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Formulation based antagonistic endophyte Amycolatopsis sp. SND-1 triggers defense response in Vigna radiata (L.) R. Wilczek. (Mung bean) against Cercospora leaf spot disease

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Abstract

In the present work*, Amycolatopsis* sp. SND-1 (SND-1) was isolated from *Cleome chellidonii* Linn. (*C. chellidonii*) was performed as biocontrol and resistance elicitor in *Vigna radiata* (L.) R. Wilczek (mung bean) plants against Cercospora leaf spot causing pathogen *Cercospora canescens* (*C. canescens*). The SND-1 isolate showed 74% of inhibition against *C. canescens* in dual culture and *in-vitro* plant growth trials exhibited production of indole acetic acid, gibberellic acid, cytokinin, ammonia, hydrogen cyanide, and siderophore and phosphate solubilization. *In-vivo* study with talcum formulation of SND-1 revealed a significant increase in plant root length, shoots length, root and shoot fresh weight, and reduced the disease severity in treated mung bean plants. Triggering of resistance by SND-1 formulation was studied by histochemical depositions and biochemical defense enzymes resulted that the acceleration in defense response in comparison with control plants. The bioactive endophytic *Amycolatopsis* sp. SND-1 enhanced the defense against *C. canescens* infection; hence it can be used as a biological control agent in mung bean cultivars.

Introduction

Vigna radiata (L.) R. Wilczek (Mung bean) has become a major crop in South as well as Southeast Asia. About 90% of the contribution to total global production is from South Asia. According to the estimation annually 1.5 to 2.0 million tons of mung bean production are produced in India which covers 3 to 4 million hectares (Pandey et al. 2018). The cultivation of mung bean is also extended in Australia, the United States of America, and China (Jadhav et al. 2016). In the dietary profile, mung bean contains high minerals and vitamins, and became a dietary protein source for marginal people and vegetarians (Keatinge et al. 2015). Apart from its dietary importance mung bean has antimicrobial, anti-inflammatory, antioxidant, and antitumor efficacy and is also actively involved in the regulation of lipid metabolism (Tang et al. 2014). In farming practices, mung bean cultivation faces several abiotic and biotic stresses which resulted in a significant reduction in the yield all over the world. The effect of biotic stress resulted in the decreased economy of mung bean, it was estimated that maximum 60% reduction in the total yield in different corners of the world (Wenhao et al. 2010). Among the various biotic stresses the fungal diseases namely Cercospora leaf spot, Anthracnose, Web blight, Macrophomina blight, and Powdery mildew resulted in more than 50% of yield loss in mung bean (Ryley et al. 2010). Among the fungal diseases, Cercospora leaf spot became a major affecting entity in mung beans due to its adverse effect on the normal functioning of the plant and yield.

Cercospora leaf spot is a type of foliar disease caused by the fungi *Cercospora canescens*; this disease was first reported in Delhi, India (Naresh et al. 2020) and became prevalent in several countries. In 1995 this disease resulted in 61.5% of yield loss in Pakistan (Iqbal et al. 1995), and also the pathogen can induce up to 96% yield loss in a suitable epiphytotic environment (Kaur et al. 2007). Histopathology and symptomology of Cercospora disease show water-soaked spots with grey-colored borders at earlier stages and become dirty grey mass with brown margin (Jamadar et al. 1988). Later the pathogen can affect the stems, petioles as well as pods. The pathogen cause defoliation, premature defoliation in the severe stage, wrinkling of leaf shape, and decreases in the size of pods. The severity of pathogen increases due to the production of toxic chemicals namely perylenequinone-based cercosporin which can affect plants, microbes, and animals (Naresh et al. 2020). The pathogen can spread easily through rain splashes and results in the dispersal of the conidia. The control and management of Cercospora disease are practiced through the use of harmful chemical fungicides due to the application of these synthetic fungicides significant chemical contamination can accumulate in agricultural soil, and shows a negative impact on soil microbes, and continuous application of fungicides creates resistance in pathogen (Athakorn et al. 2022). An eco-friendly protocol for the control and management of Cercospora disease and increasing resistance in mung bean plants becomes an alternative approach in sustainable agriculture.

Endophytic microbes are the beneficial organisms that colonize within the endosphere and can accelerate plant growth and induce the defense system against invading phytopathogens. Among the diversity of the endosphere, the isolation of actinomycetes showed tremendous perspective in pharmaceuticals, agriculture, and industries (Passari et al. 2017). Many studies have proved the involvement of these actinomycetes in the enhancement of plant growth and acceleration of induced systemic resistance (Dina *et al*., 2020). Actinomycetes are promising plant growth enhancers due to their ability to produce phytohormone, phosphate solubilization, siderophore, and hydrogen cyanide (Sumaira et al. 2016). Induced systemic resistance (ISR) at cellular level through the accumulation of defensive barriers such as lignin, hydrogen peroxide, callose, phenol, suberin, and minerals (Silicon) by actinomycetes in different crops plants is an effective strategy to sophisticate the plant health against pathogens. The strain *S. anulatus* primed grapevine plants showed a significant acceleration in defense response against grey mold infection (Parul et al. 2017).

The elevation in biochemical defense through enhancing the concentration of defense enzymes such as phenylalanine ammonia-lyase (PAL), β-1, 3-Glucanase (GLU), and Peroxidase (POX) is also effectively inhibiting the pathogens. A study of three actinomycetes from endophytic origin exhibited acceleration of cell wall degrading enzymes and reduction damping off disease in cucumber (El-Tarabily et al. 2009). Cheng et al. (2014) reported that *Streptomyces felleus* YJ1 showed antagonistic activity and enhanced level of defense enzymes like POX, Polyphenol Oxidase (PPO), and PAL in oilseed rape against *S. sclerotiorum*. *Amycolatopsis* sp. BCA-696 isolates exhibited a broad spectrum of plant growth-promoting attributes and increased the yield in chickpea and sorghum (Gottumukkala et al. 2016), Ratchanee et al. (2014) reported that *Streptomyces* and non *Streptomyces* such as *Amycolatopsis*, *Micromonospora*, *Microbispora*, and *Nanomuraea* revealed antagonistic potential against *Xnthomonas campestris* pv. glycines.

The plant *Cleome chellidonii* Linn. (*C. chellidonii*) is a member of Cleomaceae and grows in wet places as a weed. This plant is distributed in India, Indo china, and Myanmar and has a broad spectrum of therapeutic applications which scientifically validated (Mathew et al. 1982). The literature revealed these plant parts are used to cure dysentery, otitis, rheumatism, and headache. Applications of *C. chellidonii* also exhibited anti-inflammatory, antipyretic, antimicrobial potential and in the treatment of skin diseases (Kavitha et al. 2020). Apart from therapeutics application, many *Cleome* species exhibited colonization of beneficial microbes in their endosphere and those microbes showed significant antagonistic and plant growth promoting attributes. Miyada et al. (2015) reported that the endophytic actinomycetes strain from *Cleome africana* exhibited antagonistic and plant growth promotion efficacy. *Streptomyces* CA2, CA6, CA9, and CA11 strains isolated from *Cleome Arabica* showed positive IAA production and enhanced tomato plant growth (Yacine et al. 2013). Keeping the view on tremendous agricultural applications of endophytic actinomycetes and *Cleome* plants, the present investigation was carried out to isolate endophytic actinomycetes from *C. chellidonii* and evaluation of their biocontrol potential against *Cercospora canescens* and plant growth promoting attributes in mung bean plants.

Materials And Methods

Collection of plants and isolation of actinomycetes

C. chellidonii plants were collected in different locations at Dharwad district, Karnataka, India. The collected plants were brought laboratory in sterile polythene bags and plant samples were kept at 4°C. Isolation of endophytic actinomycetes was done by adopting the method of Yacine et al. (2013). Briefly, collected plant parts (Root, stem, and leaves) were subjected to surface sterilization process. Initially all the collected plant parts were subjected to multiple washes with tap water and sterilized water for the removal of soil and debris. Then the sequence immersion of finely chopped (1 cm) plant parts with Tween 20 (0.1%) for 1 min, Ethanol (70%) for 2 min, NaOCl (0.4%) for about 1 min, and followed by 2-3 washes with sterilized distilled water. Finally the samples were dried in laminar flow hood, and then samples were grinded after removing of outer tissue layer in autoclaved pestle and mortar. Aliquots of 100 µL from root, stem and

leaves were spread on petridishes containing Starch Casein Agar (SCA), Actinomycetes Isolation Agar (AIA) and International Streptomyces Project-2 (ISP2) media. The plates were kept for incubation at 28 ℃ for 21 days. After incubation the grown actinomycetes colonies were subjected for subculture and pure culture techniques.

In- vitro antagonism assay

All the isolated actinomycetes were subjected for their antagonism efficacy by standard dual culture assay. The pure isolates were inoculated vertically on one edge of the petriplate containing Glucose yeast extract media (GYM) and the plates were incubated at 28℃. After the 5 days of incubation, a 5mm mycelial plug of *C. canescens* (5 days old) was spot inoculated at the opposite edge of actinomycetes isolates. The plates without actinomycetes were served as control. All the plates were incubated for 5 to 7 days at 28℃ and after incubation; the percentage (%) of inhibition was measured by colony radius in comparison with the control plate.

Morphological, biochemical and molecular characterization

The most potent SND-1 isolate was characterized morphologically by observing through gram staining technique for color of aerial and substrate mycelium, pigmentation, and colony texture. Scanning electron microscopy (SEM, JSM-IT500 LA, In Touch Scope Scanning Electron Microscope, Tokyo, Japan) was done for the observation of hyphal spore-bearing structures. Biochemical characterization was done by using the BCL card of the VITEK-2 compact (Biomerieux, Durham, USA). Each micro-well has a colorimetric reagent with individual substrates. The isolated SND-1 suspension with appropriate turbidity was prepared in NaCl (0.45%). The prepared suspension was adjusted to McFarland (0.5) standards and characterization was carried out with 46 tests to evaluate the carbon utilization, alkalization, enzymatic activities, and antibiotic resistance.

Molecular characterization of purified SND-1 isolate was done with amplification of genomic DNA using Polymerase Chain Reaction (PCR) with universal primers, Forward primer 27F and reverse primer 1492 R in PCR (Applied Biosystems 2720, Thermal Cycler) instrument. After the amplification, the BLAST analysis of the resultant 16S rRNA sequence was performed on the NCBI Gene bank website and most similar *Amycolatopsis* species were aligned using the software Clustal-W. The phylogenetic tree was constructed with the neighbor-joining method with the help of MEGA7 software.

In-vitro plant growth promoting attributes

Production of phytohormones

Indole acetic acid (IAA) production

IAA production was evaluated according to the method by Gordon et al. (1951). Pure culture of SND-1 isolate was cultured on Glucose Yeast Malt (GYM) with the amendment of L-tryptophan and plates were incubated for 7 days at 28 °C. After incubation, the culture was centrifuged for 20 min to get clear supernatant at 5000 rpm. The 1 mL of supernatant was treated with 4 mL of Salkowski reagent (1m of 0.5 M $FeCl_3$ in 50 mL of 35% perchloric acid) and 2 drops of orthophosphoric acid. After 1 h of incubation, the development of pink color was observed and quantification of IAA was done by measuring the absorbance in a spectrophotometer (Model-UV 9600 A METASH, SHANGHAI) at 535 nm (72, 96, and 120 h, respectively) and concentration expressed in µg/mL in comparison with standard IAA.

Production of gibberellic acid (GA3)

The GA3 production was performed according to the protocol by Miyada et al. (2017), the culture supernatant from 7-day-old SND-1 was recovered and extraction was carried out with phosphate buffer (pH 8.0). Centrifugation was done

at 10.000 rpm for 3 min and obtained supernatant was acidified (pH 2.5) by adding hydrochloric acid (5 N) and extraction was done 5 times with ethyl acetate with equal volumes. Then the final mixture of 15 mL of prepared extract, 2 mL zinc acetate, and potassium ferrocyanide (10% w/v and 1% w/v) was centrifuged at 10.000 rpm for 10 min, and further, the supernatant was mixed with hydrochloric acid (30% v/v) and kept for incubation at 20 ℃ for 75 min. Finally, the quantification of gibberellic acid was done by measuring absorbance at 254 nm (72, 96, and 120 h, respectively) and the amount of gibberellic acid was calculated by comparing the standard graph (Sigma-Aldrich) of GA3 solution.

Production of cytokinin

Quantification of cytokinin was done by a modified method by Tisha et al. (2017). Briefly, the SND-1 isolate cultured on International Streptomyces Project -2 (ISP-2) was supplemented with biotin (2 pg), Casamino acids (0.2 %), and thiamine (0.01 %) per liter (Himedia). 1 mL of culture was inoculated on 250 mL of prepared media and incubated for 5 days at 28 ℃. After incubation, the quantification of cytokinin was done spectrophotometrically at 665 nm (72, 96, and 120 h, respectively) and ISP-2 without SND-1 isolate was served as control.

Production of siderophore

The production of siderophore by SND-1 was done according to the procedure described by Schwyn et al. (1997).

SND-1 isolate was spot inoculated on agar plates amended with chrome azurol S (CAS). The plates were incubated for 7 days at 28℃ and after incubation observation was made for the formation of orange /yellow color zone around the colony.

Phosphate solubilization

Phosphate solubilization of isolated SND-1 isolate was qualitatively determined according to Subba Rao et al. 1999, the SND-1 isolate was inoculated on Pikovskaya's agar plate and incubation was done for 5 days. The phosphate solubilization was observed by the formation of a clear zone around the actinomycetes colony. The quantification of amount of phosphate solubilization was done by growing SND-1 isolate on 50 mL of National Botanical Research Institute's phosphate growth medium (NBRIP) for 15 days, and then an equal volume of ammonium molybdate and ammonium vandate were added to the culture free supernatant of NBRIP grown SND-1 isolate. Finally the quantification was done spectrophotometrically by measuring the absorbance of the developed yellow color at 420 nm.

Hydrogen cyanide (HCN) production

HCN production was qualitatively determined by inoculating SND-1 isolate on Luria-Bertani (LB) broth amended with 4.4g/L glycine (Himedia, Mumbai India). The lid of the Petri dish was covered by Whatman No 1 filter paper flooded with 2% sodium carbonate and 0.5% picric acid and plates were incubated for 7 days at 28℃. After incubation positive HCN production was observed by the change in the color of filter paper from yellow to orange (Yacine et al. 2013).

Ammonia production

Ammonia production was qualitatively determined by inoculating SND-1 isolate on 10 mL of peptone water and incubation was done for 1 week. After incubation, ammonia production was observed by the change in the color of peptone water from yellow to orange (Kenza et al. 2021).

In-vivo Biocontrol Assay

Preparation pathogen inoculum

To get mass production of *C. canescens* pathogen, the infected mung bean leaves were collected from experimental soil fields of the University of Agriculture Sciences (UAS) Dharwad. The collected diseased spots on leaves were carefully drawn out and placed on autoclaved PDA medium and incubation was done for 10 days at 28 ℃. After incubation, the pathogen was confirmed by its morphology, sporulation, and mycelia structure. Inoculum of the pathogen was prepared according to Yacine et al. 2013, spores were directly recovered in sterilized distilled water (SDW) and the final spore load (10^{-6} mL^{-1}) was adjusted.

Preparation of carrier-based formulation of SND-1 isolate

The preparation of formulation was begun by adjusting the spore load (Recovered from 0.05% v/v Tween-20 solution) from 10-day-old ISP2 grown SND-1 isolate to ≈10^6 CFU ml^{-1}. Wettable talcum powder was used for the preparation of carrier formulation with SND-1 isolate. A 25 mL of prepared spore suspension of SND-1 was finely mixed with 100 g of sterilized talcum (Fisher Scientific) powder, 10 g of carboxymethyl cellulose, and about 1.5 g of calcium carbonate (Miyada et al. 2017). After the drying the mixture overnight in a laminar flow hood, the talcum formulation was stored in dark at room temperature. The stored formulation was subjected to check its purity and viability of SND-1 was done by spreading the prepared formulation on ISP2 media every 15 days up to 6 months of storage period.

Evaluation of seed germination and vigor index

Mung bean seeds (moderately Cercospora susceptible variety DGGV-2) were washed with tap water and 1% NaOCl (Sodium hypochlorite). Then several washes were made with sterilized distilled water (SDW). A standard paper towel method was performed to evaluate the seed germination and vigor index in mung bean seeds treated with (1) Control (seeds treated with sterilized distilled water), (2) C+CC (Control + pathogen *C. canescens*), (3) SND-1 (seeds treated with SND-1 alone) and (4) SND-1 + CC) seeds treated with talcum formulation of SND-1 and challenge inoculation with the pathogen). The SND-1 alone treatment was prepared by a cell pellet of 7-day old ISP2 broth grown culture of SND-1 by centrifugation at 10.000 rpm and 4 ℃. The 40 seeds from both treated and untreated were placed on a germination paper towel, rolled up, and tied with rubber bands. Then the germination paper towels were placed in Bio-Oxygen Demand (BOD) incubator at 24 ℃ for 7 days. After the incubation, the seed germination and vigor index were calculated by using the following formula (Lukacova et al. 2021).

Seed vigor index = (mean of shoot length + mean of root length) × (percentage of germination)

In- vivo plant growth promotion attributes

In- vivo biocontrol assay was performed in greenhouse conditions with different treatments in mung bean seeds. Primarily seeds of mung bean susceptible (DGGV-2) variety were surface sterilized with sodium hypochlorite (2% NaOCl) for 2 minutes and after with SDW. After the surface sterilization, the seeds were dried and treated with talcum formulation with adhesive 10% carboxy methyl cellulose, CMC (Himedia, Mumbai, India) and dried for 2-3 h. For the greenhouse experiment, the pots (15 × 10 cm) with 1.5 kg of sterilized soil, sand, and Farm yard manure (2:1:1) was used. The biocontrol assay was performed by following treatments (1) Control (seeds treated with SDW), (2) C+CC (Control + pathogen *C. canescens*), (3) SND-1 (seeds treated with SND-1 alone), and (4) SND-1+CC (seeds treated with talcum formulation of SND-1 and challenge inoculation with the pathogen). The pre-treated mung bean seeds were transferred to pots arranged in a complete randomized design (CRD) with four seeds per pot. The prepared pathogen inoculums were sprayed for three weeks old mung bean plants and all the pots were watered on alternative days with non-sterilized water. All the pots were maintained in regulated greenhouse conditions (14 h light and 10 h dark with a temperature of 23 ±2 ℃). After 35 days the mung bean plants from all the treatments were uprooted and evaluated the plant growth promoting attributes like root length, shoot length, shoot fresh weight, root fresh weight, shoot dry weight,

and root dry weight. The disease incidence in all the treated plants was calculated by the following formula (Mayee et al. 1986).

Evaluation of histochemical response in formulation treated mung bean plants upon *C. canescens* infection

Detection of lignin deposition

Histochemical responses in formulation treated mung bean plants against *C. canescens* infection were evaluated by deposition of lignin, hydrogen peroxide, and phenol in leaf epidermal cells at different time intervals 0, 4,12,24,36, and 72 hours post inoculation (hpi). Lignin deposition was performed according to the protocol by Sherwood et al. 1976. Epidermal leaf peelings from all four treatments were stained with 2% phloroglucinol in 95 % ethanol and observed under a microscope for intense coloration and the percentage lignification was calculated by counting the lignified cells.

Detection of hydrogen peroxide (H_2O_2) deposition

The 3, 3-diamino benzidine (DAB) staining method was performed for the deposition of hydrogen peroxide (H_2O_2) in mung bean plants treated with all four treatments. Epidermal leaf peelings were first placed in DAB solution (1mg at pH 3.8) on a clean glass slide and kept for 8 h under the white light at room temperature. Then the samples were boiled for 4 h in ethanol and preservation was achieved in ethanol. After the preservation, the samples were observed under a microscope and deposition of H_2O_2 (Brown color staining) was observed and the percentage of H_2O_2 deposition was calculated by counting the stained cells (Abhayashree et al. 2017).

Detection of phenol deposition

Phenol deposition was studied by staining the epidermal leaf peelings with toluidine blue in phosphate buffer (0.1M and pH 5.5). After the staining, the epidermal peelings were observed under a microscope and the percentage of phenol deposition was calculated (Borden et al. 2002).

Evaluation of biochemical defense response

Biochemical defense response in formulation-treated mung bean plants against *C. canescens* infection was studied in different time intervals (0, 4, 12, 24, 36, and 72 hpi). The biochemical defense enzymes such as phenylalanine ammonia-lyase (PAL), peroxidase (POX), and β-1, 3-glucanase (GLU) were estimated according to Beaudoin et al. 1985. 1 g of each uprooted mung bean biomass was homogenized with 1 ml Tris buffer (25 mM) at pH 8.8 in β-mercaptoethanol in Mortar and pestle (pre-chilled). Then centrifugation of the extract was done at 10.000 rpm at 4 ℃ for 25 min and obtained supernatant was considered as an enzyme source (Abhayashree et al. 2017).

Estimation of phenylalanine ammonia-lyase (PAL)

The PAL activity was estimated by incubating the reaction mixture of 1 mL Tris-HCl buffer (25mM) with 0.5 mL enzyme extract (pH 8.8) and 1.5 mL L-phenylalanine with the same buffer at 40 ℃ for 2 h. PAL activity was estimated based on the rate of L-phenylalanine to trans-cinnamic acid conversion at 290 nm and the activity of the enzyme was expressed as mol trans-cinnamic acid mg/protein/h (Abhayashree et al. 2017).

Estimation of β-1, 3-glucanase (GLU)

Quantification of GLU activity in all four treatments was estimated according to the method Kini et al. (2000). The prepared enzyme source was treated with 0.1 % laminarin and GLU expression was measured spectrophotometrically at

540 nm and expressed as mg/protein/h.

Estimation of Peroxidase (POX)

To estimation of the POX activity was done according to the procedure described by Hammerschmidt et al. (1982) was followed, 1 g of biomass from all four treatments of mung bean plants was macerated in mortar and pestle with sodium phosphate buffer (0.2 mM). The centrifugation was done to the homogenized mixture at 10,000 rpm at 4 ℃ and obtained supernatant was utilized and treated as a source of enzyme. A crude extract of the enzyme was added to the reaction mixture containing 25% guaiacol (v/v) in potassium phosphate buffer (10 mM) consisting of hydrogen peroxide (10 mM). The quantification of POX was done spectrophotometrically at 470 nm and expressed as units/mg/protein/min.

Data analysis:

All the performed experiments were subjected to triplicate and results were expressed in mean ± SD. The software SPSS version 22.0 was adopted and $P \leq 0.05$ was considered a statistical significance.

Results

Isolation and antagonistic activity of endophytic actinomycetes:

A total of 12 actinomycetes were isolated from collected *C. chellidonii* plants (Fig. 1). The pure cultures of all isolated organisms were tested for their antagonistic potential against *C. canescens* by dual culture assay and results showed all the isolated actinomycetes isolates were showed antagonistic nature (**Fig. 2**) and maximum activity 74 % was observed by isolate SND-1 isolate (**Fig. 3 a, b**) followed 57.48%, 57.35% by SND-14, SND-28 and lowest activity was observed 35.06 % in SND-24. The SEM analysis of inhibition area of *C. canescens* by SND-1 showed inhibition pattern through the destruction of mycelia (**Fig. 3 c, d**). Based on the antagonistic potential the isolate SND-1 was selected for further analysis.

Morphological, biochemical, and molecular characterization

The morphological characterization revealed that SND-1 isolate with white-colored aerial mycelium and substrate mycelium with light brown color and grey visualization on maturation. SEM analysis showed a clear indication of mycelia with sporulation resembles the *Amycolatopsis* sp. (**Fig. 4 a, b)**. Biochemical characterization of SND-1 isolate hinted the 22 positives out of 46 biochemical tests for carbon utilization, alkalization, enzymatic activities, and antibiotic resistance **(Table 1)**. Molecular characterization was done by 16S rRNA sequence and results showed similarity index in BLAST (BLASTN) analysis with *Amycolatopsis* sp. isolates (Accession No. OM807224). The highest similarity was obtained with *Amycolatopsis* sp. isolate 26-4 (99.09 %), *Amycolatopsis* sp. isolate CBMA229 (98.71 %), *Amycolatopsis* sp. isolate 102113 and *Amycolatopsis* sp. isolate R12-7.on the basis of phylogenetic analysis the isolated SND-1 was identified as *Amycolatopsis* sp. strain SND-1 (SND-1). The rest of the similarity index of phylogeny with *Amycolatopsis* sp. isolate was displayed in **Fig. 5.**

Table 1

Biochemical characterization of isolate SND-1

Tests	Result	Tests	Result
BETA-XYLOSIDASE	-	D-MANNITOL	-
L-Lysine-ARYLAMIDASE	-	D-MANNOSE	+
L-Aspartate ARYLAMIDASE	+	D-MELEZITOSE	-
Leucine ARYLAMIDASE	+	N-ACETYL-D-GLUCOSAMINE	-
Phenylalanine ARYLAMIDASE	+	PALATINOSE	-
L-Proline ARYLAMIDASE	+	L-RHAMNOSE	+
BETA-GALACTOSIDASE	-	BETA-GLUCOSIDASE	+
L-Pyrrolidonyl-ARYLAMIDASE	+	BETA-MANNOSIDASE	+
ALPHA-GALACTOSIDASE	+	PHOSPHORYL CHOLINE	(-)
Alanine ARYLAMIDASE	+	PYRUVATE	+
Tyrosine ARYLAMIDASE	+	ALPHA-GLUCOSIDASE	+
BETA-N-ACETYL-GLUCOSAMINIDASE	-	D-TAGATOSE	-
Ala-Phe-Pro ARYLAMIDASE	+	D-TREHALOSE	-
CYCLODEXTRIN	-	INULIN	+
D-GALACTOSE	+	D-GLUCOSE	-
GLYCOGEN	-	D-RIBOSE	-
myo-INOSITOL	-	PUTRESCINE assimilation	-
METHYL-A-D-GLUCOPYRANOSIDE acidification	-	GROWTH IN 6.5% NaCl	-
ELLMAN	+	KANAMYCIN RESISTANCE	(+)
METHYL-D-XYLOSIDE	-	OLEANDOMYCIN RESISTANCE	-
ALPHA-MANNOSIDASE	-	ESCULIN hydrolyse	+
MALTOTRIOSE	+	TETRAZOLIUM RED	-
Glycine ARYLAMIDASE	+	POLYMIXIN_B RESISTANCE	-
BETA-XYLOSIDASE	+	D-MANNITOL	-
L-Lysine-ARYLAMIDASE	-	D-MANNOSE	+

In-vitro plant growth promoting attributes

The most potential antagonistic SND-1 was subjected to *in-vitro* antagonistic activity. Results showed positive IAA production, siderophore, HCN production, phosphate solubilization, and ammonia production (**Fig. 6 a-f**). The quantification of IAA revealed highest 14.02 ± 0.9 µg/mL, 10.72 ± 0.5 µg/mL of GA3 and maximum cytokinin 4.71 ± 0.5 µg/mL at 72, 92 and 120h respectively (**Table** 2).

Siderophore production was observed by the formation of a yellow zone around the SND-1 on chrome azurol S agar media. The highest phosphate solubilization was recorded at 7.8 ± 0.32 mg/mL on Politkovskaya's agar media. The SND-1 showed positive HCN production which was observed by the color change in picric acid flooded Whatman No.1

filter paper from yellow to orange. The confirmation of ammonia production was done by the change in the color of peptone water inoculated with SND-1 isolate from light yellow to orange.

Table 2

Phytohormone production by SND-1 at different time intervals. All the values were means of triplicate determinations ± S.D

	Time intervals in hours		
Phytohormones (µg/mL)	**72**	**96**	**120**
IAA	14 ± 0.9	12.38 ± 0.6	10.56 ± 0.7
Gibberellin	10.71 ± 0.5	10.72 ± 0.5	10.5 ± 0.9
Cytokinin	4.39 ± 0.5	3.73 ± 0.5	4.71 ± 0.5

In-vivo biocontrol assay

Evaluation of seed germination and vigor index

The seed germination percentage was tested in mung bean seeds treated with four treatments before the greenhouse study. The results exhibited the highest of 94 % of seed germination in SND-1+ CC treated seeds, followed by SND-1 alone 88.34 % and SDW treated seeds 80.71 %. The decreased seed germination was observed in C+CC treated seeds which showed 65.47 % germination. The maximum vigor index of 370.57 was noted in SND-1+ CC treatment, followed by strain SND-1 alone 338.83 and SDW treatment 331.91. A delayed seed vigor index was observed in C + CC treated seeds which exhibited 270.62 (**Table 3**).

In-vivo plant growth promotion analysis

The purity and viability of prepared talcum SND-1 formulation exhibited a significant viability throughout the storage period. The greenhouse study was terminated after 35 days. The effectiveness of each treatment was carried out by evaluating the root length, shoot length, shoot fresh weight, fresh root weight, and total dry weight in treated mung bean plants **(Fig. 7a)**. Among the four, the formulation treated and challenge inoculation with pathogen treatment *Amycolatopsis* sp. strain SND-1+CC was exhibited maximum root length 8.7 ± 0.4 cm followed by *Amycolatopsis* sp. strain SND-1 alone 6.3 ± 0.4 and control SDW 6.3 ± 0.45 cm. The decreased root length was noted in pathogen-treated C+ CC, which showed 4.5 ±0.35 cm. The highest shoot length, 29.54 ± 0.65 cm, was observed in SND-1 + CC, whereas in SND-1 alone and control, SDW-treated plants exhibited 27 ± 0.26 and 26.36 ± 0.45 cm. A delayed shoot length of 20.86 ± 1.11 cm was noted in pathogen-treated C+ CC plants. Further significant increase in shoot fresh weight 2.4 ± 0.19 g was observed in SND-1+ CC treated plants in comparison with SND-1 alone 1.9 ± 0.15 g and control SDW treated plants 1.6 ± 0.15 g, whereas in pathogen treated C+CC plants exhibited 1.01 ± 0.09 g. The maximum root fresh weight of 0.9 ± 0.3 g was exhibited in formulation treated SND-1+ CC plants in comparison with SND-1 alone 0.6 ± 0.1 g and control SDW plants 0.43 ± 0.15 g. Only pathogen-treated plants showed 0.3 ± 0.1 g of fresh root weight. Total dry weight was evaluated in all the treatments it was observed that the maximum dry weight was 0.7 ± 0.1 g in SND-1+CC treated plants, 0.5 ± 0.1 in SND-1 alone treated plants, 0.4 ± 0.1 g in control SDW treated plants exhibited, and whereas in pathogen treated plants it was observed that low

weight of 0.18 ± 0.07 g. The results showed minimum disease incidence of 45.53 % was observed in plants treated with SND-1+ CC, followed by SND-1 alone 61.71 % and SDW treatment 62 .12 %. The highest disease incidence

was noted in C+CC treated plants, about 89.93 % **(Table 3)**. The severity of Cercospora infection in all the treatments was observed by comparing the diseased spots on the mung bean leaves (**Fig. 7b**).

Table 3

In-vivo plant growth parameters in mung bean plants upon *C. canescens* infection. Control (Treated with SDW); C+CC (Control treated with *C. canescens*); SND-1(Treated with SND-1 alone) and SND-1+CC (Treated with SND-1 with challenge inoculation of *C. canescens*). All the values are means of triplicate determinations ± S.D

Treatment	Germination (%)	Vigor index	Root length(cm)	Shoot length(cm)	Shoot fresh weight(g)	Root fresh weight(g)	Total dry weight(g)	Disease severity (%)
Control	80.71	331.91	6.3 ± 0.45	26.36 ± 0.45	1.6± 0.15	0.43 ± 0.15	0.4±0.1	62.12
C+CC	65.47	270.62	4.5 ± 0.35	20.86 ± 1.11	1.01 ±0.09	0.3 0.1	0.18±0.07	89.93
SND-1	88.34	338.83	6.3 ± 0.4	27 ± 0.26	1.9 ± 0.15	0.6 ± 0.1	0.5±0.1	61.71
SND-1+CC	94	370.57	8.7 ± 0.4	29.54 ± 0.65	2.4 ± 0.19	0.9 ± 0.3	0.7±0.1	45.53

Detection of lignin deposition

The histochemical defense in mung bean plants was studied by observing the deposition of lignin and phenol in the leaf epidermal cells at different time intervals of 24, 36, and 48 hpi by differential staining technique **(Fig. 8)**. The results showed the highest deposition 86 % of lignin in SND-1 +CC treated plants at 36 hpi, whereas in SND-1 alone treated plants exhibited 74 % at 36 hpi. Epidermal cells in control SDW treated plants showed 34% of lignin deposition at 24, 36, and 48 hpi, whereas very low lignin accumulation of 28 % was observed in only pathogen-treated C+CC plants at 36 hpi.

Detection of hydrogen peroxide (H_2O_2) deposition

Early (3 hpi) deposition of H_2O_2 was observed and reached the maximum of 74 % in SND-1+CC treated plants at 36 hpi, whereas in SND-1 alone, it was observed that delayed accumulation (6 hpi) and highest 46% of H_2O_2 deposition at 36 hpi. Only pathogen-treated C+CC plants offered 29 % H_2O_2 accumulation; a delayed deposition of 21 % was detected in control SDW-treated plants **(Fig. 8)**.

Detection of phenol deposition

Phenol deposition in mung bean plants upon *C. canescens* infection was carried out by aniline blue staining at 0, 4,12,24,36, and 72 hpi. The results revealed a maximum accumulation of phenol 83 % in SND-1+CC treated plants at 36 hpi followed by 72 % in *Amycolatopsis* sp. strain SND-1 alone treated plants. The delayed deposition of phenol 43% was observed in control SDW-treated plants at 36 hpi, and a very low accumulation of phenol 26 % was noted in only pathogen-treated C + CC plants at 72 hpi **(Fig. 8)**.

Assessment of biochemical defense enzymes

All the performed treatments were assessed for biochemical defense upon Cercospora infection. The PAL activity in SND-1 + CC treated began early with the occurrence of infection (0h), and a maximum of 65.83 U and 62.33 U of PAL activities were found at 24 and 36 hpi. An almost similar trend was found in SND-1 alone at 24 and 36 hpi (47.56 u and 50.16 U). Whereas in pathogen-treated C + CC plants, 7.53 U was found at 36 hpi, and comparatively delayed activity of 2.03 U was recorded in control SDW treated plants **(Fig. 9 a)**.

The GLU activity was recorded in all the treatments, and results showed the GLU activity was started at early hours upon infection in SND-1 + CC plants; it was found that maximum GLU activity 11.33 U at 4 hpi and showed maximum activity 15.23 U and 14.63 U at 24 and 36 hpi respectively. Whereas in SND-1 alone, treated plants average GLU activity of 12.39 U and 9.96 U at 36 and 72 hpi was detected. The pathogen primed C + CC plants exhibited low GLU activity 4.26 U and 6.5 U at 12 and 24 hpi. There was a slight increase in GLU activity 11.3 U at 36 hpi, and this was decreased to 9.46 U at 72 hpi. Compared to all treatments, the SDW received plants showed very low GLU activity 7.33 U and 3.56 U at 36 and 72 hpi **(Fig. 9 b)**.

The highest POX activity, 24.20 U at 24 hpi, was detected in *Amycolatopsis* sp. strain SND-1 + CC treated plants, and a slight decrease of 23.12 U and 20.96 U (at 36 and 72 hpi) was observed. Whereas in *the Amycolatopsis* sp. strain, SND-1 alone treated plants offered 22.16 U at 36 hpi and a decrease of 20.53 U at 72 hpi. Control SDW plants exhibited very delayed POX activity 2.8 U and 2.16 U (36 and 72 hpi). In pathogen alone, received plants C + CC showed maximum pox activity of 6.16 U at 24 hpi, and this was reduced to 4.36 U at 72 hpi **(Fig. 9 c)**.

Discussion

Due to the emergence of virulence in fungal phytopathogens by continuous application of chemical fungicides resulted in the loss of agricultural productivity and eradication of soil microflora and fauna by increasing the chemical load in soil. The present study was designed to exploit endophytic actinomycetes' efficacy in controlling Cercospora leaf spot disease and induction of systemic resistance in mung bean plants. The endophytic actinomycetes were isolated from the medicinal weed *C. chellidonii*, and a total of 12 actinomycetes were recovered. All the isolates showed sufficient growth on starch casein agar (SCA) and international streptomyces project-2 (ISP-2) media. The morphological observation revealed different actinomycetes such as *Streptomyces*, *Nocardia*, *Amycolatopsis* mycelia texture, and variation in spore production. The results suggested that *the C. chellidonii* plant as a source of different beneficial actinomycetes. The result was consistent with an earlier report, where 46 endophytic actinomycetes isolates were recovered from *Camellia* sp., which includes *Streptomyces*, *Kribbella*, *Amycolatopsis*, *Saccharomonospora*, *Nocardia*, *Pseudonocardia*, *Actinomadura*, and *Microbispora* (Atlanta et al. 2020).

Among the 12 isolates, the isolate SND-1 exhibited maximum antagonistic nature against *C. canescens* compared with other isolates. The antagonistic potential is due to the production of different classes of secondary metabolites. The result was in accordance with the antifungal potential of *Amycolatopsis* sp. showed 60% of inhibition against *M. phaseolina* (Tassi) Goid (Gopalakrishnan et al. 2019). The antagonistic nature of actinomycetes is facilitated by the ability to produce IAA, HCN, and siderophores which involves in suppression of pathogen (Tokala et al. 2002). A similar study was conducted where isolated *Streptomyces* sp. MM140 exhibited maximum biocontrol potential against Cercospora of legumes (Mariana et al. 2016).

Based on the antagonistic potential, the isolate *Amycolatopsis* sp. strain SND-1 was selected for further screening of in-vitro plant growth promoting attributes. The results suggested that the isolate SND-1 was able to produce IAA in a time-dependent manner; it was observed that after 96 h, the production decreased. IAA plays a significant role as a signal molecule to cell differentiation, cell division, and organogenesis (Raut et al. 2017). The result was in accordance with a considerable amount of IAA production recorded from *Amycolatopsis* sp. 4GM4 (Ting et al. 2021). The GA3 is known

for the formation of seed dormancy as well as shoot growth. GA3 plays a vital role in the stimulation of plant growth under different abiotic stress (Ahmed et al. 2010) and is also known for stomatal resistance and decreasing salinity stress (Iqbal et al. 2013). Another important phytohormone cytokinins influence crop plants' growth and physiology and plant-pathogen interactions. Several studies reported cytokinin-mediated resistance in different plants such as *Tobacco* (Grosskinsky et al. 2011) and *Arabidopsis* (Choi et al. 2010). In the present study, the *Amycolatopsis* sp. strain SND-1 exhibited moderate production of all the three phytohormones that are involved in the strengthening of plants and hinted the indirect plant growth promoting attributes.

The qualitative production of siderophore, HCN, ammonia, and phosphate solubilization was observed in *Amycolatopsi*s sp. strain SND-1, indicating the ability to accelerate growth in plants. Similar findings were reported that isolated endophytic *Amycolatopsis pretoriensis* DSM 44654 exhibited positive production of siderophore, HCN, ammonia phosphate solubilization and resulted in growth enhancement in wheat (Sumaira et al. 2016). Siderophores have the capacity to enhance the antagonism against phytopathogens against their invading nature; HCN is directly involved in the disease suppression as part of induced systemic resistance (Passari et al. 2015). Ammonia production and phosphate solubilization are the indicators of the supply of high ammonia to the plants that involve in the growth process like root, shoot elongation, and triggering factor for various pathogens which causes the disease in favorable conditions. Phosphate solubilization is an indication of the ability to phosphate supply, synthesis of inorganic acids, organic acids as well as polysaccharides (Sumaira et al. 2016).

In-vivo biocontrol and plant growth promoting assay was performed with a talc-based formulation of *Amycolatopsis* sp. strain SND-1 isolate with challenge inoculation of Cercospora leaf spot pathogen (*C. canescens*) in mung bean plants. The assay was begun with the seed germination and vigor index in mung bean susceptible (DGGV-2) seeds. It was observed that formulation-soaked seeds exhibited the maximum seed germination and vigor index with increased root and shoot length compared with other treatments (SDW, C + CC, and SND-1). The results suggested that in the presence of the pathogen, the selected *Amycolatopsis* sp. strain SND-1 isolate significantly enhanced the germination and acceleration in vigor index. Further, the 35 days greenhouse study was conducted with four different treatments, and it showed significant enhancement of growth parameters (root length, shoot length, shoot fresh weight and fresh root weight, dry weight) in mung bean plants treated with formulation and challenge inoculation of the pathogen (SND-1 + CC). The attribute of production of growth parameters such as IAA, HCN, siderophore, and ammonia and phosphate solubilization of *Amycolatopsis* sp. strain SND-1 enhanced the maximum growth parameters. The results were aligned with findings, where significant enhancement of 32.26%, 84.60% shoot length and 13.38%, 61.94%, root length in mung bean plants primed with *P. aeruginosa* BHU B13-398 and *B. subtilis* BHUM (Punam et al. 2018). Another report suggested that isolated actinomycete isolate resulted acceleration in fresh and dry weights of mung bean plants (Krisana et al. 2018). *Amycolatopsis* sp. has been reported to produce biocontrol, and PGP traits such as siderophore, HCN, and IAA were reported to disease suppression (Alekhya et al. 2016).

Histological and biochemical changes in plants are considered the fundamental process toward the induced systemic resistance against phytopathogens. In the present study, the response in formulation-treated mung bean plants upon Cercospora pathogen infection at the histological and biochemical levels was studied. The results showed significant histological (lignin, H_2O_2, and phenol) accumulation at 12 to 24 hpi in *Amycolatopsis* sp. strain. SND-1 formulation treated mung bean plants with challenge inoculation of Cercospora pathogen. Similar results were recorded that histochemical depositions in plants were associated with the process of induced systemic resistance (ISR) upon pathogen infection (Joshi et al. 2021; Patil et al. 2020). Suganthagunthalam et al. (2014) concluded that lignin and phenol localization in plant cells could reduce the severity of infection caused by pathogens. In our study, bioprimed mung bean plants with *Amycolatopsis* sp. strain. SND-1 showed maximum lignin and phenol deposition in epidermal layers and exhibited a strong defense system against *C. canescens* infection. In another report, the authors confirmed

that the temporal manner of maximum lignification and H_2O_2 deposition increased the resistance in *Trichoderma* spp. primed pearl millet and grapevine plants against downy mildew infection (Boregowda et al. 2017; Milan et al. 2021). The study was well supported by the report that lignin and phenol deposition triggers the defense response in plants against infections (Tronchet et al. 2010). A similar report observed that the maximum accumulation of H_2O_2 by actinomycete isolate in grapevine plants induced the systemic defense against grey mold infection (Parul et al. 2017).

In the present study, biochemical defense enzymes such as PAL, GLU, and POX were quantified in *Amycolatopsis* sp. strain SND-1 primed mung bean plants, and results revealed that up-regulation of these antioxidant enzymes in comparison with control. The maximum enzyme activity suggested induced protection in mung bean plants against Cercospora infection. The results were aligned with the report that a consortium of bacterial inoculants significantly enhanced the biochemical defense enzymes (PAL, POX, and GLU) in mung bean plants against *M. phaseolina* infection (Sangeeta et al. 2018). The maximum activity of PAL and POX (67 and 40 U) in chili plants exhibited a significant defense response against *C. capsici* (Abhayashree et al. 2017). In another report, authors concluded that acceleration in PAL, GLU, and POX enzyme levels in pearl millet significantly decreased the invasion of downy mildew pathogen (Sudisha et al. 2011). In the current study, the stimulation of PAL, GLU, and POX strongly reduced the invasion of *C. canescens* pathogen in *Amycolatopsis* sp. strain SND-1 primed mung bean plants. In line with the current investigation, acceleration of defense enzymes in cucumber plants treated with three actinomycetes showed increased defense response against *P. aphanidermatum* (El-Tarabily et al. 2009).

Conclusion

The present study demonstrates the potential applications of endophytic *Amycolatopsis* sp. strain SND-1 as antifungal, plant growth promoter, and induced systemic resistance elicitor in mung bean plants against Cercospora infection. Isolated *Amycolatopsis* sp. strain SND-1 revealed maximum antifungal activity against *C. canescens* and showed positive production of IAA, siderophore, HCN, ammonia, and phosphate solubilization. *In-vivo* study of talc-based formulation of SND-1 exhibited maximum disease protection upon *C. canescens* infection and enhanced the total plant growth under the greenhouse trials in comparison with control plants. Further, the SND-1 treated mung bean plants exhibited the acceleration in induced systemic resistance through cell wall depositions (lignin, H2O2, and phenol) upon *C. canescens* infection. A significant enhancement of biochemical defense enzymes (PAL, GLU, and POX) was observed in *Amycolatopsis* sp. strain SND-1 primed plants with challenge inoculation of pathogen compared to control plants. The study concluded that the *Amycolatopsis* sp. strain SND-1 has the potential to counteract the significant biotic stress in mung beans and to be explored as an eco-friendly strategy against Cercospora infection in mung bean cultivars.

Declarations

Author contribution

The concept of the present research was designed and supervised by **SN**; all the experiments were performed by **DSB**. **RSK** helped in conceptualization, data validation, and initial manuscript preparation along with **SN** and **DSB**. The methodology, formal analysis, editing, and original draft were prepared by **DSB**, and final manuscript was constructed by **SN** along with **RSK**.

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Data availability statement

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Declarations Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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Figures

Figure 1

Plant habitat of *C. chellidonii*

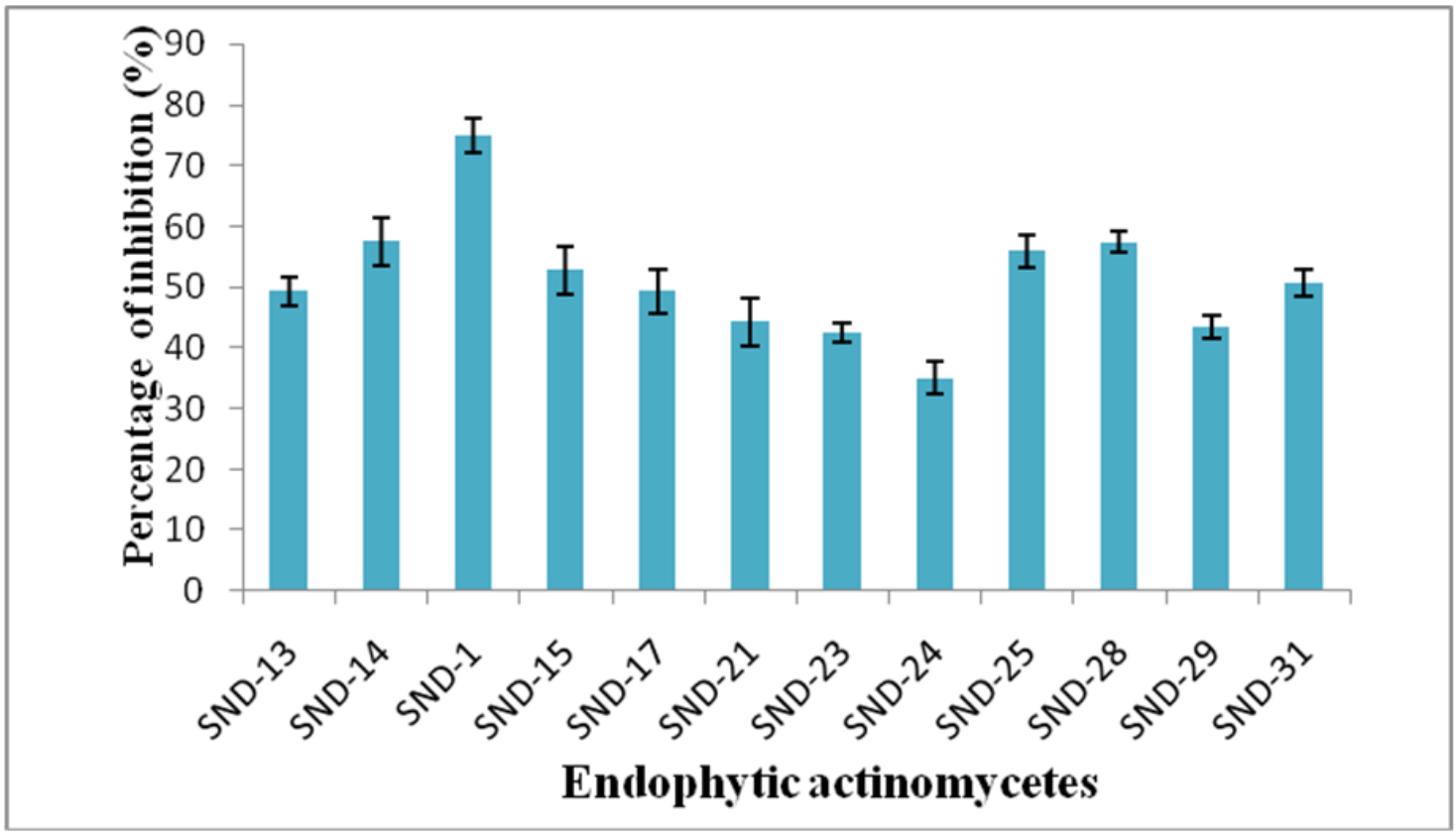

Figure 2

Antagonistic activity of isolated actinomycetes strains against *C. canescens*

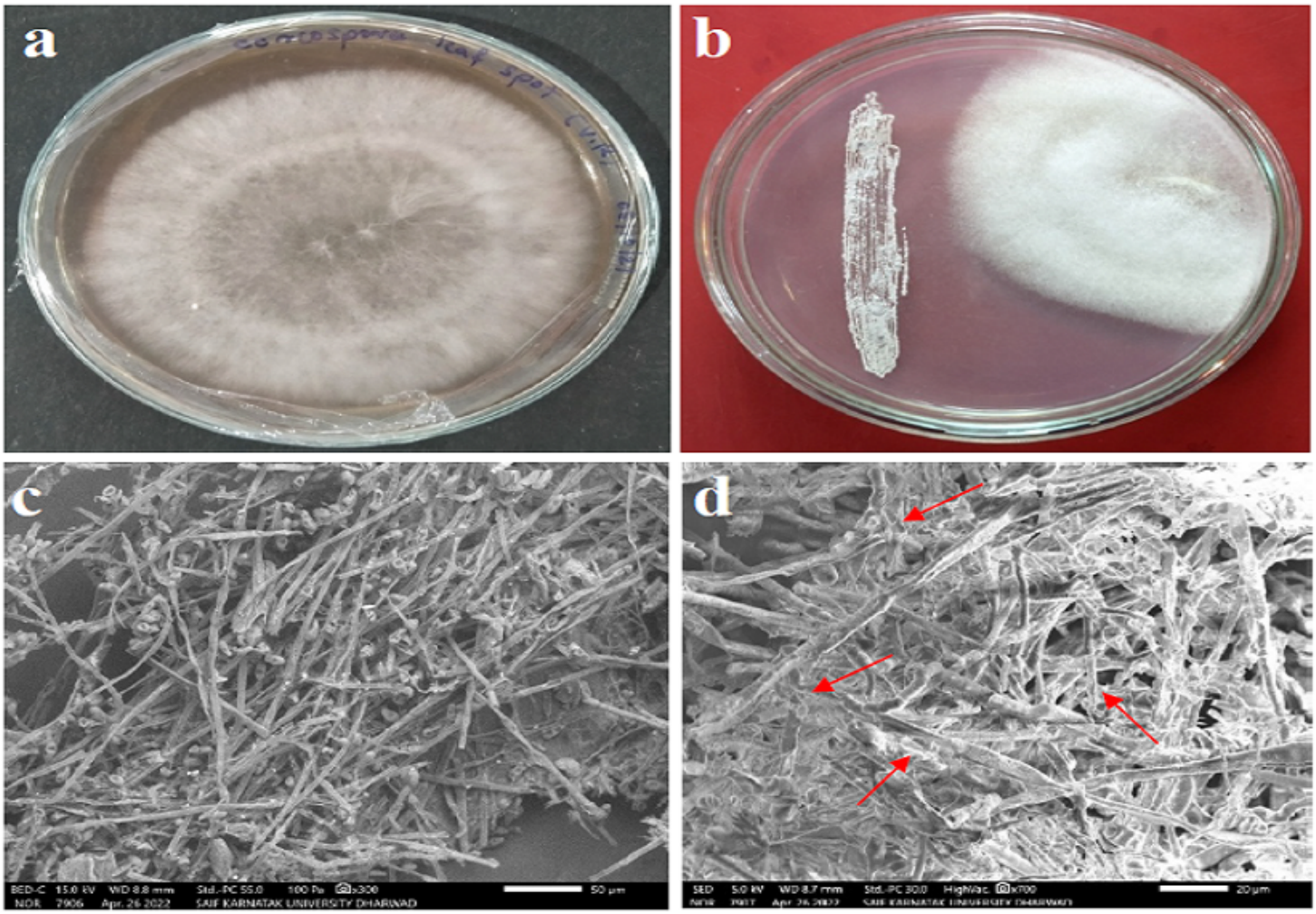

Figure 3

Dual culture assay of antagonistic activity of isolate SND-1against *C. canescens*. **(a)** *C. canescens* control, **(b)** Inhibition of *C. canescens* by isolate SND-1, **(c)** Mycelial morphology of *C. canescens* control and **(d)** Zone of inhibition area showing destruction of mycelia

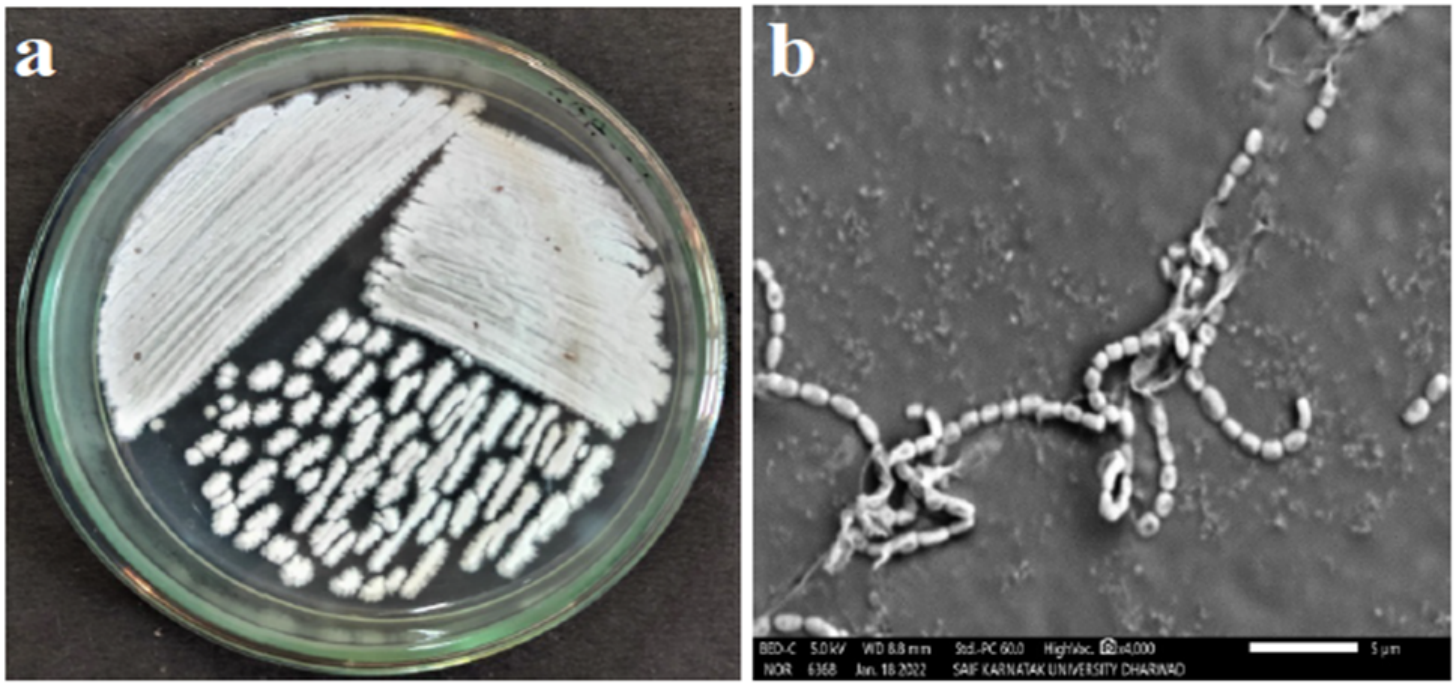

Figure 4

Morphology characterization of SND-1; **(a)** Growth on starch casein agar media and **(b)** SEM analysis showing spore morphology

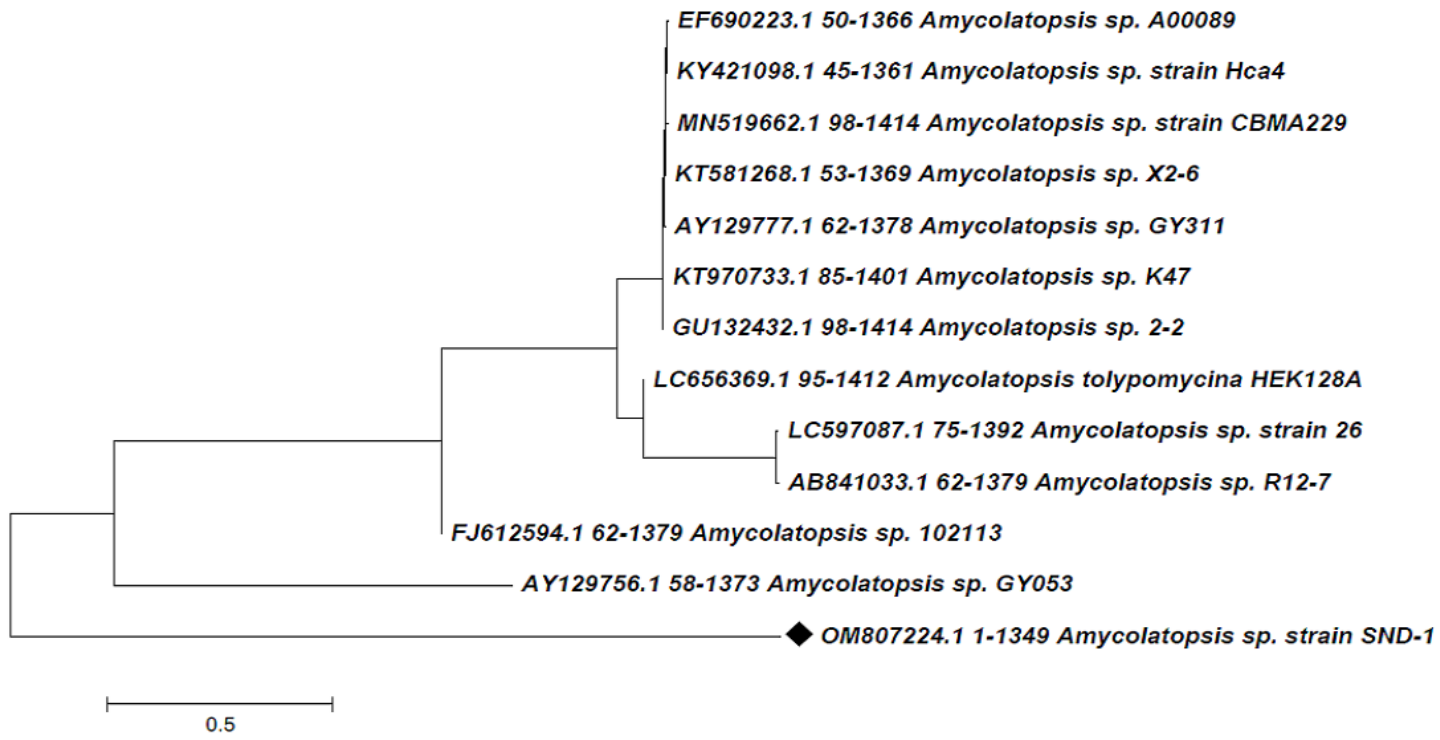

Figure 5

Phylogenetic analysis of isolate SND-1showing similarity with *Amycolatopsis* species

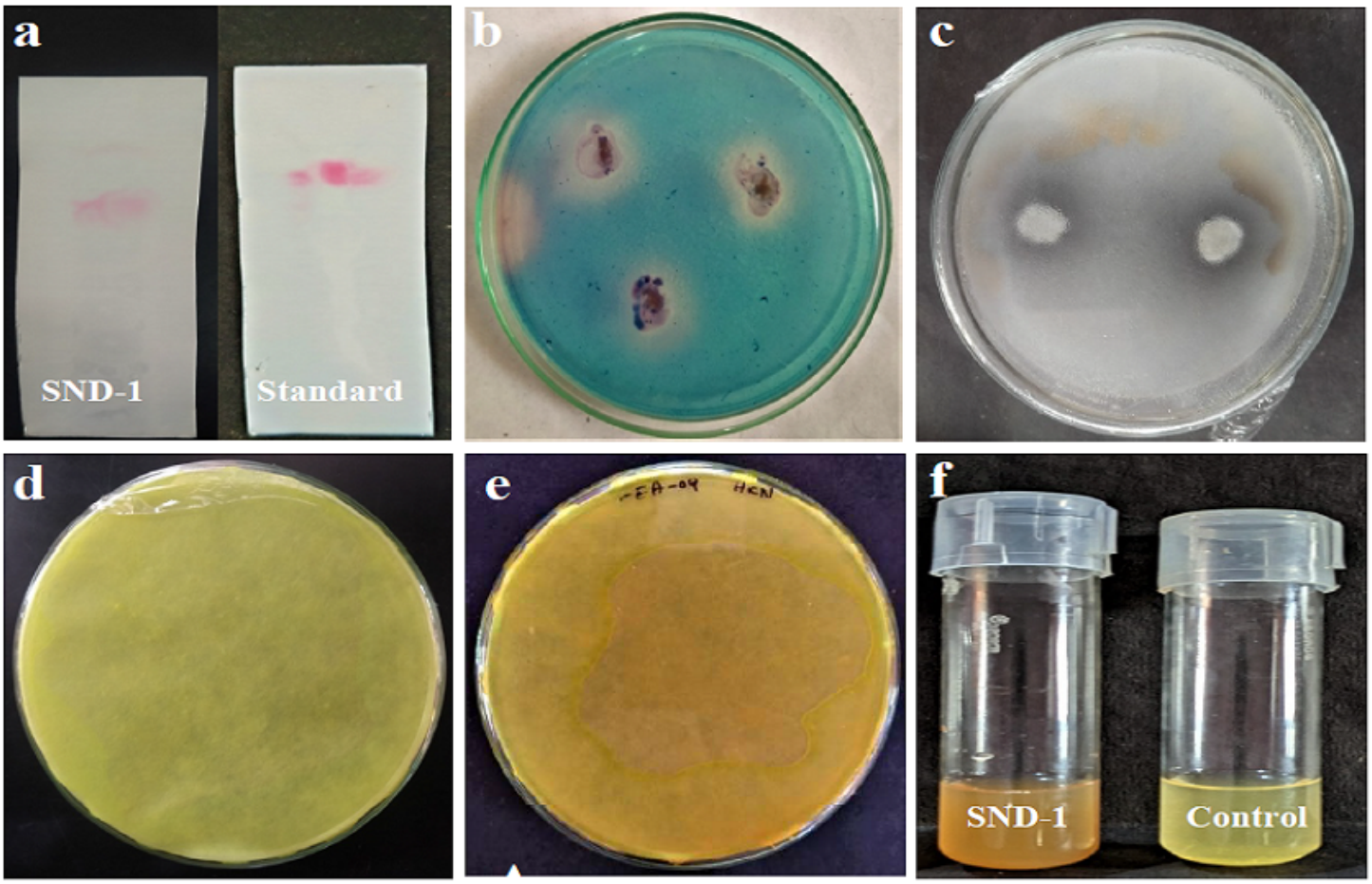

Figure 6

In-vitro plant growth promoting attributes of SND-1, **(a)** Thin layer chromatography showing production of IAA by development of pink spot, **(b)** Production of siderophore, **(c)** Phosphate solubilization, hydrogen cyanideproduction, **(d)** Control **(e)** SND-1 and **(f)** Production of ammonia showing color change in the peptone water

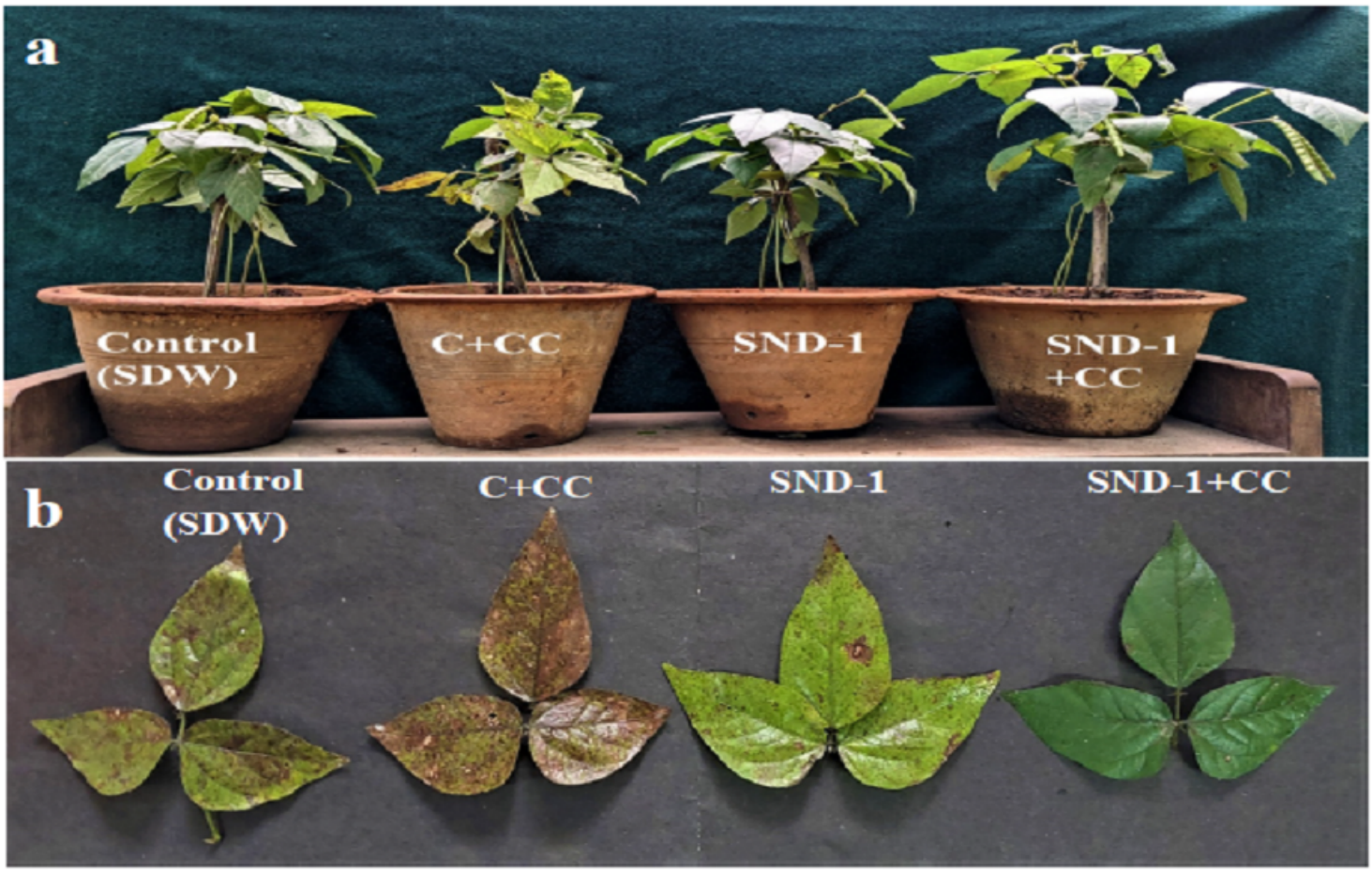

Figure 7

Effect of talcum formulation of SND-1 with challenge inoculation of *C. canescens* in mung bean plants. **(a)** Effect of different treatments after 35 days of green house experiment, **(b)** Disease severity on mung bean leaves in different treatments. Control (Treated with SDW); C+CC (Control treated with *C. canescens*); SND-1(Treated with SND-1 alone) and SND-1+CC (Treated with SND-1 with challenge inoculation of *C. canescens*

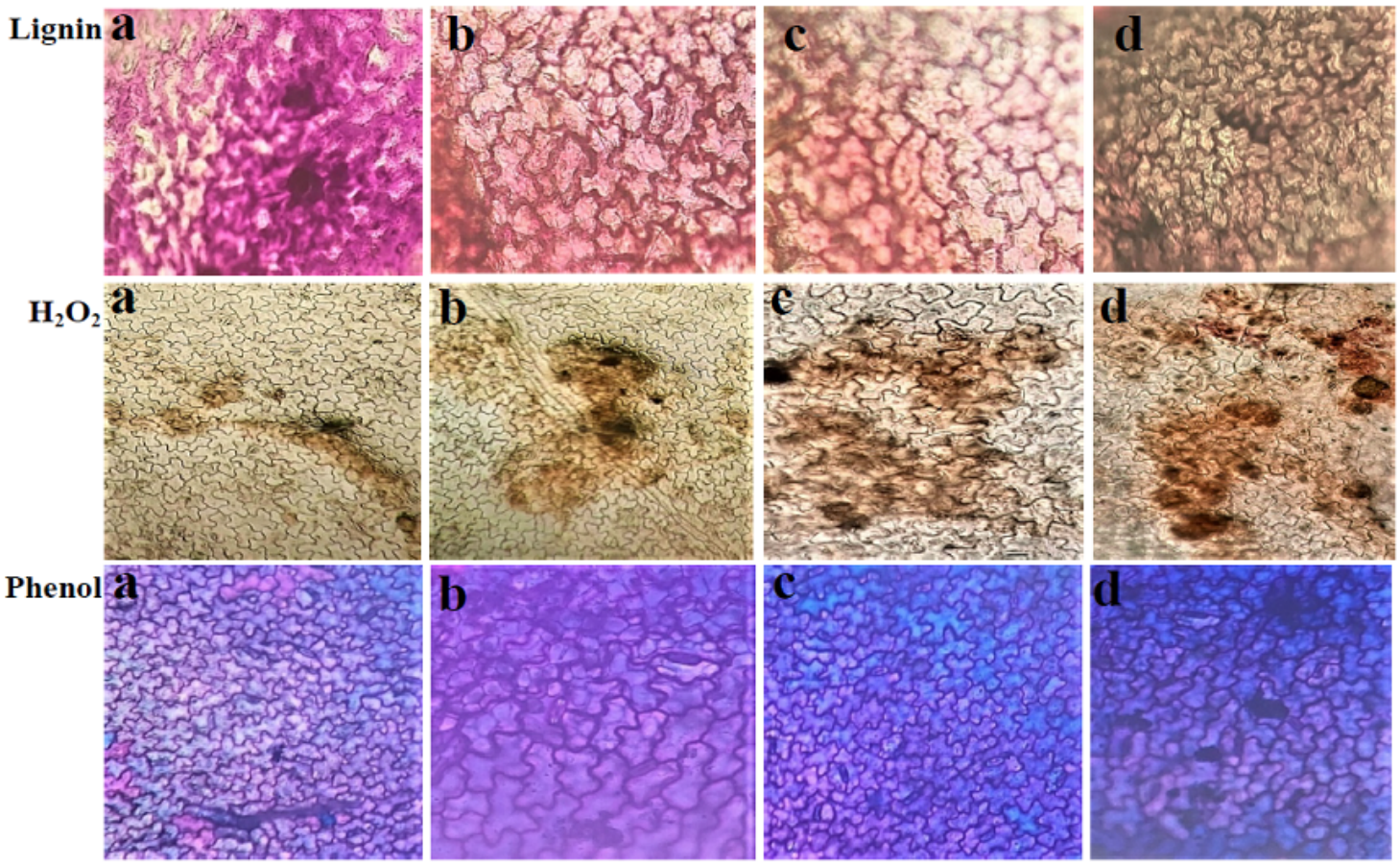

Figure 8

8 Histochemical defense response (Lignin, H_2O_2 and Phenol deposition at 36 and 24 hpi respectively) in mung bean leaf epidermis treated with talcum formulation of SND-1 and challenge inoculation with *C. canescens*. **(a)** Control (Treated with SDW), **(b)** C+CC (Control treated with *C. canescens*), **(c)** SND-1(Treated with SND-1 alone) and **(d)** SND-1+CC (Treated with SND-1 with challenge inoculation of *C. canescens*)

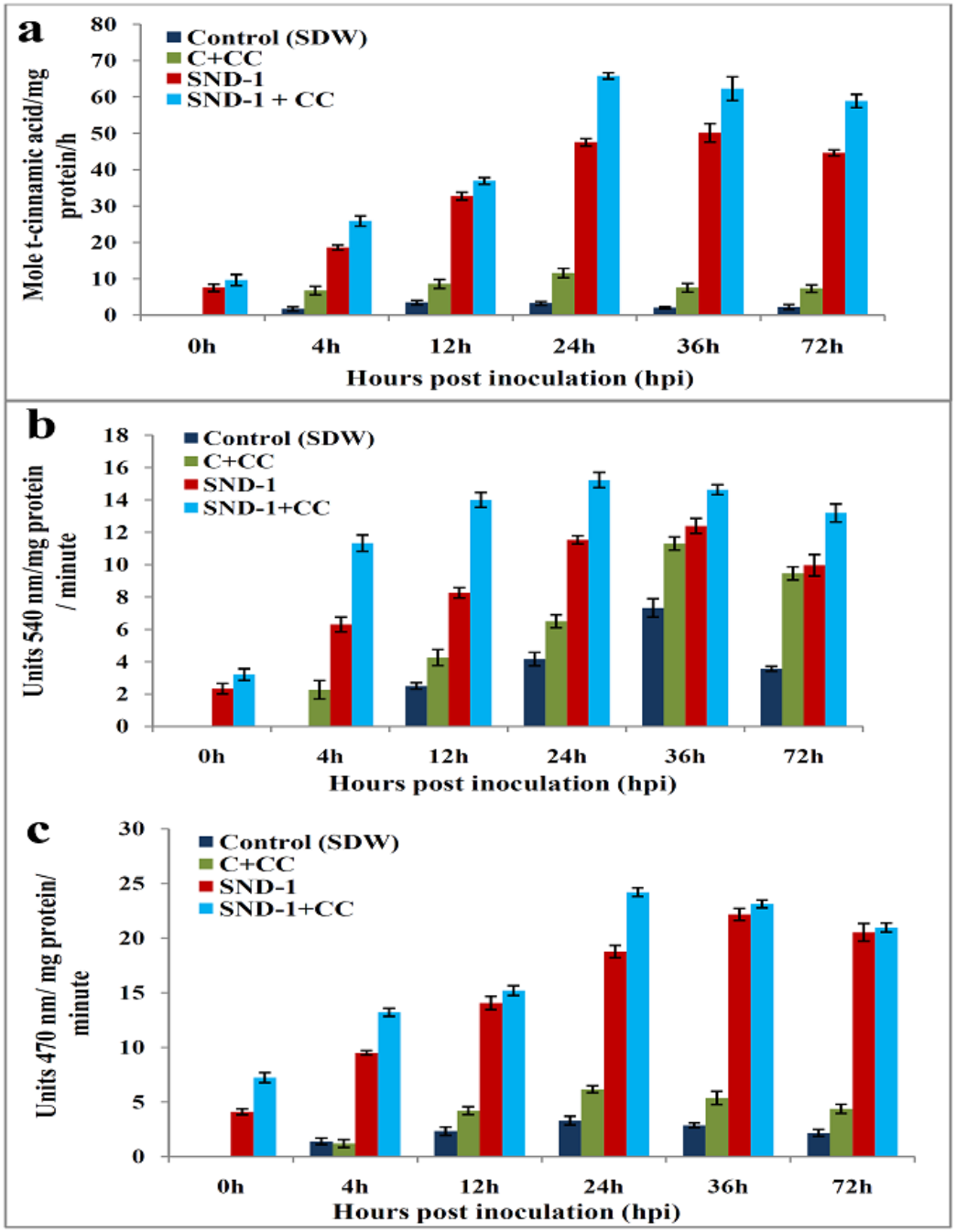

Figure 9

Biochemical defense response in mung bean leaves treated with talcum formulation of SND-1 and challenge inoculation with *C. canescens*. **(a)** Phenylalanine ammonia –lyase (PAL), **(b)** β-1, 3-glucanase (GLU) and **(c)** Peroxidase (POX) at 0. 4, 12, 24, 36 and 72 hpi. Control (Treated with SDW), C+CC (Control treated with *C. canescens*), SND-1(Treated with SND-1 alone) and SND-1+CC (Treated with SND-1 with challenge inoculation of *C. canescens*)