

HOSTED BY



Contents lists available at ScienceDirect

Journal of King Saud University – Science

journal homepage: www.sciencedirect.com

Original article

Acceleration of histopathological and biochemical defense patterns in mung bean with biopriming of *Amycolatopsis* sp. SND-1 against *Cercospora* leaf spot disease



Dhanyakumara Shivapoojar Basavarajappa^a, Raju Suresh Kumar^b, Muthuraj Rudrappa^a, Halaswamy Hiremath^a, Abdulrahman I. Almansour^b, Karthikeyan Perumal^c, Gireesh Babu Kantli^d, Sreenivasa Nayaka^{a,*}

^a P.G. Department of Studies in Botany, Karnatak University, Dharwad 580003, Karnataka, India

^b Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

^c Department of Chemistry and Biochemistry, The Ohio State University, 151 W. Woodruff Ave, Columbus, OH 43210, USA

^d Department of Life Sciences, PIAS, Parul University, Vadodara 391760, Gujarat, India

ARTICLE INFO

Article history:

Received 21 June 2023

Revised 25 July 2023

Accepted 11 September 2023

Available online 15 September 2023

Keywords:

Amycolatopsis SND-1

Mung bean

Cercospora leaf spot

Induced systemic resistance

Histochemical defense

Biochemical defense enzymes

ABSTRACT

Objectives: Failure of chemical control methods in conferring protection against *Cercospora* leaf spot (CLS) disease in mung bean crop led to the investigation of biocontrol agents for effective management of the CLS. The current study was focused on increased defense response (histochemical and biochemical) in mung bean plants by alginate pellet formulation of endophytic *Amycolatopsis* sp. SND-1 (SND-1) against CLS disease.

Methods: The sodium alginate bioformulation of SND-1 was prepared by standard method using CaCl₂ and evaluated for viability and contamination. Greenhouse study of prepared SND-1 formulation was performed on mung bean plants against CLS infection. Further, the acceleration of histochemical depositions (lignin, H₂O₂ and phenol) by differential staining methods and biochemical defense enzymes like PAL, GLU, and POX, phenolic, flavonoid and chlorophyll contents and cell death analysis were evaluated through microscopic and spectroscopic studies.

Results: The viability assay of the prepared formulation exhibited a negligible reduction in SND-1 spores (99.5% to 86.55%) throughout the six months of storage period. In-vivo (Greenhouse) study exhibited increased growth parameters in alginate pellet formulation of SND-1 challenged with *Cercospora* pathogen (SND-1+Pathogen) treated mung bean plants in comparison with Control (SDW) and only pathogen infested plants. Consequently, the plants with SND-1+Pathogen treatment showed significant reduction in disease severity up to 56%. Furthermore, the microscopic evaluation of histochemical defense markers depicted increased deposition of lignin (82.43±0.31%), H₂O₂ (47.97±0.66%), and phenol (76.74±0.79%) and cell death analysis through microscopic observation exhibited lesser dead cells of 31% in SND-1+Pathogen treatment in comparison with other treatments. Defense patterns at biochemical level hinted the elevation in activities of defense enzymes like PAL, GLU and POX at 12 and 24 h post pathogen inoculation (hpi) in SND-1+Pathogen treatment. The formulation treatment with pathogen (SND-1+Pathogen) also substantiated that enhanced total phenolic, flavonoid, and chlorophyll contents at different time intervals.

* Corresponding author.

E-mail addresses: dhanyakumarsb@gmail.com (D.S. Basavarajappa), sraju@ksu.edu.sa (R.S. Kumar), rmuthuraj20@gmail.com (M. Rudrappa), hmalaswamy2063@gmail.com (H. Hiremath), almansor@ksu.edu.sa (A.I. Almansour), pkarthikjaya@gmail.com (K. Perumal), gibugiri@gmail.com (G.B. Kantli), sreenivasankud@gmail.com (S. Nayaka).
Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

<https://doi.org/10.1016/j.jksus.2023.102903>

1018-3647/© 2023 The Author(s). Published by Elsevier B.V. on behalf of King Saud University.

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Conclusions: Overall the present investigation showed that *Amycolatopsis* sp. SND-1 formulation confers the protection in mung bean upon *Cercospora* infection and it might be an effective eco-friendly approach towards sustainable agriculture.

© 2023 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Mung bean is a foremost legume crop growing predominantly in Asia and worldwide. Over 7 million hectares were recorded for the mung bean crop alone (Nair et al., 2019). The seeds of mung bean are rich source of proteins, antioxidants, and fiber. The sprout contains abundant niacin, thiamine, and ascorbic acid profile. The seeds and sprouts of the mung bean are excellent source of minerals such as magnesium, phosphorus, calcium and iron (Itoh et al., 2006). A recent estimation of yield consistency on mung bean exhibited a reduction in the total production from 2.5 to 3.0 tons/hectare to 0.5 tons/hectare, and abiotic and biotic constrain were considered as significant cause of the yield reduction (Pratap et al., 2019). Among the biotic stresses the CLS disease caused by the deuteromycete fungus *Cercospora canescens* (*C. canescens*) is the most devastating entity which shows maximum yield loss. The CLS is a foliar disease and showed high yield loss in 50–70% and up to 96% in mung bean cultivation (Rafiq et al., 2022). The symptoms shows water soaked spots with grey borders and can affect leaf, petiole, pods and cause severe damage. Apart from the aggressiveness of the pathogen, the susceptible nature of the mung bean varieties and long term survival capability (Dormant mycelium in plant debris as primary inoculums) of the pathogen play significant affecting nature. The application of chemical fungicides poses chemical contamination and pollution, and repeated adaptation of these fungicides develops resistance in pathogens. An alternative eco-friendly method for controlling these diseases and improving the resistance in mung bean plants is a much-needed strategy to achieve sustainable cultivation. Biological control is the eco-friendly strategy of controlling the plant diseases that shows employment of beneficial living organism to counteract the specific or targeted plant pathogen with. This strategy includes the pathogen and disease control through the antibiosis, parasitism and resource competition (El-Tarabily et al., 2009).

Bioformulations developed with beneficial microbes (Bacteria, actinomycetes, and non-pathogenic fungi) can trigger the defense mechanisms and reduce the pathogen's multiplication and severity of diseases. Several studies reported that actinomycetes (Soil and endophytic) play an essential role as antifungal agents and elicit the induced systemic resistance in different crop plants (Bhat et al., 2022; Sreenivasa et al., 2020). Endophytic actinomycetes colonize the different tissues (obligate or facultative) in plants and create the symbiotic relationship. Furthermore, the colonized actinomycetes play significant role in plant defense enhancement and survival strategies during biotic and abiotic stresses (Gopalakrishnan et al., 2019). The development bioformulations through the encapsulation of plant growth promoting microbes exhibits the protection, prolonged and controlled release of the formulated microbes' spores. The encapsulation of microbes through sodium alginate is the most convenient for its rapid diffusion of the spores and compounds preferable for large scale application. Moreover, the alginate bioformulations shows high adsorption, excellent carrier and harmless to the environment (Miyada et al., 2017). El-Tarabily et al. (2009) reported increased plant growth and decreased severity of *Pythium* disease in cucumbers by glucanase-producing endophytic actinomycetes through the acceleration of induced protection. In another study, significant plant growth enhancement was observed when plants were exposed to stress conditions with the endophytic *Amycolatopsis pittospori* sp.

nov., *Amycolatopsis* sp. BCA-696, and *Streptomyces rochei* strain PTL2 in rice, sorghum and tomato plants (Onuma and Christopher, 2021; Gopalakrishnan et al., 2019; Ting et al., 2017; Miyada et al., 2017). In another report Sabarathanam et al. (2015) studied the significant biocontrol activity of *Streptomyces* sp. strain Di-944 formulation (Alginate-Kaolin-based) against damping-off severity in tomato plants. Several actinomycetes based formulations such as 'Mycostop' (*Streptomyces griseoviridis* K61 strain) for soil and seed borne fungal diseases, 'Actinovate' registered for control of *Pythium ultimum* and *Rhizoctonia solani* and PGP trials, 'Mykocide KIBC' registered in South Korea for controlling of brown patch, powdery mildew and grey mold diseases and 'Actofit', 'Incide SP' and 'Actin' are used for the control of diverse diseases of the crop plants in Ukraine, USA and in India were commercially available in the market (Churasia et al. 2018).

Plant defense response is initiated at the cellular level by depositing frontline defense barriers (lignin, hydrogen peroxide and phenol) in cell walls (Sudisha et al., 2020). Deposition of increased lignin in plant's cell walls was reported to block the pathogen invasion (Milan et al., 2021). Another barrier, H₂O₂, and phenol effectively induced the mechanism of programmed cell death of plant pathogens (Singh et al., 2021). Several researchers have found that acceleration in the activity of these enzymes can significantly counteract the pathogen. In another report, formulation of endophytic *Streptomyces* spp. elicited the PAL, GLU and POX resulted in the suppression of *S. rolfisii* in chickpea, *M. phaseolina* in mung bean, *Phytophthora megakarya* in cocoa plants (Singh et al., 2021; Sangeetha et al., 2018; Aristide et al., 2022). The *S. rimosus* and *S. monomycini* increased the phenolic and flavonoid contents that showed effective inhibition of damping-off disease causing pathogen *P. drechsleri* in cucumber plants (Akram et al., 2017). Considering the noteworthy literature of actinomycetes formulations, the current study performs the effect of bioformulation of endophytic *Amycolatopsis* sp strain SND-1 on the acceleration of plant growth against *Cercospora* disease in mung bean and elicitation of resistance at histochemical and biochemical levels.

2. Materials and methods

2.1. Collection of actinomycetes strain, pathogen, and mung bean variety

For the current study, the previously identified *Amycolatopsis* sp. strain SND-1 (SND-1) with Accession No-OM807224 and characterized for production of indole acetic acid (IAA), gibberellic acid (GA3), Cytokinin, Siderophore, hydrogen cyanide (HCN), Ammonia and phosphate solubilization and plant growth promotion in mung bean was selected (Basavarajappa et al., 2023). The highly susceptible variety of mung bean seeds (DGGV-2) and pathogen *Cercospora canescens* were collected from University of Agricultural Sciences (UAS), Dharwad, Karnataka, India.

2.2. Preparation of sodium alginate pellet formulation

Final density of SND-1 spores $\approx 10^6$ CFU mL⁻¹ was adjusted by the cell count method. The sodium alginate pellets formulation of SND-1 was prepared according to Miyada et al., (2017). Finally, the

dried SND-1 pellets were evaluated for the spores at $\approx 2 \times 10^6$ CFU g^{-1} . The prepared sodium alginate SND-1 formulation was stored with the double distilled water in the sterilized container for further experimental use.

2.3. Purity and viability of pellet formulation

The purity and viability assay of SND-1 formulation was done according to Miyada et al., (2017), by cell counting plate method (CFU g^{-1}). All the viability trials were performed in triplicates for statistical significance (Miyada et al., 2017).

2.4. Pathogen inoculums preparation

Inoculums of *C. canescens* pathogen were prepared according to (Gopalakrishnan et al., 2019) and final spore load (10^5 CFU mL^{-1}) was adjusted.

2.5. Greenhouse study

The growth promotion and biocontrol assay was conducted under controlled greenhouse (*in-vivo*) conditions during the pre monsoon season (May to July) with the pots (15×10 cm) filled with sterilized soil, farm yard manure and sand (1:1:2). The dried SND-1 alginate pellets (0.14 g) were added to the soil around the seed inoculation area by soil drench method. Then the mung bean seeds were surface sterilized with 2% sodium hypochlorite (NaOCl) for 2–5 min, and then multiple times with SDW and sowed (4 seeds per pot). The inoculation of the pathogen was performed for two weeks old mung bean plants by spraying the final spore load for 5 consecutive days at early morning conditions. The triplicate trials of each treatment were considered for statistical analysis. After 35 days, the plants belonging to individual treatments were uprooted and subjected to evaluate growth parameters. Disease severity assessed by employing the 5-class scale which includes measurements such as 0=no cercospora symptoms, 1=0–

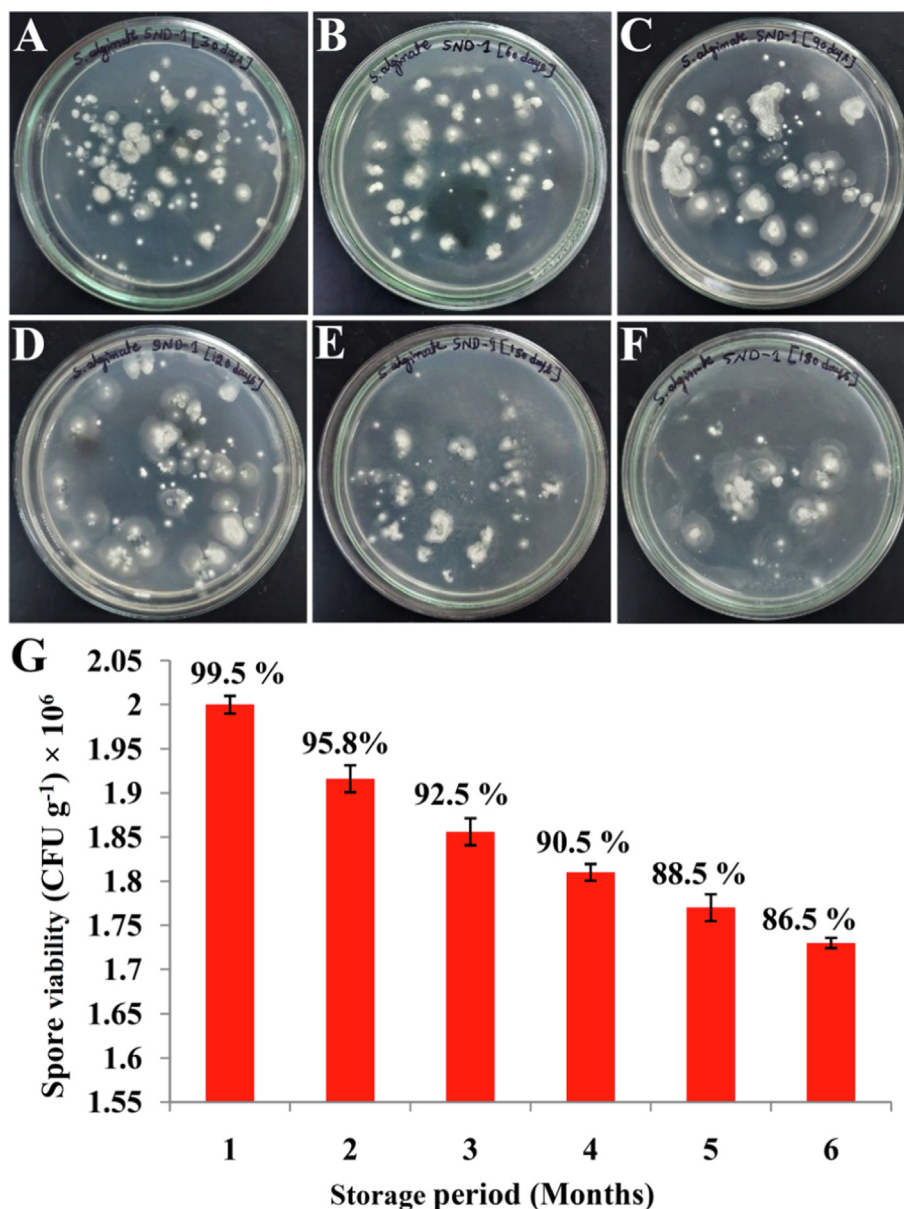


Fig. 1. Purity and viability assay: (A-F) Spore viability of sodium alginate formulation of SND-1 strain, evaluated up to 6 months (1 month interval) of storage period. (G) Histogram representing % of viability of sodium alginate formulation of SND-1.

25% of Cercospora symptoms, 2=26–50% of Cercospora symptoms, 3=51–75% Cercospora symptoms and 4=76–100% Cercospora symptoms and the values were converted into percentage. Finally, each treatment’s disease severity was measured using the following formula.

$$DSI\% = \sum (R \times N) \times 100 / HT \times T$$

(R = Disease rating and N = Number of mung bean plants. H = Highest disease rating, and T = Total number of plants).

2.6. Assessment of induced systemic resistance

The induced systemic resistance in treated mung bean plants was analyzed histochemically through the localization of lignin, hydrogen peroxide (H₂O₂), and phenol. Biochemical defense pat-

terns were evaluated by measuring the activity of antioxidant enzymes like PAL, POX and GLU in the leaf epidermis. Each experiment was performed with fresh leaves (1 g) from each treatment at specified time intervals of hours after post pathogen inoculation (hpi).

2.7. Microscopic observation of cell wall depositions

Deposition of lignin was assessed according to Milan et al. (2021). The leaf peelings were observed for lignin deposition (reddish brown depositions) under a microscope (OLYMPUS, CX23) at 40 X magnification and deposition percentage (cells) was calculated. The H₂O₂ deposition was examined by using 3, 3-Diaminobenzidine (DAB) and phenol deposition in each treatment was performed according to Milan et al. (2021) using 0.05% tolu-

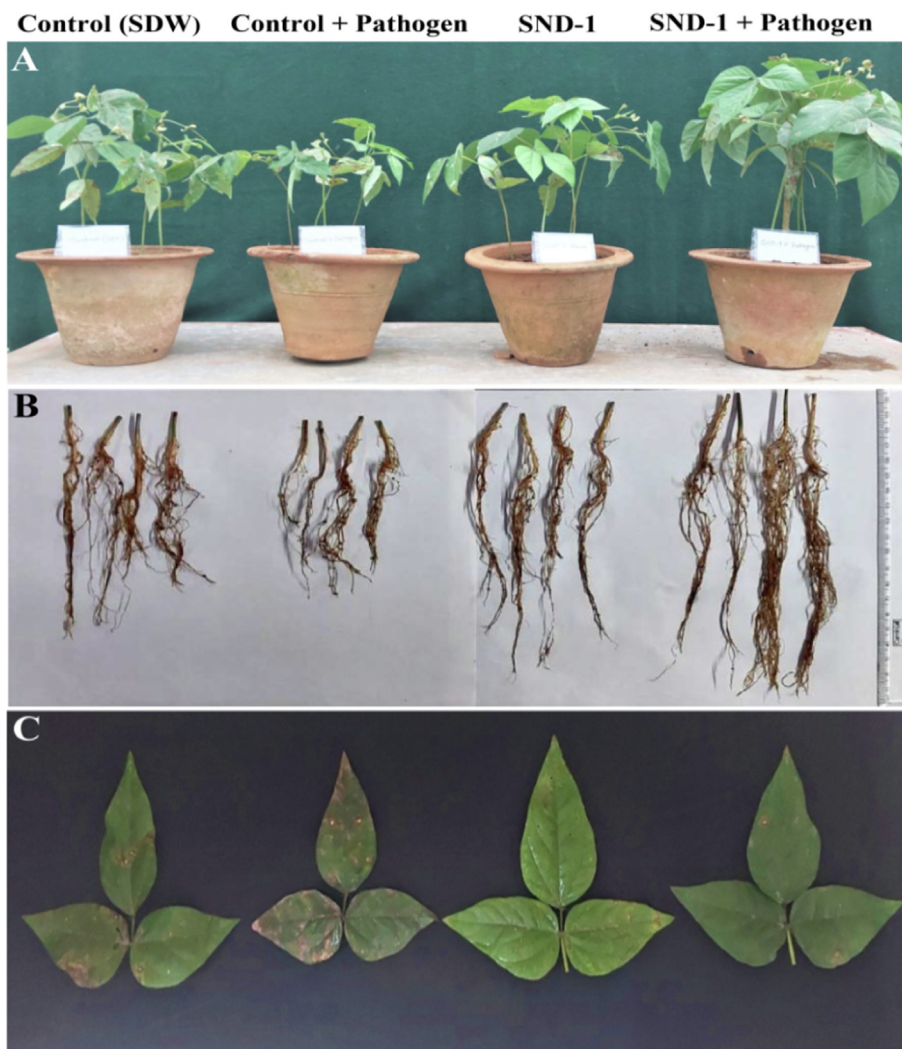


Fig. 2. Effect of SND-1 sodium alginate formulation on mung bean plants under greenhouse conditions: (A) Growth patterns of mung bean plants upon different treatments, (B) Increased root length among the different treatments and (C) Disease severity among the different treatments.

Table 1
Effect of SND-1 sodium alginate formulation on mung bean growth parameters under controlled greenhouse conditions.

Treatments	Root length (cm)	Shoot length (cm)	Root fresh weight (g)	Shoot fresh weight (g)	Total dry weight (g)	DiseaseSeverity(%)
Control (SDW)	10.6 ± 0.3	27.06 ± 0.25	1.21 ± 0.02	2.23 ± 0.15	0.59 ± 0.03	55.2
Control+Pathogen	8.3 ± 0.30	19.93 ± 0.55	0.81 ± 0.02	1.97 ± 0.03	0.42 ± 0.03	88.19
SND- 1	11.27 ± 0.29	27.62 ± 0.52	1.53 ± 0.28	2.62 ± 0.25	0.62 ± 0.04	8.85
SND-1+Pathogen	13.9 ± 0.04	30.05 ± 0.31	1.79 ± 0.05	3.34 ± 0.55	0.76 ± 0.03	43.63

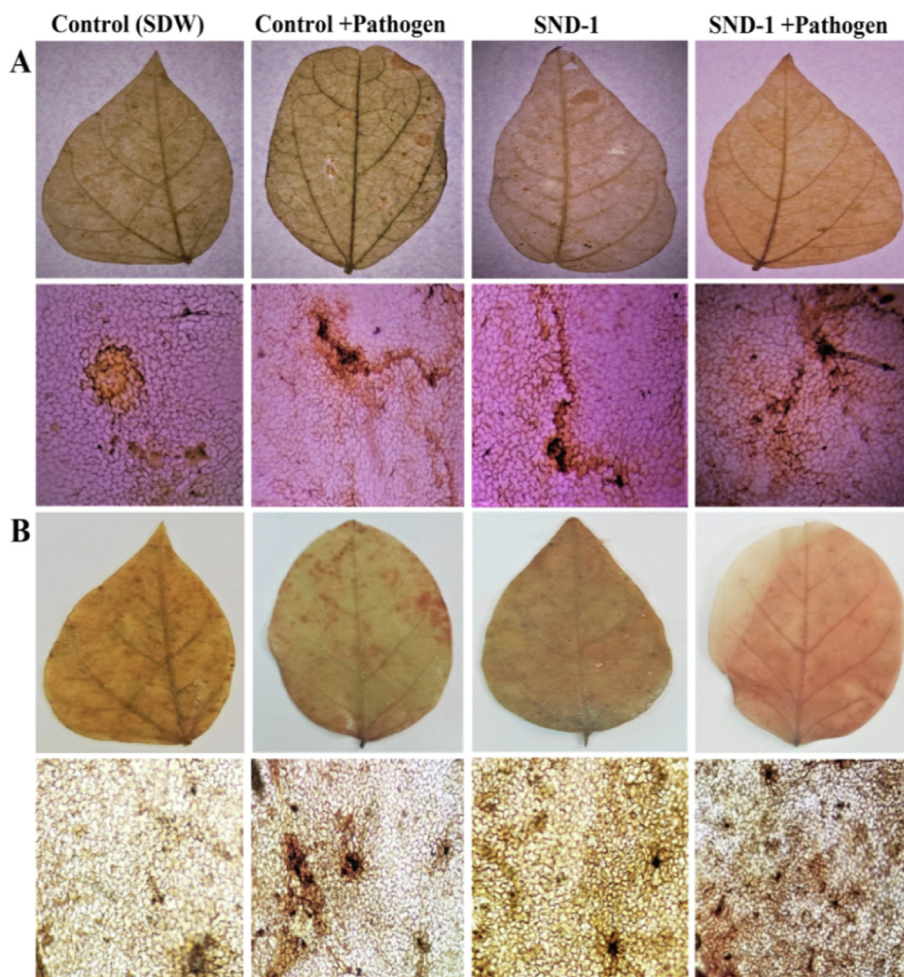


Fig. 3. Histochemical depositions in treated mung bean plants: **(A)** Lignin deposition in leaves and leaf epidermis (reddish brown deposition along the cell wall) stained with phloroglucinol at 24 hpi. **(B)** Visualization of hydrogen peroxide (H_2O_2) deposition (brown color deposition along the cell wall) in leaves and leaf epidermis upon staining with DAB at 24 hpi.

idine blue staining technique. The % cell death was evaluated using trypan blue staining method (blue color depositions).

2.8. Estimation antioxidant defense enzymes

The enzyme extract obtained from the homogenized leaf samples was used as an enzyme source, and at respective time intervals. The Phenylalanine ammonia-lyase (PAL) activity was estimated using a spectrophotometer (Shanghai Metash Instruments Co., Ltd., Shanghai, China) at 290 nm and β -1,3-glucanase (GLU) activity was evaluated spectroscopically at 540 nm using laminarin (1%). The Peroxidase (POX) quantification was done using the substrate guaiacol (25%) and the activity was at 470 nm. Peroxidase and GLU activities were expressed in units/mg protein/min (Milan et al., 2021).

2.9. Estimation of phenolic, flavonoid, and chlorophyll content

The phenolic content was quantified spectrophotometrically according to Vernon et al. (1993) by Folin-Ciocalteu (FC) method by measuring the absorbance at 765 nm. Flavonoid quantification was performed using aluminum chloride method and recorded the absorbance at 425 nm. The chlorophyll content was estimated using *N,N*-dimethylformamide (DMF) and absorption spectra was recorded between 350 and 750 nm (Natale et al., 2023).

2.10. Statistical analysis

The viability assay, histochemical depositions and biochemical tests were performed in triplicates trials and results were tabulated as Mean \pm Standard deviation (SD).

3. Results

3.1. Purity and viability of pellet formulation

The prepared sodium-alginate pellet formulation of SND-1 exhibited alginate beads with the 0.3 mm of approximate size and stored in sterilized container with distilled water. The SND-1 pellet formulation showed there is no contamination and a slight decrease up to 6 months storage period (Fig. 1 A-F). It was recorded 2×10^6 CFU g^{-1} (99.5%) up to 30 days from the day of formulation preparation and reached 1.73×10^6 CFU g^{-1} (86.5%) after 6 months of storage period (Fig. 1 G).

3.2. Greenhouse study

In-vivo growth promoting and biocontrol assay (after 35 days of the greenhouse study) showed significant increase in growth parameters and less disease severity (DSI) in alginate pellet formulation treated mung bean plants (Fig. 2A) in comparison with con-

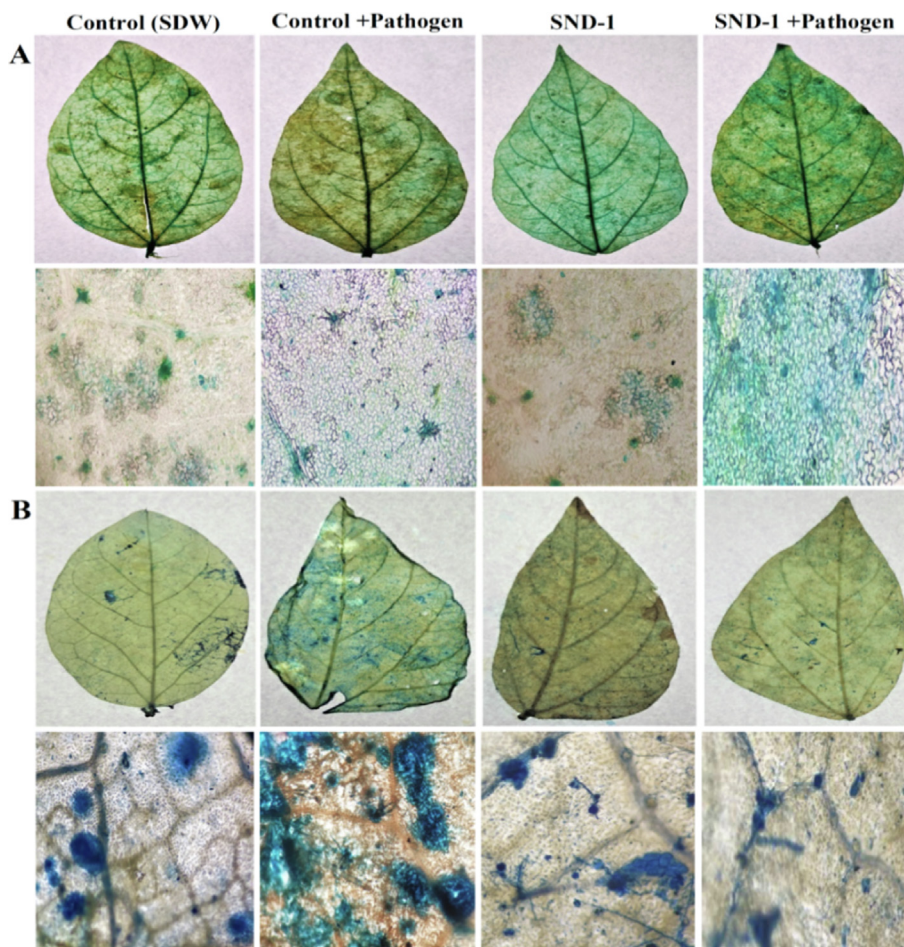


Fig. 4. Histochemical deposition and cell death analysis in mung bean plants: **(A)** Deposition of phenol in mung bean leaves and leaf epidermis (greenish blue deposition along the cell wall) upon staining with toluidine blue at 24 hpi. **(B)** Detection of cell death in mung bean leaves and leaf epidermis staining with trypan blue (dark blue stain represents dead cells). The results were displayed by considering the necrosis spot and compared with complete leaf blade surface.

Control and pathogen treatments (Table 1). The plants treated with SND-1+Pathogen exhibited root length 13.9 ± 0.04 cm, shoot length 30.05 ± 0.31 cm, root fresh weight 1.79 ± 0.05 g, shoot fresh weight 3.34 ± 0.55 g and 0.76 ± 0.03 g of dry weight (Fig. 2B). Whereas minimum root length of 8.3 ± 0.30 cm, shoot length of 19.93 ± 0.55 cm, root fresh weight of 0.81 ± 0.02 g, shoot fresh weight of 1.97 ± 0.03 g and 0.42 ± 0.03 g of total dry weight. In the treatment with only SND-1 formulation and Control (SDW) the moderate growth parameters were observed. The biocontrol efficacy of SND-1 alginate formulation was assayed by calculating the disease severity (Table 1) in comparison with all the treatments and very low severity 43.63% in SND-1+Pathogen was recorded. In only SND-1 formulation treatment it was observed 8.85%, and disease severity of 55.2% was noticed in Control (SDW) plants and highest 88.19% of disease severity was found in only pathogen treated plants (Fig. 2C).

3.3. Evaluation of cell wall depositions

Microscopic observation of stress-responsive defense markers such as lignin, H_2O_2 and phenol deposition resulted maximum deposition of lignin $82.43 \pm 0.31\%$ at 24 hpi in SND-1+Pathogen treatment and in only SND-1 formulation treated plants it was found that $68.07 \pm 0.27\%$ at 24 hpi. Lignin localization in Control +Pathogen treatment showed $33.16 \pm 0.27\%$ lignin at 24 hpi and Control (SDW) plants exhibited a very low accumulation of $27.17 \pm 0.30\%$ at 24 hpi (Fig. 3A). The early deposition of H_2O_2 was

observed at 4 hpi and reached $47.97 \pm 0.66\%$ at 24 hpi was observed in SND-1+Pathogen treatment and in SND-1 formulation only it was found that $43.08 \pm 0.19\%$ at 24 hpi (Fig. 3B). The plants received Control+Pathogen showed maximum $23.28 \pm 0.43\%$ of deposition of H_2O_2 at 24 hpi and in control (SDW) treatment it was exhibited $21.12 \pm 0.18\%$ of H_2O_2 accumulation at 24 hpi.

The deposition of phenol was noticed early (at 4 hpi) and maximum phenol accumulation $76.74 \pm 0.79\%$ at 24 hpi was observed in SND-1+Pathogen received plants (Fig. 4A). A fair amount of phenol deposition with $45.33 \pm 0.34\%$ and $37.00 \pm 0.26\%$ was detected in only SND-1 formulation as well as in Control+pathogen treated plants. The Control (SDW) treated plants showed delayed accumulation of phenol $33.48 \pm 0.45\%$ at 24 hpi. Trypan blue staining method revealed that highest percentage of cell death (65%) in pathogen treated and 53% in Control (SDW) mung bean plants (Fig. 4B). Whereas in only SND-1 formulation treatment 41% of cell death and significant reduction in cell death 31% was observed in SND-1+Pathogen treatment. The time course analysis of histochemical depositions was graphically represented in Fig. 5 A-C.

3.4. Estimation of antioxidant defense enzymes

The PAL activity in SND-1+Pathogen was initiated at early (0 h) after pathogen inoculation and maximum activity 45.48 U was observed at 24 hpi (Fig. 6A). The only SND-1 formulation treated plants showed 42.25 U of PAL activity at 24 hpi. PAL activity in Control+Pathogen treated plants showed no activity up to 4 hpi

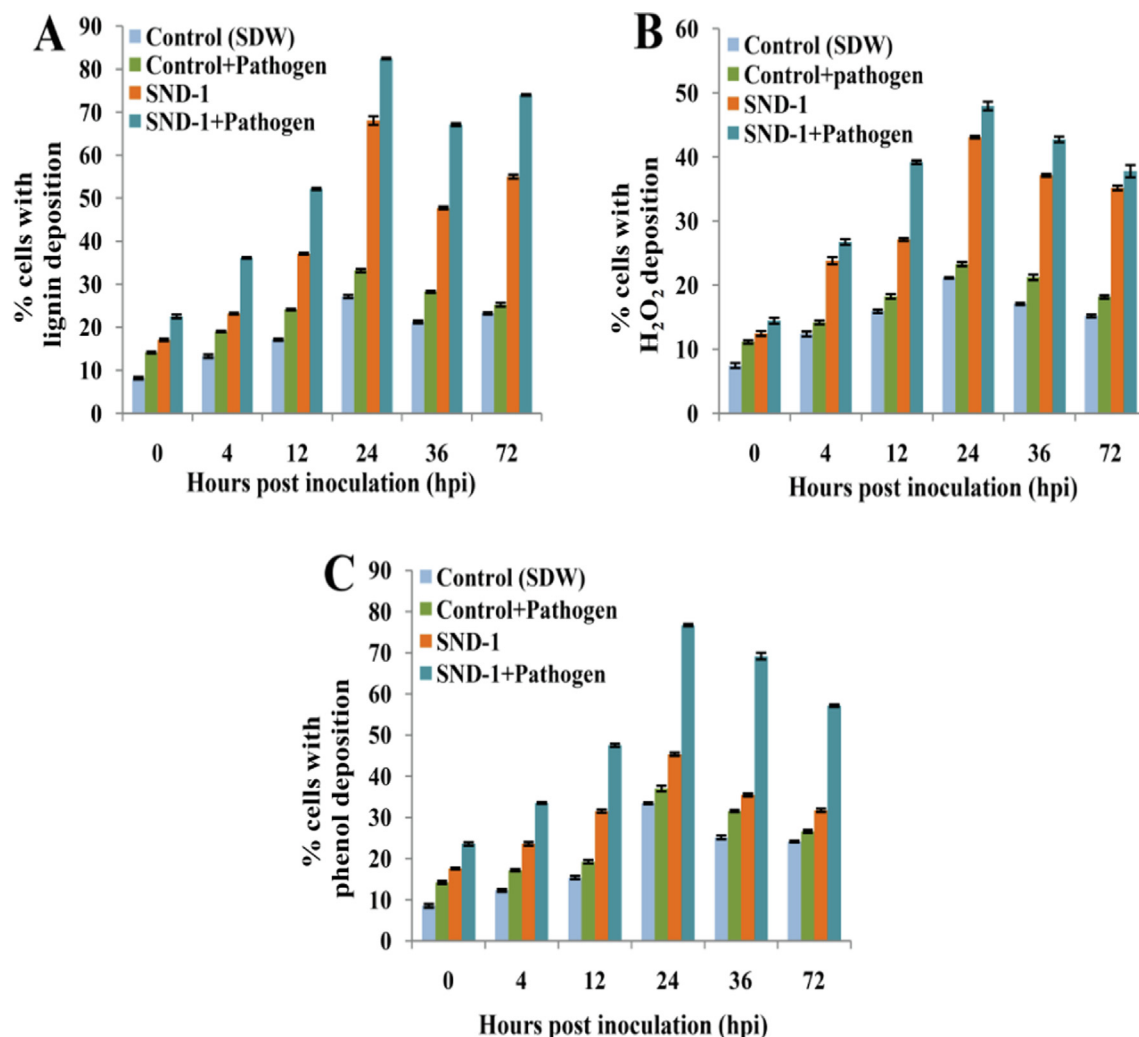


Fig. 5. Histogram representing histochemical depositions in treated mung bean plants: (A) Time course analysis of lignin deposition. (B) Time course analysis of H₂O₂ deposition. (C) Time course analysis of phenol deposition.

and maximum activity 9.54 U was recorded at 24 hpi. Whereas in Control (SDW) treatment the highest PAL activity was recorded 4.79 U at 24 hpi. The varied activity of GLU was found in performed treatments and highest GLU activity 14.54 U at 24 hpi was exhibited in plants treated with SND-1+Pathogen at 24 hpi (Fig. 6B). A progressive increase in GLU activity and maximum 12.13 U was recorded in SND-1 alone treatment at 24 hpi. The plants treated with Control+Pathogen showed 9.05 U of GLU and in control (SDW) showed delayed activity of GLU 6.02 U at 24 hpi and was declined drastically at later time intervals. The increased 21.21 U of POX activity at 24 hpi was obtained in the SND-1+pathogen treatment (Fig. 6C). Whereas SND-1 only treatment exhibited maximum POX activity 19.17 U at 24 hpi respectively. Control+Pathogen treatment displayed 5.2 U of POX activities at 24 hpi, whereas Control (SDW) treatment the elevated POX activity 3.32 U was observed at 24 hpi and later there was gradual decrease in the POX activity was noted up to 72 hpi.

3.5. Estimation of total phenolic, flavonoid, and chlorophyll contents

The highest phenolic content 3.64 ± 0.11 mg at 24 hpi was recorded in plants treated with SND-1+Pathogen (Fig. 7A). A fair phenolic content of 3.46 ± 0.05 , 2.70 ± 0.05 , and 1.73 ± 0.06 mg/g fresh weight at 24 hpi was recorded in only SND-1 formulation,

Control+Pathogen and control SDW plants. The maximum flavonoid 1.01 ± 0.06 mg was observed in mung bean plants with the treatment of SND-1+Pathogen at 24 hpi (Fig. 7B). Plants treated with only SND-1 formulation, Control+Pathogen and Control (SDW) plants offered 0.80 ± 0.02 , 0.60 ± 0.01 and 0.43 ± 0.02 mg/g fresh weight of flavonoid content. The chlorophyll content 1.16 ± 0.03 mg was observed SND-1+Pathogen treatment and 0.82 ± 0.02 mg/g fresh weight in only SND-1 formulation treatment. A fair amount of chlorophyll 0.6 ± 0.02 mg/g fresh weight in Control (SDW) treated and lowest chlorophyll content 0.4 ± 0.01 mg/g fresh weight was exhibited by Control+Pathogen treated mung bean plants (Fig. 7C).

4. Discussion

The formulated actinomycetes products were excellent alternatives of chemical products and proven for their biocontrol, growth enhancement and ISR with beneficial actinomycetes in different crop plants (Chakraborty et al., 2022). Furthermore, the chemical fungicides creates the tremendous negative impacts on soil microflora and reduction in soil nutrient profile results the decreased soil quality. The *Amycolatopsis* sp. SND-1 was previously isolated from *Cleome chellidonii* Linn. plant and characterized for its *in-vitro* plant growth and potential antagonistic action against *Cercospora*

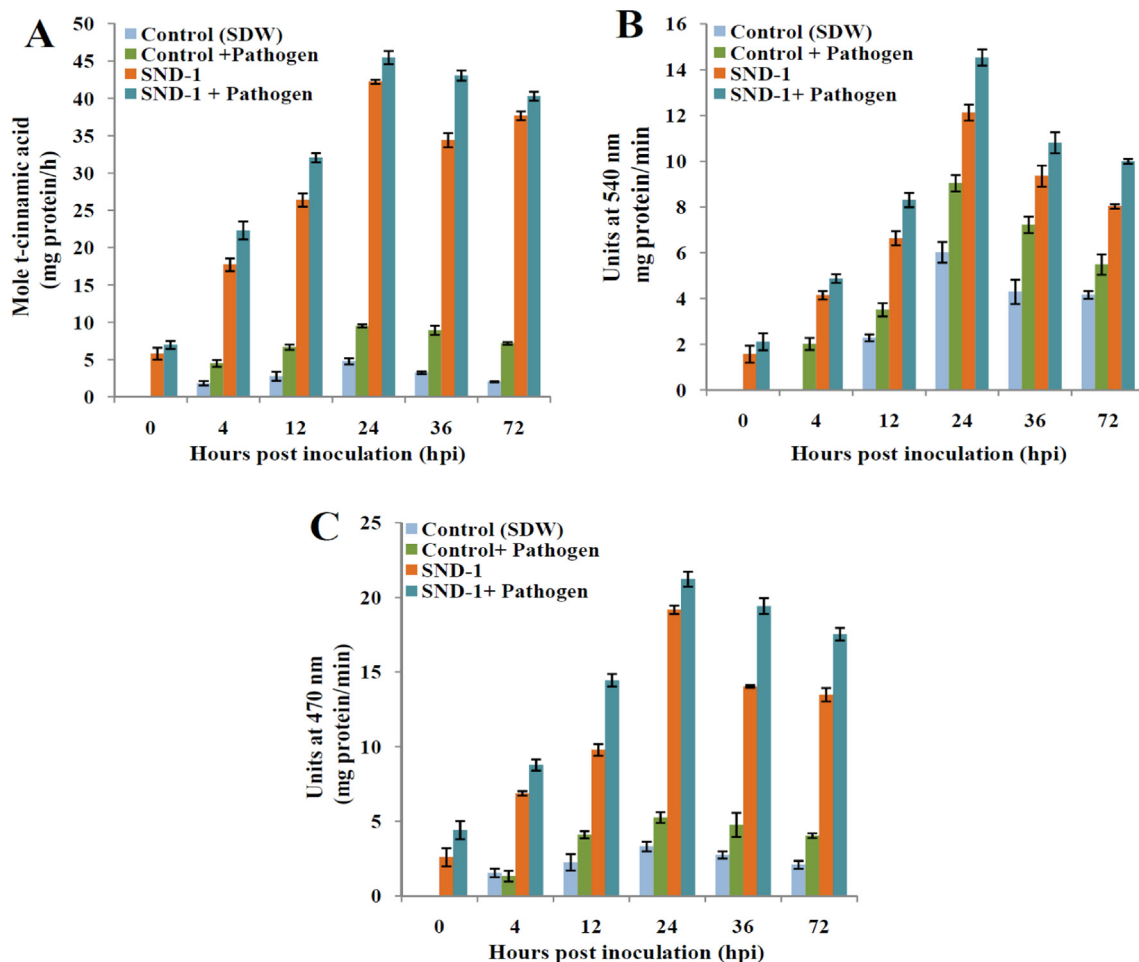


Fig. 6. Temporal expression of antioxidant enzymes (biochemical defense) in SND-1 formulation treated mung bean plants: (A) Phenylalanine ammonia-lyase (PAL), (B) β 1, 3-glucanase (GLU) and (C) Peroxidase (POX).

pathogen was utilized for extended biocontrol efficacy in the sodium alginate pellet formulation. The prepared SND-1 formulation showed clear distribution of spores, purity and viability test depicted no contamination and also a negligible decrease in the viability of spores up to six months of storage period. Similar study on *Streptomyces rochei* PTL2 alginate formulation displayed reduction from 2×10^6 CFU g^{-1} to 1.7×10^6 CFU g^{-1} of viability of spores at 2 months intervals up to one year of storage period (Miyada et al., 2017).

The greenhouse study resulted significant increase in growth parameters of mung bean and minimum disease severity in SND-1 formulation challenged with *C. canescens* pathogen when compared with untreated plants. The results were compared with the studies, where alginate formulation of *Streptomyces* sp. Di-944 and *Streptomyces palmae* CMU-AB204^T exhibited significant biocontrol activity up to 94% against *Rhizoctonia solani* and 96 to 97% against *Ganoderma boninense* infections, and increased the growth in tomato and palm plants up to 30 to 50 cm in comparison with the control and pathogen treatments (Sabarathanam et al., 2002; Kanaporn et al., 2020).

The SND-1 formulation challenged with pathogen significantly enhanced the histochemical defense markers such as lignin, H_2O_2 and phenol compared with other treatments. Milan et al. (2021) reported that increased depositions of lignin and H_2O_2 in leaf epidermis of the pathogen *P. viticola* infected *Vitis vinifera* L. plants treated with talcum formulation of *Trichoderma harzianum* and showed potentially up to 82.9% protection from the downy mildew

disease. In their study, the histochemical depositions in the *Trichoderma harzianum* treated *Vitis vinifera* L. plants exhibited gradual increase at early hours (0, 4, 12 and 24 h) of the of the pathogen infection and decreased at later time intervals (at 36, 48 and 72 h). Similarly, the rhizobacteria treated *Vigna radiata* (L.) R. Wilczek plants effectively conferred the *M. phaseolina* infection through instigating maximum histochemical depositions (Boubakri et al., 2012; Javed et al., 2021). Lignin, H_2O_2 and phenol accumulations can acts as mechanical barrier, signal molecule, and synthesis of specific substrates that alters modifications of defense enzymes that decrease the diffusion of toxins by pathogen. Lignin (aromatic polymer) deposition increases the strength and imperviousness, perturbations and high level of lignin biosynthesis induce phenylpropanoid pathway and the corresponding defense related enzymes results in controlling the invasion of the pathogen. The H_2O_2 acts as stress tolerance and higher concentrations leads to oxidation of cell components such as lipids, proteins and nucleic acids which shows enhancement in defense state in pathogen affected cells (Milan et al., 2021). Another function of H_2O_2 in plant defense is it involves in the mechanism like superoxide dismutation (SOD) and it's higher accumulation leads to the increased and regeneration of enzymatic activity such as catalase and Glutathione reductase (GSH reductase) which significantly involved in the upregulation of antioxidant and oxidation reactions during the pathogen suppression. Similarly phenol accumulations at pathogen infected sites slowdown the pathogen proliferation through the mechanisms like synthesis of stress related simple

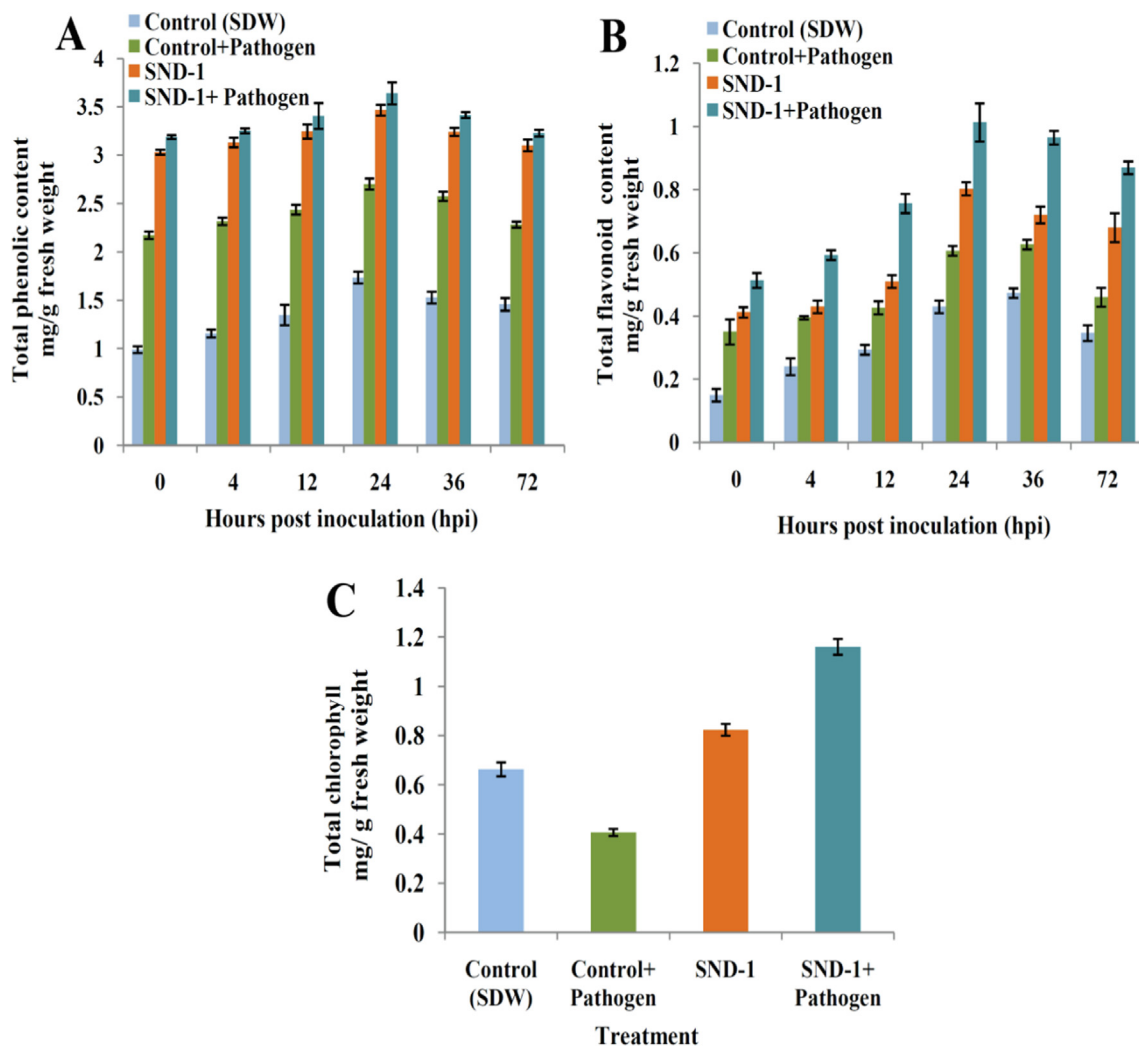


Fig. 7. Histogram representing (A) Total phenolic content, (B) Total flavonoid content and (C) Total chlorophyll contents in treated mung bean plants.

phenols, phytoalexins and hydroxycinnamic acids etc. (Lee et al., 2019).

The upregulation of defense enzymes in SND-1 formulation with the inoculation of *Cercospora* pathogen exhibited maximum induced protection by the associated PAL, POX and GLU antioxidant enzyme activities. The similar study reported the enhanced activity of PAL, GLU and POX in *T. virens*-Tv4 treated mung bean plants exhibited maximum protection against *R. solani* (Alfi et al., 2020). The accelerated activity of these defense enzymes can counteract the pathogen at the time of infection and plays a significant role in induction of resistance patterns in plants through the biosynthesis of cell wall thickenings such as lignin, H_2O_2 and phenol which play vital role as defense barriers. PAL initiates primary enzyme metabolism (phenylpropanoids) which leads to increased synthesis of defense related phenols and lignins. The GLU is the polysaccharide that counteracts the pathogen cell wall and hydrolyzes the chitin a major component of the fungal cell wall (Javed et al., 2021). POX is the oxidoreductive enzyme which directly correlates with the suppression of pathogen through cell wall cross-linking, phytoalexin synthesis, suberization, auxin metabolism and phenol oxidation (Manjula et al., 2015).

The PAL, GLU and POX acts as key enzymes that regulates synthesis of phenolic compounds, flavonoids, and hydrocinnamates also plays a significant role in lignin synthesis, creates oxidative stress, and ROS during pathogen infection and significant impact

on plant physiology and expression of defense genes (Solekha et al., 2019; Rudrappa et al., 2022; Hammerschmidt et al., 1982). These enzymes play a vital in biochemical defense mechanism through increasing the cells wall deposition by activating the genes related to synthesis of lignin, H_2O_2 , callose, phenol and suberin. A maximum phenolic content in the plants treated with SND-1 formulation and pathogen in comparison with other treatments. In previous report by Alfi et al. (2020) and Umar et al. (2019), where significant defense response was observed through maximum accumulation of phenolic content after *R. solani* and MYMV pathogen infection in mung bean plants. Increased phenolic content correlated with resistance and also proper functioning of plant tissues (Sidra et al., 2017). The several studies reported that antifungal action of different flavonoids against phytopathogens such as *F. oxysporum*, *S. sclerotium*, *A. flavus* and *R. solani* (Morkunas et al., 2005; Katsumata et al., 2018). The accelerated phenolic and flavonoid content significantly involves in the synthesis of esters, lignin, tannins and flavonoids are chelating molecules of metal stresses (Ullah et al., 2017).

The maximum chlorophyll content in plants treated with SND-1 bioformulation and challenged with *Cercospora* pathogen showed the survival and adaptation of mung bean plants in stressful (Pathogen infestation) conditions. However, the exogenous application of bioformulations can induce the accelerated activity of physiological conditions (Enzymatic levels) that strengthen the

synthesis of chlorophyll in leaves (Li et al., 2018). Djebaili et al. (2021) reported there was a significant increase of chlorophyll content in *Triticum durum* by the application of different actinomycetes formulations. As the chlorophyll increases, the plants get proper photosynthesis process and high survival capability during the biotic stresses (Umar et al., 2019).

5. Conclusion

In the present study, the application of *Amycolatopsis* sp. SND-1 alginate bioformulation significantly triggered the growth patterns and histochemical and biochemical defense patterns in the *Cercospora* infected in mung bean plants. Further, the induction of systemic resistance study unveiled the involvement of SND-1 formulation in the acceleration of histochemical depositions such as lignin and H₂O₂ and phenol with decreased cell death and biochemical defense-related enzymatic levels (PAL, GLU, and POX) that resulted in significant defense patterns in mung bean plants. An increased phenolic, flavonoid, and chlorophyll content in SND-1 formulation treated mung bean plants showed the higher levels of the physiological and survival potential. Finally, we concluded that *Amycolatopsis* sp. SND-1 formulation has the ability to counteract the *Cercospora* pathogen stress in mung bean plants through the enhancement of cellular and enzymatic defense processes that render significant adaptability under pathogen stress in mung bean plants.

Funding

This research was funded by Researchers Supporting Project (Project Number- RSP2023R231), King Saud University, Riyadh, Saudi Arabia.

Availability of data and materials

The datasets presented made available from the corresponding author upon reasonable request.

CRedit authorship contribution statement

Dhanyakumara Shivapoojar Basavarajappa: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Raju Suresh Kumar:** Software, Writing – review & editing. **Muthuraj Rudrappa:** Methodology, Writing – review & editing. **Halaswamy Hiremath:** Methodology, Writing – review & editing. **Abdulrahman I. Almansour:** Funding acquisition, Writing – review & editing. **Karthikeyan Perumal:** Writing – review & editing. **Gireesh Babu Kantli:** Writing – review & editing. **Sreenivasa Nayaka:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors grateful to the P. G. Department of Studies in Botany for granting access to laboratory and instrumentation facility. The University Scientific Instrumentation Centre (USIC) and Sophisticated Analytical Instrumentation Facility (SAIF), Karnatak University, Dharwad, Karnataka, India for providing instrumentation facilities. The project was funded by Researcher Supporting

Project number (RSP2023R231) King Saud University, Riyadh, Saudi Arabia.

References

- Akram, S., Parisa, K., Hossein, A., Ebrahim, K., Ali, R.A., 2017. Plant growth promotion and suppression of *Phytophthora drechsleri* damping-off in cucumber by cellulase-producing *Streptomyces*. *Bio. Control.* 62, 805–819. <https://doi.org/10.1007/s10526-017-9838-4>.
- Alfi, I., Liliek, S., Luqman, Q.A., Eriyanto, Y., 2020. *Trichoderma virens*-Tv4 enhances growth promoter and plant defense-related enzymes of mungbean (*Vigna radiata*) against soil-borne pathogen *Rhizoctonia solani*. *Biodivers.* 21, 2410–2419. <https://doi.org/10.13057/biodiv/d210611>.
- Aristide, D., Martial, T.T.P., Ruth, N.N.E., Grace, L.B., Ebenezer, F.T., Flore, M.P.T., Thaddee, B., 2022. Effects of a powder formulation of *Streptomyces cameroonensis* on growth and resistance of two cocoa hybrids from cameroon against *Phytophthora megakarya* (Causal Agent of Black Pod Disease). *J. Microbiol. Biotechnol.* 32, 160–169. <https://doi.org/10.4014/jmb.2110.10006>.
- Basavarajappa, D.S., Kumar, R.S., Nayaka, S., 2023. Formulation-based antagonistic endophyte *Amycolatopsis* sp. SND-1 triggers defense response in *Vigna radiata* (L.) R. Wilczek. (Mung bean) against *Cercospora* leaf spot disease. *Arch. Microbiol.* 205 (77). <https://doi.org/10.1007/s00203-023-03419-w>.
- Bhat, M.P., Nayaka, S., Kumar, R.S., 2022. A swamp forest *Streptomyces* sp. strain KF15 with broad spectrum antifungal activity against chilli pathogens exhibits anticancer activity on HeLa cells. *Arch. Microbiol.* 204, 540. <https://doi.org/10.1007/s00203-022-03147-7>.
- Boubakri, H., Wahab, M.A., Chong, J., Bertsch, C., Mliki, A., Soustre, G.I., 2012. Thiamine induced resistance to *Plasmopara viticola* in grapevine and elicited host–defense responses, including HR like-cell death. *Plant. Physiol. Biochem.* 57, 120–133. <https://doi.org/10.1016/j.plaphy.2012.05.016>.
- Chakraborty, B., Kumar, R.S., Almansour, A.I., Gunasekaran, P., Nayaka, S., 2022. Bioprospection and secondary metabolites profiling of marine *Streptomyces levis* strain KS46. *Saudi. J. Biol. Sci.* 29, 667–679. <https://doi.org/10.1016/j.sjbs.2021.11.055>.
- Churasia, A., Meena, B.R., Tripathi, A.N., Pandey, K.K., Rai, A.B., Singh, B., 2018. Actinomycetes: an unexplored microorganisms for plant growth promotion and biocontrol in vegetable crops. *World. J. Microbiol. Biotechnol.* 34, 132. <https://doi.org/10.1007/s11274-018-2517-5>.
- Djebaili, R., Marika, P., Massimiliano, R., Cinzia, F., Maria, S., Maddalena, D.G., Mahmoud, K., 2021. Characterization of plant growth-promoting traits and inoculation effects on *Triticum durum* of actinomycetes isolates under salt stress conditions. *Soil. Syst.* 5, 2–26. <https://doi.org/10.3390/soilsystems5020026>.
- El-Tarabily, K.A., Nassar, A.H., Hardy, G.J., Sivasithamparan, K., 2009. Plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber, by endophytic actinomycetes. *J. Appl. Microbiol.* 106, 13–26. <https://doi.org/10.1111/j.1365-2672.2008.03926.x>.
- Gopalakrishnan, S., Srinivas, V., Naresh, N., Alekhya, G., Sharma, R., 2019. Exploiting plant growth-promoting *Amycolatopsis* sp. for bio-control of charcoal rot of sorghum (*Sorghum bicolor* L.) caused by *Macrophomina phaseolina* (Tassi) Goid. *Archiv. Phytopathol. Plant. Protec.* 52, 543–559. <https://doi.org/10.1080/03235408.2018.1553472>.
- Hammerschmidt, R., Nuckles, E.M., Kuc, J., 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber of *Colletotrichum lagenarium*. *Physiol. Plantarum Pathol.* 20, 73–82.
- Itoh, T., Garcia, R.N., Adachi, M., Maruyama, Y., Tecson-Mendoza, E.M., Mikami, B., 2006. Structure of 8S α globulin, the major seed storage protein of mung bean. *Acta. Crystallogr. D. Biol. Crystallogr.* 62, 824–832. <https://doi.org/10.1107/S090744490601804X>.
- Javed, S., Javaid, A., Hanif, U., Bahadur, S., Sultana, S., Shuaib, M., Ali, S., 2021. Effect of necrotrophic fungus and GPGR on the comparative histochemistry of *Vigna radiata* by using multiple microscopic techniques. *Microsc. Res. Tech.* 84, 2737–2748. <https://doi.org/10.1002/jemt.23836>.
- Kanaporn, S., Wasu, P., Mihoko, M., Kazuyuki, D., Kazuro, S., Saisamorn, L., 2020. *Streptomyces palmae* CMU-AB204T, an antifungal producing-actinomycete, as a potential biocontrol agent to protect palm oil producing trees from basal stem rot disease fungus, *Ganoderma boninense*. *Biol. Control.* 148, 1–12. <https://doi.org/10.1016/j.biocontrol.2020.104307>.
- Katsumata, S., Hiroaki, T., Morifumi, H., 2018. Xylosylated Detoxification of the rice flavonoid phytoalexin sakuranetin by the rice sheath blight fungus *Rhizoctonia solani*. *Molecules* 23, 276. <https://doi.org/10.3390/molecules23020276>.
- Lee, M.H., Jeon, H.S., Kim, S.H., Chung, J.H., Roppolo, D., Lee, H.J., Cho, H.J., Tobimatsu, Y., Ralph, J., Park, O.K., 2019. Lignin-based barrier restricts pathogens to the infection site and confers resistance in plants. *EMBO J.* 2 (23). <https://doi.org/10.15252/emboj.2019101948>.
- Li, Y., He, N., Hou, J., Xu, L., Liu, C., Zhang, J., Wang, Q., Zhang, X., Wu, X., 2018. Factors influencing leaf chlorophyll content in natural forests at the biome scale. *Front. Ecol. Evol.* 6 (64). <https://doi.org/10.3389/fevo.2018.00064>.
- Manjula, S., Murali, M., Shivamurthy, G.R., Amruthesh, K.N., 2015. Non-specific lipid transfer proteins (ns-LTPs) from maize induce resistance in pearl millet against downy mildew disease. *Phytoparasitica* 43, 437–447. <https://doi.org/10.1007/s12600-014-0446-x>.
- Milan, V.K., Shreya, M.J., Shiva, H., Sudisha, J., 2021. Biopriming with rhizosphere *Trichoderma harzianum* elicit protection against grapevine downy mildew

- disease by triggering histopathological and biochemical defense responses. *Rhizosphere*. <https://doi.org/10.1016/j.rhisph.2021.100398>.
- Miyada, Z., Yacine, G., Nasseridine, S., Florence, M., Abdelghani, Z., 2017. Development of formulations based on *Streptomyces rochei* strain PTL2 spores for biocontrol of *Rhizoctonia solani* damping-off of tomato seedlings. *Biocontrol. Sci. Tech.* 27, 723–738. <https://doi.org/10.1080/09583157.2017.1334257>.
- Morkunas, I., Marczak, L., Stachowick, J.S., 2005. Sucrose-induced lupine defense against *Fusarium oxysporum*: Sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to *Fusarium oxysporum*. *Plant. Physiol. Biochem.* 43, 363–373. <https://doi.org/10.1016/j.plaphy.2005.02.011>.
- Nair, R.M., Pandey, A.K., War, A.R., Hanumantharao, B., Shwetha, T., Alam, A., Pratap, A., Malik, S.R., Karimi, R., Mbeyagala, E.K., Douglas, C.A., Rane, J., Schafleitner, R., 2019. Biotic and abiotic constraints in mungbean production progress in genetic improvement. *Front. Plant. Sci.* 10, 1340. <https://doi.org/10.3389/fpls.2019.01340>.
- Natale, S., Rocca, N.L., Battistuzzi, M., Morosinotto, T., Nardini, A., Alboresi, A., 2023. Structure and function of bark and wood chloroplasts in a drought-tolerant tree (*Fraxinus ornus* L.). *Tree. Physiol.* 1–16. <https://doi.org/10.1093/treephys/tpad013>.
- Onuma, K., Christopher, M.M.F., 2021. *Amycolatopsis pittospori* sp. nov., an endophytic actinobacterium isolated from native apricot tree and genome mining revealed the biosynthesis potential as antibiotic producer and plant growth promoter. *Anton. Van. Leeuwenh.* 114, 365–377. <https://doi.org/10.1007/s10482-021-01519-5>.
- Pratap, A., Gupta, S., Basu, S., Tomar, R., Dubey, S., Rathore, M., Shankar, U.P., Singh, P., Kumari, G., 2019. Towards development of climate-smart mungbean: challenges and opportunities. In: Kole, C. (Ed.), *Genomic Designing of Climate Smart Pulse Crops*. Springer Nature, New York.
- Rafiq, H., Z-e-H. A., Anjum, T., Ali, B., Akram, W., Bashir, U., Mirza, F.S., Aftab, M., Ali, M.D., Li, G., 2022. Bio-fabrication of Zinc Oxide nanoparticles to rescue Mung Bean against *Cercospora* leaf spot disease. *Front. Plant. Sci.* 13, 1052984. <https://doi.org/10.3389/fpls.2022.1052984>.
- Rudrappa, M., Kumar, S., Kumar, R.S., Almansour, A.I., Perumal, K., Nayaka, S., 2022. Bioproduction, purification and physicochemical characterization of melanin from *Streptomyces* sp. strain MR28. *Microbiol. Res.* 263. <https://doi.org/10.1016/j.micres.2022.127130>.
- Sabarathanam, S., James, A., Traquair., 2015. Mechanism of antagonism by *Streptomyces griseocarneus* (strain D1944) against fungal pathogens of greenhouse-grown tomato transplants. *Can. J. Plant. Pathol.* 37, 197–211. <https://doi.org/10.1080/07060661.2015.1039062>.
- Sabarathanam, S., James, A., Traquair., 2002. Formulation of a *Streptomyces* biocontrol agent for the suppression of rhizoctonia damping-off in tomato transplants. *Biol. Control.* 23, 245–253. <https://doi.org/10.1006/bcon.2001.1014>.
- Sangeetha, M., Gangopadhyay, S., Jagdish, P., Panwar, P.K., 2018. Screening of mung bean varieties for resistant against *Macrophomina phaseolina* causing dry root rot. *Int. J. Trop. Agri.* 36, 147–149. <https://serialsjournals.com/abstract/1>.
- Sidra, H., Khalid, P.A., Amjad, H., Tehsen, G., Shumaila, K., Sumaira, Y., Ghulam, A., Muhammad, J.A., Nighat, S., 2017. Biochemical changes in the leaves of mungbean (*Vigna radiata*) plants infected by phytoplasma. *Turk. J. Biochem.* 42, 591–599. <https://doi.org/10.1515/tjb-2016-0304>.
- Singh, Y., Nair, A.M., Verma, P.K., 2021. Surviving the odds: From perception to survival of fungal phytopathogens under host-generated oxidative burst. *Plant. Comm.* 2, 1–17. <https://doi.org/10.1016/j.xplc.2021.100142>.
- Solekha, R., Susanto, F.A., Joko, T., Nuringtyas, T.R., Purwestri, Y.A., 2019. Phenylalanine ammonia-lyase (PAL) contributes to the resistance of black rice against *Xanthomonas oryzae* pv. *oryzae*. *J. Plant. Pathol.* 102, 359–365. <https://doi.org/10.1007/s42161-019-00426-z>.
- Sreenivasa, N., Bhat, M.P., Pallavi, S.S., Chakraborty, B., Airodagi, D., Rudrappa, M., Nagaraja, S.K., Hiremath, H., Basavarajappa, D.S., Vaishnavi, M.D., 2020. Biogenic synthesis of silver nanoparticles using *Paenibacillus* sp. in-vitro and their antibacterial, anticancer activity assessment against human colon tumour cell line. *J. Environ. Biol.* 42, 118–127. <https://doi.org/10.22438/jeb/42/1/MRN-1401>.
- Sudisha, J., Praveen, S., Savitha, D.B., Narasimhamurthy, K., Udayashankar, A.C., 2020. Exogenous priming of chitosan induces upregulation of phytohormones and resistance against cucumber powdery mildew disease is correlated with localized biosynthesis of defense enzymes. *Int. J. Biol. Macromol.* 162, 1825–1838. <https://doi.org/10.1016/j.ijbiomac.2020.08.124>.
- Ting, X., Yan, L., Xiadong., 2017. Isolation and evaluation of endophytic *Streptomyces endus* OsiSh-2 with potential application for biocontrol of rice blast disease. *J. Sci. Food. Agri.* 97, 1149–1157. <http://www.corc.org.cn/handle/1471x/6043083>.
- Ullah, C., Unsicker, S.B., Fellenberg, C., Constabel, P., Schmidt, A., Gershenzon, J., Hammerbacher, A., 2017. Flavan-3-ols are an effective chemical defense against rust infection. *Plant. Physiol.* 175, 1560–1578. <https://academic.oup.com/plphys/article/175/4/1560/6116803>.
- Umar, U.D., Din, S.B., Khan, M.F., Rehman, A., S. A. H., Naqvi, M.A., Zulfiqar, A.A., Khan N., Ilyas., 2019. Enhancing resistance level against Mungbean Yellow Mosaic virus by inducing defense related enzymes in Mung bean. *Pakistan. J. Agric. Res.* 32, 241–251. <https://doi.org/10.17582/journal.pjar/2019/32.2.241.251>.
- Vernon, L.S., Rudolf, O., Rosa, M.L.R., 1993. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Am. J. Enol. Vitic.* 25, 119.