusted to the concentration of $10^2$ CFU /ml (Kilam et al. 2015). One set of seven-day-old rice plants were subjected to sequential inoculation of *S. indica* and *SRIAz3* (Supplementary Fig. 1). A pierce was done in soil near the vicinity of roots and inoculated with 1 ml of fungal spores with concentration $5 \times 10^5$ followed by *SRIAz3* treatment on seventh day after fungus inoculation (dafi) (Supplementary Fig. 1). A similar process was followed for the individual inoculation of *S. indica* and *SRIAz3*. The sequential and single inoculated plants were utilized for the root colonization, biomass and chlorophyll content estimation along with non-inoculated control plants.

### 2.7 *S. indica* root colonization

A set of ten plants were chosen randomly for root colonization with *S. indica* and *S. indica* + *SRIAZ3* each. Three root fragments from each colonized root were taken for root colonization study. In brief, rice plants were uprooted from soil followed by mild washing in tap water to get rid of adhered soil particles. They were excised and cut into small segments, followed by heating them in 10% KOH for 15 min. Further,

the root segments were treated with 1 N HCl for 4 min and kept overnight after staining with 0.02% Trypan blue followed by destaining with 50% lactophenol. The stained spores were visualized using Nikon light microscope under 10× magnification (Tripathi et al. 2015). For percent root colonization, the methodology was adopted as described by Kilam et al. (2015). The cortex region of stained roots was analyzed for the presence of fungal spores. The following formula was used for root colonization analysis: % root colonization = [(No. of colonized root segments/ Total no. of root segments)] ×100.

### 2.8 Analysis of rice growth and chlorophyll estimation

Four randomly selected (25 day-old) plants of each treatment were uprooted from soil to estimate the length, fresh weight, and dry weight of shoot and root. The plants were placed carefully over a black cloth for measuring shoot and root length with the help of measuring scale. The root and shoot length were measured from the root-shoot junction to tip of the longest root and leaf respectively. The fresh weight and dry weight of the root and shoot were estimated as described by Arora et al. (2016). To estimate the chlorophyll content, four plants were randomly chosen and 1 g leaf tissue was soaked in 10 ml of 80% acetone and further incubated at room temperature for 24 h in dark. Absorbance was measured in a spectrophotometer at 663 and 645 nm for estimation of chlorophyll a and chlorophyll b, respectively. The formula used for calculating the total chlorophyll content is as follows: Chlorophyll a (mg/g) = (12.72A663–2.59A645) × V/(m × 1000); Chlorophyll b (mg/g) = (22.88A663–4.67A645) × V/(m × 1000), where 'm' defines the weight of leaf tissue in mg and 'V' defines volume of solvent. Total chlorophyll estimation was calculated by Chl a + Chl b (Arora et al. 2016).

### 2.9 Statistical analysis

The experiment was designed as a completely randomized design (CRD) with treatment as factors. Means were compared using Fisher's LSD test. The statistical analyses were performed using the Package for Social Sciences version 16 (SPSS16, USA).

## 3 Results

### 3.1 *SRIAz3* modulates the growth of *S. indica* in co-culture

The impact of *SRIAz3* on *S. indica* growth was studied in terms of radial growth on agar plates together with the estimation of dry cell weight and spore germination pattern in liquid cultures. Sequential inoculation of *SRIAz3* on 3 dafi resulted in restricted fungus growth (1.78 cm ± 0.03) as compared with control plate (2.15 cm ± 0.06) (Fig. 1a, b and c). Interestingly, *SRIAz3* inoculation on 7 dafi significantly stimulated the fungus growth (3.1 cm ± 0.02) in comparison to control (2.12 cm ± 0.04) (1D, E and F). In addition to this, a similar experiment of bacterial inoculation on 7 dafi was also performed in suspension culture to determine the dry cell weight. An increase of 14.24% in dry cell weight was observed in the co-culture of *S. indica* and *SRIAz3* as compared to control culture of *S. indica* only (Fig. 1g and h).

To explore the effect of *SRIAz3* on fungal growth, *S. indica* spores were stained with WGA-Alexa 488 dye followed by imaging using confocal microscopy. The results revealed that morphology of *S. indica* spores remain unaltered with *SRIAz3* interaction. Besides, fungal spore length, breadth, and height were also measured in the presence of *SRIAz3* with the help of NIS Elements software (Nikon, Japan). Analysis of spores dimensions from the control plate (*S. indica* only) and co-culture of *SRIAz3*-*S. indica* revealed that morphology of *S. indica* remains unaffected in the presence of *SRIAz3*, and no significant difference was observed in morphology of spores from both samples (Fig. 2a, b and c). Along with this, spore germination analysis was also conducted in presence of bacterial cell-free supernatant. The confocal analysis revealed enhanced fungal spore germination in the presence of the cell-free supernatant as compared to respective control (*S. indica* alone) (Fig. 2d and e). Further, the quantitative spore germination analysis also indicated that enhanced fungal spore germination in presence of bacterial cell-free supernatant (59.3%) as compared with control-*S. indica* alone (42.7%).

### 3.2 *S. indica* root colonization and effect of sequential inoculation on rice growth and chlorophyll content

Rice plants were chosen randomly across the treatments to check *S. indica* colonization. The root samples were examined under a light microscope and trypan blue stained fungal spores showed evidence of root colonization in rice roots. The spores were found to colonize the roots of rice plants treated with the co-culture as well as in individual fungus inoculation. Fungal root colonization analysis indicated a significantly higher level of colonization (65% ±0.05) in the dual culture inoculated roots (*S. indica* + *SRIAz3*) as compared with *S. indica* alone (45% ±0.09). The results of biomass measurements indicated that individual inoculation of *S. indica* or *SRIAz3* alone improved plant growth as compared with non-inoculated control plants. Plants inoculated with both microbes displayed better growth, then respective control plants (Fig. 3a). Notably, sequential combination of *S. indica* and *SRIAz3* treated plants recorded significantly increased fresh weight, dry weight, and shoot-root length as compared with *S. indica* or *SRIAz3* alone inoculated plants. (Fig. 3b–e). The fresh weight and dry weight of shoot was found to be significantly higher in

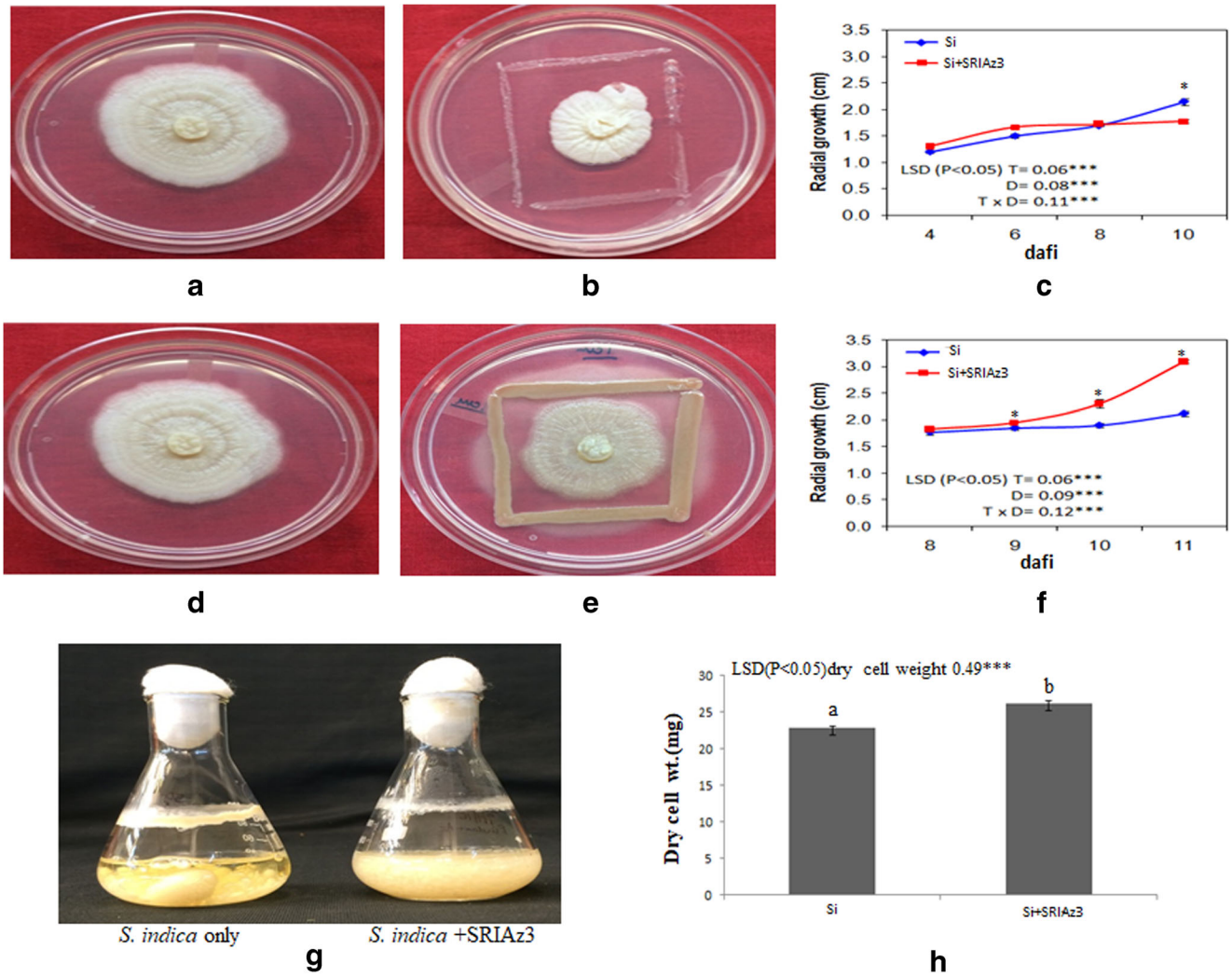


**Fig. 1** Interaction analysis of *Serendipita indica* and *Azotobacter vinelandii* strain *SRIAz3*. Growth of *S. indica* on Hill and Kaefer agar plates without *SRIAz3* inoculation (Control, **a** and **d**) and with *A. vinelandii* inoculation 3 DAFI (**b**) and 7 DAFI (**e**) of the plates. Analysis of hyphal radius at different days after 3 DAFI (**c**), and 7 DAFI (**f**) of *SRIAz3* inoculation (*indicates significant differences between axenic culture of *S. indica* and co-culture of the fungus with *A. vinelandii*; $n = 3$). *S. indica* co-culture with and without *SRIAz3* in suspension culture (**g**), and the respective dry cell weights (**h**). Values are means of 3 biological repetitions and standard errors are indicated. Significant differences among means are demonstrated by different letters. LSD represents least significant difference at significance level $P < 0.05$. DAFI: days after fungus inoculation

*SRIAz3* alone inoculated plants then *S. indica* alone treated plants. While there was no significant difference in root fresh weight and dry weight of plants inoculated individually with fungus and bacteria. Moreover, dual inoculated plants exhibited a significant increase in shoot and root length i.e., 55.2% and 22.9% respectively, as compared to non-inoculated control plants. The results of the chlorophyll estimation analysis revealed significantly increased level of chlorophyll content in *S. indica* alone (2.12 ± 0.38 mg/g) and *SRIAz3* alone (2.27 ± 0.16 mg/g) inoculated plants as compared with non-inoculated plants (1.22 ± 0.05 mg/g). Though, dual inoculated plants revealed significantly higher chlorophyll content (3.13 ± 0.57 mg/g) as compared to all other treatments (Fig. 3f).

## 4 Discussion

### 4.1 *SRIAz3* promotes hyphal growth and spore germination of *S. indica*

To address the influence of the bacterial strain on fungal growth, we investigated the impact of *A. vinelandii* strain *SRIAz3* inoculation on *S. indica* growth under in vitro conditions. Notably, restricted and augmented *S. indica* growth was observed upon sequential inoculation of *SRIAz3* on 3 dafi and 7 dafi, respectively. Further, application to rice seedlings, approved sequential application of both microbes i.e. *SRIAz3* inoculation on 7 dafi colonized rice plants as most successful.

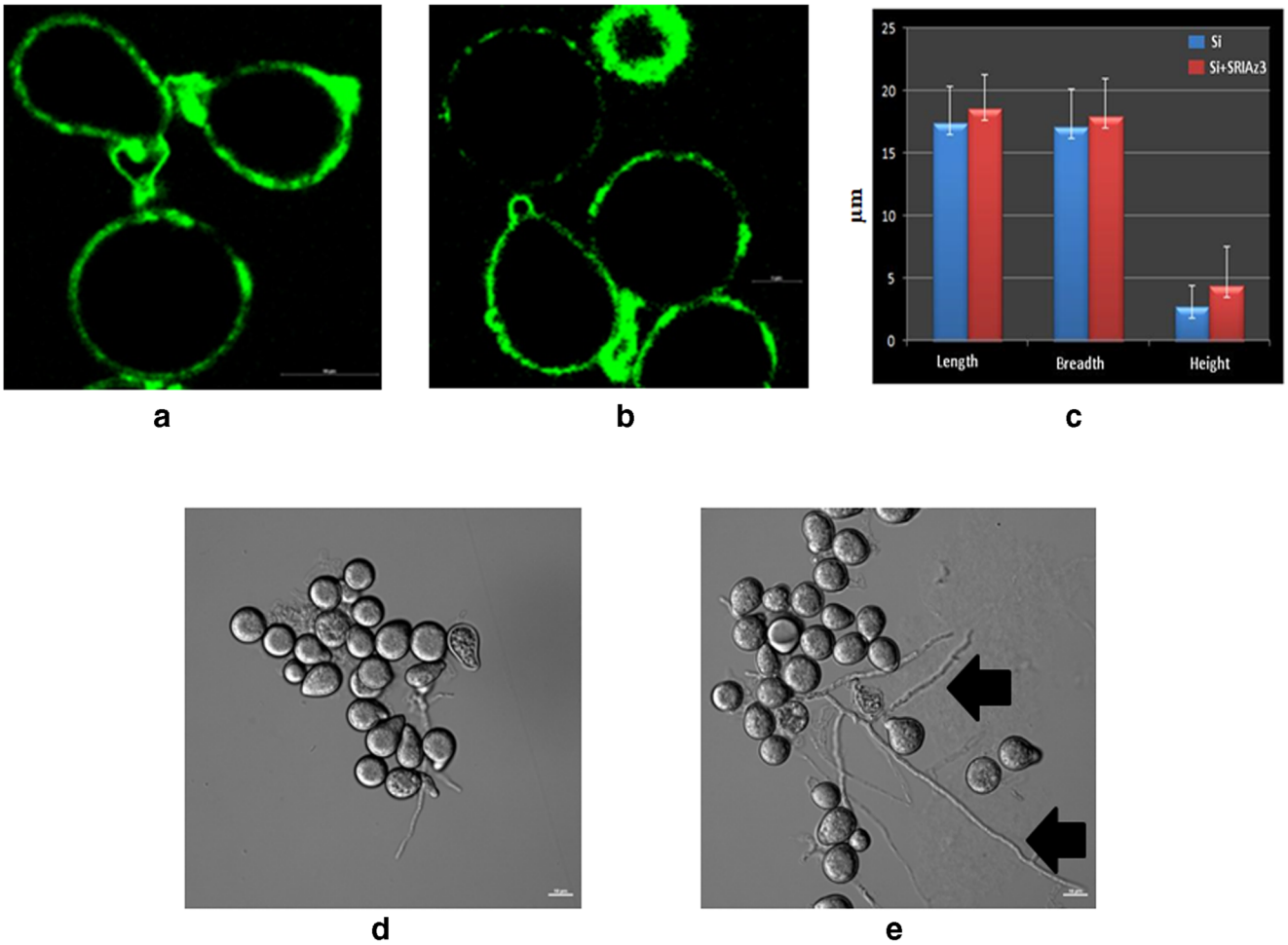


**Fig. 2** Confocal Microscopy based analysis of *S. indica* spores. Morphology of the *S. indica* spores without *SRIAz3* inoculation (**a**), and in the presence of *SRIAz3* (**b**), and their respective NIS elements software-based measurement analysis (**c**). Chlamydospore germination pattern of *S. indica* in Hill and Kafer minimal medium in the absence (**d**), and presence (**e**) of *SRIAz3* cell-free supernatants, arrow indicates hyphae of germinating spores. Scale bar: 10 µm

Recently, co-culture study of strain-specific *A. chroococcum* sequential inoculation after 3 days with *S. indica* demonstrated fungal growth inhibition and stimulation by *M4* and *WR5* respectively (Bhuyan et al. 2015). Though, bacterial counterpart can modulate the *S. indica* growth either positively (*Pseudomonas putida IsoF*, *Azospirilium* and *Bradyrhizobium*) or negatively (*Pseudomonas fluorescens* strains *WS5* and *SS101*) or impartially (*Herbaspirillum frisingense GSF30*$^T$, *Herbaspirillum lusitanum P6-12*$^T$, *Bacillus coagulans NCC235, Bacillus subtilis NCC09*) (Malla and Pokhare 2008; Varma et al. 2012; Ansari et al. 2014). Utilization of the microbial consortium in a sequential manner can be a key factor in plant growth promotion activities. In a bacterial-fungal interaction study, co-inoculation of bacteria (*Shewanella algae* or *Vibrio vulnificusn*) at different days i.e. 5th or 7th days after inoculation with the fungus *Arthrinium c.f. saccharicola* demonstrated increased bioactivity and growth of the fungal partner (Miao et al. 2006). Sequential and simultaneous inoculation of bacteria and fungus on different days indicated differential impact on fungal growth. Plant growth-promoting rhizobacterium (*Pseudomonas* sp. strain *PsJN*) inhibited growth of the fungus *Botrytis cinerea* growth on sequential (2nd day) inoculation, while simultaneous inoculation results into spreading of the fungal counterpart under in vitro condition (Barka et al. 2002). These studies corroborated with our findings of differential response of *SRIAz3* inoculation on 3 dafi and 7 dafi and further suggest sequential inoculation of these microbes.

The specific fungal growth response could be caused by specific bacterial metabolites released into the environment during co-cultivation. The fungal dry cell weight from the co-culture (*S. indica* and *SRIAz3*) was found to be significantly higher than culture of *S. indica* alone. These results go along with Bhuyan et al. (2015), where fungal dry cell weight from co-culture of *S. indica* and *A. chrococcum WR5* was also found to be significantly higher than from a culture of *S. indica* alone. To investigate the impact of the bacterial cell-free supernatant on germination of fungal spores, we

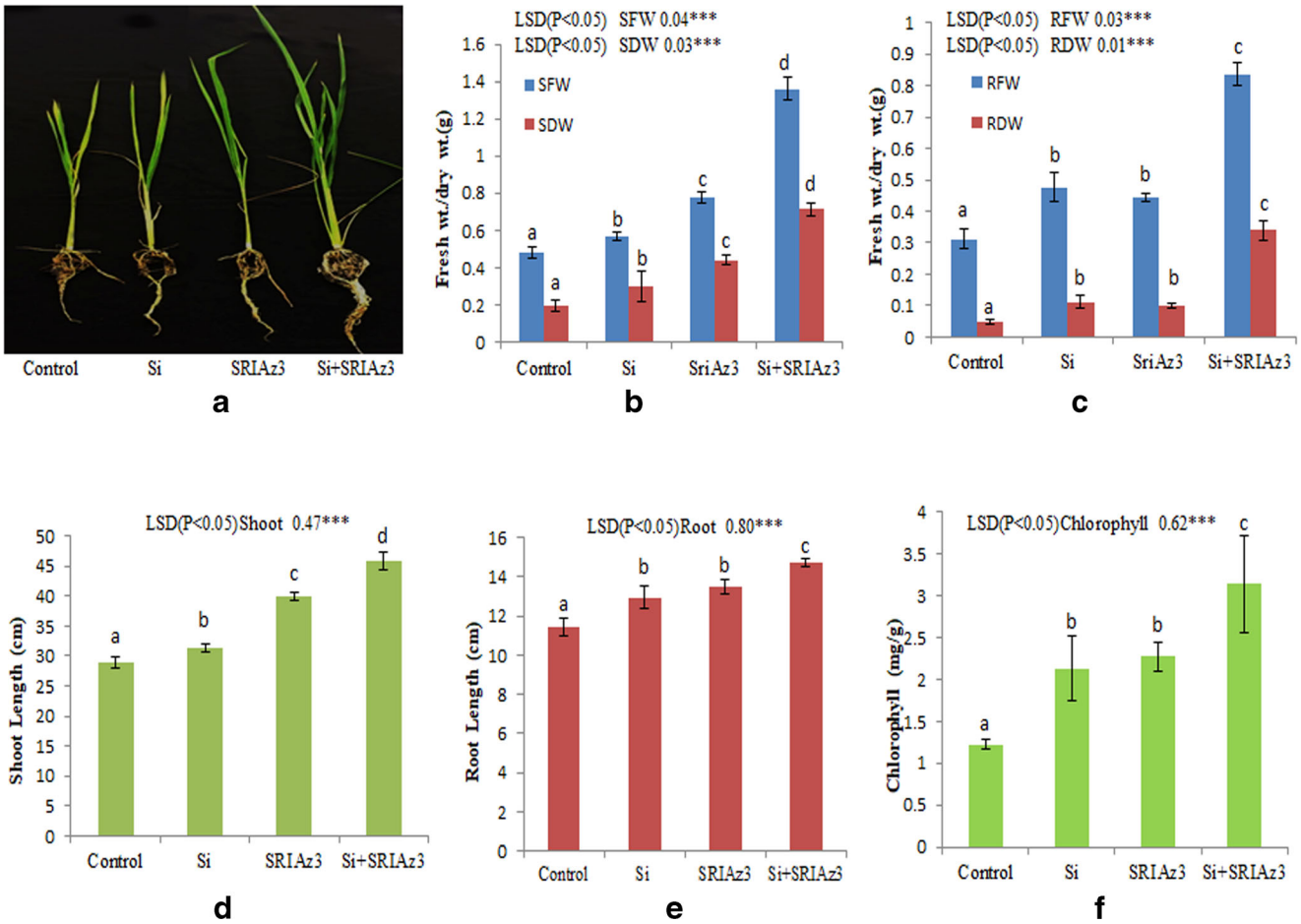


**Fig. 3** *S. indica* root colonization and effect of *S. indica* and *SRIAz3* on rice growth.Rice growth in presence of the *S. indica* and *SRIAz3* inoculation (**a**), Fresh and dry weight of Shoot (**b**) and Root (**c**), Length of Shoot (**d**) and Root (**e**) and Chlorophyll content (**f**) of rice plants in presence of the inoculation of *S. indica* and *SRIAz3* along with control. Values are means of ± SE ($n = 4$) and significant difference among means are demonstrated by different letters on the histogram. LSD represents least significant difference at significance level $P < 0.05$. Significance level: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns = non-significant. SFD: Shoot Fresh Weight, SDW: Shoot Dry Weight, RFD: Root Fresh Weight, RDW: Root Dry Weight

performed spore germination analysis in presence of *SRIAz3*. The results of enhanced spore germination in presence of *SRIAz3* suggest the existence of active bacterial metabolites, which could be responsible for specific fungal growth. These observations correspond with Bandyopadhyay et al. (2016b), where they have shown that diffusible factors from *A. chroococcum* modulated spore germination pattern. Secretory proteins are the key player of microbe-microbe or plant-microbe interactions (De-la-Pena et al. 2008; Netzker et al. 2015; Van Dam and Bouwmeester 2016). Bacterial secretion system affects the physiology of fungus by stimulating or restricting fungal growth (Nazir 2012). In a recent study, cell-free supernatant of *A. chroococcum WR5* was reported to enhance the spore germination of *S. indica*. In the presence of *WR5*, induction of fungal glutamate dehydrogenase and attenuation of cell wall degrading enzyme (α-glucosidase b) were proposed as the mechanism associated with increased spore germination (Bandyopadhyay et al. 2016b). Similar results were reported in our study with *SRIAz3* inoculation, which might indicate the involvement of the similar mechanism as with *A. chroococcum WR5*, to enhance the fungal spore germination.

## 4.2 Dual inoculation augments rice growth

In the rhizosphere zone of soil, interactions among microbes play a key role in plant growth promotion activities (Bandyopadhyay et al. 2016a). Individual inoculation of *S. indica* and *SRIAz3* are well known to stimulate plant growth and development (Varma et al. 2012; Jogawat et al. 2013; Sahoo et al. 2014b; Gill et al. 2016; Zhang et al. 2017; Dehury et al. 2018). In addition, *S. indica* mediated stress alleviation has been reported in numerous crops such as wheat (Serfling et al. 2007), barley (Waller et al. 2005; Deshmukh and Kogel 2007), tomato (Sarma et al. 2011), maize (Kumar et al. 2009), lentil (Dolatabadi et al. 2012) and rice (Das et al.

2014; Jogawat et al. 2013). Moreover, *SRIAz3* is reported to increase rice growth and salt stress tolerance (Sahoo et al. 2014b; Dehury et al. 2018). Inoculation of microbial consortium was reported to enhance plant growth and defense response (Kumar and Jagadeesh 2016). Dual inoculation of *S. indica* and bacteria has been reported to increase the plant growth viz. with *Pseudomonas striata*/*P. fluroscens* in chickpea (Gosal et al. 2010; Nautiyal et al. 2010). Co-inoculation of *S. indica* with *A. chrococcum* improved the growth of rice (Prajapati et al. 2008), *Stevia rebaudiana* (Kilam et al. 2015) and *Artemisia annua* L. (Arora et al. 2016). *S. indica* is well known to colonize the cortex region of plant roots, which further mediates early flowering, enhanced plant growth, and stress tolerance (Varma et al. 2012). In the present study, colonization experiments conducted in rice roots revealed that a combination of *S. indica* and *SRIAz3* inoculation displayed a significant increase in spore colonization as compared to roots treated with *S. indica* only. Similar results were also reported in *Artemisia annua* L. (Arora et al. 2016) and *Stevia rebaudiana* (Kilam et al. 2015), where the combination of *S. indica* and *A. chrococcum* revealed higher root colonization as compared to control.

Bacterial-fungal combination could be associated with the production of growth-promoting substances, as shown in dual inoculation of *S. indica* and *A. chrococcum* in *Artemisia annua* L. (Arora et al. 2016). Nitrogen-fixing bacteria and arbuscular mycorrhizal fungi interactions have been earlier reported to increase in plant growth and nutrient uptake (Artursson et al. 2006). Furthermore, *S. indica* and *P. fluorescens* exhibited better and improved endurance rate with enhanced growth parameters in *Chlorophytum* sp. (Gosal et al. 2010). Similarly, augmented rice growth was observed in this study by dual inoculation of *SRIAz3* after seventh day of *S. indica* treatment as compared to individual inoculation of bacteria/fungus. Moreover, Gosal et al. (2010) and Sharma et al. (2014) reported increased chlorophyll content in *S. indica* inoculated plants as compared with non-inoculated ones. Similar results were also observed in this study in terms of chlorophyll content in inoculated plants.

## 5 Conclusions

Our findings of in vitro interaction analyses concluded that inoculation of *SRIAz3* on 3 dafi inhibited the fungal growth, while stimulated the growth on 7 dafi. Individual inoculation of the *S. indica* or *SRIAz3* resulted into better rice growth. In addition, sequential inoculation of the fungus and bacteria i.e. inoculation of the *SRIAz3* on 7 dafi promote plant growth as compared with respective control. These findings clearly suggest that sequential inoculation of microbes is a worthwhile approach for sustainable agriculture.

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## Compliance with ethical standards

**Conflict of interest** Authors declare that they have no conflict of interest

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