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BIOCONTROL POTENTIAL OF BACTERIAL ISOLATES AGAINST SOIL BORNE PHYTOPATHOGEN *Sclerotinia sclerotiorum* (LIB.) DE BARY, CAUSAL AGENT OF STEM ROT IN INDIAN MUSTARD

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ABSTRACT

Stem rot caused by *Sclerotinia sclerotiorum*, poses a serious threat to *Brassica juncea* (Indian mustard), leading to significant yield losses. The non-availability of genetic resources offering resistance, necessitates the use of fungicides as a method of control. Hence, it becomes imperative to explore effective and eco-friendly biocontrol methods. In this context, we screened fifty-five bacterial isolates, finding *Pseudomonas aeruginosa* and *Serratia proteamaculans* with the potential to inhibit mycelial growth of *S. sclerotiorum* by 42.5% and 44.5%, respectively. Further, the colonization and endophytic nature of both isolates were confirmed by the presence of rod-shaped structures in the roots of ten-day-old seedlings of bacterial treated *B. juncea* through scanning electron microscopic (SEM) studies. In conclusion, our study highlights the biocontrol potential of *Pseudomonas aeruginosa* and *Serratia proteamaculans* against stem rot in Indian mustard. Future prospects include elucidating their mechanisms of action, optimizing application methods, and scaling up production for field use.

Keywords: Antagonism, Brassica, Endophytes, SEM, Stem rot

Brassica juncea (Indian mustard) is a major oilseed crop contributing 90% of total acreage in India. (Sandhu *et al.,* 2023). With a productivity of 1980 kg/ha, the rapeseed-mustard crop spans 36.59 million hectares and yields 72.37 million tonnes of output (Choudhary *et al.,* 2024). As a versatile member of the Brassicaceae family (Cruciferae), it serves various purposes including its use as vegetable, flavouring agents, livestock feed, and as a source of edible oil. However, the survival, growth, and productivity of *B. juncea* are threatened by a number of biotic stress factors such as powdery mildew, downy mildew, white rust, Alternaria leaf blight, and Sclerotinia stem rot. Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, has become more prevalent in mustard-growing regions of India such as Rajasthan, Haryana, Punjab, Uttar Pradesh and other states (Gupta *et al.,* 2020).

Sclerotinia sclerotiorum is a fungus that lives mostly in the soil with wide host range. Its infestation in crop showed serious yield reductions up to 80% in regions of Punjab and Haryana (Kang and Chahal, 2000). The pathogen's sclerotia, exhibit remarkable longevity which can persist in the soil for years (Wang *et al.,* 2024). Over 400 host species have been reported to get infected as a result of this prolonged viability (Boland and Hall, 1994). Mycelium and apothecia emerge from the germination of sclerotia. Later, the apothecia grow and mature, releasing asci with ascospores that can spread through the air. Upon contact with host tissue, these ascospores germinate, producing hyphae that infect the lower stem and roots. All above-ground plant parts are susceptible to attack, but the stem being most often affected. Once the stem is completely covered in a white mycelial growth, it begins to wilt and desiccate. According to a study by Gill *et al.* (2021), the pathogenic aggressiveness of *S. sclerotiorum isolates on B. juncea* has been correlated with their oxalic acid production. Controlling Sclerotinia ascospores has proven difficult due to their ability to disperse over large distances (Johnson and Atallah, 2014).

Numerous methods such as utilization of organic soil additives (Asirifi *et al.,* 1994), soil sterilization (Lynch and Ebben, 1986), tillage and irrigation (Bell *et al.,* 1998) as well as zero tillage and crop rotation (Duncan, 2003; Yexin *et al.,* 2011) have been employed for the control of *S. sclerotiorum.* However, these methods fall short due to their lengthy time frame to achieve efficient management and their inability to deliver a total pathogen control. The development of genetically resistant cultivars offers a sustainable, economical, and environmentally beneficial approach to disease control (Li *et al.,* 2006). However, no crop cultivars that are commercially available exhibit complete resistance, primarily because breeding for resistance to Sclerotinia stem rot is a difficult task

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because the pathogen is polygenic (Wang *et al.,* 2019). Furthermore, the presence of virulent pathotypes has hindered the development of complete host resistance (Barbetti *et al.,* 2014).

One successful approach for controlling plant diseases is the application of chemicals, notably fungicides, have proven effective in reducing stem rot in rapeseed-mustard crop. These artificial substances can destroy fungus by interfering with their vital biological functions. Fungicide application has shown a significant decrease in stem rot disease incidence in Brassica (Derbyshire and Dento-Giles, 2016). However, the widespread use of fungicides has raised concerns in agriculture due to potential toxicity to non-target organisms and adverse environmental impacts (Gikas *et al.,* 2022). Therefore, sustainable approaches are needed to mitigate the disease while minimizing harm to ecosystems and human health.

There has been growing attention towards the role of endophytic bacteria in strengthening plant defence mechanisms against pathogenic diseases. Microorganisms known as endophytes reside within healthy plants in a mutually beneficial symbiotic relationship, aid in disease resistance through the production of secondary metabolites and antibiotics, while also influencing plant growth by stimulating growth hormones. On the other hand, these endophytic bacteria thrive within the plants, where they receive resources they need to flourish. Due to their ability to prevent ascospore and sclerotia germination, these naturally occurring microorganisms have been shown to significantly reduce disease risk (Fernando *et al.,* 2007). Furthermore, these endophytes help the plant to resist a variety of insects-pests, and other harmful environmental variables (Durvasula *et al.,* 1997; Ramamoorthy *et al.,* 2001).The present study aims to screen the inhibitory action of various bacterial strains against *S. sclerotiorum,* alongside investigating their endophytic nature.

MATERIALS AND METHODS

Procurement and revival of bacterial isolates

Forty-one endophytic bacteria isolated from the different parts (roots, stem and leaves) of Indian mustard were procured from the culture repository of the Department of Microbiology, Punjab Agricultural University, Ludhiana while fourteen isolates were sourced from the Division of Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi. These bacterial isolates have been screened for plant growth-promoting activities in the earlier studies (unpublished data) and were revived on nutrient agar (comprising peptone B at 1.5g/L, sodium chloride at 5g/L, yeast extract at 1.5g/L, peptone at 5g/L, and agar at 15g/L) through the process of quadrate streaking using a flame- sterilized inoculation loop, followed by incubation at 28°C for a period of 48 h as described by Pelczar and Reid (1958).

Isolation and purification of S. sclerotiorum

The sclerotia of *S. sclerotiorum* were collected from the severely diseased mustard plants grown in experimental fields of PAU, Ludhiana, India. Surface sterilization of the sclerotium was performed by treating it with 0.1% (v/v) mercuric chloride ($HgCl_2$) for 15 seconds, followed by its subsequent washing thrice with sterile distilled water. Excess moisture was soaked using autoclaved filter paper. The sclerotium was then bisected and cut side was exposed onto the plates containing potato dextrose agar (PDA) (composed of potatoes 200 g/L, dextrose 20 g/L, agar 15g/L).The Petri plates were sealed and incubated at 25±1ºC for 3 to 4 days. The resulting mycelial plug was excised from the culture and transferred onto a fresh PDA plates and incubated again at 25±1ºC for 3 to 4 days. The cultured material thus obtained was preserved for future use.

Antagonism on solid medium by dual plate assay

Under *in vitro* conditions, the ability of bacterial endophytes to antagonize the soil-borne plant pathogenic fungi, *S. sclerotiorum*, was assessed using a dual plate culture method. The fungus was grown and maintained as a pure culture on potato dextrose agar, whereas the endophytic bacteria were cultured on nutrient agar at 28°C for 48 hours. A 5 mm thick mycelial disk from the fungus culture was placed at one end of the yeast malt extract agar plate, whereas the bacterial isolate was streaked onto the opposite end of the same plate. The yeast malt extract agar composition included yeast extract at 3g/L, dextrose at 10g/L, peptone at 5g/L, malt extract at 3g/L, and agar agar at 20g/L. Control plates comprised of the fungal pathogen, with no bacterial culture added. The plates were further incubated at 28°C for a period of 4 days. The percentage of reduction in fungal growth was calculated using the formula:

Reduction in fungal growth (%) = [(control – treated)/ control] × 100

where, control represents the diameter of fungal pathogen colony in plates lacking bacteria, and treated represents the diameter of fungal pathogen colony in plates inoculated with bacterial culture.

Preparation of bacterial inoculum

Bacterial inoculations were carried out by transferring 500 µl of bacterial cultures, previously grown at 28°C in nutrient broth for 24 hours, into 100 ml of fresh nutrient broth. The nutrient broth consisted

of glucose at 1g/L, peptone at 15g/L, sodium chloride at 6g/L, and yeast extract at 3g/L. These cultures were then incubated at 28°C and with shaking at 180 rpm for 48 hours. After incubation, the cultures were used as inoculum, and their optical density at 600nm was adjusted to 1.0, corresponding to approximately 10^9 colony forming units (CFU) per ml.

Preparation of charcoal-based bioinoculant

The charcoal was sterilized by subjecting it to a temperature of 121°C for 1 h on two alternate days. The bacterial inoculum was mixed with sterilized charcoal in a 1:2 ratio (20 ml inoculum and 40 g of sterilized charcoal) to achieve a concentration of approximately 10^7 cfu per gram of charocoal-based bio inoculant.

Seed treatment

The seeds of *B. juncea* variety PBR 357 were surface sterilized by immersing them in a 20% sodium hypochlorite solution for 3 minutes, followed by rinsing three times with sterile de-ionized water. The slurry containing the charcoal-based inoculum was mixed with seeds and then dried in the shade for half an hour, with a concentration of 25 g per kg of seeds during sowing.

Confirmation of bacterial endophyte colonization in roots of B. juncea plants

The assessment of bacterial endophyte colonization within the roots of *B. juncea* seedlings was conducted through Scanning Electron Microscopy (SEM) facility available at Sophisticated Analytical Instrumentation Facility, Panjab University, Chandigarh, India. Sterilized seeds of PBR 357 treated with a bacterized charcoal-based slurry were sown in sterilized vermiculite in 12-inch pots. Seedlings from both groups (with and without bacterial treatment) were uprooted after 10 days. Subsequently, root samples were collected and washed with sterile water. To study the colonization, root tissues were dissected into small segments, approximately 1 centimeter in length, using a sterile razor blade. These segments were then fixed in a 2.5% glutaraldehyde solution at a temperature of 4°C followed by removal of fixative after 24 hours and washing the samples thrice for 15 minutes each, with a 0.1 M sodium cacodylate buffer (pH 7.2). Subsequently, 1% osmium tetroxide (OsO_4) was added and kept for 1-2 hours at 4°C. Incubation was followed by draining of OsO_4 and subsequent washing the samples with 0.1 M sodium cacodylate solution for 15 minutes. Dehydration of the samples was achieved through a series of ethanol washes, ranging from 30% to 100% concentration, followed by drying in vaccum dessicator. The samples were then coated with gold particles in accordance with established protocol guidelines, and their morphological changes were examined at various magnifications using scanning electron microscopy (SEM) (Singh *et al.,* 2014).

Statistical Analysis

The experiment was conducted in triplicates and the data from the *in vitro* growth inhibition was analyzed statistically ($P < 0.05$) on the basis of mean values with one factor analysis and Tukey's Test using OPSTAT software.

RESULTS AND DISCUSSION

Antagonism on solid medium by dual plate assay

In the present study, fifty-five endophytic bacterial isolates were screened for their antagonistic activity against *S. sclerotiorum* using dual culture plate assay on yeast malt extract agar medium. Such assays have been widely used in various research studies to assess the antifungal potential of different endophytes against range of fungal pathogens (Helmy, 2016; Sun *et al.,* 2017; Vinodkumar *et al.,* 2017; Ribeiro *et al.,* 2021; Vyas and Singh, 2024). Out of fifty-five bacterial isolates tested, two isolates namely *Pseudomonas aeruginosa* (Accession no. OL413676) and *Serratia proteamaculans* (Accession no. OP595540) obtained from the Division of Microbiology, IARI, New Delhi and Department of Microbiology, PAU, Ludhiana, respectively, exhibited a significant growth inhibition of 42.5% and 44.5%, respectively against *S. sclerotiorum* (Table1, Fig. 1). The presence of clear inhibition zone between the bacterial culture and actively growing fungus further confirms the antagonistic activity of these isolates.

The bacterial strains displayed consistent antagonistic behaviour across repeated tests, leading to a significant reduction in fungal growth. The mean fungal diameter measurements at 4.4 cm for *S.*

Table 1. Mycelial diameter of *Sclerotinia sclerotiorum* after 4 days incubation at 28ºC

Bacterial isolate	Fungal mycelial diameter (cm)	Inhibition (%)
Pseudomonas aeruginosa	4.6±0.05[a]	42.5±0.72[a]
Serratia proteamaculans	4.4±0.12[a]	44.5±1.50[a]
Control	8.5±0[b]	0.00±0.00[b]

Data represent mean ± standard error of three replicates. Superscript letters within the same column indicate no statistical difference based on Tukey's test at $P < 0.05$

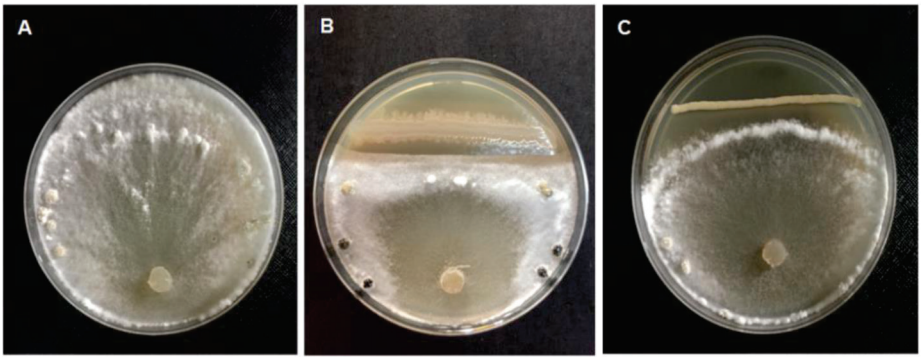

Fig. 1. Antagonism assessment of bacterial isolates against *Sclerotinia sclerotiorum* on yeast malt extract agar. (A) Control without bacteria; (B) *Pseudomonas aeruginosa* and (C) *Serratia proteamaculans* after 4 days of incubation at 28ºC

proteamaculans and 4.6 cm for *P. aeruginosa* (Table 1) further support this observation with *P. aeruginosa* and *S. proteamaculans* showing notable inhibitory effects. Bacteria belonging to both genera have been proven effective in combating Sclerotinia in oilseed rape in previous studies by Li *et al* (2011), Smolinska and Kowalska (2018), Schmidt *et al* (2021). An isolate of *P. aeruginosa* effectively suppressed the growth of *S. sclerotiorum* by substantially hindering the mycelial growth and sclerotia formation (Gupta *et al.,* 2006). This suppression is likely attributed to the production of secondary metabolites by these bacteria. Pseudomonas species are known to produce a variety of metabolites such as pyrrolnitrin, lipopeptides, volatile organic compounds (VOCs), including hydrogen cyanide (HCN), 1-undecene, 1-decene and tridecane (Montes-Osuna *et al.,* 2022, Pellicciaro *et al.,* 2022, Raio *et al.,* 2024). On the other hand, VOCs including dimethyl disulfide and 2-heptanone produced by *S. proteamaculans* is suggested to contribute the fungistatic effect (Popova *et al.,* 2014). The diversity of these molecules indicates the presence of varied molecular mechanisms responsible for the inhibition of pathogen growth. Consequently, this emphasizes the possibility of using these bacterial isolates as natural alternatives to chemical fungicides for disease management, thereby reducing the reliance on synthetic chemicals in agriculture. These findings highlight the efficacy of diverse bacterial isolates, particularly *P. aeruginosa* and *S. proteamaculans,* in suppressing the growth of *S. sclerotiorum*. Moreover, the utilization of dual plate assays has been a valuable tool in assessing antagonistic properties of these microorganisms, emphasizing its importance in microbiological research.

Colonisation of bacterial endophytes in roots of *B. juncea* seedlings

The Scanning Electron Microscopy (SEM) analysis conducted on 10-days-old *B. juncea* seedlings grown in sterilized vermiculite provided crucial insights into the endophytic nature of the selected bacterial isolates. The micrographs revealed the presence of bacterial colonization within the treated roots, characterized by distinct rod shaped structures (Fig. 2). In contrast, the root surface of un-inoculated *B. juncea* seedlings appeared smooth, devoid of any bacterial colonization. The presence of rod-shaped structures within the root tissues strongly suggests the entry and colonization of

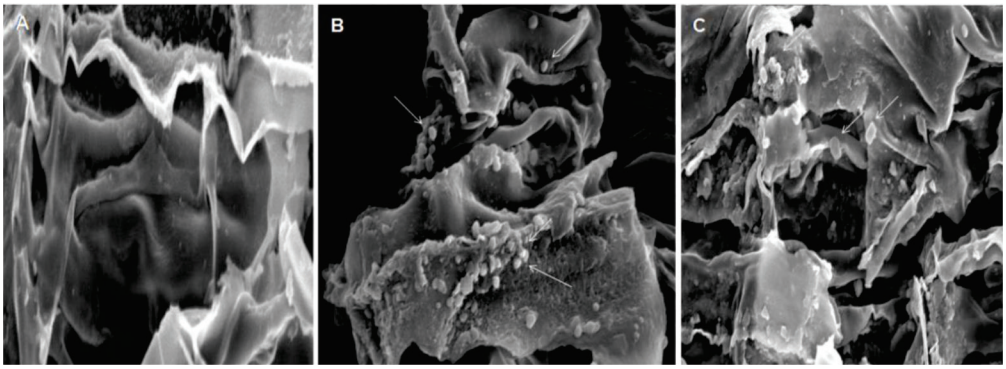

Fig. 2. Scanning Electron Micrograph of root of 10 day old seedling of *B. juncea* confirming bacterial colonization of *Pseudomonas aeruginosa* (white arrows) (B) and *Serratia proteamaculans* (white arrows) (C) in comparison to un-inoculated control (A) at different magnifications

endophytic bacteria, and were found to be consistent with previous reports (Gupta and Pandey, 2020; Shah *et al.,* 2021). Moreover, the observed structures in the root tissues closely match the morphology of the bacterial isolates used in our study, further confirming their endophytic nature (Neupane *et al.,* 2013; Diggle and Whiteley, 2020). The observations highlight the ability of the selected bacterial isolates, namely *P. aeruginosa* and *S. proteamaculans,* to establish themselves as endophytes within the root tissues of *B. juncea* seedlings. By residing within the plant tissues, these endophytic bacteria may exert their antagonistic effects against the fungal pathogen through various indirect mechanisms, such as induction of plant defense responses.

The dual culture assay enabled the identification of two best strains responsible for inhibiting the mycelial growth of *S. sclerotiorum*. The selected strains were able to colonize the roots of *B. juncea* as indicated by the micrographs of SEM, thus confirming its endophytic nature. Additional *in vivo* studies are needed to further assess their potential in enhancing the defense response against stem rot disease in *B. juncea.* Understanding their behaviour within the plant and their interactions with the pathogen will be essential for elucidating their potential as biocontrol agents.

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Authors' contribution

Conceptualization and designing of the research work (SS); Execution of lab experiments (R, SS, PV, PSS); Analysis of data and interpretation (SS, R, PV, PSS, ST); Preparation of the manuscript (SS, R & ST).

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