

# Bio-priming of paddy crop by 2,4-diacetylphloroglucinol and phenazine-containing supernatant of *Pseudomonas fluorescens* for better storage and improved germination

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## ABSTRACT

Plant growth-promoting rhizobacteria play an important role in sustainable agriculture by enhancing crop productivity and suppressing phytopathogens. The present study focused on the isolation and characterization of fluorescent *Pseudomonas* strains from the rhizosphere of paddy (*Oryza sativa* L.) cultivated in Gangoh Block, Saharanpur, Uttar Pradesh, India. Seven bacterial isolates were obtained using serial dilution and streak plate techniques, among which *Pseudomonas fluorescens* MS-05 exhibited the highest production of indole-3-acetic acid and siderophores. Biochemical characterization and 16S rRNA gene sequencing confirmed the identity of the strain, showing 99% sequence similarity with *P. fluorescens* (NCBI Accession No. PP501522.1). The antagonistic potential of *P. fluorescens* MS-05 against the storage-deteriorating fungus *Aspergillus niger* was evaluated using a dual-culture assay. The strain produced a clear inhibition zone of  $21.3 \pm 1.2$  mm after 7 days of incubation, with sustained inhibition of fungal growth observed under prolonged *in vitro* incubation. Gas chromatography–mass spectrometry (GC–MS) analysis of the bacterial metabolites identified extracellular metabolites, including 2,4-diacetylphloroglucinol and phenazine, previously reported for antifungal activity. Comparative evaluation demonstrated that the cell-free supernatant of *P. fluorescens* MS-05 exhibited significantly higher antifungal efficacy than the corresponding bacterial broth. This enhanced activity is likely due to the direct availability of extracellular antifungal metabolites, highlighting the functional advantage of metabolite-based, cell-free formulations over whole-cell preparations. Overall, the findings highlight the potential of *P. fluorescens* MS-05 and its extracellular metabolites as effective bio-priming agents for controlling seed and storage-associated fungal pathogens in paddy. The study emphasizes the importance of metabolite-based biocontrol strategies and provides a foundation for the development of eco-friendly alternatives to chemical fungicides in post-harvest disease management.

## 1. INTRODUCTION

Paddy (*Oryza sativa* L.) is one of the most important cereal crops worldwide and serves as a staple food for nearly half of the global population. Major rice-producing countries, such as China, India, Indonesia, Bangladesh, and Vietnam play a critical role in sustaining global food security, with India alone producing more than 135 million metric tons annually [1]. Despite high production levels, post-harvest losses caused by fungal contamination remain a major constraint affecting grain quality and food safety. Stored paddy grains are particularly vulnerable to infestation by fungal pathogens, such as *Aspergillus niger* and *Aspergillus flavus*, which are responsible for grain deterioration and mycotoxin contamination [2-5]. These fungi pose

serious health risks, including nephrotoxicity, immunosuppression, respiratory disorders, and carcinogenic effects, especially under prolonged exposure [6]. Increasing resistance to chemical fungicides and concerns regarding residual toxicity have intensified the need for sustainable and environmentally safe alternatives for post-harvest disease management. Plant growth-promoting rhizobacteria (PGPR) have been widely investigated as eco-friendly biological agents for improving crop productivity and suppressing phytopathogens. Among them, fluorescent *Pseudomonas* species have gained particular attention due to their ability to produce a diverse array of antifungal metabolites, including siderophores, hydrolytic enzymes, volatile organic compounds, phenazines, pyoverdine, pyrrolnitrin, and 2,4-diacetylphloroglucinol (DAPG) [7,8,26]. These metabolites inhibit fungal pathogens through multiple mechanisms, such as iron sequestration, disruption of fungal membrane integrity, interference with respiratory metabolism, and induction of oxidative stress [31,34].

Bio-priming, defined as the treatment of seeds with beneficial microorganisms before sowing or storage, is widely recognized

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as a sustainable strategy for improving seed germination, seedling vigor, and disease resistance [15,26]. Numerous studies have demonstrated that bio-priming with live PGPR, such as *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Trichoderma harzianum*, enhances early plant establishment and reduces seed- and soil-borne fungal infections [12,15]. Bio-priming with *Pseudomonas* spp. has been reported to suppress fungal pathogens, including *Aspergillus* and *Fusarium*, through effective seed surface colonization and *in situ* production of antifungal metabolites, such as DAPG, phenazines, and siderophores [4,5,24]. Similarly, *Bacillus*- and *Trichoderma*-based bio-priming approaches have been shown to improve disease resistance through competitive exclusion, production of lytic enzymes, and induction of host defense responses [6-8]. However, the efficacy of conventional bio-priming strategies relying on live microbial inocula is strongly influenced by environmental factors, microbial survival during storage, and inconsistent colonization of the seed surface or rhizosphere [9,26]. These limitations are particularly pronounced in post-harvest and storage environments, where conditions may not favor microbial persistence or metabolic activity. As a result, despite extensive research on live-cell bio-priming, the direct application of cell-free, metabolite-based formulations remains relatively underexplored, especially for managing storage-associated fungal deterioration of paddy grains.

Extracellular antifungal metabolites produced by PGPR, particularly DAPG and phenazines from *Pseudomonas* spp., are well documented for their broad-spectrum antifungal activity against phytopathogens, such as *Fusarium*, *Rhizoctonia*, and *Aspergillus* [24,30,34]. Nevertheless, most studies have focused on metabolite identification or rhizosphere-mediated disease suppression during early plant growth stages, rather than evaluating the functional efficacy of metabolite-rich supernatants as standalone bio-priming agents. The comparative effectiveness of whole-cell bacterial formulations versus extracellular metabolite-based treatments under post-harvest or storage-stage conditions, therefore, remains insufficiently addressed. In this context, the present study aims to bridge this knowledge gap by isolating and characterizing a locally adapted *P. fluorescens* strain and functionally comparing the antifungal efficacy of its bacterial broth and cell-free, metabolite-rich supernatant against storage-associated fungal pathogens of paddy. By focusing on a supernatant-mediated, metabolite-based bio-priming strategy, this work evaluates an alternative approach that may offer greater consistency and applicability for post-harvest fungal management, thereby contributing to the development of sustainable and eco-friendly alternatives to chemical fungicides.

## 2. MATERIALS AND METHODS

### 2.1. Sampling Area and Cultivar

The experimental fields were located in the Gangoh Block of Saharanpur District, Uttar Pradesh, India. Throughout the study, a commonly cultivated paddy variety, “Pusa Basmati-1509”, was used.

### 2.2. Isolation and Characterization of Rhizobacteria and their Plant Growth Promoting Traits

Soil samples were collected from the rhizospheric zone of paddy crops. Bacteria were isolated from loosely attached soil particles using the serial dilution method, and dilutions ranging from  $10^{-1}$  to  $10^{-6}$  were prepared [9,10]. From each dilution, 100  $\mu$ L was spread onto selective fluorescent *Pseudomonas* medium (HiMedia) plates in triplicate and incubated at 29 °C for 48 h. Seven bacterial strains were isolated and purified using the streak plate method. All isolates were examined

for fluorescence under UV light. Further, kit-based biochemical examination was completed using the KB-002 biochemical test kit. Each well of the KB-002 kit was inoculated with a standardized 0.5 McFarland suspension and incubated at  $28 \pm 2$  °C for 24–48 h. Results were interpreted based on colour changes according to the manufacturer’s instructions. *Pseudomonas fluorescens* MTCC 2421 served as the positive control, while uninoculated wells served as negative controls. All isolates were screened for siderophore production using the chrome azurol S (CAS) assay [11]. Additionally, indole-3-acetic acid (IAA) production was analyzed as described by [12]. Biochemical traits were further compared with the reference strain *P. fluorescens* MTCC 2421 to confirm identification. Among the isolated strains, the most potent strain, MS-05, was further analyzed by 16S rRNA gene sequencing for species-level identification following the method described in [13]. The fluorescent *Pseudomonas* strain was submitted to the NCBI database to obtain an accession number.

### 2.3. Fungal Deteriorating Agent

The fungal deteriorating agent for *Aspergillus niger* was isolated from 1-year-old stored paddy grains following the method of Chitranshi and Arora [14]. Briefly, infected rice grains were placed on freshly prepared PDA plates and incubated for 7–10 days at 25–29°C. Further, the fungal strain was preserved on potato dextrose agar plates at 4°C in the departmental laboratory.

### 2.4. Assessment of the Biocontrol Potential of *P. fluorescens* against Fungal Pathogens

#### 2.4.1. Dual culture assay

The dual culture assay was performed in triplicate to assess the antifungal activity of potent bacterial strains against selected pathogenic fungi, with minor modifications from Sultana and Hossain [15]. Briefly, a 5 mm disc from a 1-week-old fungal culture was placed 1.5 cm from the left edge of a PDA-containing Petri dish. The bacterial culture was then inoculated 1.5 cm from the right edge of the same plate. A control plate containing only the fungal inoculum was also prepared. In addition, a positive control was maintained using 0.2% Mancozeb, a standard fungicide with broad-spectrum antifungal activity, while the negative control consisted of plates with only the fungal culture without bacterial inoculation. All plates were incubated at 29°C for 1 week in separate incubators to prevent potential cross-contamination, ensuring independent and sterile growth conditions for each treatment. The antagonistic activity of the bacterial strains was evaluated by observing the inhibition of fungal growth.

#### 2.4.2. Supernatant spray assay

The collected solvent layer was evaporated under sterile airflow and the dried extract was reconstituted in sterile distilled water (10 mg/mL). The extract was then passed through a 0.22  $\mu$ m syringe filter to ensure complete removal of bacterial cells. For the spray assay, 1 mL of the filtered supernatant was uniformly applied to each 90 mm PDA plate using a sterile atomizer. All plates received equal volume, and the treated plates were air-dried for 10 min in a laminar flow hood before fungal inoculation.

#### 2.4.3. GC-MS analysis for bacterial metabolite identification

For the detection of metabolites produced during the dual culture assay, *P. fluorescens* MS-05 was analyzed further by Gas Chromatography and mass spectrometry (GC-MS) technique as described by Abed *et al.* [16]. The GC-MS analysis was carried out using an Agilent 7890A Gas Chromatograph coupled with a 5975C Mass Selective

**Table 1:** Biochemical identification of isolates by KB-002 biochemical test kit Hi-Media (Gram-positive Rods) (+) denotes positive results and (-) denotes negative results for biochemical characterization.

Biochemical tests	MS-01	MS-02	MS-03	MS-04	MS-05	MS-06	MS-07
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
H <sub>2</sub> S production	--	--	--	--	--	--	--
Nitrate reduction	+	+	--	--	+	--	--

Detector. Separation of metabolites was achieved on an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was set at 250°C, the injection volume was 1 μL, and the split ratio was 10:1. The oven temperature program started at 60°C (2 min hold), increased to 280°C at 10°C/min, and was held for 10 min. The ionization mode was electron impact (EI) at 70 eV, and mass spectra were recorded over the m/z range 50–600. Compound identification was performed by comparing retention times and mass spectra with those in the NIST 14 library and with authentic standards of DAPG and phenazine-1-carboxylic acid (PCA). Briefly, *P. fluorescens* MS-05 was cultured in nutrient broth medium (Hi-media) in Erlenmeyer flasks, the flasks were placed on a rotary shaker incubator at room temperature (approximately 25–30°C) with 110 rpm for 7 days. Further, the culture broth was filtered, and the filtered substances were mixed separately with chloroform and ethanol absolute (research grade) in a 1:1 ratio. The addition of chloroform resulted in the formation of two layers in the culture broth; the solvent layer was separated using a separating funnel and stored in sterile vials. The ethanol-added culture broth was kept as an aqueous extract.

## 2.5. Statistical Analysis

All experiments were conducted in triplicate ( $n = 3$ ). Data presented as mean ± SD; significance determined by one-way ANOVA ( $P < 0.05$ ).

## 3. RESULTS AND DISCUSSION

### 3.1. Morphological and Biochemical Identification of Rhizobacteria

The morphological identification of seven bacterial isolates through a compound microscope (Olympus 14C0504) shows that all isolates MS-01, MS-02, MS-03, MS-04, MS-05, MS-06, and MS-07, were Gram-negative and rod-shaped. Moreover, these isolates were further analyzed for various bio-chemical activities [Table 1]. All the strains were further checked for IAA and siderophore activity test and found that bacterial strain MS-05 shows the best IAA production, including positive siderophore production [Table 2]. Bacterial strain MS-05 shows the best results in all the kit-based bio-chemical test specifically for *P. fluorescens*. Similar observation in IAA activity was reported earlier by the researchers [17,18]. Further, it was observed that within a short period of time, considerable enhancement in the germination was observed in case of the crop treated with IAA [19]. Siderophore production by beneficial microorganisms is known to play an essential role in natural biocontrol by restricting iron availability to fungal pathogens [20-22].

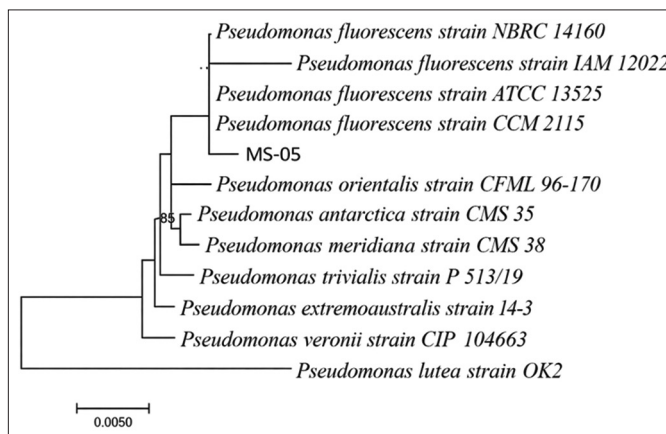
### 3.2. Molecular Characterization

Antagonistically active bacterial strain MS-05 was screened further on various basic tests among all other seven strains. The strain was Gram-negative and rod-shaped, which shows positive oxidase and catalase

**Table 2:** Qualitative analysis of isolates for PGP activity (+) weak (++) moderate and (+++) high and (--) negative results, IAA (indole-3-acetic acid).

Sample ID	Qualitative		Quantitative
	IAA	Siderophore	IAA
MS-01	+	--	0.746±0.29
MS-02	+	--	01.164±0.42
MS-03	+	--	0.918±0.38
MS-04	+	--	2.465±0.90
MS-05	+++	+++	10.925±3.69
MS-06	+	--	1.14±0.43
MS-07	++	--	4.668±1.90

IAA: Indole-3-acetic acid



**Figure 1:** Phylogenetic trees of bacteria MS-05 (MEGA-X).

tests with fluorescent property under UV light. Further, in 16s rRNA sequencing (Sanger method) strain was showing 99% similarities with *Pseudomonas* [23]. Moreover, a comparative study with the available database on NCBI and the phylogenetic analysis [Figure 1] of that strain MS-05 shows the direct relation with *P. fluorescens*. Further, the sequence was submitted to the NCBI database with Accession No: PP501522.1.

### 3.3. Anti-Fungal Assay

The antagonistic potential of *P. fluorescens* MS-05 was evaluated against the fungal deteriorating agent AN-03 (*Aspergillus niger*, NCBI Accession No. OR083359) using a dual-culture antifungal assay. The assay was performed in triplicate ( $n = 3$ ), and the inhibition zone was measured after 7 days of incubation. The mean zone of inhibition recorded for *P. fluorescens* MS-05 was  $21.3 \pm 1.2$  mm, whereas the control plate (fungus only) showed unrestricted mycelial growth. Observations taken at 10, 20, and 30 days revealed complete growth

suppression of *A. niger* in plates treated with the bacterial supernatant, while minimal growth was observed in plates treated with bacterial broth [Table 3]. These results demonstrate that the cell-free supernatant of *P. fluorescens* MS-05 exhibits superior antifungal efficacy compared with the bacterial suspension. The efficacy observed in this study aligns with earlier reports of *Pseudomonas* spp. producing DAPG, phenazines, pyoluteorin, and pyrrolnitrin that inhibit fungal growth and promote plant health [24-26]. Comparable inhibition was reported for *P. protegens* ML15 [27,28] and *P. chlororaphis* KNU17Pc1 [36]. *P. fluorescens* is widely recognized as an effective biocontrol agent, and the present findings further support its ability to suppress storage-associated fungal pathogens. A representative image of the dual-culture assay showing inhibition of *A. niger* by *P. fluorescens* MS-05 is presented in Figure 2.

### 3.4. Metabolite Production Analysis

*P. fluorescens* MS-05 provided strong protection to paddy grains against *A. niger* during the spray assay. However, the supernatant of *P. fluorescens* strain MS-05 shows the strongest inhibition in comparison to the broth spray. Further, the supernatant was analyzed through GC-MS technique. A peak shown at a retention time of 6.780 min confirms the presence of DAPG in *P. fluorescens* strain MS-05 [Figure 3]. Similarly, the DAPG standards displayed a retention time of 6.76 min in GC-MS [29]. Moreover, it was observed that DAPG producing *P. fluorescens* strain Q8r1-96, have high biocontrol activity [30]. *P. fluorescens* and closely related species are well known for their ability to produce antimicrobial compounds, including DAPG, phenazines [31]. Similarly, it was observed that

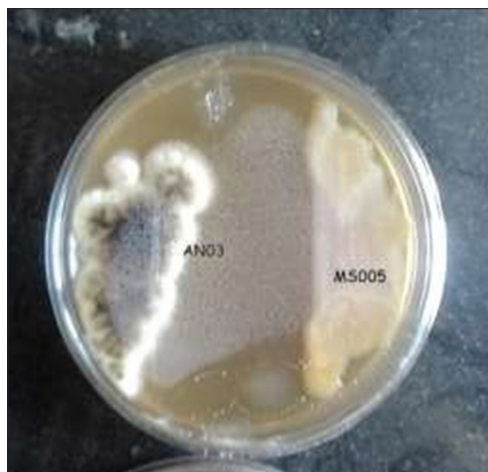
volatile organic compounds VOCs released by *P. fluorescens* MS-05 suppress the growth of fungi [32]. In addition, another peak observed at a retention time of 18.679 min confirmed the presence of PCA. According to researchers, phenazines are redox-active secondary metabolites produced by *Pseudomonads*, exhibiting antibacterial, antifungal, and antitumor properties [33]. Phenazines suppress fungal growth, including *A. niger*, by disrupting respiration and biofilm formation, playing a key role in agricultural disease management [34]. Similarly, it was reported by Shrivastava et al. [35] in their GC-MS findings, identified PCA and phenazine-1,6-dicarboxylic acid in *P. fluorescens*, reinforcing their importance in suppressing soilborne fungal pathogens [36]. The presence of these antimicrobial compounds, confirmed by GC-MS analysis, underscores the strain's effectiveness in suppressing *Aspergillus niger* and other fungal pathogens. The antifungal activity observed in the present study is consistent with the reported biochemical activities of these metabolites in earlier studies. DAPG interferes with fungal membrane integrity and inhibits key respiratory enzymes, leading to disruption of energy metabolism and cell lysis. PCA functions as a redox-active compound that generates reactive oxygen species within fungal cells, thereby impairing spore germination and biofilm formation. The combined activity of these metabolites produces a synergistic effect, resulting in strong and sustained fungal suppression. Similar inhibitory mechanisms have been reported in other *Pseudomonas* strains, where phenazine derivatives and DAPG jointly contribute to biocontrol efficacy and plant disease resistance [34,35]. Thus, *P. fluorescens* MS-05 emerges as a promising candidate for sustainable plant disease management in paddy cultivation [Table 4].

The objective of the present study was to identify extracellular antifungal metabolites produced by PGPR strains and to evaluate their direct antifungal effectiveness when applied as a cell-free formulation. Accordingly, the comparison between bacterial broth and metabolite-rich supernatant was designed as a functional assessment of application strategy rather than a mechanistic investigation. The use of cell-free supernatant allows direct exposure of fungal pathogens to bioactive secondary metabolites without variability arising from bacterial growth, colonization efficiency, or nutrient competition. GC-MS analysis was therefore employed for qualitative identification of key antifungal metabolites, and the observed superior efficacy of the supernatant reflects the functional activity of these extracellular compounds.

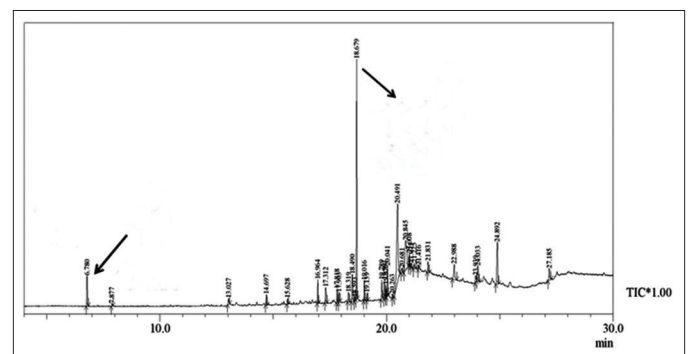
This study was designed as an *in vitro* functional evaluation of metabolite-based bio-priming and therefore does not include seed germination assays, long-term storage trials, or quantitative

**Table 3:** Dual culture assay on PDA for detection of antagonistic activity of bacteria against deteriorating fungal pathogen in Mm,  $\pm$  standard deviation (SD) from three independent replicates ( $n=3$ ).

Treatment	2 <sup>nd</sup> day (mm)	5 <sup>th</sup> day (mm)	7 <sup>th</sup> day (mm)	Mean zone of inhibition (mm $\pm$ SD, $n=3$ )
Plate A	15.3	26.4	56.3	21.3 $\pm$ 1.2
Plate B	12.5	27.2	48.2	20.8 $\pm$ 1.4
Plate C	14.2	29.3	52.6	21.5 $\pm$ 1.0
Control (fungus only)	–	–	–	0.0



**Figure 2:** Dual culture assay *Aspergillus niger* versus *Pseudomonas fluorescens* MS-05.



**Figure 3:** Gas chromatography-mass spectrometry analysis showing the presence of 2-4 DAPG, phenazine.

**Table 4:** Broth spray assay inhibitory effect of *Pseudomonas fluorescens* MS-05 on the growth of *Aspergillus niger* under *In Vitro* (+) = Slow growth of pathogen, (++) = Medium growth (+++) High growth, while (-) denotes no growth of pathogen.  $\pm$  standard deviation (SD) from three independent replicates ( $n=3$ ).

Treatment	10 days (mm)	20 days (mm)	30 days (mm)	Mean fungal growth (mm $\pm$ SD, $n=3$ )
T1 (Bacterial broth spray)	5.8 $\pm$ 0.4	7.2 $\pm$ 0.5	9.1 $\pm$ 0.6	7.4 $\pm$ 0.5
T2 (Supernatant spray)	3.1 $\pm$ 0.3	2.2 $\pm$ 0.2	0.0 $\pm$ 0.0	1.8 $\pm$ 0.3
T3 (Control fungus only)	18.6 $\pm$ 1.1	25.3 $\pm$ 1.4	32.5 $\pm$ 1.6	25.5 $\pm$ 1.3
T4 (Solvent control)	17.9 $\pm$ 1.0	24.6 $\pm$ 1.3	31.8 $\pm$ 1.4	24.8 $\pm$ 1.2

potency ranking against purified standards, such as MIC or MFC determination. The antifungal observations are thus qualitative and correlative. These aspects represent important next steps toward translational application and will be addressed in future studies using purified metabolites, crude supernatant, and controlled storage and germination models.

#### 4. CONCLUSION

The present study demonstrates that *P. fluorescens* strain MS-05 effectively suppresses the growth of fungal pathogens, particularly *Aspergillus niger*, associated with stored paddy grains. The supernatant of *P. fluorescens* MS-05 showed stronger antifungal activity than the bacterial broth, highlighting the functional effectiveness of extracellular metabolites when applied directly. These findings highlight the potential of this strain as a promising biocontrol agent for managing post-harvest fungal deterioration in paddy.

However, further validation under controlled storage and field conditions is required to assess the long-term effects of this treatment on seed morphology, chemical stability, and germination performance. The use of such bio-based control approaches offers an eco-friendly and sustainable alternative to chemical fungicides for protecting stored food grains and promoting safer agricultural practices. This study primarily focused on *in vitro* antifungal evaluation. Quantitative seed germination and storage trials are planned as part of follow-up studies to validate the observed protective effects of the supernatant under controlled and field conditions.

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#### 6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

#### 7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

#### 8. ETHICAL APPROVALS

All experimental procedures involving storage crops and microbial isolates were conducted in accordance with institutional bio-safety guidelines. The study did not involve any human or animal subjects, and following standard laboratory and safety protocols.

#### 9. DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article. Additional datasets are available from the corresponding author upon reasonable request.

#### 10. PUBLISHER'S NOTE

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#### 11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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