



## Development of low-cost plant probiotic formulations of functional endophytes for sustainable cultivation of *Coleus forskohlii*



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### ABSTRACT

Deployment of plant endophytes at field level is reported to make an impact on agricultural crop productivity; development and deployment of suitable crop specific plant probiotics in a suitable delivery matrix is a value-added task. In our study, we attempted to develop bioformulations of native, fungal endophytes of *Coleus forskohlii* to improve plant yield using two different carrier-based materials (talc and wheat bran). Initially, fungal endophytes (RF1, SF1, and SF2) were grown on sterilized wheat bran under solid state condition and their growth kinetics and pattern were analyzed by ergosterol content and scanning electron microscope, respectively. 10-day-grown fungal endophytic cultures were used for the development of two types of formulations (wheat bran and talc-based formulations) and tested for their efficacy on host plant, *C. forskohlii* under field conditions. Interestingly, application of wheat bran-based endophytic formulations significantly ( $p < 0.01$ ) enhanced plant height (12–29%), number of branches (51–63%), root biomass (26–33%), photosynthetic pigments (32–101%), and forskolin content (35–56%) compared to talc-based formulations under field conditions. Shelf life of endophytes (RF1, SF1, and SF2) in both formulations revealed spore viability in wheat bran-based formulations for 6 months storage period as compared to talc-based formulations. Overall, the present investigation envisages developing plant probiotic bioformulations of functional endophytes of *C. forskohlii* to enhance root biomass and *in planta* forskolin content.

### 1. Introduction

Endophytic fungi are endosymbiotic microorganisms that reside in throughout the plant system without deleterious consequences, and establish beneficial interactions with the host. The study of beneficial interactions between specific microbial species and particular plants is not a recent concept (Lareen et al., 2016). Importance of interaction between plants and endophytes in current research addresses a broad scientific scope to reveal the mode and complexity of interactions. Endophytic interactions contribute to beneficial effects in plants in terms of plant growth and disease management (Khare et al., 2018). Plant-associated microorganisms can be used in preparation of bioformulations to make environment-friendly and sustainable cultivation of important crops (Kalra et al., 2010; Lobo et al., 2019). Application of endophytes to plant system establishes beneficial effects in plants, like improving nutrients uptake (P and N), plant productivity, and also prevention of colonization of foreign parasitic organisms in the host plant (Bamisile et al., 2018). For instance, colonization of *Colletotrichum*

*tofieldiae* significantly improves phosphorus uptake into its host, *Arabidopsis thaliana*, in order to increase fertility and boost plant growth under phosphate-deficient conditions (Hiruma et al., 2016). The other roles of endophytes include production of plant hormones and protection of plant from pathogens (Waqas et al., 2012; Rabha et al., 2014).

During stress conditions, endophytes provide tolerance in plants to heavy metals, drought, and salinity stress, as well as help plants in mitigating stress by exhibiting ACC deaminase activity and subsequent siderophore formation (Ullah et al., 2015; Kong et al., 2015). In addition to above-mentioned beneficial effects, endophytes also play a crucial role in production of pharmaceutically important medicinal compounds like taxol (Kumaran et al., 2010), vincristine (Kuriakose et al., 2016), hypericin (Kusari et al., 2008), and podophyllotoxin (Eyberger et al., 2006). Besides *in vivo* production, *in planta* enhancement of secondary metabolites is also possible by successfully colonized-microorganisms as bioinoculants (Wani et al., 2017). It has been previously reported that the inoculation of *Bacillus megaterium*, *Glomus intraradices*, *Trichoderma harzianum* ThU, and their co-inoculation

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significantly enhances bacoside content in *Bacopa monnieri* (Gupta et al., 2015); while, inoculation of chitinolytic microbes such as *Chitiniphilus* sp. MTN22 and *Streptomyces* sp. MTN14, alone as well as when co-inoculated, significantly upregulates the biosynthetic pathway genes of bacoside A and modulates systemic defense mechanism against nematode, *Meloidogyne incognita* in *Bacopa monnieri* (Gupta et al., 2017). Similarly, earlier studies on endophyte-mediated *in planta* secondary metabolite enhancement also confirmed that inoculation of endophytes enhances vindoline content in *Catharanthus roseus* (Pandey et al., 2016a), and benzyl isoquinoline alkaloids in *Papaver somniferum* (Pandey et al., 2016b). An earlier study reported that the application of bio-inoculants such as *Glomus mosesae* and *Glomus fasciculatum* significantly enhances root yield and forskolin content in *C. forskohlii* (Singh et al., 2009).

*C. forskohlii* is a perennial, branched, medicinal plant, belonging to the family Lamiaceae, and distributed in many places of the world including India, China, Africa, Pakistan, and Sri Lanka (Shukla et al., 2018). An extract from this plant has been used for centuries in Ayurvedic medicine to treat various health problems (Ding et al., 2005). Tuberos roots of the plant contain a diterpene compound, forskolin, which is a main active ingredient of the plant, and is used for various disorders. Forskolin activates adenylate cyclase, a membrane bound enzyme that is responsible for various biological mechanisms in reproductive organs, endocrine system, urinary system, olfactory system, nervous system, platelet aggregating system, gastrointestinal tract, respiratory tract, skin, bones, eyes, and smooth muscles; hence, the activation of this enzyme by forskolin may regulate various mechanisms in the body (Alasbahi and Melzig, 2012). In addition, forskolin also regulates other membrane proteins like glucose transporter, voltage-gated potassium channel, and ligand-gated ion channel (Joost and Steinfelder, 1987; Zhang et al., 2015). Due to its activation efficiency on adenylate cyclase, forskolin is considered as a significant tool for studying the role of cAMP in the body to treat various disorders (Alasbahi and Melzig, 2012). In general, forskolin shows multifaceted pharmacological activities, e.g. stimulating thyroid hormones (T4 and T3) (Laurberg, 1984), lowering blood pressure (Jagtap et al., 2011), increasing the rate of lipolysis (Loftus et al., 2015), lowering aqueous humor flow in eyes (Burstein et al., 1984), reducing brain microglial response (Owona et al., 2016), lowering inflammation response (Dahle et al., 2005), and controlling glucose level in blood (Ríos-Silva et al., 2014). However, less content of forskolin is present in roots of *C. forskohlii* (Pateraki et al., 2017). Purification of natural compounds from plants has proven infeasible in many cases due to low yield (Nielsen et al., 2014). Hence, enhancement of forskolin content within the root system significantly increases the production, profit and reduces the investment and cost.

A few endophytes of microbial origin are now available for commercial use. Generally, a major constraint of this potential sector is the delivery of promising endophytes into farmers' field. Green approaches like application of crop specific endophytes in a suitable formulation matrix could be an economical approach during sustainable cultivation of medicinal plants. In view of the above facts, efforts were made to develop suitable carrier-based formulations of three functional fungal endophytes *Fusarium redolens* (RF1), *Phialemoniopsis cornearis* (SF1), and *Macrophomina pseudophaseolina* (SF2) for cultivation of *C. forskohlii* and their evaluation under field conditions.

## 2. Materials and methods

### 2.1. Source of endophytes and base materials

In our previous studies, the endophytic fungi RF1, SF1, and RF1 were isolated from *C. forskohlii* and identified as *Fusarium redolens* KY992586 (RF1), *Phialemoniopsis cornearis* MK408657 (SF1) and *Macrophomina pseudophaseolina* MF351729 (SF2) (Fig. S1), and were deposited at National Collection of Industrial Microorganisms (NCIM),

Pune with accession numbers NCIM-1402, NCIM-1404, and NCIM-1403, respectively (Mastan et al., 2019). The above three endophytes were used for the development of low-cost plant probiotic formulations in wheat bran and talcum powder purchased from the local market, as explained below.

### 2.2. Preparation of wheat bran and talc-based endophytic formulations

Initially, 60 g of wheat bran was taken in 500 mL conical flask and 48 mL of water was added to attain 80% moisture level. The wheat bran containing flasks were autoclaved and cooled to room temperature. After cooling, five circular agar plugs (6 mm in diameter) containing pure endophytic fungi RF1, SF1, and RF1 were inoculated into sterilized wheat bran. The inoculated flasks were incubated at 28 °C for 10 days; during the incubation period, from 4th to 10th day, 1 g of the sample was collected each day and subjected to ergosterol estimation. On 6th day, 1 g of sample was collected and subjected to scanning electron microscopy (SEM), to analyze growth pattern of fungal endophytes on wheat bran substrate. For SEM analysis, RF1, SF1 and SF2 grown on wheat-bran and empty wheat-bran (control) were fixed separately overnight at 4 °C by adding formaldehyde to a final concentration of 7%. The critical-point dried wheat bran-grown mycelial samples were mounted on scanning electron microscope stubs, sputter-coated with gold, and viewed on a FEI Quanta 200 3D dual beam scanning electron microscope.

After 10 days of incubation, total CFU of wheat bran-grown fungal endophytes (SF2, SF1 and RF1) were determined to be  $38 \times 10^{12}$ ,  $14 \times 10^{11}$ , and  $21 \times 10^{12}$  CFU g<sup>-1</sup>, respectively. 50 g of wheat bran-grown fungal endophytes was collected, dried and powdered. Finally, 20 g of dried endophyte-containing wheat bran powder was mixed with 80 g of sterilized wheat bran or 80 g of sterilized talcum powder to prepare wheat bran-based or talc-based formulations, respectively. These developed endophyte formulations were finally normalized for  $8-12 \times 10^{10}$  CFU g<sup>-1</sup> of carrier material and packaged in high-density polyethylene (HDPE) covers and stored at 30 °C for shelf life analyses up to 6 months.

### 2.3. Fungal endophyte growth analysis by ergosterol assay

Ergosterol (sterol) is present in fungal cell membrane and absent in plants. Hence, in order to estimate the growth kinetics between fungal endophytes on wheat bran solid medium, ergosterol-estimation assay was performed (Ng et al., 2008). During their growth, 1 g of fermented medium was collected every day for 10 days and ergosterol was estimated by TLC method. Initially, 1 g of sample was macerated in 1 mL of extraction buffer containing chloroform, methanol, and 2% phosphoric acid (1:2:1) and vortexed for 30 min. After vortex, the homogenate was centrifuged and the upper aqueous layer was removed. The chloroform layer, which contains ergosterol, was collected in a 1.5 mL eppendorf tube and extraction was repeated twice. The obtained chloroform layer was concentrated in vacuum drier (V-AL mode) for 20 min at 30 °C, and the obtained sample was re-constituted in 40 µL chloroform. From this, 10 µL sample was loaded on aluminium pre-coated silica gel 60 F254 plates (Merck). The sample loaded TLC plates were developed in mobile phase containing diethyl ether, petroleum ether and acetic acid (30:70:1). The developed plates were visualized under UV at 254 nm before staining the plates. The completely dried plates were dipped in neutral lipid staining (0.315 g MgCl<sub>2</sub>, 30 ml methanol, 30 ml H<sub>2</sub>O, and 2 ml con. H<sub>2</sub>SO<sub>4</sub>), and the plates were charred for 10 min at 110 °C in hot air oven. The charred plates showed ergosterol in brown to dark blue color bands. Quantification of ergosterol in each day sample was carried out using standard graph of 98% ergosterol purchased from Sigma Aldrich. Using Spot Densitometric Analyzable Software AlphaEaseFC (version 4.0.0, Alpha Innotec), the intensity level of the total band size of ergosterol was measured (Verbitski et al., 2008).

## 2.4. Viability analysis of bioformulations

To analyze the viability of fungal endophytes in the prepared wheat bran and talc-based formulations, standard plate count method was performed every month until 6 months of storage period. For standard plate count, 1 g of each bioformulation sample was dissolved in 10 mL of sterilized distilled water and serially diluted up to  $10^{-11}$  and the sample spread plated on potato dextrose agar (PDA). The plates were incubated at 28 °C for 5 days; after incubation, the formed fungal colonies on PDA were measured in terms of CFU  $g^{-1}$  of formulation.

## 2.5. Efficacy of wheat bran-based fungal formulations on *C. forskohlii* under field conditions

To analyze the effect of wheat bran-based endophyte formulations on *C. forskohlii*, individual field experiments were carried out in the experimental farm of CIMAP Research Centre, Bangalore, India. Initially the plants were propagated by stem cuttings in polythene bags. Further, the rooted cuttings were transplanted into field conditions where the plants were raised in experimental plots (dimension 3 × 3 m) comprising four rows (each row containing 4 plants). Under field conditions, 60 cm distance between plant to plant as well as plant to ridge was maintained, and each bed was separated by 40 cm guard ridge to lessen the effect of adjoining plots. After 30 days of transplantation, 2 g of endophyte (RF1, SF1 and SF2)-containing wheat bran formulations ( $11 \times 10^9$  CFU  $g^{-1}$ ) were inoculated around the root zone of each plant. *C. forskohlii* ( $n = 16$ ) plants of one complete bed without any endophyte treatment served as control. Each bed contained 16 plants in 4 rows; however, four central plants were considered as non-peripheral plants for taking the growth measurements. Plant growth parameters including plant height and number of branches were recorded. After 150 days of transplantation, the plants were harvested by manually uprooting the complete plant without harming the roots and fresh shoot, and root weight was measured. Forskolin content of roots were analyzed from shade dried roots.

## 2.6. Application of Talc-based fungal formulations on *C. forskohlii* under field conditions

In order to analyze the effect of talc-based endophyte formulations on plant growth, plant yield, and forskolin content of *C. forskohlii*, field level experiments were performed at CSIR-CIMAP, Bangalore, India. The experimental bed was initially transplanted with 16 rooted-plants, half of the bed (8 plants) was used for endophyte inoculation, while the other half (8 plant), without any endophyte treatment, was considered as control. Two parts of the bed were separated by 30 cm guard ridge to prevent cross contamination. Each plant was inoculated with 2 g ( $11 \times 10^9$  CFU  $g^{-1}$ ) talc-based endophyte formulation (RF1, SF1 and SF2). After 150 days post-inoculation, growth parameters and forskolin content were analyzed.

## 2.7. Quantification of forskolin

Efficacy of wheat bran and talc-based formulations of endophytes on forskolin content was analyzed by thin layer chromatography (TLC). Dried root samples of *C. forskohlii* from field experiment were powdered and 100 mg crude powder was macerated in 500  $\mu$ L of ethyl acetate. After 30 min vortex, the samples were incubated for 2 h at room temperature and supernatant was separated by centrifugation for 4 min at 10,000 rpm. The supernatant was concentrated in vacuum drier (V-AL mode) for 30 min at 30 °C and the obtained concentrate finally dissolved in 50  $\mu$ L methanol. 2  $\mu$ L of each sample was used to load on aluminium pre-coated silica gel 60 F254 plates (Merck). The reference standard forskolin (95%) was purchased from Natural remedies and used for this study. The plates were developed in a presaturated (15 min with tissue paper) chamber with mobile phase, ethyl acetate and

hexane (3:7 ratio). Developed plates were dipped in anisaldehyde reagent for 5 s and dried in hot air oven at 110 °C for 10 min to visualize the forskolin bands. The stained plates showed forskolin bands in pink color and the plates subjected immediately to scanning of the TLC image. The forskolin bands were quantified using standard graph, which was developed by varying concentrations of forskolin (95%) ranging from 2 to 100  $\mu$ g. Using AlphaEaseFC (Spot Densitometric Analyzable Software, version 4.0.0, Alpha Innotec), the intensity level of the total band size of forskolin was quantified (Verbitski et al., 2008). Variation of forskolin content in formulations treated and control *C. forskohlii* plants was compared by referring to the standard graph.

## 2.8. Photosynthetic pigments estimation

After 150 days of inoculation, the healthy leaves from both wheat bran-based, talc-based formulations treated plants and control *C. forskohlii* plants were collected and analyzed for photosynthetic pigments (chlorophyll *a* and *b* and carotenoids). Uniform leaf area was collected from each treatment using cork borer (6 mm in diameter). Samples were macerated using motor and pestle and extracted using 1 mL of ice-cold methanol. The whole extraction process was carried out under low luminous condition. The samples were then incubated in a refrigerator at 4 °C for overnight. Thereafter, supernatant was collected using centrifugation at 5000 rpm for 5 min. Absorbance values of samples were measured at 470 nm, 653 nm, and 666 nm from three replicates. For pigment estimation, the obtained readings were substituted in their respective formulas (Lichtenthaler and Wellburn, 1971). Chlorophyll *a* =  $(15.65 A_{666} - 7.34 A_{653})$ , Chlorophyll *b* =  $(27.05 A_{653} - 11.21 A_{666})$ , and Carotenoids =  $[1000 A_{470} - (2.86 \text{ Chl } b - 129.2 \text{ Chl } a)/245]$ .

## 2.9. Statistical analysis

Mean and standard deviation of each individual experiment were calculated. Graphical data was performed by Sigma Plot software (Version 10). The difference in photosynthetic pigments and forskolin yield between control and endophyte treated plants were analyzed from three biological replicates. Significant difference of plant biomass, forskolin content and photosynthetic pigments between control and endophyte treated plants were analyzed at  $p < 0.01$  and  $p < 0.05$  by one-way ANOVA analysis with Dunnett multiple comparisons test using Graph Pad prism Software (GraphPad version 6.0, San Diego, CA).

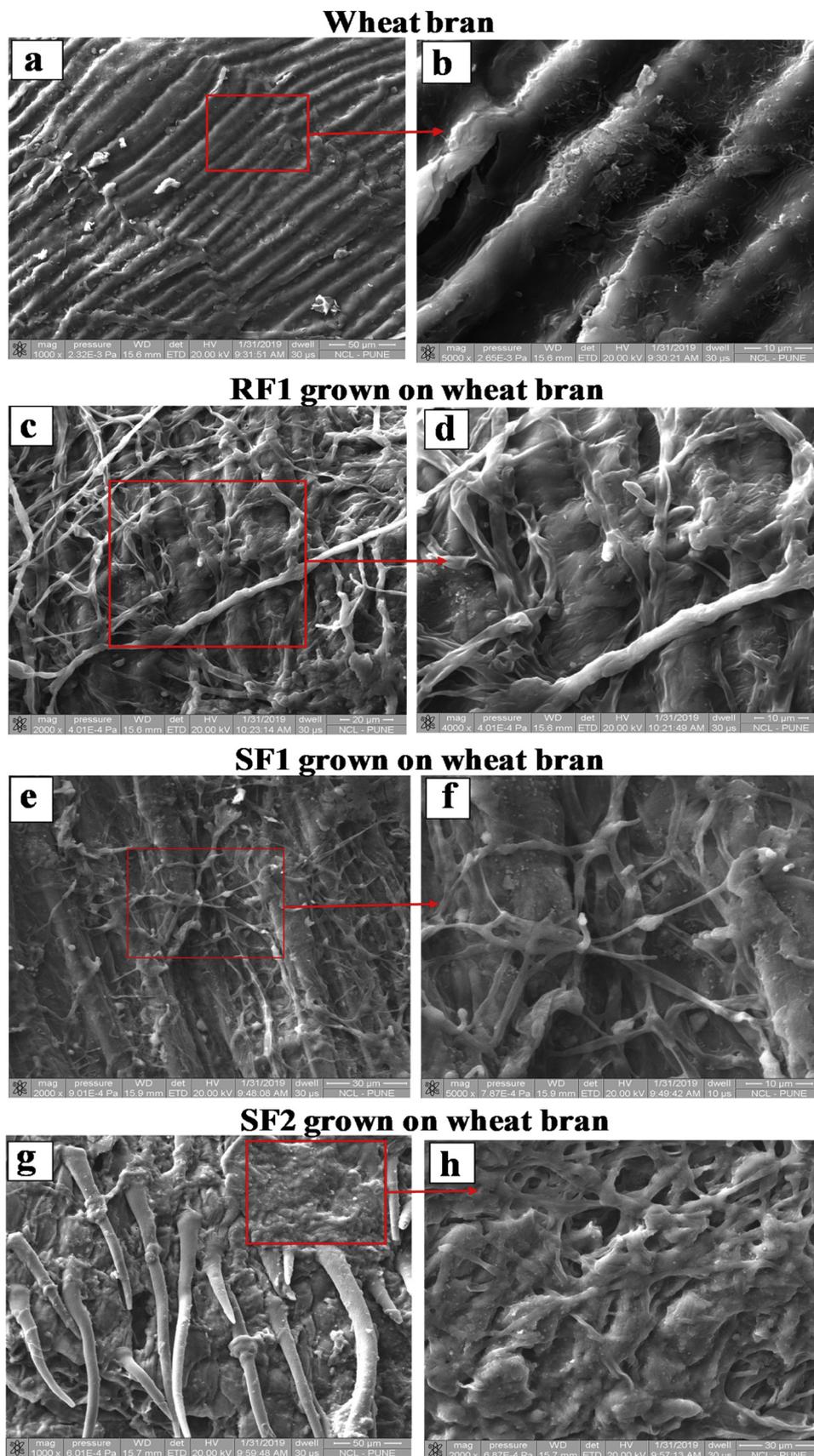
## 3. Result

### 3.1. Development of fungal endophytic formulations

Endophytes are endosymbiotic microorganisms and considered to have a plethora of plant growth promoting and plant metabolite stimulating traits. In our previous studies, we found three functional fungal endophytes for improving plant growth and forskolin content of *C. forskohlii* and identified as *Fusarium redolens* (RF1), *Phialemoniopsis cornearis* (SF1), and *Macrophomina pseudophaseolina* (SF2). However, delivery of these promising endophytes into farmers' field is a major constraint. Hence, in the present study the wheat bran- and talc-based formulations of fungal endophytes (RF1, SF1, and SF2) were developed and separately stored in high-density polyethylene (HDPE) covers (Fig. S2) and used for further studies.

### 3.2. Scanning electron microscopy of fungal endophytes grown on carrier matrix

Wheat bran-grown endophytes via scanning electron microscopy clearly showed endophyte growth pattern on wheat bran matrix after 6 days of solid-state fermentation (Fig. 1). However, growth variation between endophytes was noticed with SF2 and RF1, having more growth on wheat bran substrate compared to SF1 endophyte.



**Fig. 1. Screening of endophytes growth on wheat bran particles by SEM.** Wheat bran particles without endophyte inoculation (a and b), growth of RF1 endophyte on wheat bran particles (c and d), growth of SF1 endophyte on wheat bran particles (e and f), and growth of SF2 endophyte on wheat bran particles (g and h). Scale bar 50  $\mu\text{m}$  (a and g); 10  $\mu\text{m}$  (b, d, and f); 20  $\mu\text{m}$  (c); 30  $\mu\text{m}$  (e and h).

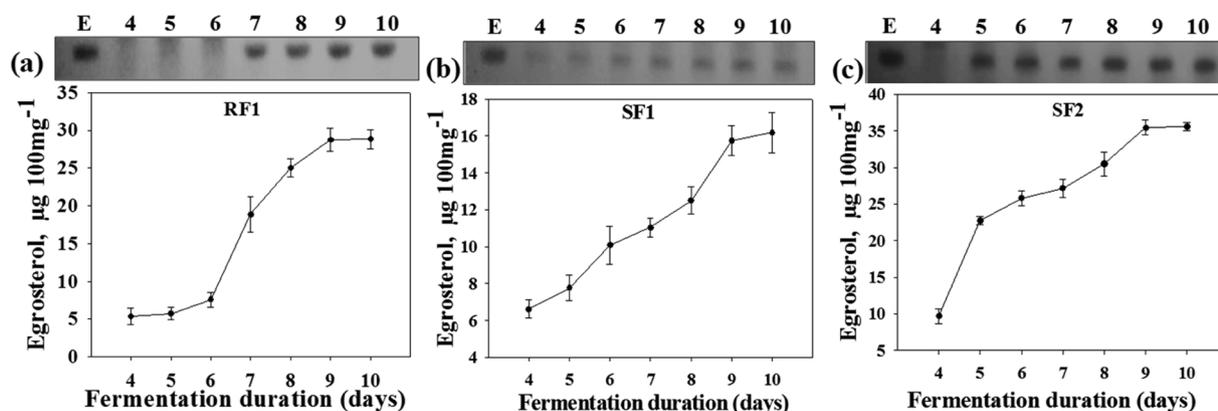


Fig. 2. Estimation of endophyte growth by ergosterol assay during solid state fermentation. (a) growth progression of RF1, (b) SF1 growth progression, and (c) SF2 endophyte growth progression during fermentation. Error bars from average of three replicates. TLC plates above the graphical images indicate ergosterol content ( $\mu\text{g } 100 \text{ mg}^{-1}$  medium) from 4th to 10th day of endophytes growth. E: ergosterol standard and numbers above plate indicate days of the fermentation.

Interestingly, this result was directly correlated with pattern of ergosterol content in fungal mycelium during their growth (Figs. S5, S6 and S7) and initial spore count of RF1 ( $21 \times 10^{12} \text{ CFU g}^{-1}$ ), SF1 ( $14 \times 10^{11} \text{ CFU g}^{-1}$ ), and SF2 ( $38 \times 10^{12} \text{ CFU g}^{-1}$ ) at 10 days of growth.

### 3.3. Fungal growth estimation by ergosterol assay

Ergosterol is the main sterol found in fungal cell membrane, and estimation of ergosterol is a rapid tool to measure fungal growth (Ng et al., 2008). Ergosterol estimation during solid state (wheat bran) fermentation showed significant variation between growth of the fungal endophytes (Fig. 2). The SF2 inoculated medium showed significant growth and continuous enhancement of ergosterol content from 5th to 10th day ( $22\text{--}35 \mu\text{g } 100 \text{ mg}^{-1}$  substrate). However, SF2 endophyte growth saturation was noticed on the 9th day of fermentation (Figs. 2c; S7). In case of RF1, during early fermentation period (1st–6th day), no significant growth was observed in wheat bran medium; interestingly, after 6 days of fermentation, significant growth and ergosterol content was noticed ( $17\text{--}27 \mu\text{g } 100 \text{ mg}^{-1}$  mg). Saturation of RF1 growth was detected between 9th to 10th day of fermentation (Figs. 2a; S5). Growth of SF1 endophyte showed continuous enhancement of ergosterol from 6th to 10th day of fermentation duration ( $9$  to  $15 \mu\text{g } 100 \text{ mg}^{-1}$ ), which is, however, an indication of significantly low growth compared to other endophytes (SF2 and RF1) (Fig. 2b; S6). For development of formulations, 10th day of fermented media was used and CFU of all endophytes were normalized ( $8$  to  $12 \times 10^{10} \text{ g}^{-1}$ ) for field level evaluation and shelf life estimation.

### 3.4. Viability and shelf life of fungal endophytes in formulation matrix

Standard plate count revealed variations in viability of endophytes (RF1, SF1 and SF2) in developed wheat bran-based and talc-based formulations during the period of 6 months storage. All the endophytes in talc-based formulations showed significant reduction in their viability compared to wheat-bran based formulations during the storage (Fig. 3). Interestingly, RF1 endophyte showed significant viability followed by SF1 in wheat bran-based formulations compared other formulations (Fig. 3a). The endophyte, SF2, showed continuous reduction of spore viability in both wheat bran and talc-based formulations. However, SF2 endophyte in wheat bran-based formulations maintained considerable spore viability ( $8 \times 10^6 \text{ CFU g}^{-1}$ ) for 6 months, which is more than the viability level ( $< 2 \times 10^6 \text{ CFU g}^{-1}$  minimum level) recommended by the Central Insecticides Board Act, Govt. of India (Tripathi et al., 2015). In talc-based formulation, all endophytes lost the recommended viability within 4 months of storage period (Fig. 3b). Overall, all endophytes in wheat bran-based formulation showed significant or considerable viability up to 6 months of storage compared to

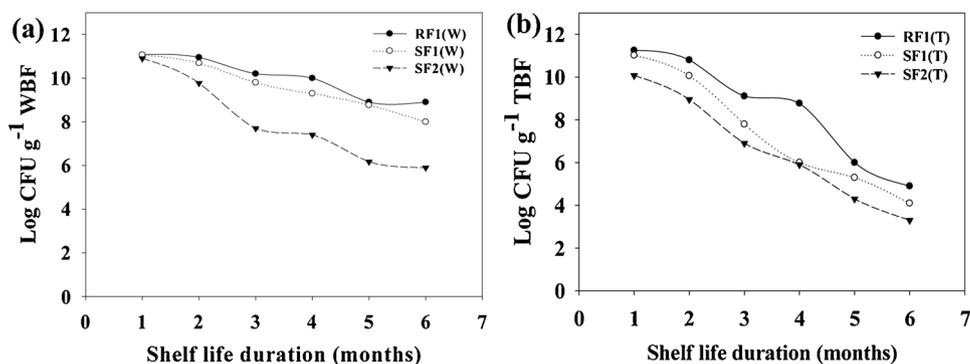
talc-based formulations.

### 3.5. Evaluation of wheat-bran based endophyte formulations for *C. forskohlii* cultivation under field conditions

Inoculation of wheat bran-based endophyte formulations (RF1, SF1, and SF2) differently enhanced the plant growth parameters like plant height, number of branches, and fresh and dry weight of shoot and root (Figs. 4; S3). Treatment of plants with RF1 formulations significantly enhanced plant height (29%) and number of branches (63%) compared to untreated control plants (Fig. 4a, b). In case of SF1 endophyte treated plants, less significant ( $p < 0.05$ ) enhancement of plant height (12%) and more significant ( $p < 0.01$ ) enhancement of number of branches (51%) were noticed. Similarly, inoculation of SF2 endophyte formulations revealed significant result in plant height (18%) and number of branches (54%) over the control plants. In case of plant biomass, the plants treated with RF1 formulations showed significant enhancement in fresh shoot (50%) and root weight (89%) as well as dry shoot (22%) and root weight (27%) compared to untreated plants (Fig. 4c, d). While SF1 endophyte treatments enhanced only dry root yield (33%) more significantly compared to control plants, interestingly, SF2 endophyte treatments enhanced fresh and dry weight of root (82 and 26% respectively) more significantly than fresh and dry weight of shoot. Overall, all the three endophyte treatments significantly enhanced plant growth parameters compared to control plants. However, RF1 treated plants showed more significant enhancement in all plant growth parameters compared to other treatments as well as untreated control plants.

### 3.6. Evaluation of talc-based endophyte formulations for *C. forskohlii* cultivation under field conditions

Effect of talc-based endophyte formulations was tested on *C. forskohlii* under field conditions (Fig. S4). Interestingly, none of the talc-based endophyte formulations showed significant enhancement in plant height and branch number, though less significant enhancement ( $p < 0.05$ ) of branch number was noticed only in RF1 endophyte treated plants (22%). The shoot endophytes (SF1 and SF2) treated plants did not show any significant effect in branch number (Fig. 5a, b). In case of plant biomass, inoculation of talc-based RF1 formulations only enhanced the fresh shoot biomass in less significant amount (20%), but enhanced neither fresh root weight nor dry shoot or root weight (Fig. 5c, d). However, the other two endophyte (SF1 and SF2) formulations did not show any significant enhancement in fresh shoot and root weight or dry shoot and root weight. Overall, the plants treated with wheat bran-based formulations significantly enhanced all plant growth parameters compared to talc-based formulations.



**Fig. 3.** Endophyte viability study in wheat bran and talc-based formulations for 6 months of storage period. CFU g<sup>-1</sup> were log-transformed (log CFU g<sup>-1</sup>) to improve homogeneity of viability variances. (a) viability of endophytes (RF1, SF1, and SF2) in wheat bran-based formulations. (b) viability of endophytes (RF1, SF1, and SF2) in talc-based formulations.

**3.7. Impact of endophytic formulations on forskolin content**

Application of wheat bran-based formulations and talc-based formulations of endophytes (RF1, SF1, and SF2) were evaluated for their efficiency in forskolin enhancement under field conditions (Figs. 6; S8). Among these, wheat bran formulations of RF1 (780 μg 100 mg<sup>-1</sup> dry root), SF2 (676 μg 100 mg<sup>-1</sup>) and talc-based formulations of RF1 (690 μg 100 mg<sup>-1</sup>) significantly enhanced the forskolin content by 56, 35% and 38%, respectively compared to other formulations as well as uninoculated control plants (497 μg 100 mg<sup>-1</sup>). However, application of wheat bran formulations of RF1 showed more enhancement of forskolin compared to wheat bran-based SF2 formulations and talc-based RF1 formulations. Overall, application of wheat bran-based endophyte formulations showed more significant enhancement in forskolin content compared to talc-based formulations.

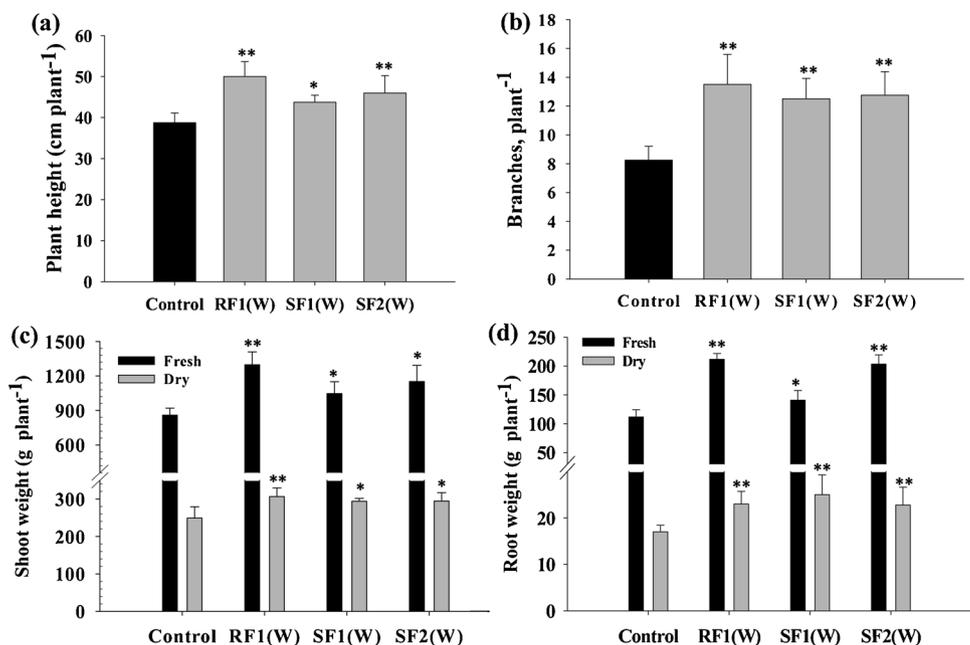
**3.8. Impact on photosynthetic pigments**

In plants, photosynthesis is the main process for providing carbon-based molecules, which are the main precursors for synthesis of primary and secondary metabolites. Hence, impact of wheat bran and talc-based formulations of endophytes on photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoid) was evaluated under field conditions. Interestingly, application of wheat bran-based endophyte formulations (RF1 and SF2) significantly enhanced chlorophyll a (96 and 65% respectively), chlorophyll b (90 and 32% respectively), and carotenoid (101 to 65%, respectively) content compared to control

plants (Fig. 7). Similarly, inoculation of SF1 endophyte enhanced the carotenoid content (32%) and there is no significant effect on chlorophyll a and b content. In case of talc-based formulations, only RF1 formulations enhanced chlorophyll a and b content but not carotenoid compared to other talc-based formulations and control plants (Fig. 8). Overall, the application of wheat bran-based formulations of endophytes significantly enhanced the photosynthesis compared to talc-based formulations.

**4. Discussion**

Endophytes establish beneficial interactions with their host plants and contribute positive effects in terms of plant growth and secondary metabolite enhancement. Application of fungal endophytes can improve plant fitness and productivity. So far, with several practical inoculation modes, significant colonization of fungal endophytes was noticed in various important crops, which include *Opium poppy* by *Beauveria bassiana* (Quesada-Moraga et al., 2006), wheat by *Metarhizium brunneum* (Jaber, 2018), rice by *Piriformospora indica* (Mohd et al., 2017), Soybean by *Cladosporium sphaerospermum* (Hamayun et al., 2009), and *Ocimum basilicum* by *Serendipita indica* (Sabra et al., 2018). Application of plant growth promoting *Streptomyces corchorusii* through powder formulations significantly enhanced the shoot length, weight of shoot and root, total grain yield and weight of grains in rice plants (Tamreihao et al., 2016). Similarly, incorporation of solid substrate formulations of *Rhizoctonia* spp. in potting mix significantly reduced the severity of damping-off of diseases in *Capsicum annuum* by inhibiting



**Fig. 4.** Effect of wheat bran-based endophyte formulations on primary plant productivity of *C. forskohlii* under field conditions. After 150 days of post inoculation (dpi) plant height (a), number of branches (b), fresh and dry weight of shoot (c), fresh and dry weight of root (d) were measured. The mean value and standard deviation of 16 plant of each treatment. The asterisks above the bars indicate significant difference. *P* significant values were represented as follows \**p* < 0.05 and \*\**p* < 0.01.

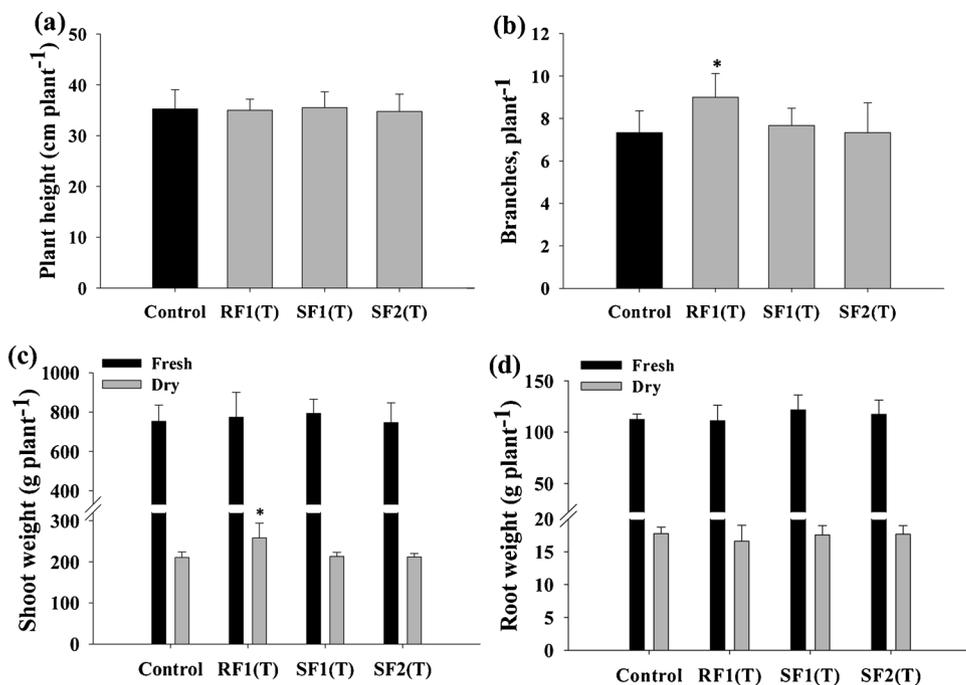


Fig. 5. Effect of talc-based endophyte (RF1, SF1, and SF2) formulations on primary plant productivity of *C. forskohlii* under field conditions. After 150 dpi, height (a), number of branches (b), fresh and dry weight of shoot (c), fresh and dry weight of root (d) were measured. The average mean value and standard deviation of 8 plant of each treatment. The asterisk above the bars indicates significant difference ( $*p < 0.05$ ).

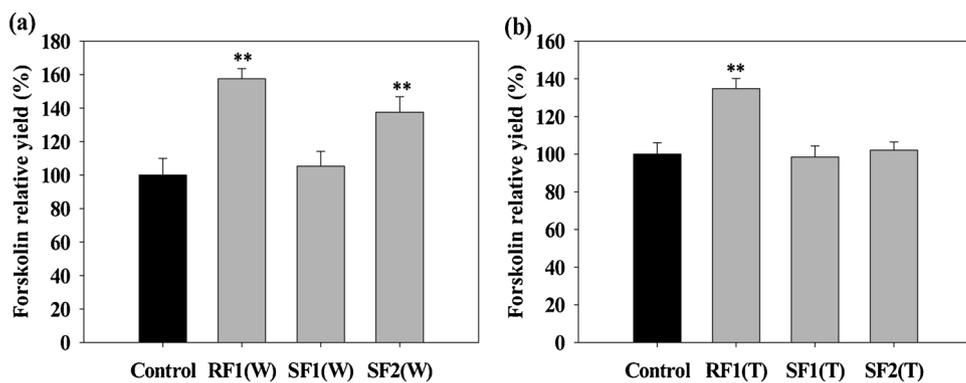


Fig. 6. Relative yield of forskolin in wheat bran-based and talc-based endophyte formulations and their comparison with control plants grown under field conditions were analyzed by TLC method. Intensity of total forskolin spot measured using AlphaEaseFC 4.0 software. (a) Forskolin variation between control and wheat bran-based endophyte formulation treated plants (b) Forskolin variation between control and talc-based endophyte formulation treated plants. Standard deviation of mean (SD) between two biological replicates. Asterisks above the error bars indicate a significant difference ( $*p < 0.05$ ,  $**p < 0.01$ ).

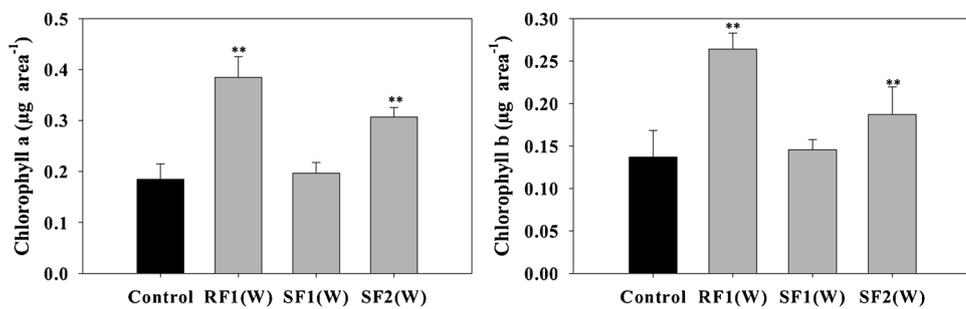
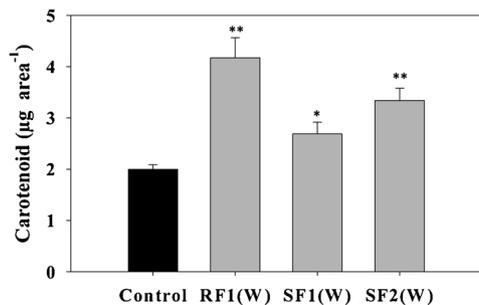


Fig. 7. Estimation of photosynthetic pigments per unit leaf area of control and wheat bran-based endophyte (RF1, SF1, and SF2) formulations treated *C. forskohlii* plants at 150 dpi. (a) chlorophyll a, (b) chlorophyll b, and (c) carotenoid. Each treatment was carried out with three biological replicates. Asterisks above the error bars represent significant difference of mean ( $\pm$  SD) between control and treatments, according to Dunnett multiple comparisons test ( $*p < 0.05$  and  $**p < 0.01$ ).



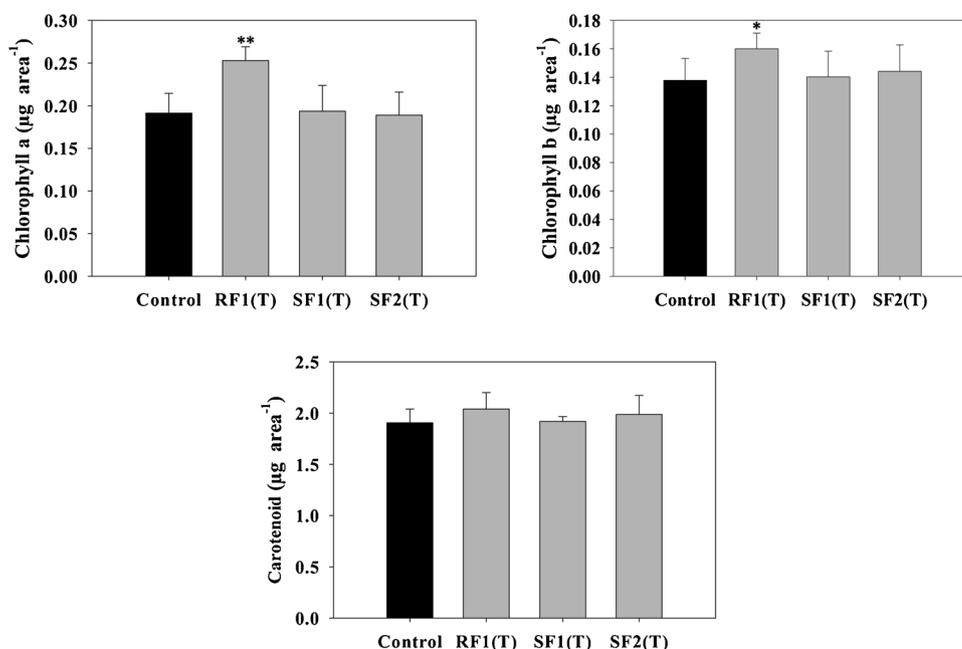


Fig. 8. Estimation of photosynthetic pigments in talc-based endophyte (RF1, SF1, and SF2) formulation treated *C. forskohlii* plants at 150 dpi. (a) chlorophyll a, (b) chlorophyll b, and (c) carotenoid in per unit leaf area of control and talc-based endophyte formulations treated plants. Each treatment was carried out with three biologicals replicates. Asterisks above the error bars represent significant difference of mean ( $\pm$  SD) between control and treatments, according to Dunnett multiple comparisons test (\* $p < 0.05$  and \*\* $p < 0.01$ ).

the growth of plant pathogens, *Rhizoctonia solani* and *Pythium* spp. (Harris, 2000). In our previous study, from *C. forskohlii*, a total of nine fungal endophytes were isolated and analyzed for their beneficial effects on their host plant under pot conditions. Among them, only three fungal endophytes (RF1, SF1, and SF2) under both pot and field conditions have shown disease management, plant probiotic effects and forskolin enhancement (Mastan et al., 2019). Hence, here we report, for the first time, development of suitable formulations for delivery of functional fungal endophytes as potential biostimulants for *C. forskohlii* cultivation.

Ergosterol is one of the major components for distinguish the fungi from plant-based materials since it is present in fungal cell membrane (Yang et al., 2015) and absent in plants (Klemptner et al., 2014). The previous literatures stated that ergosterol assay is rapid and very sensitive method to analyze the degree of fungal growth in plant-based materials compared to other methods (Martin et al., 1990; Ng et al., 2008). In our study, we used wheat bran (Plant-based substrate) and endophytes (Fungi) for developing the bioformulations. During solid state fermentation, the ergosterol content in fermented media was quantified to analyze the degree of endophyte growth. During fermentation, SF2 endophyte showed significant growth and contentous enhancement of ergosterol in fermented medium. However, moderate growth and ergosterol content was noticed in RF1 and SF1 fermented medium. Thus, ergosterol estimation by TLC method provided us with rapid result to normalize the growth-difference between fungal endophytes for developing the carrier-based formulations.

Commercially, formulations are available in various forms, which include powder, pellets, tablets, granules, gel beads and balls (Lewis, 1991; Kaewchai et al., 2009). As per earlier reports, scientists used inorganic carrier-based (talc, vermiculite, calcium sulphate, copper sulphate, and perlite) and organic carrier-based (peat and charcoal) formulations to develop bioformulations to promote plant growth and control various diseases (Chakraborty et al., 2009; Khabbaz and Abbasi, 2014; Bashan et al., 2014; Balakrishnan et al., 2017; Pahari et al., 2017; Berninger et al., 2018). In addition, development of liquid fermentation-based formulations is also common in agriculture applications; however, liquid fermentation-based formulations are more vulnerable to desiccation compared to solid state fermentation-based formulations (Sriram et al., 2011). Considering these aspects, in this study, we attempted to develop suitable endophyte (RF1, SF1, and SF2) formulations using two types of carrier-based materials such as inorganic base

material (talc) and organic base material (wheat bran) for sustainable shelf life of endophytes. According to previous studies, the successful colonization of functional endophytes can significantly improve the *C. forskohlii* plant growth and root yield (Das et al., 2014). In our study, inoculation of endophytes through wheat bran-formulations showed better results in terms of plant growth and root yield of *C. forskohlii* under individual field conditions compared to talc-based endophyte formulations. In addition, we also found that longevity of fungal endophyte formulations was significantly influenced by specific type of carrier-based materials. The viability of endophytes in developed formulations showed longer period in wheat bran-based formulations compared to talc-based formulations.

Endophytes are known to affect plant primary productivity by increasing chlorophyll content and photosynthetic rate (Khan et al., 2012). Colonization of fungal endophyte, *Epichloë typhina* results in improved photosynthetic efficiency by enhancing photosynthetic pigments in its host orchard grass, *Dactylis glomerata* (Rozpądek et al., 2015). Field trails of *C. forskohlii* with wheat bran-based endophyte formulations improved chlorophyll content significantly as an indication of higher photosynthetic rate, but talc-based endophyte formulation treated plants did not show a significant enhancement compared to untreated control plants. From this data, it can be concluded that application of wheat bran-based formulations has a prominent role in improvising chlorophyll content and secondary metabolites along with root biomass, whereas, SF1 in both formulations did not improve the photosynthetic pigments compared to control.

Endophytes can act as both producers and bio-stimulants for secondary metabolites. For instance, endophyte *Talaromyces radicus* isolated from *Catharanthus roseus* produces vincristine and vinblastine in fermentation media (Palem et al., 2015). While colonization of endophytes *Curvularia* sp. and *Choanephora infundibulifera* in their host plant, *C. roseus* enhanced the vindoline content as bio-stimulant (Pandey et al., 2016a), in our investigations, two types of endophyte formulations were evaluated for *in-plant* enhancement of forskolin as bio-stimulants under field conditions. Interestingly, the application of wheat bran-based fungal endophyte formulations significantly enhanced the forskolin content compared to talc-based endophyte formulations, therefore making compatibility variations between wheat bran and talc material evident for development of fungal endophyte formulations.

## 5. Conclusion

Our study highlights the development of formulations of three fungal endophytes (RF1, SF1, and SF2), which were previously characterized for plants growth and forskolin enhancement as well as disease management properties in *C. forskohlii* under field conditions. Shelf life analysis up to six months showed good stability of endophyte viability in the wheat bran-based formulations compared to talc-based formulations. Overall, our study reinforces that the deployment of developed wheat bran-based fungal endophytic formulations under field conditions could improve primary plant productivity and forskolin content in roots during sustainable cultivation of *C. forskohlii*.

## Authors' contribution

CSV designed the experiments. AM and DR performed the bench work and AM, CSV, DR and SGD analysed the data. AM and CSV wrote the manuscript.

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## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.micres.2019.126310>.

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