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# Mutagenesis driven enhancement of chitinase production in *Stenotrophomonas maltophilia* and its application in the biocontrol of *Fusarium oxysporum* in chickpea

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**ABSTRACT**

A chitinase-producing strain of *Stenotrophomonas maltophilia* BSUC-16 was subjected to physical and chemical mutagenesis to improve its chitinolytic and antifungal potential. The resulting mutant strain, BSUCM-24, exhibited significantly increased chitinase activity of 520.16 ± 2.38 mU/ml, representing a 36.8% improvement over the parent strain. The parental BSUC-16 and mutant BSUCM-24 strains exhibited antifungal activity against live mycelia of *Fusarium oxysporum* f. sp. *ciceris* (FOC), as demonstrated by inhibition zones in the agar well diffusion assay, with the mutant strain showing enhanced antifungal activity compared with the parental strain. The median infectious dose, ID50, of FOC was determined to be $1.005 \times 10^5$ conidia/g of soil, which was used to induce effective disease incidence in chickpea seeds. Pot assay experiments demonstrated enhanced biocontrol efficacy of BSUCM-24, with 93.3% plant survival in the presence of FOC, compared with 53.3% plant survival observed with the parental strain BSUC-16. Additionally, plants treated with the mutant strain showed improved growth parameters, suggesting its plant growth-promoting potential. Defense-related enzyme analysis indicated elevated levels of defense-related enzymes, while SEM (Scanning Electron Microscopic) analysis suggested reduced fungal colonization and pathogen-induced damage in treated plants. Overall, the findings demonstrate that mutagenesis successfully generated a strain with enhanced chitinase production that was associated with improved biocontrol performance against Fusarium wilt in chickpea, highlighting its potential as a promising eco-friendly biocontrol agent.

## 1. INTRODUCTION

Chickpea (*Cicer arietinum* L.), commonly known as garbanzo bean or Bengal gram, belongs to the tribe Cicereae of the family Fabaceae and the papilionoid subfamily [1]. According to the Food and Agriculture Organization of the United Nations (FAO) (2025), over the past 5 years, chickpea has consistently ranked as the third most important pulse crop worldwide, after dry beans and peas. Chickpea is grown across tropical, subtropical, and temperate regions, including South and West Asia, southern Europe, North and East Africa, North and South America, and Australia. Asian countries account for 83.6% of global chickpea production, with India being the largest producer. Chickpea is cultivated on approximately 14.12 million hectares worldwide, of which India alone accounts for about 9.58 million hectares, representing nearly 68% of the global chickpea-growing area. Although India possesses the largest chickpea harvested area globally and ranks first among the top ten chickpea-growing countries, followed by Australia, Pakistan, Turkey, Iran, Russia, Myanmar, Ethiopia, the United States, and Canada, it remains considerably behind in yield performance, ranking approximately 8th in terms of production per hectare (tonnes per hectare) [2]. Several factors contribute to this yield gap, among which plant diseases play a significant role. One of the most devastating diseases affecting chickpea production is *Fusarium* wilt. Optimizing plant disease management practices could enable the maintenance of current production levels using less cultivated land, thereby allowing the reallocation of land resources to other crops or vegetation and contributing to sustainable agricultural intensification.

*Fusarium* wilt is caused by the seed- and soil-borne fungal pathogen *Fusarium oxysporum* f. sp. *ciceris* (FOC). Infected chickpea plants exhibit a variety of symptoms, including reduced growth at the seedling stage. In mature plants, symptoms include drying of shoot tips followed by the shedding of twigs and leaves. This organism induces vascular wilt, cortical tissue decay, and causes loss of chickpea yield of up to 10%–100% depending on varietal susceptibility and climatic conditions. It is reported that the disease is more predominant in India, Spain, Ethiopia, Tunisia, Turkey and the U.S. [3]. Chemical fungicides remain the primary approach for managing plant diseases; however, their excessive use has raised significant environmental and biological

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concerns. Repeated application of site-specific chemical fungicides promotes the emergence of resistant pathogen strains, reducing their long-term effectiveness. In addition, fungicide residues contaminate soil, air, and water, posing risks to human and animal health. These chemicals can also negatively impact beneficial organisms such as pollinators, earthworms, natural predators, and soil microorganisms, ultimately disturbing soil health and ecological balance [4].

Chitinolytic microorganisms are well-known biocontrol agents. Chitinase-mediated biocontrol involves the enzymatic degradation of chitin, a major component of fungal cell walls, leading to the weakening or complete lysis of fungal hyphae and spores. This enzymatic attack disrupts fungal integrity, inhibits growth, and reduces pathogen infectivity [5]. The successful management of plant diseases requires an integrated and sustainable approach that minimizes dependence on chemical inputs and enhances the resilience of cropping systems. Within this framework, microbial chitinases have emerged as important components of eco-friendly disease suppression strategies and potential alternatives to chemical fungicides, offering the dual benefits of pathogen control and plant growth promotion. Therefore, the objective of the present study was to evaluate the potential of the chitinase-producing strain *S. maltophilia* BSUC-16 and its mutant BSUCM-24 against the phytopathogenic fungus FOC, the causal agent of chickpea wilt.

## 2. MATERIALS AND METHODS

### 2.1. Test media, Strains, and Reagents

Colloidal chitin agar (CCA) medium was prepared with 0.6% $Na_2HPO_4$, 0.3% $KH_2PO_4$, Yeast Extract, 1% Colloidal Chitin as the sole carbon source, and 2.5 g of Agar powder. The test strains included S. maltophilia BSUC-16, which was previously isolated from rhizospheric soil and identified through whole-genome sequencing analysis. The strain yielded 380.25 ± 2.7 mU/ml of chitinase activity after media optimization through statistical methods [6]. The bacterial strain was maintained on CCA plates. The other strain included was phytopathogenic fungi FOC which was gifted by the Plant Pathology Department, Anand Agricultural University, Anand, Gujarat, India. The fungal strain was maintained on Potato Dextrose Agar (PDA) plates. To enhance the chitinase activity of *S. maltophilia* BSUC-16, the strain was subjected to mutagenesis.

### 2.2. Chitinase Activity

Chitinase production was carried out in the 1% (w/v, dry weight basis) colloidal chitin broth and the amount of enzyme was estimated using the dinitrosalicylic acid (DNS) procedure. A 4% inoculum was inoculated in colloidal chitin broth and incubated at 28°C for 120 hours. The broth was then centrifuged, and the supernatant was collected to estimate the enzyme activity following the procedure described previously [7]. The 3 ml reaction system contained 1 ml of the undiluted enzyme-containing supernatant, 1 ml of 5% colloidal chitin (substrate), 1 ml of acetate buffer (50 mM, pH 5). The system was allowed to react for 60 minutes at 50°C in a water bath. After incubation, the reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid to the reaction mixture, and then N-acetyl-D-Glucosamine (NAG) as a product was detected at 530 nm. All the experiments were performed in triplicate. One unit (U) of chitinase activity was defined as the amount of enzyme releasing 1 µmol of NAG equivalent reducing sugars per minute under the assay conditions.

### 2.3. Mutagenesis

Mutagenesis was employed as a strategy to induce genetic variability in *S. maltophilia* BSUC-16. Physical and chemical mutagens were used to generate mutants with enhanced chitinase production for improved biocontrol efficiency.

#### *2.3.1. Physical mutagenesis*

A bacterial suspension of the chitinase-producing strain *S. maltophilia* BSUC-16 was prepared in sterile distilled water and adjusted to a concentration of approximately $10^8$ CFU/ml. From this suspension, 0.1 ml was aseptically spread onto CCA plates for ultraviolet (UV) mutagenesis. The inoculated plates were exposed to deuterium lamp with short-wavelength UV radiation at a distance of 15 cm for different time intervals (0, 0.5, 1.0, 1.5, and 2.0 minutes). After exposure, the plates were incubated at 28°C for 48 hours to allow colony development. To avoid photoreactivation, all handling following UV exposure was performed under dark condition [8]. Mutants were screened on the basis of clearance zone/ colony size (CZ/CS) ratio and chitinase activity. The minimum lethal dose (MLD) was determined by calculating the survival percentage at each exposure time, and mutants were selected at approximately 0.1% survival. Distinct colonies appearing under MLD conditions were isolated. The obtained mutants were subsequently screened for enhanced chitinase production by estimating enzyme activity using the DNS method.

#### *2.3.2. Chemical mutagenesis*

The selected UV-induced mutant strain, BSUCM-1 of *S. maltophilia*, was subjected to chemical mutagenesis using ethyl methanesulfonate (EMS) to enhance chitinase production. An actively growing culture ($10^8$ CFU/ml) was harvested, washed with 0.1 M Tris-HCl buffer (pH 7.0), and resuspended to obtain a concentrated cell suspension. Aliquots (1 ml) were treated with 0.05M EMS per ml of cell suspension and incubated at 37°C for varying time intervals (0, 15, 30, 45 and 60 minutes). Following treatment, cells were washed to remove residual mutagen and allowed to recover in fresh medium for 24 hours. The cultures were then serially diluted, plated on CCA, and incubated. Surviving colonies were screened based on clear zone formation and chitinase activity, and mutants with the highest CZ/CS ratio and chitinase activity were selected for further studies [9].

The phenotypic stability of the finally selected mutant, BSUCM-24, was assessed through successive subculturing in colloidal chitin broth. The mutant strain was initially inoculated into colloidal chitin broth and incubated for 120 hours under standard growth conditions. At the end of each incubation cycle, the culture was centrifuged and the resulting cell pellet was transferred aseptically into fresh colloidal chitin broth. This procedure was repeated for six successive cycles, with each transfer occurring after 120 hours of incubation. Following each subculture, chitinase activity was quantified using the standard assay method to monitor consistency in enzyme production. The ability of the mutant to maintain comparable chitinase activity levels across all successive transfers would indicate the absence of phenotypic reversion or significant loss of enzyme-producing capability.

### 2.4. Assessment of Antifungal Activity of BSUC-16 and BSUCM-24

The antifungal activity of the parental strain BSUC-16 and the final mutant strain BSUCM-24 was evaluated against live mycelia of FOC using the agar well diffusion assay. A mycelial suspension of FOC was prepared and uniformly spread over the surface of PDA plates to obtain an even fungal lawn. Wells of uniform diameter were then punched aseptically into the agar immediately after spreading. Crude culture supernatants containing chitinase produced by the parental strain BSUC-16 and mutant strain BSUCM-24 were aseptically dispensed into the respective wells. The plates were incubated at 28°C

to allow diffusion of the culture supernatants. Following incubation, the antifungal activity of the respective culture supernatants was evaluated by measuring the diameter of the clear zone of inhibition formed around each well.

### 2.5. Determination of Inoculum Density ($ID_{50}$) for FOC infection on chickpea plants

The median infectious dose of FOC was determined following Baker [10] and Marois and Mitchell [11]. FOC was cultured on PDA at 28 °C for 7 days, and conidia were harvested using sterile distilled water containing 0.01% Tween 20. The conidial suspension was filtered, quantified using a Neubauer hemocytometer, and adjusted to prepare inoculum densities ranging from $10^1$ to $10^6$ conidia/g of soil. Sterilized soil was artificially infested with the respective conidial concentrations and transferred to pots. Five pre-germinated chickpea seedlings were sown in each pot, with three biological replicates (pots) ($n$ = 3) for each inoculum density, resulting in a total of 15 plants per inoculum dose. Disease incidence was recorded after 20 days. The proportion of infected plants (X) was calculated for each inoculum density. For $ID_{50}$ estimation, disease incidence was transformed using the logarithmic transformation, and the transformed values were plotted against $\log_{10}$ inoculum density. Linear regression was used to estimate the inoculum density corresponding to 50% disease incidence ($ID_{50}$).

### 2.6. Pot Assay to Evaluate the Biocontrol Potential of *S. maltophilia* BSUC-16 and Mutant BSUCM-24 on Chickpea Plant

A pot assay was conducted to evaluate the biocontrol efficacy of the parental strain BSUC-16 and its mutant derivative BSUCM-24 against FOC, the causal agent of *Fusarium* wilt in chickpea. Surface-sterilized chickpea seeds were soaked in different treatments during germination on sterile plates as follows:

(i) Control: Seeds germinated in sterile distilled water.
(ii) Pathogen control: Seeds treated with FOC with $10^5$ conidia/g of soil.
(iii) Culture control:
  (a) Seeds treated with $10^8$ CFU/ml of *S. maltophilia* BSUC-16 (parental strain).
  (b) Seeds treated with $10^8$ CFU/ml of *S. maltophilia* BSUCM-24 (mutant strain).
(iv) Chemical pesticide control: Seeds treated with chemical pesticide (SAAF) (12% Carbendazim + 63% Mancozeb) + 105 conidia/g of soil of pathogen FOC.
(v) Biocontrol treatment:
  (a) Seeds treated with $10^8$ CFU/ml of *S. maltophilia* BSUC-16 (parental strain) + $10^5$ conidia/g of soil of pathogen FOC.
  (b) Seeds treated with $10^8$ CFU/ml of *S. maltophilia* BSUCM-24 (mutant strain) + $10^5$ conidia/g of soil of pathogen FOC.

Following germination, uniformly sprouted seeds were transferred to pots containing sterile soil. The respective treatments, including the pathogen FOC and bacterial inoculants (BSUC-16 and BSUCM-24), were applied to the rhizospheric region at every 15 days interval. The experiment was conducted as a single experimental run with three biological replicates ($n$ = 3) per treatment. For each treatment, three separate pots were used, with five seeds sown in each pot. Each pot was considered one biological replicate, while the five seeds within each pot were considered subsamples. Plants were maintained under controlled conditions and watered regularly for a period of 2 months. This experiment was performed following the method described by Khanna *et al.* [12], with appropriate modifications.

At the end of the experimental period, plants were assessed for growth performance and disease symptoms. Parameters including plant survival, disease severity, biocontrol index, and growth performance were recorded to determine the effectiveness of the bacterial strains in suppressing *Fusarium* wilt and promoting plant growth. Plant survival was assessed by recording the number of healthy and diseased plants in each treatment. Plant growth promotion was evaluated by measuring shoot length of healthy and infected plants. Disease severity index (DSI) was determined based on the percentage of infected leaves per plant using a 0–5 disease rating scale, where 0 = no symptoms, 1 = 1%–25% of leaves infected, 2 = 26%–50% of leaves infected, 3 = 51%–75% of leaves infected, 4 = 75%–100% of leaves infected, and 5 = completely dead or no growth. Percentage DSI and biocontrol index were calculated by the following formulas.

$$\text{Percentage disease severity} = \frac{\text{(Disease Rating x No.of plants in that disease rating)}}{\text{(Maximum disease rating x Total No.of plants)}} \times 100$$

$$\text{Biocontrol Index} = \frac{\text{DSI of infected control - DSI of treatment}}{\text{DSI of infected control}} \times 100$$

Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test to determine significant differences among treatments at $p < 0.05$. Treatments sharing the same letter were considered not significantly different, whereas treatments with different letters differed significantly according to Tukey's HSD test.

### 2.7. Defense-Related Enzyme Analysis

To evaluate the induced systemic resistance (ISR) and defense response, biochemical analyses were performed on chickpea leaves from each treatment. Enzymatic activities of phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), peroxidase (POX), β-1,3-glucanase, and superoxide dismutase (SOD) were measured using standard spectrophotometric method [13]. These analyses collectively determined the effectiveness of both parental and mutant strain in promoting plant growth and suppressing FOC-induced wilt in chickpea. The analyses were conducted using samples obtained from all three independent biological replicates. The resulting data were expressed as mean ± SD and subjected to one-way ANOVA followed by Tukey's HSD test at $p < 0.05$.

### 2.8. Scanning Electron Microscopy Analysis

SEM analysis was performed to examine the internal structure of xylem vessels and to assess fungal colonization within vascular tissues. Comparative observations were carried out among healthy, pathogen infected, and biocontrol treated plants. Stem segments (~5 mm in length) were excised from plants at approximately 2 cm above the root-stem junction. The samples were gently washed three times with sterile distilled water to remove adhering soil particles and debris, followed by air drying under aseptic conditions. The dried samples were mounted onto aluminum stubs and prepared for microscopic examination.

### 2.9. Preliminary Biosafety Assessment of BSUC-16 and BSUCM-24 Through Antibiotic Sensitivity Profiling

Antibiotic sensitivity assessment is essential for evaluating the biosafety of the strains intended for bioformulation. The presence

of multidrug resistance may pose ecological and public health risks, thereby limiting regulatory approval and field application. Therefore, determining the antibiotic susceptibility pattern provides a descriptive assessment of the strains' responses to the tested antibiotics; however, further studies are necessary to confirm their safety for humans, animals, and the environment. The antibiotic sensitivity of *S. maltophilia* BSUC-16 and BSUCM-24 was evaluated against a range of antibiotics, including ceftriaxone, vancomycin, chloramphenicol, clindamycin, tetracycline, and erythromycin, using the standard disc diffusion method. The zones of inhibition were measured to describe the responses of the isolates to the tested antibiotics.

## 3. RESULTS AND DISCUSSION

### 3.1. Physical Mutagenesis Results

To assess the impact of UV irradiation on *S. maltophilia* BSUC-16, a survival curve was generated by exposing the bacterial culture to UV light for 0, 0.5, 1, 1.5, and 2 minutes. A gradual decline in colony forming units was observed with increasing exposure time. The highest survival was recorded at 0 minutes (control), while the lowest colony count was noted at 2 minutes of UV exposure, indicating increased lethality with prolonged irradiation (Fig. 1a). Following UV mutagenesis, 20 mutant colonies were isolated and screened based on CZ/CS ratio. Among these, three mutants, BSUCM-1, BSUCM-2, and BSUCM-3, obtained at 2 minutes of UV exposure (Fig. 1b), exhibited significantly enhanced chitinase activity compared to the parental strain. BSUCM-1 exhibited the highest chitinase activity of 482.15 ± 1.59 mU/ml and was selected for further rounds of mutagenesis and functional characterization (Fig. 1c). The increase in chitinase activity among selected mutants suggests that UV mutagenesis was successful in enhancing enzyme production, likely by inducing mutations in regulatory or structural genes involved in chitinase expression.

### 3.2. Chemical Mutagenesis Results

To further improve chitinase production, EMS mutagenesis was performed on the UV-induced mutant BSUCM-1, which had previously shown elevated chitinase activity. The EMS survival curve was generated by treating the bacterial suspension with EMS for 0, 15, 30, 45, and 60 minutes. A progressive decline in colony forming units was observed with increased exposure time, consistent with the cytotoxic nature of EMS. Notably, only 0.1% survival was recorded at 45 minutes of exposure, indicating effective mutagenic pressure (Fig. 2a). Based on this treatment, EMS mutagenesis yielded 18 mutant colonies (designated BSUCM-21 to BSUCM-38), which were screened for chitinase overproduction using the CZ/CS ratio (Fig. 2b). The UV mutant BSUCM-1 was used as a control for comparative evaluation. Among the EMS-induced mutants, BSUCM-24 exhibited the highest chitinase activity, reaching 520.16 ± 2.38 mU/ml (Fig. 2c), a substantial improvement over the parental and UV-mutant strains. The significant increase of 36.8% in chitinase production in BSUCM-24 suggests that EMS mutagenesis successfully introduced beneficial mutations that likely enhanced gene expression and improved enzyme stability.

To evaluate the phenotypic stability of the chitinase overproducing mutant BSUCM-24, the strain was subjected to six consecutive subcultures in fresh colloidal chitin broth medium. The mutant consistently retained high levels of chitinase activity and remained stable, with an average value of 520.19 ± 4.54 mU/ml, showing no significant variation between transfers (Fig. 3).

This suggests that the mutation responsible for elevated chitinase production is phenotypically stable and not prone to reversion or suppression under standard laboratory conditions. The reproducibility of chitinase output over multiple generations supports the suitability of BSUCM-24 for potential long-term applications, such as biocontrol. Phenotypic stability during serial cultivation is a critical attribute for strains intended for application purposes, and the performance of BSUCM-24 under repeated transfer confirms its robustness.

### 3.3. Antifungal Activity of BSUC-16 and BSUCM-24 Against Live Fungal Mycelia

The antifungal activity of the parental strain BSUC-16 and the final mutant strain BSUCM-24 was evaluated against live mycelia of FOC on PDA plates using the agar well diffusion assay. Crude culture supernatants containing chitinase produced by the respective strains were dispensed into the wells. Both strains exhibited notable antifungal activity against FOC, as evidenced by the formation of distinct zones of inhibition surrounding the wells. *S. maltophilia* BSUC-16 produced a zone of inhibition of 25.6 ± 1.3 mm, whereas the mutant strain BSUCM-24 exhibited a significantly larger inhibition zone of 28.7 ± 1.2 mm, indicating enhanced antagonistic activity against the pathogen. However, the mutant strain BSUCM-24 produced a slightly larger zone of inhibition than the parental strain BSUC-16, indicating its enhanced antifungal efficacy. The antifungal activity of the parental and mutant strains against FOC is illustrated in Figure 4.

### 3.4. ID-50 of FOC on Chickpea Seeds

The pathogenicity of FOC was evaluated using increasing inoculum densities ($10^1$-$10^6$ conidia/g soil) to determine the $ID_{50}$ in chickpea. Each treatment consisted of three biological replicates, with five seedlings per pot, resulting in 15 plants per inoculum dose. Disease incidence increased progressively from 0% at $10^1$ and $10^2$ conidia/g soil to 86.67% at $10^6$ conidia/g soil (Table 1). The observed disease proportions (X) were first transformed using $\log_e [1/(1-X)]$ for graphical visualization (Fig. 5). For quantitative estimation of $ID_{50}$, the transformed values were further converted to $\log_{10}\{\log_e[1/(1-X)]\}$ and subjected to linear regression against $\log_{10}$ inoculum density (Table S1). Since no disease incidence was observed at $10^1$ and $10^2$ conidia/g soil, the corresponding $\log_{10}\{\log_e[1/(1-X)]\}$ values were undefined and these two observations were excluded from the regression analysis. Therefore, the $ID_{50}$ was estimated by linear regression using the remaining four inoculum densities ($10^3$-$10^6$ conidia/g soil). The regression equation was $y = 0.4928x - 2.6241$ ($R^2 = 0.9977$) (Fig. S1), which was used to estimate $ID_{50}$.

At 50% disease incidence (X = 0.5), the transformed response (y) was calculated as $y = \log_{10}[\log_e (1/ (1\text{-}0.5))] = \log_{10}(\log_e 2) = -0.1592$. Substitution of this value into the regression equation yielded an $ID_{50}$ of $1.005 \times 10^5$ conidia/g soil, approximately equivalent to $10^5$ conidia/g soil. This inoculum density was subsequently selected as the pathogen challenge dose for the pot assay.

### 3.5. Evaluation of Biocontrol Potential of Parental Strain BSUC-16 and Mutant Strain BSUCM-24 on Chickpea Plant

To evaluate the biocontrol potential of *S. maltophilia* parent (BSUC-16) and mutant (BSUCM-24) strains, a pot experiment was conducted using pre-germinated chickpea seeds treated under different combinations, as outlined in the Materials and Methods. The experiment was conducted as a single experimental run with three biological replicates ($n = 3$) per treatment, with each biological replicate consisting of one pot containing five seeds. Measurements from the five seeds were

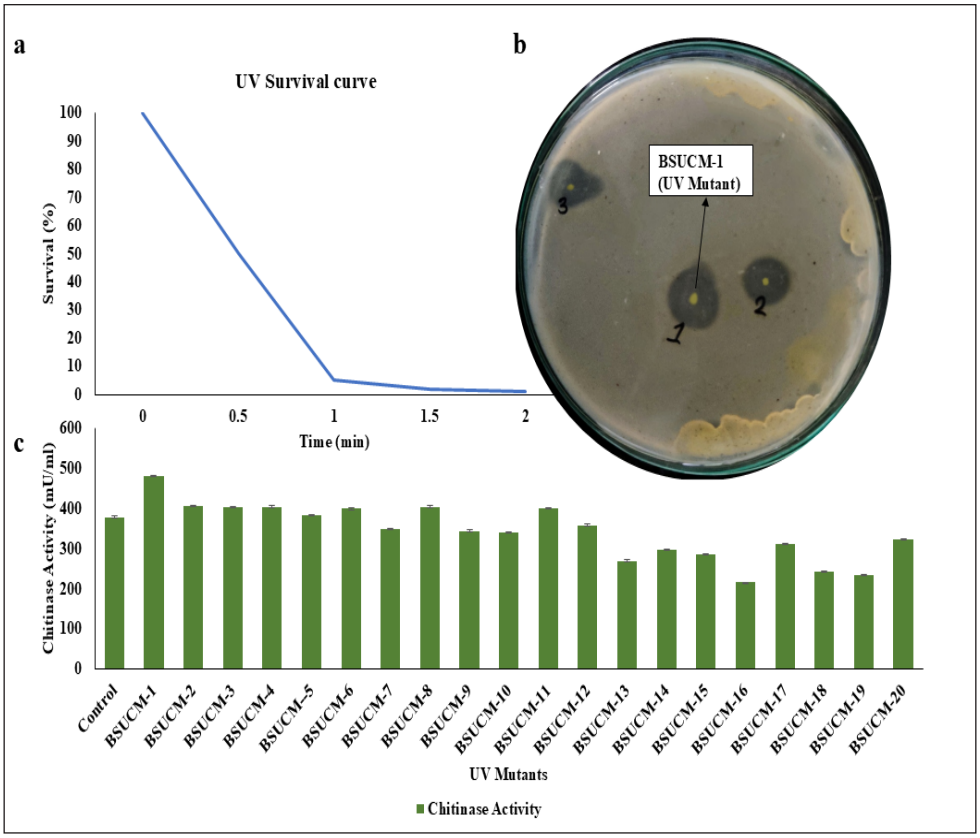



**Figure 1.** UV mutagenesis results: (a) UV survival curve. (b) Selected UV-mutant showing zone of inhibition on CCA plate, and (c) Chitinase activity of UV mutants.

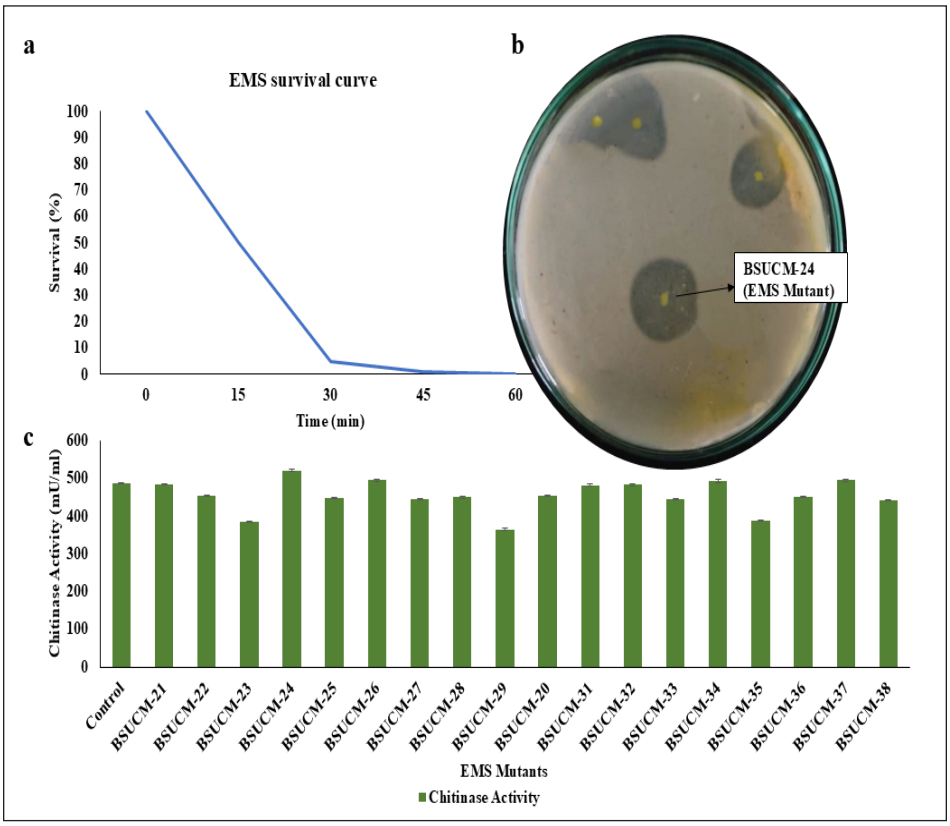



**Figure 2.** EMS mutagenesis results: (a) EMS survival curve, (b) Selected EMS-mutant showing zone of inhibition on CCA plate, and (c) Chitinase activity of EMS mutants.

averaged to obtain one value for each biological replicate, and these replicate-level means were used for statistical analysis. Treatment effects were analyzed using one-way ANOVA, followed by Tukey's HSD test for multiple comparisons. The results of pot assay are shown in Figure 6. Based on pot assay, parameters including plant survival, disease severity index, biocontrol index and growth promotion were calculated for all treatments to determine the effectiveness of the bacterial strains in controlling *fusarium* wilt.

### *3.5.1. Control*

In the uninoculated control, 100% healthy plant growth was observed with no visible disease symptoms. Similarly, the culture control treatments inoculated with BSUC-16 and BSUCM-24 alone exhibited 100% plant survival and healthy growth with 0% disease severity, indicating the non-pathogenic and plant-compatible nature of the bacterial strains. In contrast, the infected control inoculated only with FOC showed severe wilt symptoms with 93.3% plant mortality and a DSI of 94.67%, confirming the pathogenic potential of FOC under pot conditions (Fig. 7).

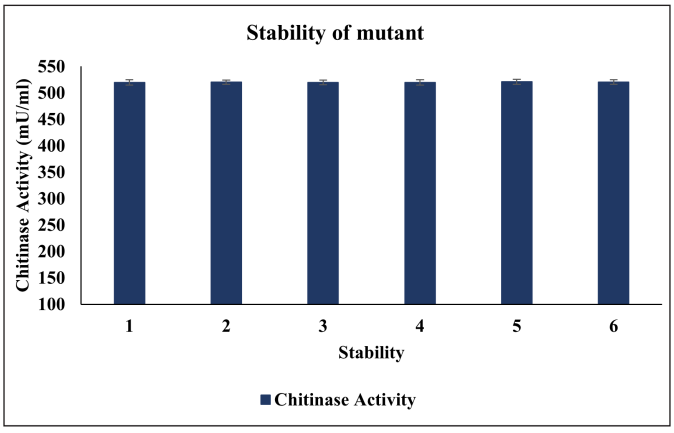



**Figure 3.** Stability of chitinase producing capability of selected mutant BSUCM-24.

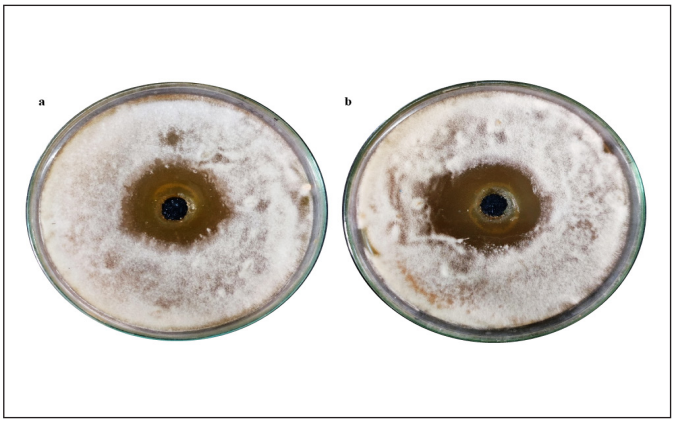



**Figure 4.** Antifungal activity of (a) Parent (BSUC-16) and (b) Mutant (BSUCM-24) against live fungal mycelia.

### *3.5.2. Biocontrol treatment results*

In the biocontrol treatments, both BSUC-16 and the EMS mutant strain BSUCM-24 effectively suppressed *Fusarium* wilt disease caused by FOC. The BSUC-16 + FOC treatment resulted in 53.3% surviving/non-wilted plants, whereas BSUCM-24 + FOC resulted in a substantially higher proportion of surviving/non-wilted plants (93.3%) compared with the infected control (Fig. 7a). The infected control exhibited a disease severity index of 94.67%, whereas treatment with the parental strain BSUC-16 + FOC reduced the disease severity index to 33.33%. Notably, treatment with the mutant strain BSUCM-24 + FOC resulted in a further reduction in disease severity, with a disease severity index of only 2.67%, indicating greater disease suppression by the mutant strain than by the parental strain (Fig. 7b). The biocontrol indices of BSUC-16 and BSUCM-24 were recorded as 64.79% and 97.18%, respectively, indicating enhanced antagonistic efficacy of the mutant strain against FOC (Fig. 7c). In addition to disease suppression, both strains promoted chickpea growth under pathogen stress conditions. BSUC-16 enhanced plant growth by 23%, while BSUCM-24 promoted growth by 34.5% compared to the uninoculated control, demonstrating the dual role of these chitinolytic bacterial strains in disease management and plant growth promotion (Fig. 7d). Chitinolytic bacteria have been widely reported as effective biocontrol agents against *Fusarium oxysporum*, and their chitinase-producing ability has been associated with reduced disease incidence and improved plant growth under pot conditions [14–16]. These results clearly indicate the superior biocontrol potential of the mutant strain BSUCM-24 against FOC, highlighting its association with reduced *Fusarium* wilt incidence in chickpea plants.

The chemical pesticide treatment effectively controlled *Fusarium* wilt and markedly reduced disease incidence; however, it did not exhibit any significant additional plant growth-promoting effect compared to the bacterial treatments. The enhanced performance of the chitinolytic bacterial strains may be associated with their ability to sustainably produce chitinase and other antagonistic metabolites in response to the phytopathogen FOC. This may contribute to prolonged disease suppression along with additional plant growth-promoting benefits. In contrast, chemical pesticides primarily suppress the pathogen without contributing to sustained enzymatic activity or plant growth promotion.

The present study demonstrated that the mutant strain BSUCM-24 exhibited enhanced chitinase production along with improved antifungal and biocontrol activity against *F. oxysporum*. However, the observed enhancement in disease suppression cannot be attributed exclusively to increased chitinase production, as random mutagenesis may alter multiple physiological and metabolic traits simultaneously. Therefore, the current findings indicate an association between enhanced

**Table 1.** Percent infected chickpea seeds with different dose of FOC.

| Inoculum density (conidia/g soil) | Pots (n) | Plants/pot | Total plants | Diseased plants | Disease incidence (%) |
|---|---|---|---|---|---|
| $10^1$ | 3 | 5 | 15 | 0 | 0.00 |
| $10^2$ | 3 | 5 | 15 | 0 | 0.00 |
| $10^3$ | 3 | 5 | 15 | 1 | 6.67 |
| $10^4$ | 3 | 5 | 15 | 3 | 20.00 |
| $10^5$ | 3 | 5 | 15 | 8 | 53.33 |
| $10^6$ | 3 | 5 | 15 | 13 | 86.67 |

**Table S1.** Transformation of disease incidence for graphical presentation and regression analysis.

| Inoculum density (conidia/g soil) | Disease incidence (%) | Disease proportion (X) | $\log_e (1/(1-X))$ | $\text{Log}_{10}$ [$\log_e(1/(1-X))$] |
|---|---|---|---|---|
| $10^1$ | 0.00 | 0.000 | – | Undefined |
| $10^2$ | 0.00 | 0.000 | – | Undefined |
| $10^3$ | 6.67 | 0.0667 | 0.06899 | −1.1612 |
| $10^4$ | 20.00 | 0.2000 | 0.22314 | −0.6515 |
| $10^5$ | 53.33 | 0.5333 | 0.76214 | −0.11797 |
| $10^6$ | 86.67 | 0.8667 | 2.01490 | 0.3043 |

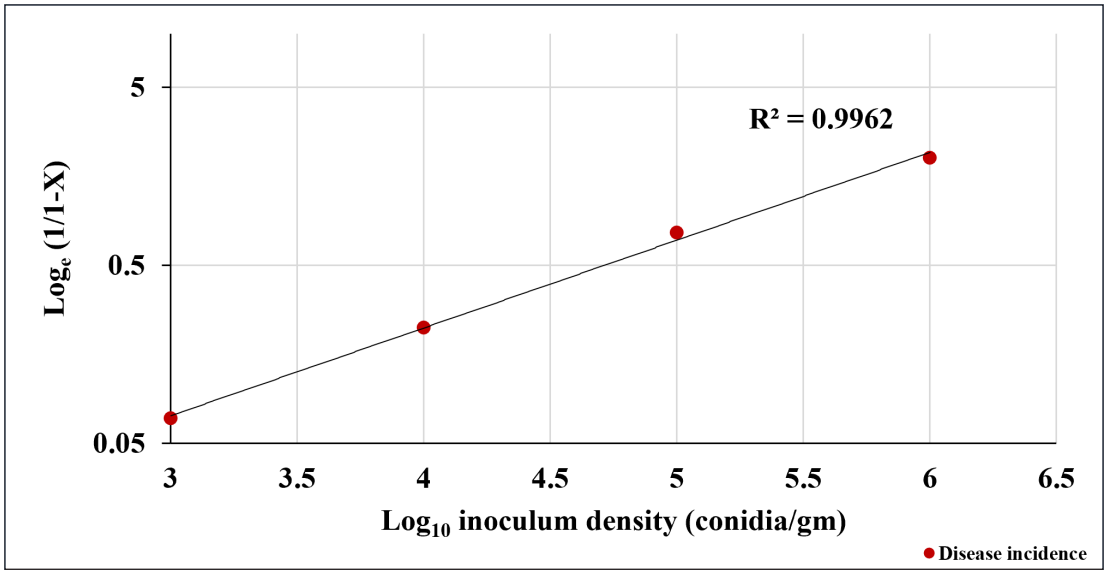



**Figure 5.** Relationship between $\log_{10}$ inoculum density (conidia/g soil) of FOC and transformed disease incidence [$\log_e (1/(1-X))$] in chickpea plants.

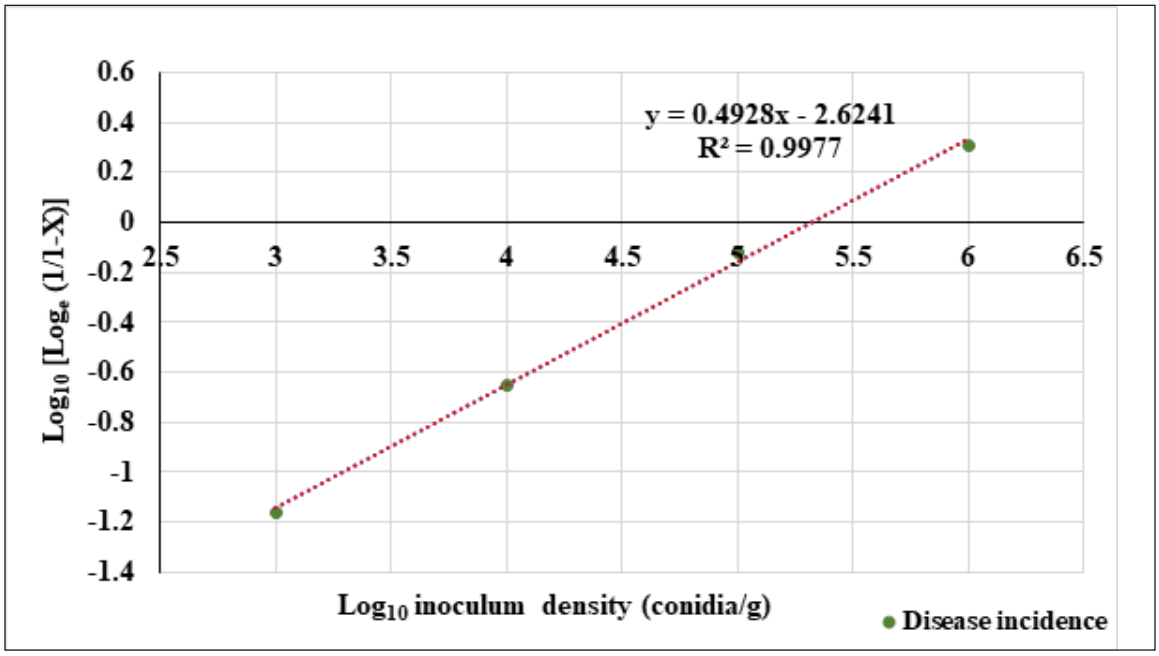



**Figure S1.** Regression plot showing the relationship between $\log_{10}$ inoculum density and $\log_{10}[\log_e(1/(1-X))]$ transformed disease incidence for estimation of the $ID_{50}$ of FOC in chickpea. The fitted regression equation was y = 0.4928x − 2.6241, with a coefficient of determination ($R^2$) of 0.9977.

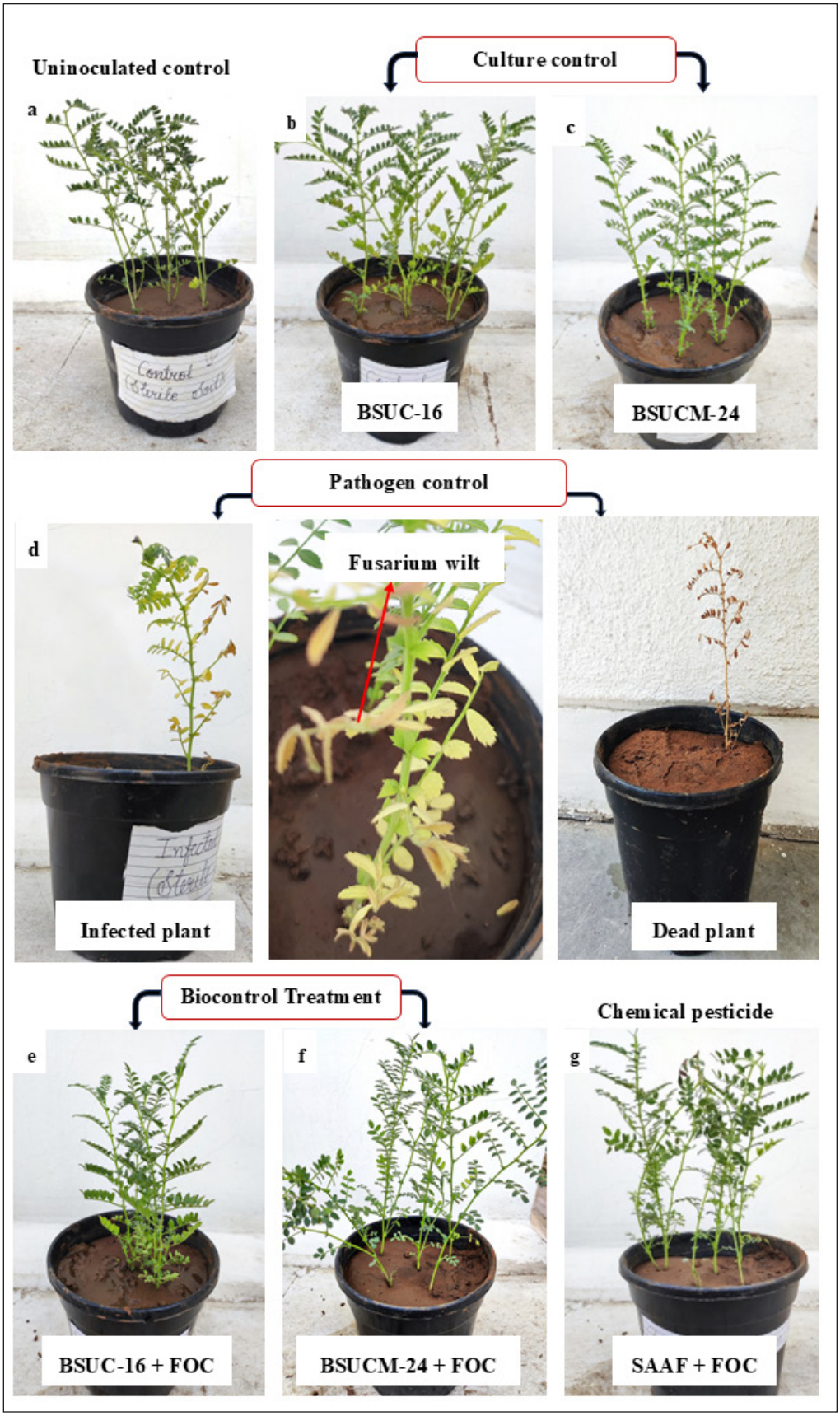



**Figure 6.** Chickpea pot assay results showing the biocontrol potential of *S. maltophilia* BSUC-16 and its mutant BSUCM-24 against FOC. (a) Uninoculated control, (b) Chickpea plants treated with BSUC-16, (c) Chickpea plants treated with BSUCM-24, (d) FOC-infected plants, (e) FOC-infected plants treated with BSUC-16, (f) FOC-infected plants treated with BSUCM-24, (g) FOC-infected plants treated with chemical pesticide (SAAF)

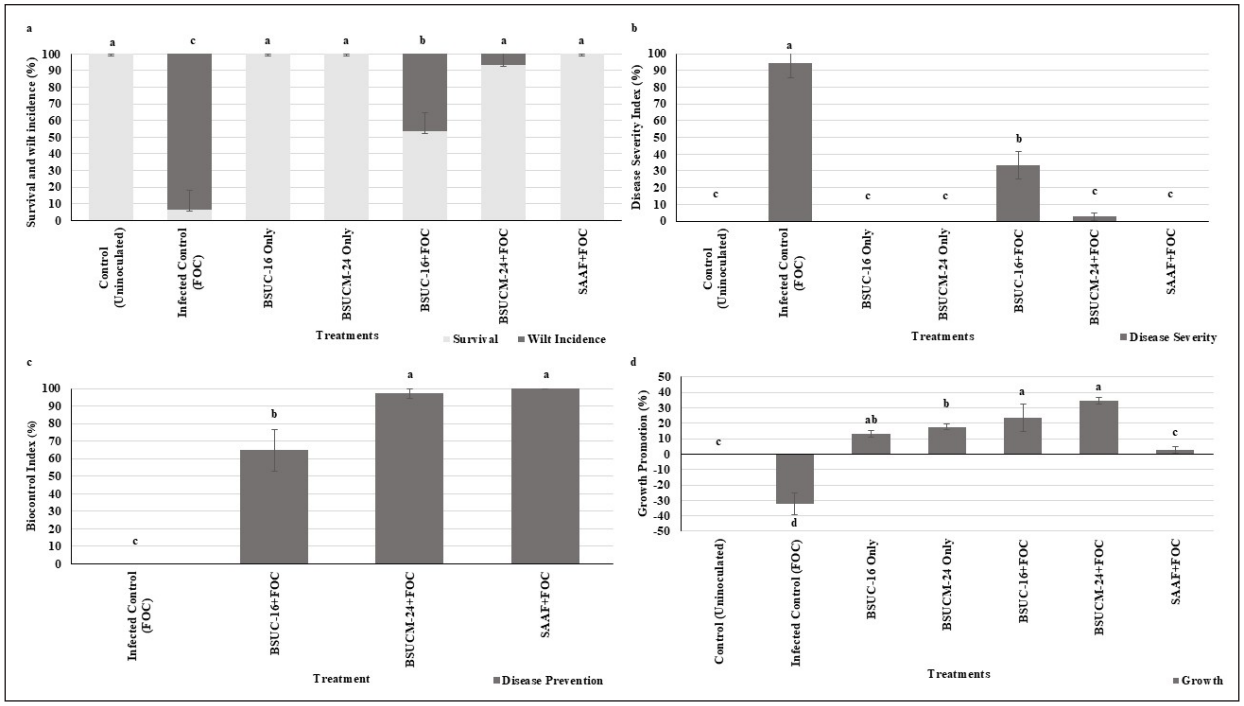



**Figure 7.** Plant growth parameters determined on the basis of pot assay results: (a) Survival and wilt incidence, (b) Disease severity, (c) Biocontrol index, and (d) Growth promotion. Bars represent mean ± SD of three biological replicates ($n = 3$). Different lowercase letters above the bars indicate significant differences among treatments according to Tukey's HSD test at $p < 0.05$. Bars sharing the same letter are not significantly different.

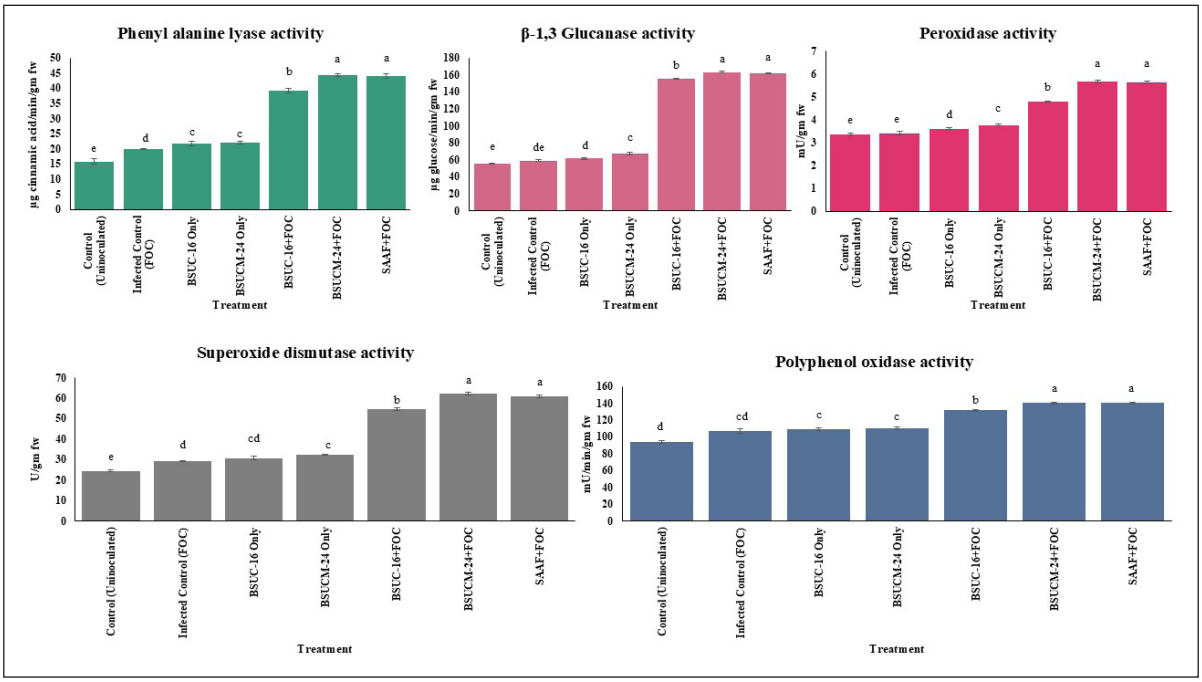



**Figure 8.** Defense related enzyme activity in infected and biocontrol-treated chickpea plants. Bars represent mean ± SD of three biological replicates ($n = 3$). Different lowercase letters above the bars indicate significant differences among treatments according to Tukey's HSD test at $p < 0.05$. Bars sharing the same letter are not significantly different.

chitinolytic activity and improved biocontrol performance rather than a definitive causal relationship. Further molecular and mechanistic studies are required to elucidate the specific factors contributing to the enhanced biocontrol efficacy of BSUCM-24.

### 3.6. Defense-Related Enzyme Analysis

Defense-related enzyme analyses, including PAL, β-1,3-glucanase, POX, SOD, and PPO activities, were performed to evaluate the induction of plant defense responses. These enzymes are key components of the

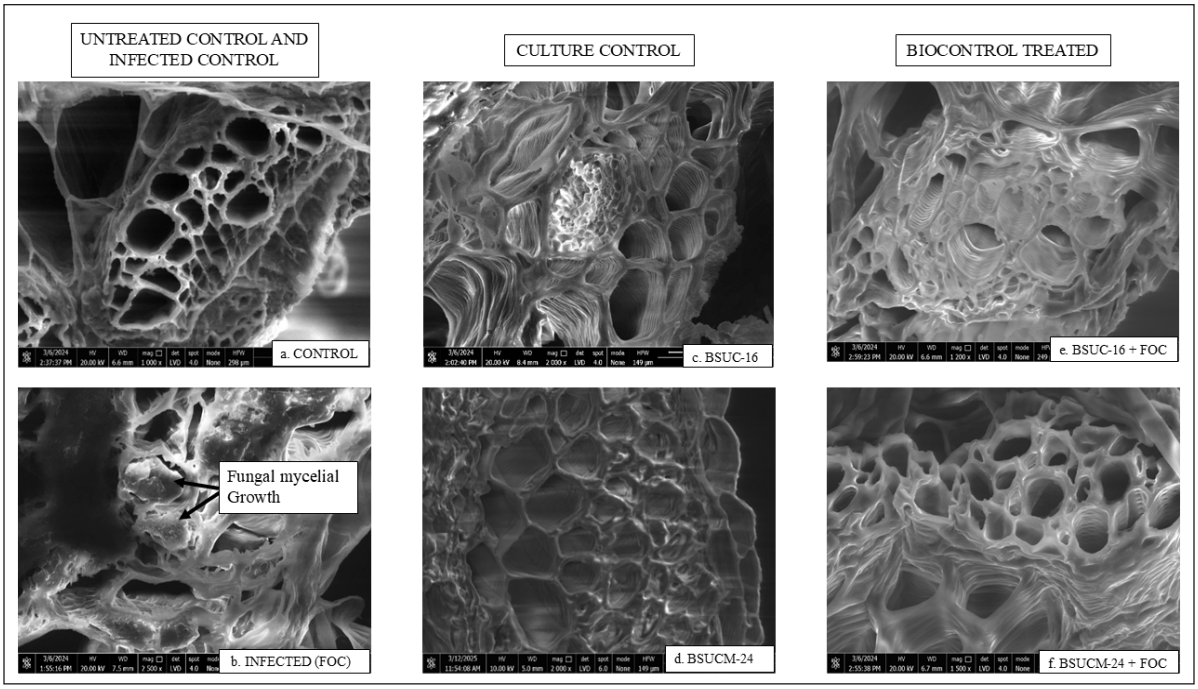



**Figure 9.** SEM analysis of infected and treated chickpea plant stems.

**Table 2.** Inhibition-zone diameters of BSUC-16 and BSUCM-24 against the tested antibiotics.

| Antibiotic | Zone of inhibition (mm) | |
|---|---|---|
| | BSUC-16 | BSUCM-24 |
| Ceftriaxone | 29 | 28 |
| Chloramphenicol | 19 | 20 |
| Clindamycin | 0 | 0 |
| Tetracycline | 20 | 20 |
| Erythromycin | 15 | 20 |
| Vancomycin | 10 | 22 |

plant's defense system and are typically upregulated during pathogen attack. PAL is involved in the phenylpropanoid pathway leading to the synthesis of phenolics and lignin, which strengthen plant cell walls. Several studies have reported an increase in PAL activity following pathogen infection, suggesting its important role in the activation of plant defense responses [17–19 ]. β-1,3-glucanase contributes to the degradation of fungal cell walls. The activity of β-1,3-glucanase has been reported to increase during pathogen infection, indicating its role in plant defense responses [20]. POX and PPO are associated with lignification and oxidative defense reactions, which can contribute to limiting pathogen spread. Previous studies have reported increased POX and PPO activities following pathogen infection, suggesting their important roles in plant defense mechanisms [12,21–24 ]. SOD plays a crucial role in scavenging reactive oxygen species, thereby protecting plant cells from oxidative damage. Bharathi reported the elevated levels of SOD in castor in resistant cultivars during pathogen infection [25].

All biocontrol treatments significantly enhanced the activities of defense-related enzymes in chickpea plants, as determined by Tukey's HSD test using mean values of three biological replicates ($n = 3$), with enzyme activities expressed per gram fresh weight (fw) (Fig. 8). The highest PAL activity (µg cinnamic acid/minute/g fw) was observed in infected plants treated with the mutant strain BSUCM-24 (M = 44.24), followed by BSUC-16 (M = 39.14), whereas considerably lower activities were recorded in the uninoculated control (M = 15.81) and infected control (M = 20.07). Similarly, β-1,3-glucanase activity (µg glucose/minute/g fw) was markedly elevated in the BSUCM-24 treated plants (M = 163.11), followed by BSUC-16 treatment (M = 155.54), compared to the uninoculated control (M = 55.62) and infected control (M = 59.12). POX activity (mU/g fw) was also significantly higher in BSUCM-24 treated plants (M = 5.67), followed by BSUC-16 treatment (M = 4.78), whereas lower activities were observed in the uninoculated control (M = 3.35) and infected control (M = 3.41). Likewise, SOD activity (U/g fw) was highest in infected plants treated with BSUCM-24 (M = 62.34), followed by BSUC-16 (M = 54.49), in comparison to the uninoculated control (M = 24.47) and infected control (M = 29.26). PPO (mU/minute/g fw) activity was also substantially enhanced in BSUCM-24 treated plants (M = 140.17), followed by BSUC-16 treatment (M = 131.64), compared to the uninoculated control (M = 94.27) and infected control (M = 106.92). The chemical pesticide treatment produced enzyme activities comparable to those observed with the mutant strain BSUCM-24, indicating strong induction of host defense responses. However, the biocontrol treatments additionally offer the advantage of sustainable biological disease suppression along with plant growth-promoting potential.

The enhanced activity of these enzymes in treated plants indicates that the biocontrol agents stimulated plant defense mechanisms, likely through ISR, thereby improving the plant's ability to resist pathogen infection. Our results demonstrated significantly elevated levels of defense related enzymes in chickpea plants treated with the biocontrol agents (BSUC-16 and BSUCM-24) compared to pathogen FOC infected plants. The maximum increase was observed in plants treated with the mutant strain BSUCM-24, indicating its enhanced ability to induce plant defense responses and confer improved resistance against pathogen infection.

### 3.7. SEM Analysis of Infected and Treated Chickpea Plants

SEM analysis of chickpea stem tissues further supported the protective association of the parent and mutant strains of *S. maltophilia* against FOC. In infected control plants where the plants were infected with FOC, the stem sections exhibited clear signs of fungal hyphae colonization within vascular tissues, along with xylem blockage, cellular disintegration, and tissue collapse, which are characteristic symptoms of *Fusarium* wilt. In contrast, stems from plants treated with parent BSUC-16 and mutant strain BSUCM-24 in presence of FOC showed well-preserved vascular integrity. Reduction in fungal structures within the xylem and surrounding tissues was observed, while the overall cellular architecture remained intact, suggesting reduced pathogen-induced damage and enhanced plant defense responses (Fig. 9). These results suggest that BSUC-16 and BSUCM-24 were associated with reduced systemic colonization and pathogen-induced tissue damage.

### 3.8.Antibiotic Response Profiling of BSUC-16 and BSUCM-24

In view of the reported opportunistic pathogenicity of *S. maltophilia* in clinical settings, antibiotic sensitivity profiling of BSUC-16 and BSUCM-24 was performed as a preliminary biosafety assessment. Both strains were found to be sensitive toward chloramphenicol, ceftriaxone, vancomycin, erythromycin, and tetracycline. The zones of inhibition (mm) exhibited by BSUC-16 and BSUCM-24 against all tested antibiotics are presented in Table 2. The results are reported descriptively because validated interpretive criteria specific to *S. maltophilia* were not applied. However, further biosafety assessments are necessary to establish their suitability for agricultural application and confirm their safety for humans, animals, and the environment.

## 4. CONCLUSION AND FUTURE PROSPECTS

In the present study, a mutant strain of *S. maltophilia* (BSUCM-24) was successfully developed through mutagenesis, exhibiting significantly enhanced chitinase activity (520.16 ± 2.38 mU/ml) compared to the parental strain (BSUC-16). The mutant also demonstrated enhanced antifungal activity against live FOC mycelia, indicating improved potential for suppressing the phytopathogen. The $ID_{50}$ of FOC was determined to be approximately $10^5$ conidia/g of soil, which was used to establish effective disease incidence in subsequent pot assays. Evaluation under pot conditions confirmed the biocontrol potential of both strains, with BSUCM-24 showing increased *Fusarium* wilt disease protection in chickpea plants. Plants treated with the mutant strain BSUCM-24 exhibited enhanced growth performance along with higher survival rates, and an improved biocontrol index compared to other treatments with reduced disease severity. Furthermore, defense-related enzyme analysis revealed elevated levels of defense-related enzymes, indicating enhanced plant defense responses associated with BSUCM-24 treatment. Overall, the results suggest an association between enhanced chitinolytic activity of BSUCM-24 and improved antifungal and biocontrol potential against *Fusarium* wilt in chickpea. However, since random mutagenesis may alter multiple physiological traits simultaneously, the enhanced biocontrol efficacy observed in BSUCM-24 cannot be attributed exclusively to increased chitinase production alone. Further mechanistic and molecular studies are required to elucidate the specific factors contributing to its improved biocontrol performance. This highlights its promising application as a sustainable and eco-friendly alternative to chemical pesticides in agricultural disease management.

Future studies should focus on the purification and characterization of chitinase from the mutant strain BSUCM-24 to elucidate its functional properties. Additionally, efforts may be directed toward the development of a stable and efficient bioformulation to improve field applicability and shelf life of the biocontrol agent. Further field trials are required to validate its biocontrol efficacy under natural environmental conditions. Successful field evaluation could facilitate the translation of this strain into a commercially viable product. It may further pave the way for its potential patenting and large-scale application as an eco-friendly biocontrol agent for the management of *Fusarium* wilt in chickpea. However, according to several reports, *S. maltophilia* has been recognized in clinical settings as an opportunistic human-associated bacterium. Therefore, careful consideration of biosafety evaluation and regulatory assessment are essential prior to commercialization and large-scale field deployment, despite its promising biocontrol potential.

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## 7. CONFLICTS OF INTEREST

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

## 8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

## 9. AUTHOR 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 an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## 10. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

## 11. DISCLOSURE OF USING AI TOOLS

During the preparation of this manuscript, the authors used Quillbot (QuillBot, Inc., USA) to assist with language editing, grammatical correction, and improvement of clarity and readability. The tool was not used to generate or analyses data, perform statistical analyses, formulate scientific conclusions, or replace the authors' independent interpretation of the results. All AI-assisted content was reviewed and verified by the authors, who take full responsibility for the accuracy, integrity, and final content of the manuscript.

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