Food Control 168 (2025) 110929

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Exploration of antimicrobial activities and mechanisms of biocontrol agent *Serratia nematodiphila* BC-SKRU-1 against *Penicillium digitatum* in tangerine fruit

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ARTICLE INFO

Keywords:
Biological control
Serratia nematodiphila
Penicillium digitatum
Mechanism of action
Membrane integrity

ABSTRACT

The isolate BC-SKRU-1, identified as *Serratia nematodiphila* from fungal culture contamination, was investigated for its biocontrol potential against *Penicillium digitatum in vitro* and on tangerine fruit, with a focus on its mechanisms of action. *In vitro* tests on PDA plates revealed BC-SKRU-1's broad-spectrum antifungal activity, achieving up to 74.68% inhibition against seven plant pathogenic fungi, including *P. digitatum* NKP4321. Bacterial culture filtrates from BC-SKRU-1 (BCF BC-SKRU-1) at 5–10% concentrations inhibited NKP4321 mycelial growth in PDB medium (78.41–83.68%) more effectively than in PDA medium (46.20–72.61%), with complete suppression at 15% (v/v) in both media. BCF BC-SKRU-1's efficacy was comparable to chemical fungicides such as propiconazole®, prochloraz®, and mancozeb®. Notably, BCF BC-SKRU-1 retained its antifungal activity after dilution (1/1000), autoclaving (at 121 °C), and storage (at −20 °C). *In vivo* evaluations on tangerine fruit showed significant reduction in the severity of postharvest green mold disease with BC-SKRU-1 treatments, especially at a concentration of 10^8 CFU mL^{-1}. Both preventive and curative applications were effective, with curative treatments being more successful. Mechanistic studies indicated that BCF BC-SKRU-1 reduces intracellular ergosterol content, compromises plasma membrane integrity, and attenuates antioxidant defense activities (SOD, CAT, GSH, GSSG, and GSH/GSSG ratio). These findings highlight BC-SKRU-1 and its metabolites as promising biocontrol agents against green mold in tangerine fruits and provide insights into their antifungal mechanisms.

1. Introduction

Citrus fruits, a significant source of nutrients like vitamin C, are crucial to both fruit-dependent industries worldwide (Li et al., 2024) and human health (Liu et al., 2022; Lu et al., 2023; Peer et al., 2023). Despite their nutritional benefits, the citrus industry faces major challenges from postharvest diseases, notably caused by pathogens such as *Penicillium digitatum*, *P. italicum*, and *Colletotrichum gloeosporioides*. These diseases occur during picking, packing, storage, and transportation (Ferreira et al., 2020) and are a significant concern, especially *P. digitatum*, which causes green mold disease and is the most prevalent and severe cause of postharvest decay in citrus fruits (Cheng et al., 2020). Such issues lead to extensive global losses, accounting for up to 90% of all citrus postharvest losses (Cheng et al., 2020; Li et al., 2024).

Control of green mold disease in citrus fruits is currently managed through the application of chemical fungicides, including widely used agents such as prochloraz, thiabendazole, imazalil, pyrimethanil, and fludioxonil, during storage (Bhatta, 2022; Fadda et al., 2021; Sánchez-Torres & Gandía, 2022). However, the prolonged and frequent use of these fungicides can lead to resistance in target pathogens, potentially resulting in diminished control efficacy or complete failure (Oiki et al., 2022; Zhong et al., 2021). Moreover, chemical fungicides pose significant health, environmental, and food safety risks (Koch et al., 2018; Patle et al., 2018; Zhong et al., 2021). Therefore, identifying viable alternatives is crucial for effectively managing green mold disease, reducing dependency on chemical fungicides, and mitigating their

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https://doi.org/10.1016/j.foodcont.2024.110929
Received 6 June 2024; Received in revised form 22 September 2024; Accepted 29 September 2024
Available online 1 October 2024

associated risks to human health and the environment.

Biological control employing antagonistic microorganisms stands out as an effective, safe, and eco-friendly strategy for managing various plant diseases. Extensive research has focused on *Bacillus* spp. (Ferreira et al., 2023; Khadiri et al., 2023; Li et al., 2024; Liu et al., 2023), *Pseudomonas* spp. (Ferreira et al., 2023; Hu et al., 2021; Wang et al., 2021), *Streptomyces* spp. (Boukaew et al., 2020; Tian et al., 2024), *Paenibacillus* spp. (Lai et al., 2012), *Aureobasidium* spp. (Liu et al., 2007), *Lactobacillus* spp. (Jahantigh et al., 2023), *Serratia* spp. (Firdu et al., 2022; Khoa et al., 2016; Soenens & Imperial, 2020), and yeasts such as *Wickerhamomyces* spp., *Metschnikowia* spp., *Aureobasidium* spp., and *Saccharomyces* spp. (Parafati et al., 2017), *Nodulisporium* spp. (Yeh et al., 2021), *Clavispora* spp. (Pereyra et al., 2022), *Rhodotorula* spp. (Ahima et al., 2019a, 2019b), revealing their potential in combating plant pathogens. While the concept of utilizing microorganisms for biological control has been a longstanding proposition (Erdogan & Benlioglu, 2010; Mizumoto et al., 2007), the predominant biocontrol agents demonstrating effectiveness against green mold disease in citrus fruits are *Bacillus* sp. and yeasts. Therefore, there is an ongoing imperative to further investigate and identify additional advantageous microorganisms to effectively counteract the occurrence of green mold disease in citrus fruits.

The genus *Serratia*, part of the Enterobacteriaceae family, comprises Gram-negative, facultatively anaerobic bacteria. These bacteria are notable for their chemoorganotrophic nature and the production of the red pigment prodigiosin. They thrive in diverse natural habitats, including soil, water, and plant surfaces. *Serratia* is celebrated for its robust secondary metabolism and its ability to synthesize a broad range of natural bioactive compounds (Kai et al., 2007; Matilla et al., 2015; Domik et al., 2016a, 2016b). Many species within this genus are identified as opportunistic human pathogens (Grimont & Grimont, 2009). However, *S. nematodiphila* is valued for its use as a biocontrol agent, bio-stimulant, and bio-fertilizer (Dastager et al., 2011; Kang et al., 2015; Zhang et al., 2009). Research conducted by Khoa et al. (2016) demonstrated that *S. nematodiphila* CT-78 could suppress bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. Furthermore, Firdu et al. (2022) revealed the inhibitory effects of *S. nematodiphila* B2 on the germination of *Colletotrichum siamense* and *Alternaria alternata* conidia, attributing this to chitinolytic, glucanolytic, and cellulolytic mechanisms, alongside prodigiosin production. Additionally, Bahadou et al. (2018) reported over 74% suppression of fire blight in apple cultivars caused by *Erwinia amylovora* using *S. nematodiphila* HC4. Gondil et al. (2017) found that prodigiosin isolated from *S. nematodiphila* RL2 exhibited antimicrobial activity against several bacterial pathogens, including *Listeria* sp., *Pseudomonas* sp., *Yersinia* sp., and *Shigella* sp. Notably, the application of *S. nematodiphila* in these contexts is facilitated by the absence of officially published documentation concerning its pathogenic potential to humans and its environmental impact.

As previously noted, information regarding the antifungal capabilities of the *Serratia* genus in combatting post-harvest green mold diseases in citrus fruit caused by *P. digitatum* is currently lacking. This study seeks to address this gap by examining the impact of *S. nematodiphila* BC-SKRU-1 on the control of *P. digitatum* in tangerine fruit. Initially discovered as a contaminant in a fungal culture plate, this bacterium exhibited robust antagonism against the mycelial growth of various fungi. Consequently, our investigation delves into the potential of BC-SKRU-1 as a biocontrol agent for gray mold, undertaking pertinent research in biological control. The exploration of BC-SKRU-1's effectiveness in managing post-harvest green mold diseases in citrus fruit, coupled with a comprehensive understanding of the underlying inhibition mechanisms, holds significant value in advancing biocontrol strategies. The main objectives of this study are to: (i) evaluate the biocontrol efficacy of BC-SKRU-1 against *P. digitatum* both *in vitro* and in tangerine fruit, and (ii) elucidate the inhibitory mechanisms of BC-SKRU-1 against this pathogen.

2. Material and methods

2.1. Sources of materials

2.1.1. Microorganisms and inoculum preparation

The antagonistic strain BC-SKRU-1 was inadvertently obtained as a contaminant from a fungal culture plate. Following its isolation, the strain was cultured on nutrient agar medium (NA) (28 g L^{-1}; Himedia Laboratories Pvt. Lts.) at 30 °C for 24 h. Subsequently, for the preparation of a bacterial suspension, the strain was inoculated onto 50 mL of nutrient broth medium (NB) (13 g L^{-1}; Himedia Laboratories Pvt. Lts.) and incubated at 30 °C for 24 h. After cultivation, the bacterial suspension was adjusted with sterilized water to achieve a final concentration of 10^5–10^8 CFU mL^{-1} using the McFarland standard.

The green mold pathogen *Penicillium digitatum* NKP4321 was obtained from the Center of Excellence in Microbial Diversity and Sustainable Utilization at Chiang Mai University. The rice pathogen *Rhizoctonia solani* (AG-1 IA) was collected from rice plants in the field and sourced from the Phatthalung Rice Research Center of Thailand. The anthracnose pathogen *Colletotrichum gloeosporioides* PSU-03 was isolated from postharvest anthracnose-infected chili fruit, displaying aggressive behavior on chili fruit (Boukaew et al., 2021). Aflatoxin-producing fungi, namely *Aspergillus parasiticus* TISTR 3276 and *A. flavus* PSRDC-4, were acquired from the Microbiology Laboratory of the International Program in Biotechnology at Prince of Songkla University, Thailand, and the Phitsanulok Seed Research and Development Center, Department of Agriculture, Ministry of Agriculture and Cooperative, Thailand, respectively. *Curvularia oryzae*, the causal agent of severe oil palm leaf spot disease, was sourced from the Suratthani Oil Palm Research Center, operated under the Department of Agriculture, Ministry of Agriculture and Cooperative in Thailand. *Phomopsis* sp., causing durian leaf spot, was provided by Assist. Prof. Dr. Maneerat Koohapitagtam. They were cultured on potato dextrose agar (PDA) at 30 °C for a period ranging from 3 to 10 days, depending on the rate of fungal growth.

For antifungal suppression tests, mycelial plugs of *R. solani*, *Phomopsis* sp., *C. oryzae*, and *C. gloeosporioides* PSU-03 were incubated on PDA at 30 °C for 3–10 days. In parallel, spore suspensions of *P. digitatum* NKP4321, *A. parasiticus* TISTR 3276, and *A. flavus* PSRDC-4 were cultured on PDA at the same temperature for 5 days. After this period, spores from NKP4321, TISTR 3276, and PSRDC-4 were harvested by scraping them off the agar and were then suspended in distilled water. The spore concentrations were adjusted using a hemocytometer to achieve the required levels for subsequent experiments.

2.1.2. Commercial chemical fungicides

For the meticulous preparation of chemical fungicides, six distinct formulations—prochloraz® (45% (w/v)), metalaxyl® (25% (w/v)), azoxystrobin® (25% (w/v)), propiconazole® (25% (w/v)), thiram® (80% (w/w)), and mancozeb® (80% (WP)) —were precisely crafted in accordance with the recommended guidelines provided by their respective companies. Prochloraz® was synthesized by combining 30 mL with 20 L of sterile distilled water, while propiconazole® and azoxystrobin® were expertly formulated using 20 mL in 20 L of sterile distilled water. Thiram® was meticulously prepared with the addition of 20 g to 20 L of sterile distilled water, and metalaxyl® was carefully concocted by introducing 40 g to 20 L of sterile distilled water. Furthermore, Mancozeb® was skillfully crafted by incorporating 70 g into 20 L of sterile distilled water.

2.1.3. Tangerine fruits and fruit preparation

Tangerine fruits (*Citrus tangerina* Tanaka) cv. Sai Num Phung were purchased from a supermarket in Hat Yai, Songkhla province, Thailand. Fruits, chosen for their absence of injury and uniform size, were washed with tap water and surface disinfected using 2% (v/v) commercial sodium hypochlorite for 3 min. After disinfection, the tangerine fruits were

rinsed with sterile distilled water and dried in a laminar flow cabinet.

2.2. Identification of BC-SKRU-1

The identification of BC-SKRU-1 involved a comprehensive analysis encompassing morphological, biochemical, and molecular aspects. Initial characterization relied on the observation of colony characteristics, defining cultural and morphological properties. Subsequent biochemical analyses were conducted, with results compared against established standards (Holt et al., 1994).

For the molecular study, the bacteria were cultivated in 5 mL of nutrient broth (NB) in 18 × 180 mm test tubes, subjected to shaking at 150 rpm on an orbital shaker in the dark. After 48 h, bacterial cells were collected via centrifugation at 11,000 rpm and underwent a triple wash with sterile distilled water. Genomic DNA extraction utilized the DNA Extraction Mini Kit (FAVORGEN, Taiwan), following the manufacturer's protocol. The amplification of the 16S ribosomal RNA (rRNA) gene was achieved through polymerase chain reactions (PCR) using 27F/1492R primers (Lane, 1991; Stackebrandt & Liesack, 1993). PCR thermal conditions included 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min on a peqSTAR thermal cycler (PEQLAB Ltd., UK). The PCR product underwent verification and purification using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). Sequencing was performed by the 1ST Base Company (Kembangan, Malaysia), and the obtained sequences were utilized for phylogenetic analysis. Sequence alignment was accomplished with MUSCLE (Edgar, 2004), manually adjusted using BioEdit v.6.0.7 (Hall, 2004). The phylogenetic tree construction employed maximum likelihood (ML) with RAxML v7.0.3, supported by 1000 bootstrap (BS) replications (Felsenstein, 1985; Stamatakis, 2006).

2.3. Determination of the antifungal capability of BC-SKRU-1

The inhibitory effect of the antagonistic BC-SKRU-1 on the mycelial growth of seven pathogenic fungi was assessed using a dual culture method. A loop of BC-SKRU-1 was streaked on both sides of a PDA plate. In the center of each plate, either a 5-mm-diameter mycelial plug of *R. solani*, *Phomopsis* sp., *C. oryzae*, and *C. gloeosporioides* PSU-03, or 10 μL of a 10^5 spores mL^{-1} suspension of *P. digitatum* NKP4321, *A. parasiticus* TISTR 3276, and *A. flavus* PSRDC-4, was inoculated. The plates were incubated at 30 °C for 3–10 days. For control plates, either a 5-mm mycelial plug or 10 μL of a 10^5 spores mL^{-1} suspension of the respective phytopathogenic fungus was inoculated at the center of a PDA plate without the antagonistic strain. After incubation, the radial growth of each fungal colony was measured. Each experimental setup was replicated three times. The percentage of mycelial growth inhibition by BC-SKRU-1 was calculated using the formula proposed by Parizi et al. (2012): Percent inhibition of growth = [(Control - Treated)/Control] × 100.

2.4. Suppression of mycelial growth of NKP4321 by BCF BC-SKRU-1

Bacterial culture filtrates were produced by cultivating BC-SKRU-1 under submerged conditions at 30 °C on a rotary shaker set at 150 rpm, in a 250 mL flask containing 100 mL of NB. After six days of incubation, the culture broth was centrifuged at 8880×*g* for 20 min to separate the cell-free supernatant from the pellet. The supernatant, referred to as BCF BC-SKRU-1, was then filtered through a 0.45 μm pore size filter to remove any remaining bacteria. These culture filtrates were subsequently used to assess antifungal activity against NKP4321 in both solid and liquid culture bioassays.

For the solid culture assays, PDA medium with varying water contents of 95%, 90%, 85%, 80%, and 75% (v/v) was prepared in separate conical flasks. Each medium was autoclaved at 121 °C for 15 min and allowed to cool to 60 °C. Subsequently, BCF BC-SKRU-1, in concentrations ranging from 5% to 25% (v/v), was added to each flask, mixed thoroughly, and then poured into sterile Petri dishes. Control plates containing PDA with sterile water were also prepared. A 10 μL aliquot of a 10^5 spores mL^{-1} suspension of NKP4321 was inoculated at the center of each plate, which was then incubated at 30 °C. After 7 days, the diameters of the colonies were measured, and the percentage inhibition of hyphal growth was calculated. Three replications were maintained for each assay.

For the liquid culture assays, PDB medium with varying water contents of 95%, 90%, 85%, 80%, and 75% (v/v) was prepared in separate conical flasks. Each medium was autoclaved at 121 °C for 15 min and then cooled to 60 °C. BCF BC-SKRU-1, ranging from 5% to 25% (v/v), was then added to each flask. Control flasks containing only PDB and sterile water were also prepared. A 10 μL aliquot containing 10^5 spores mL^{-1} of NKP4321 was introduced into each of the conical flasks. These were incubated at 30 °C on a rotary shaker set at 150 rpm. After 7 days, the mycelial mats were filtered, dried, and weighed to determine their mass (Li et al., 2011). The percentage inhibition of hyphal growth was calculated based on these measurements. Three replications were maintained for each assay.

2.5. Comparison of the suppression of mycelial growth of NKP4321 using BCF BC-SKRU-1 and six chemical fungicides

The effect of BCF BC-SKRU-1 and six chemical fungicides on the mycelial growth of NKP4321 was assayed in PDB medium. PDB medium with varying water content at 85% (v/v) and 99% (v/v) was prepared in separate conical flasks, autoclaved at 121 °C for 15 min, and allowed to cool to 60 °C. BCF BC-SKRU-1 (at 15% (v/v)) or each chemical fungicide (at 1% (v/v)) were added to each flask. The controls consisted of PDB conical flasks without BCF BC-SKRU-1 or chemical fungicides. A 10 μL aliquot of 10^5 spores mL^{-1} of NKP4321 was transferred into PDB conical flasks and incubated at 30 °C on a rotary shaker (at 150 rpm). After 7 days, mycelial mats were filtered, dried, and weighed. The percentage inhibition of hyphal growth was then calculated. Three replications were maintained for each assay.

2.6. Suppression of NKP4321 spores using BCF BC-SKRU-1

The effects of BCF BC-SKRU-1 on the spore germination of NKP4321 were evaluated under various conditions, including undiluted, 1/10 diluted, 1/100 diluted, 1/1000 diluted, autoclaved at 121 °C for 15 min, stored at 4 °C for 15 min, and stored at −20 °C for 15 min. For each test, 100 μL of BCF BC-SKRU-1 under one of these conditions and 100 μL of a 10^5 spores mL^{-1} suspension of the pathogen were added to glass tubes containing 1.8 mL of PDB medium. All tubes were then incubated on a rotary shaker at 150 rpm at 30 °C for 12 h. The germination was determined by randomly observing more than 150 spores under a microscope and counting the number of spores with germ tubes equaling or exceeding the length of the spore (Zadoks & Schein, 1979, p. 427). Each assay was replicated three times.

Equal volumes of NKP4321 spore suspensions were mixed with BCF BC-SKRU-1 under various conditions. Sixty seconds after mixing, 100 μL of each suspension was sampled and plated on PDA. The fungal colony-forming units (CFUs) were counted after 48 h of incubation at 30 °C. Each assay was replicated three times.

2.7. In vivo biocontrol assay of BC-SKRU-1 against NKP4321 in tangerine fruit

2.7.1. Effect of different concentrations of BC-SKRU-1 on NKP4321 in tangerine fruit

To prepare tangerine fruits (obtained from section 2.1.3) for infection, each fruit was punctured twice with a sterile needle (3 mm deep × 5 mm diameter), and the surrounding tissue was removed. Each wound was then inoculated with a spore suspension of NKP4321 (20 μL of 10^5

spores mL^{-1}). After allowing the fruits to air-dry, the wounds were further inoculated with 20 μL of live bacterial cells from BC-SKRU-1, adjusted to concentrations ranging from 10^5 to 10^8 CFU mL^{-1}. Additionally, 20 μL of sterile distilled water was used as a negative control for each fruit.

Infected tangerine fruits were placed in plastic containers with water-soaked paper at the bottom to maintain high relative humidity (90–95%) (Parafati et al., 2017; Perez et al., 2017; Zhang et al., 2021). After a 12-day incubation period, disease severity (DS) was determined as the average lesion diameter for each treatment. The percentage of biocontrol efficacy by BC-SKRU-1 was calculated using the formula: Biocontrol efficacy (%) = [(DS of control - DS of treated)/DS of control] × 100. The experiment involved three biological replicates, with each replicate comprising five fruits (Tian et al., 2020).

2.7.2. Comparison of the biocontrol efficacy of BC-SKRU-1 with six chemical fungicides against NKP4321 in tangerine fruit

The biocontrol efficacy of BC-SKRU-1 was compared with six chemical fungicides on wounded tangerine fruits. Each wound was first treated with a 20 μL spore suspension (10^5 spores mL^{-1}) of NKP4321. After allowing the fruits to air-dry, 20 μL of each antifungal treatment was applied to the respective wounds. The treatments included: (A) 10^8 CFU mL^{-1} of BC-SKRU-1; (B) BCF BC-SKRU-1; (C) Propiconazole®; (D) Prochloraz®; (E) Metalaxyl®; (F) Azoxystrobin®; (G) Thiram®; and (H) Mancozeb®. A control group received sterile distilled water on their wounds.

Infected tangerine fruits were placed in plastic containers with water-soaked paper at the bottom to maintain high relative humidity (90–95%) (Parafati et al., 2017; Perez et al., 2017; Zhang et al., 2021). The experimental procedures and disease severity were followed as described above for infected fruits. The experiment involved three replicates of antifungal agent application, with each replicate comprising five fruits (Tian et al., 2020).

2.8. Preventive and curative efficacy of BC-SKRU-1 against NKP4321 in tangerine fruit

To evaluate the preventive efficacy of live bacterial cells BC-SKRU-1 (10^8 CFU mL^{-1}) on tangerine fruits, each pruning wound was treated with 20 μL of the bacterial suspension. This was followed by the inoculation of the wound with 20 μL of a NKP4321 spore suspension (10^5 spores mL^{-1}) at time intervals of 0, 2, 4, or 6 h after applying the BC-SKRU-1 treatment. The control group received a treatment of sterile distilled water.

To investigate the curative potential of live bacterial cells BC-SKRU-1 (10^8 CFU mL^{-1}) against NKP4321 on tangerine fruits, the procedure was adjusted so that each pruning wound first received an inoculation of 20 μL of a NKP4321 spore suspension (10^5 spores mL^{-1}). Treatment with 20 μL of live bacterial cells BC-SKRU-1 followed the inoculation at intervals of 0, 2, 4, or 6 h. A control group was treated with sterile distilled water to provide a baseline for comparison.

The experimental procedures, including both preventive and curative effects on disease severity, were conducted as described previously for infected fruits. The experiment consisted of three biological replicates, with each replicate comprising five fruits (Tian et al., 2020).

2.9. Antifungal mode of action of BCF BC-SKRU-1 on NKP4321 cells

After introducing BCF BC-SKRU-1 at concentrations of 0% (control), 5%, and 10% (v/v) into PDB (as described in section 2.4), the resulting mycelial mats were analyzed to determine effects on plasma membrane ergosterol biosynthesis and both enzymatic and non-enzymatic defense systems. These mechanisms are further elaborated upon in subsequent sections.

2.9.1. Determination of ergosterol content in NKP4321 cells treated with BCF BC-SKRU-1

The ergosterol content in NKP4321 cells treated with BCF BC-SKRU-1 was quantified using the method established by Das et al. (2020), supplemented with additional procedural details from Tian et al. (2012). Fungal sample preparation for ergosterol analysis followed the protocol described by Boukaew et al. (2023). Each treatment was conducted in triplicate.

2.9.2. Effect of BCF BC-SKRU-1 on oxidative impairment in NKP4321 cells

After treatment with BCF BC-SKRU-1 at concentrations of 0% (control), 5%, and 10% (v/v), the biomass of NKP4321 was prepared for analysis of antioxidant defense activities. Cellular reactive oxygen species (ROS) were quantified using the method described by Keston and Brandt (1965). Catalase (CAT) activity was measured according to the protocol by Beers and Sizer (1952), while superoxide dismutase (SOD) levels were determined using the procedures established by Lowry et al. (1951) and Kostyuk and Potapovich (1989). Additionally, the analysis of glutathione in both its reduced (GSH) and oxidized (GSSG) forms, as well as the GSH/GSSG ratio, was conducted following the methodology set forth by Hissin and Hilf (1976). All assays were performed in accordance with the detailed procedures outlined by Das et al. (2020). Each treatment was conducted in triplicate.

2.9.3. Measurement of electrical conductivity

Cellular leakage was assessed using a modified protocol adapted from Lewis and Papavizas (1987). Initially, spores of NKP4321 were inoculated into 100 mL of PDB to achieve a concentration of 10^5 spores mL^{-1}. This culture was incubated at 30 °C on a rotary shaker at 150 rpm for 3 days. Post-incubation, the mycelia were collected, thoroughly rinsed with sterile distilled water, and 2.5 g (wet weight) of the rinsed mycelia were suspended in 25 mL of sterile distilled water with BCF BC-SKRU-1 at concentrations of 0% (control), 5%, and 10% (v/v). This suspension was then incubated on a rotary shaker at 30 °C for 0, 2, 4, 6, and 8 h, following modifications from Liu et al. (2017). After incubation, the mycelia were filtered through sterile cheesecloth. The resulting filtrates were then analyzed for electrolyte leakage using a conductivity meter (model EC 700, APENA), following the procedures optimized by Lee et al. (1998). Each measurement was conducted in triplicate.

2.10. Statistical analysis

All experiments were conducted in triplicate, and the data were analyzed for mean ± standard deviation using SPSS software. Significant variations among the data sets were assessed using ANOVA and Tukey's Honest Significant Difference (HSD) test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Characteristics and molecular identification of BC-SKRU-1

Colony characteristics of BC-SKRU-1 on NA agar after 48 h were observed as circular, milky white, small, entire, and shiny. Microscopic examination revealed Gram-negative, motile short rods measuring 0.8–1.36 × 0.6–0.7 μm. The strain tested negative for oxidase but positive for catalase. It exhibited sugar fermentation with acid production for maltose, sorbitol, glucose, sucrose, inulin, galactose, dextrin, and mannitol. Additionally, it showed positive results for biochemical tests including Voges-Proskauer, lysine decarboxylase, gelatin hydrolysis, and nitrite reductase, while tests for indole and methyl red were negative.

The 16S rRNA gene sequence, consisting of 1371 base pairs, from BC-SKRU-1 has been submitted to the GenBank database with the accession number PP267069. The phylogenetic analysis placed the bacterial strain SKRU-01 within the identical clade as *Serratia nematodiphila*, including

the type species (DZ0503SBS1), with robust support (bootstrap value of 100%). Consequently, the strain BC-SKRU-1 has been conclusively identified as *S. nematodiphila*, as illustrated in Fig. 1.

3.2. Antagonism of BC-SKRU-1 to various plant pathogenic fungi

To assess the antifungal capability of BC-SKRU-1, we conducted tests measuring its inhibitory effects on seven different fungal pathogens (*R. solani*, *Phomopsis* sp., *C. oryzae*, *A. flavus* PSRDC-4, *A. parasiticus* TISTR 3276, *C. gloeosporioides* PSU-03, *P. digitatum* NKP4321) and calculated the corresponding inhibition rates. Utilizing the dual-culture test, BC-SKRU-1 significantly ($p < 0.05$) inhibited the growth of fungal mycelia, with variations observed among the different pathogen species (Table 1). Notably, BC-SKRU-1 exhibited the highest antagonistic activity against *P. digitatum* NKP4321, resulting in an inhibition rate of 74.68%. Consistent inhibitory effects were observed against *Phomopsis* sp., *C. oryzae*, *A. flavus* PSRDC-4, *A. parasiticus* TISTR 3276, and *C. gloeosporioides* PSU-03 (approximately 69–71%). However, the inhibitory effect on *R. solani* was relatively weaker at 61.67%. Consequently, *P. digitatum* NKP4321 was selected for further testing due to its heightened susceptibility to BC-SKRU-1.

3.3. Suppression of mycelial growth of NKP4321 by BCF BC-SKRU-1 in solid and liquid cultures

The study thoroughly investigated the inhibitory effects of various concentrations (0–25% (v/v)) of BCF BC-SKRU-1 on the mycelial growth of NKP4321, as outlined in Table 2. Both solid and liquid culture were scrutinized, revealing a significant ($p < 0.05$) role of mycelial growth inhibition in mitigating NKP4321-induced disease in solid culture. The presence of different concentrations of BCF BC-SKRU-1 distinctly diminished the fungal colony diameter compared to corresponding controls. Specifically, concentrations ranging from 5 to 10% (v/v) resulted in 46.20–72.61% inhibition of the pathogen's radial growth, while complete growth inhibition (100%) was observed at 15% (v/v) of BCF BC-SKRU-1.

The inhibitory effect of BCF BC-SKRU-1 in liquid culture is confirmed and detailed in Table 2. The results in liquid culture mirrored those in solid culture, with diverse concentrations of BCF BC-SKRU-1 markedly reducing mycelial growth compared to respective controls. Notably, concentrations of 5–10% (v/v) yielded 78.41–83.68% inhibition of mycelial growth, surpassing the inhibition observed in solid culture

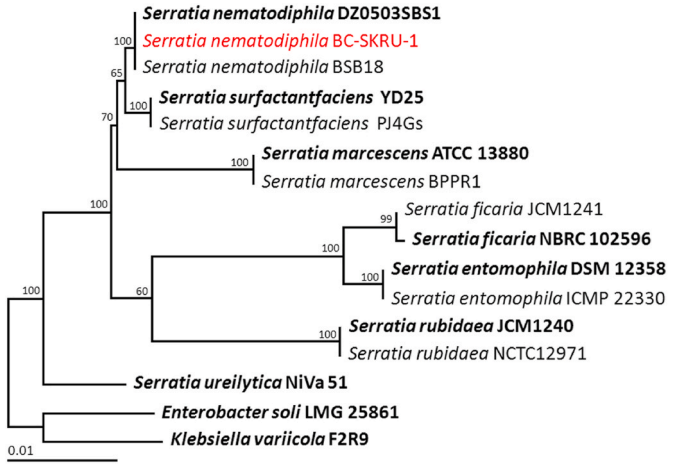
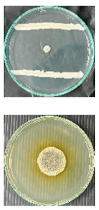
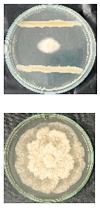
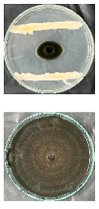
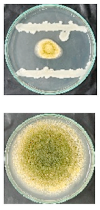
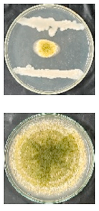


Fig. 1. Phylogenetic tree derived from maximum-likelihood analysis of 16S rRNA genes of 16 bacterial strains *Enterobacter soli* and *Klebsiella variicola* present as the outgroup. Numbers above branches represent bootstrap percentages. Scale bar represents 0.01 substitutions per nucleotide position. Strain obtained in this study is in red. Type species are in bold.

Table 1
Assessing the impact of *Serratia nematodiphila* BC-SKRU-1 on the growth of *Rhizoctonia solani*, *Phomopsis* sp., *Curvularia oryzae*, *Aspergillus flavus* PSRDC-4, *Aspergillus parasiticus* 3276, *Colletotrichum gloeosporioides* PSU-03, and *Penicillium digitatum* NKP4321 using a dual culture assay on PDA medium after incubation at 30 °C.

Parameters	*R. solani*		*Phomopsis* sp.		*C. oryzae*		*A. flavus*		*A. parasiticus*		*C. gloeosporioides*		*P. digitatum*	
	Control	Dual culture assay	Control	Dual culture assay	Control	Dual culture assay	Control	Dual culture assay	Control	Dual culture assay	Control	Dual culture assay	Control	Dual culture assay
Colony diameter (cm)	9.00 ± 0.0	3.45 ± 0.10	9.00 ± 0.0	2.75 ± 0.30	9.00 ± 0.0	2.63 ± 0.61	8.50 ± 0.0	2.57 ± 0.39	9.00 ± 0.0	2.58 ± 0.51	4.90 ± 0.66	1.45 ± 0.33	3.08 ± 0.10	0.78 ± 0.10
Inhibition of mycelial growth (%)	61.67^{c} ± 1.11		69.44^{b} ± 3.33		70.78^{b} ± 2.75		69.76^{b} ± 4.64		71.33^{b} ± 1.62		70.41^{b} ± 2.05		74.68^{a} ± 3.79	
Colony morphology and growth														

Note: The presented data represent the mean of three replicates ± standard deviation (SD). Values within the same row that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

Table 2
The mycelial growth of *Penicillium digitatum* NKP4321 in the PDA and PDB medium was assessed after 7 days of incubation at 30 °C, revealing distinct impacts of *Serratia nematodiphila* BC-SKRU-1 culture filtrates (BCF BC-SKRU-1) at concentrations ranging from 5 to 25% (v/v).

Parameters	In PDA medium						Parameters	In PDB medium					
	BCF BC-SKRU-1 concentration (% v/v)							BCF BC-SKRU-1 concentration (% v/v)					
	Control	5	10	15	20	25		Control	5	10	15	20	25
Colony diameter (cm)	3.03^{a} ±0.06	1.63^{b} ± 0.15	0.83^{c} ±0.06	0.0^{d} ± 0.0	0.0^{d} ± 0.0	0.0^{d} ± 0.0	Mycelial dry weight (mg/50 mL)	230.97^{a} ± 0.03	49.87^{b} ± 0.02	37.70^{bc} ± 0.05	0.0^{c} ±0.0	0.0^{c} ±0.0	0.0^{c} ±0.0
Inhibition of mycelial growth (%)	–	46.20	72.61	100	100	100	Inhibition of mycelial growth (%)	–	78.41	83.68	100	100	100

Note: The presented data represent the mean of three replicates ± standard deviation (SD). Values within the same row that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

(46.15–72.53%). However, at 15% (v/v) of BCF BC-SKRU-1, complete inhibition (100%) of pathogen growth was evident, consistent with the findings in solid culture.

3.4. Comparison of mycelial growth suppression of NKP4321 by BCF BC-SKRU-1 and six chemical fungicides

The comparison of BCF BC-SKRU-1 (15% (v/v)) with six chemical fungicides (prochloraz®, metalaxyl®, azoxystrobin®, propiconazole®, thiram®, and mancozeb®) at a concentration of 1% (v/v) on the mycelial growth of NKP4321 in PDB medium was summarized in Table 3. It was observed that BCF BC-SKRU-1 (100%) significantly ($p < 0.05$) inhibited the mycelial growth of the pathogen to a greater extent than thiram® (79.22%) and azoxystrobin® (16.38%). However, there was no significant difference ($p > 0.05$) when compared to prochloraz® (100%) propiconazole® (100%), and mancozeb® (100%). On the other hand, metalaxyl® was found to be ineffective in inhibiting strain NKP4321, as evidenced by its mycelial growth (235.20 mg) exceeding that of the control (218.97 mg). The results obtained indicate that BCF BC-SKRU-1 exhibit a strong antifungal effect against NKP4321.

3.5. Effect of BCF BC-SKRU-1 on germination and survival of NKP4321 spores in vitro

Table 4 shows that the spore germination of NKP4321 in PDB was significantly inhibited ($p < 0.05$) by BCF BC-SKRU-1 under various conditions. These conditions included undiluted, 1/10 diluted, 1/100 diluted, and 1/1000 diluted BCF BC-SKRU-1, as well as BCF BC-SKRU-1 autoclaved at 121 °C for 15 min and stored at both 4 °C and −20 °C for 15 min after 12 h of incubation. Compared to the control, all treatments significantly ($p < 0.05$) reduced spore germination to below 50%. Undiluted BCF BC-SKRU-1 exhibited the most effective spore germination inhibition of the pathogen, showing only 24.00% spore germination compared to the control (74.22% spore germination). Furthermore, BCF BC-SKRU-1 exhibited a robust antifungal effect even when diluted to 1/1000 and autoclaved at 121 °C, with both conditions resulting in less than 50% germination of NKP4321 spores. Similar results were observed in the survival of the tested pathogen's spores on PDA across various treatment conditions of BCF BC-SKRU-1 and the control.

Table 4
Effect of various conditions of *Serratia nematodiphila* BC-SKRU-1 culture filtrates (BCF BC-SKRU-1) on the germination rate in PDB and survival of *Penicillium digitatum* NKP4321 on PDA, incubated at 30 °C for 12 h and 48 h, respectively.

Treatments	Spore germination in PDB (%)	Survival on PDA (CFU/plate)
Control	74.22^{a} ± 4.13	85.67^{a} ± 3.61
Undiluted BCF BC-SKRU-1	24.00^{c} ± 1.90	31.56^{e} ± 4.93
1/10 diluted BCF BC-SKRU-1	25.78^{c} ± 3.51	33.67^{bc} ± 1.70
1/100 diluted BCF BC-SKRU-1	34.67^{bc} ± 1.57	39.89^{c} ± 5.86
1/1000 diluted BCF BC-SKRU-1	44.44^{b} ± 2.81	49.89^{b} ± 3.21
Stored BCF BC-SKRU-1 at 4 °C	26.00^{c} ± 2.65	33.56^{de} ± 4.62
Stored BCF BC-SKRU-1 at −20 °C	28.00^{c} ± 3.20	31.89^{de} ± 2.52
Autoclaved BCF BC-SKRU-1 at 121 °C	46.44^{b} ± 1.70	49.33^{b} ± 4.36

Note: The presented data represent the mean of three replicates ± standard deviation (SD). Values within the same column that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

3.6. Efficacy of BC-SKRU-1 in inhibiting green mold disease caused by NKP4321 in tangerine fruit

The lesion area (Fig. 2A), biocontrol efficacy (Fig. 2B), and disease symptoms (Fig. 2C) of postharvest green mold caused by NKP4321 in tangerine fruit, treated with varying concentrations of BC-SKRU-1 (ranging from 10^5 to 10^8 CFU mL^{-1}), exhibited significant reductions ($p < 0.05$) in disease severity, decreasing from 3.71 cm^2 to 0.89 cm^2 compared to control fruits (4.97 cm^2) afflicted by NKP4321 (Fig. 2).

Table 3
The comparison of *Serratia nematodiphila* BC-SKRU-1 culture filtrates (BCF BC-SKRU-1) (15% (v/v)) with six chemical fungicides (prochloraz®, metalaxyl®, azoxystrobin®, propiconazole®, thiram®, and mancozeb® (1% v/v) on the mycelial growth of *Penicillium digitatum* NKP4321 in PDB medium was assessed after 7 days of incubation at 30 °C.

Parameters	Treatments							
	Control	BCF BC-SKRU-1	Propiconazole®	Prochloraz®	Metalaxyl®	Azoxystrobin®	Thiram®	Mancozeb®
Mycelial dry weight (mg/50 mL)	218.97^{b} ± 0.28	0.0^{e} ± 0.0	0.0^{e} ± 0.07	0.0^{e} ± 0.0	235.20^{a} ± 0.04	183.10^{c} ± 0.08	45.50^{d} ± 0.10	0.0^{e}±0.0
Inhibition of mycelial growth (%)	–	100	100	100	−7.41	16.38	79.22	100

Note: The presented data represent the mean of three replicates ± standard deviation (SD). Values within the same row that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

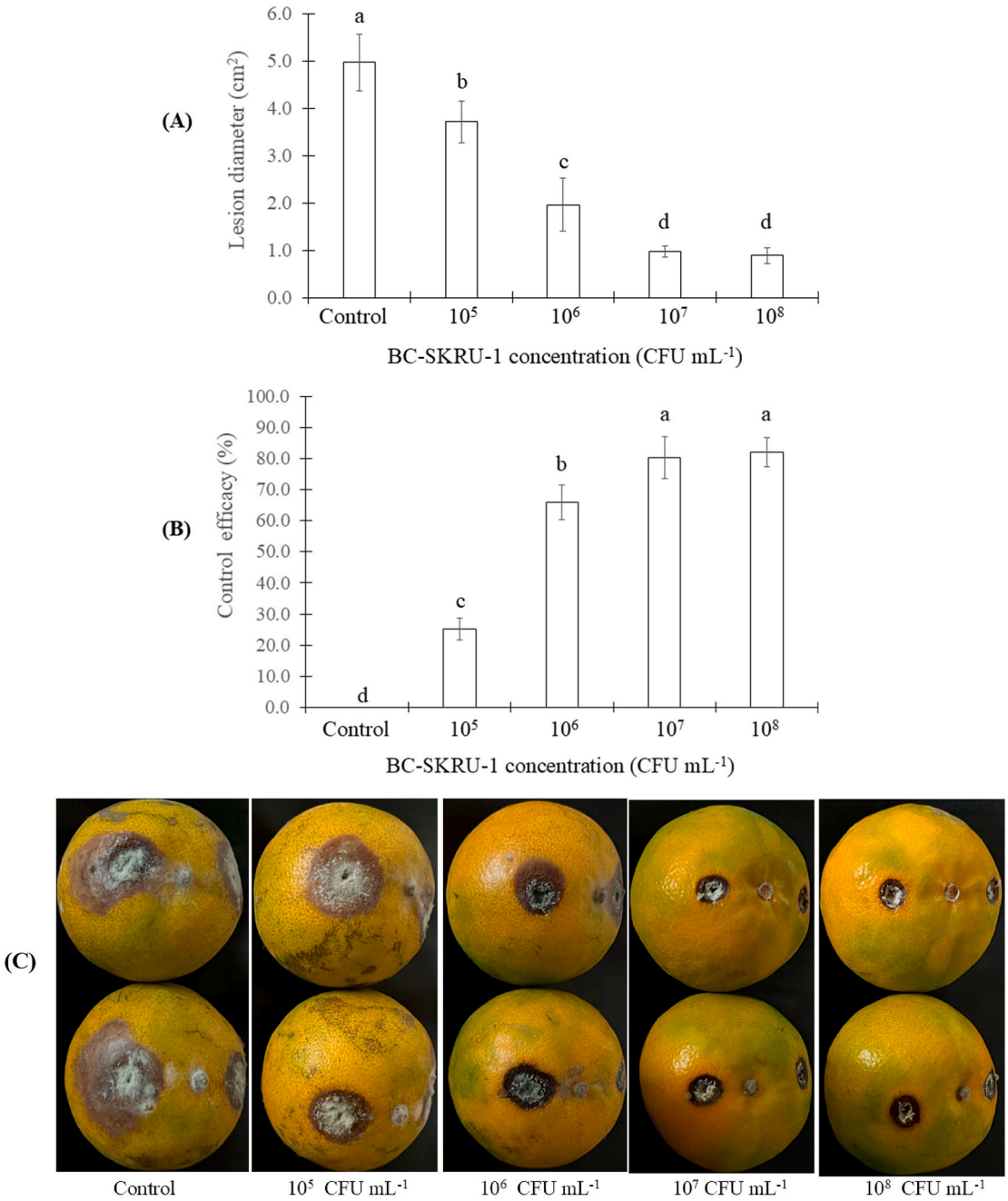


Fig. 2. Impact of *Serratia nematodiphila* BC-SKRU-1 concentration (10^5–10^8 CFU mL^{-1}) on green mold development induced by *Penicillium digitatum* NKP4321 in tangerine fruit after a 12-day storage period at 30 °C under humid conditions. Lesion diameters **(A)**, Control efficacy **(B)**, Disease symptoms **(C)**. The presented data represent the mean of three replicates ± standard deviation (SD) (n = 15). Values within the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

Notably, among the tested concentrations, treatment with 10^8 CFU mL^{-1} demonstrated the most effective control against green mold disease, with a recorded lesion area of 0.89 cm^2 and a control efficacy of 82.08%, which was not significantly different ($p > 0.05$) from the application of 10^7 CFU mL^{-1} (lesion area = 0.98 cm^2 and control efficacy = 80.26%). Therefore, the concentration of 10^8 CFU mL^{-1} of BC-SKRU-1 was selected for its superior biocontrol efficacy compared to the six chemical fungicides.

The biocontrol efficacy of bacterial cells and BCF BC-SKRU-1 was compared with six synthetic fungicides (propiconazole®, prochloraz®, metalaxyl®, azoxystrobin®, thiram®, and mancozeb®) against NKP4321 in tangerine fruit, as summarized in Table 5. Significant differences ($p < 0.05$) were observed between bacterial BC-SKRU-1 and chemical fungicides. The application of 10^8 CFU mL^{-1} of BC-SKRU-1 showed considerable potential against NKP4321, with a recorded lesion area of 0.87 cm^2 and a control efficacy of 82.24%, although this was lower compared to propiconazole®, prochloraz®, and mancozeb® (lesion area = 0 cm^2 and control efficacy = 100%). Additionally, BCF BC-SKRU-1 (lesion area = 1.88 cm^2 and control efficacy = 61.63%) significantly ($p < 0.05$) inhibited NKP4321, albeit less effectively than the bacterial suspension. However, it was more effective than thiram® (lesion area = 3.13 cm^2 and control efficacy = 36.12%) and metalaxyl® (lesion area = 4.40 cm^2 and control efficacy = 10.20%), and equally effective as azoxystrobin® (lesion area = 1.74 cm^2 and control efficacy = 64.49%).

Table 5
Comparison of the biocontrol efficacy of *Serratia nematodiphila* BC-SKRU-1 with six chemical fungicides on green mold development caused by *Penicillium digitatum* NKP4321 in tangerine fruit after a 12-day storage period at 30 °C under humid conditions.

Parameters	Treatments							
	Control	*S. nematodiphila* BC-SKRU-1		Chemical fungicides				
		10^8 CFU mL^{-1}	BCF BC-SKRU-1	Propiconazole®	Prochloraz®	Metalaxyl®	Azoxystrobin®	Thiram®	Mancozeb®
Lesion diameter (cm^2)	$4.90^a \pm 0.47$	$0.87^e \pm 0.0$	$1.88^d \pm 0.46$	$0.0^f \pm 0.0$	$0.0^f \pm 0.0$	$4.40^b \pm 0.41$	$1.74^d \pm 0.22$	$3.13^c \pm 0.31$	$0.0^f \pm 0.0$
Control efficacy (%)	–	82.24	61.63	100	100	10.20	64.49	36.12	100
Disease symptoms									

Note: The presented data represent the mean of three replicates ± standard deviation (SD) (n = 15). Values within the same row that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

3.7. Preventive and curative efficacy of BC-SKRU-1 against NKP4321 in tangerine fruit

The efficacy of BC-SKRU-1 against NKP4321 in tangerine fruit was evaluated through both preventive and curative applications, as detailed in Table 6. Preventive treatments involved applying BC-SKRU-1 bacterial suspensions (10^8 CFU mL^{-1}) to tangerine fruits before inoculation with NKP4321. Curative treatments were administered at various time intervals post-inoculation (simultaneously, 2 h, 4 h, and 6 h). Results are summarized in Table 6, with significant differences ($p < 0.05$) observed for both treatment types. For preventive applications, where fruits were inoculated with NKP4321 after BC-SKRU-1 treatment, significant control of green mold disease was achieved, with efficacy ranging from 79.28% when applied simultaneously to 8.46% when applied 6 h before inoculation. In contrast, curative applications resulted in a decrease in efficacy from 79.28% to 52.43% at 6 h post-inoculation. Application of BC-SKRU-1 simultaneously with NKP4321 showed the best control efficacy (79.28%). Overall, curative applications of BC-SKRU-1 proved more effective than preventive treatments in controlling green mold disease in wounded tangerine fruits.

3.8. Antifungal mode of action of BCF BC-SKRU-1 on NKP4321 cells

3.8.1. Effect of BCF BC-SKRU-1 on ergosterol content in NKP4321 cells

BCF BC-SKRU-1 inhibited plasma membrane ergosterol biosynthesis in NKP4321 in a dose-dependent manner. At a 5% (v/v) concentration, ergosterol biosynthesis was reduced by 58.00%. When the concentration of the culture filtrates was increased to 10% (v/v), the inhibition of ergosterol biosynthesis in NKP4321 rose to 83.90%.

3.8.2. Effect of BCF BC-SKRU-1 on cellular reactive oxygen species and antioxidant enzyme activities in NKP4321 cells

Various biochemical oxidative defense markers, such as ROS, SOD, CAT, and cellular glutathione, were evaluated to investigate changes in the biochemical profile of NKP4321 cells. This analysis revealed the antifungal action of BCF BC-SKRU-1. The effects of these filtrates at concentrations of 0% (control), 5%, and 10% (v/v) on the enzymatic and nonenzymatic defense systems are shown in Fig. 3. Treatment with BCF BC-SKRU-1 resulted in increased ROS levels, with concentrations of 2.39 μM/mg protein at 5% (v/v) and 3.91 μM/mg protein at 10% (v/v), compared to 1.29 μM/mg protein in the control (Fig. 3A). Furthermore, there was a significant increase ($p < 0.05$) in the activity of cellular antioxidant enzymes. SOD and CAT levels were 0.30 units/min/mg protein and 66.09 units/min/mg protein in the control, respectively. These levels increased to 0.95 and 124.15 units/min/mg protein at 5% (v/v), and to 1.36 and 202.29 units/min/mg protein at 10% (v/v) (Fig. 3B and C). Additionally, significant changes ($p < 0.05$) were observed in the levels of GSH, GSSG, and the GSH/GSSG ratio in the NKP4321 cells treated with the culture filtrates. At 5% (v/v), there was a noticeable increase in GSH levels to 0.72 μM/mg protein, though changes at 10% (v/v) were not statistically significant ($p > 0.05$) (0.75 μM/mg protein) (Fig. 3D). The GSH/GSSG ratio similarly increased, while GSSG levels decreased in response to treatments of 5% and 10% (v/v) (Fig. 3E and F).

3.8.3. Effect of BCF BC-SKRU-1 on NKP4321 cell membrane permeability

Our investigation has demonstrated that treatment with BCF BC-SKRU-1 affects the growth of NKP4321. Specifically, we examined the impact of these filtrates at concentrations of 5% and 10% (v/v) on the electrical conductivity, which was used to determine changes in the permeability of fungal cell membranes in NKP4321. As shown in Fig. 4, no significant changes in electrical conductivity were observed in the control group throughout the 8-h observation period. However, exposure to a 5% (v/v) concentration of BCF BC-SKRU-1 led to a noticeable increase in cellular content leakage from the NKP4321. Furthermore, a progressive increase in electrical conductivity was observed with

Table 6
Timing of preventive or curative measures by *Serratia nematodiphila* BC-SKRU-1 for protecting against green mold development caused by *Penicillium digitatum* NKP4321 in tangerine fruit after a 12-day storage period at 30 °C under humid conditions.

Parameters	Treatments							
	Control	BC-SKRU-1 and NKP4321 applied simultaneously	Preventive effect			Curative effect		
			BC-SKRU-1 applied 2 h before NKP4321	BC-SKRU-1 applied 4 h before NKP4321	BC-SKRU-1 applied 6 h before NKP4321	BC-SKRU-1 applied 2 h after NKP4321	BC-SKRU-1 applied 4 h after NKP4321	BC-SKRU-1 applied 6 h after NKP4321
Lesion diameter (cm^2)	$4.73^{a} \pm 0.38$	$0.98^{f} \pm 0.12$	$1.88^{e} \pm 0.16$	$3.22^{c} \pm 0.30$	$4.33^{b} \pm 0.38$	$1.55^{e} \pm 0.07$	$2.29^{d} \pm 0.16$	$2.25^{d} \pm 0.12$
Control efficacy (%)	–	79.28	60.25	31.92	8.46	67.23	51.59	52.43

Note: The presented data represent the mean of three replicates ± standard deviation (SD) (n = 15). Values within the same row that share the same letter are not considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

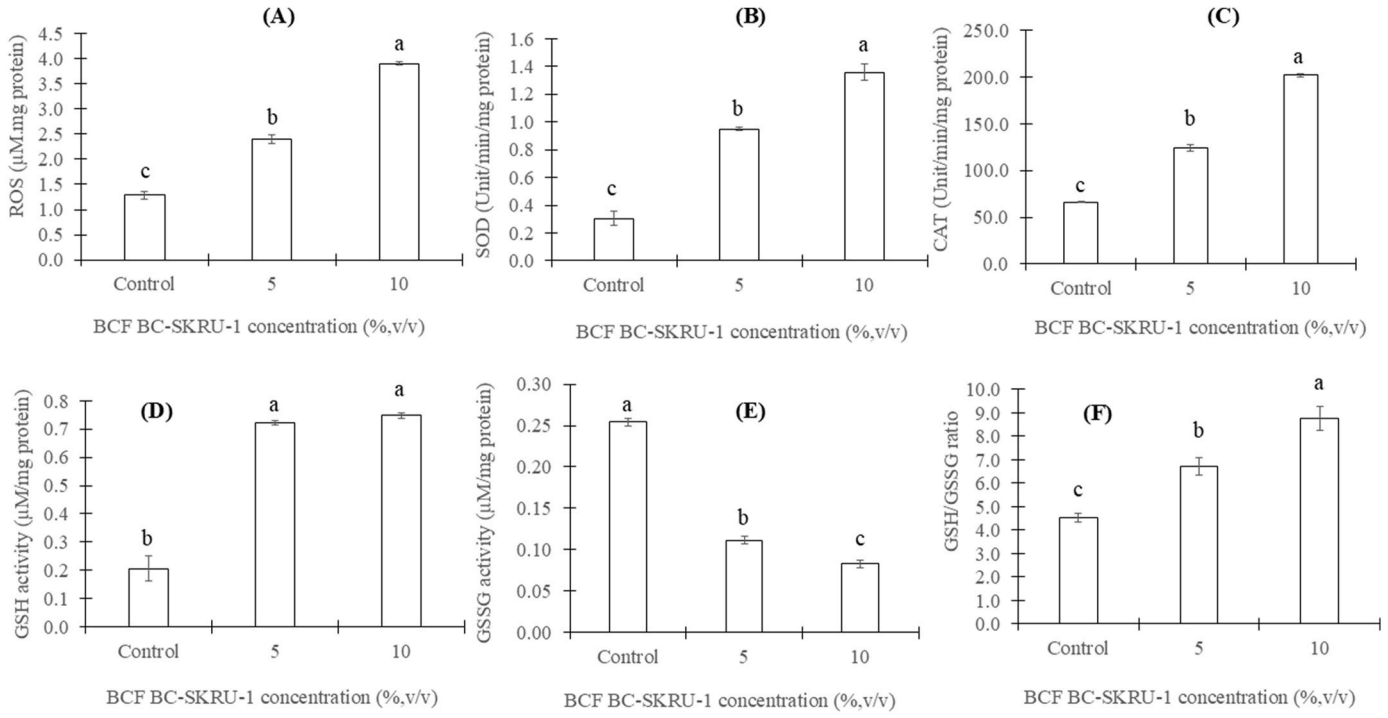


Fig. 3. The effect of *Serratia nematodiphila* BC-SKRU-1 culture filtrates (BCF BC-SKRU-1) concentrations (at 0% (control), 5%, and 10% (v/v)) on the levels of reactive oxygen species (ROS) **(A)**, superoxide dismutase (SOD) **(B)**, catalase (CAT) **(C)**, reduced glutathione (GSH) activity **(D)**, oxidized glutathione (GSSG) activity **(E)**, and GSH/GSSG ratio **(F)** was assessed in *Penicillium digitatum* NKP4321 cells cultured in potato dextrose broth (PDB) at 30 °C for seven days. The presented data represent the mean of three replicates ± standard deviation (SD). Values not sharing the same letter are considered significantly different, as determined by ANOVA following Tukey's HSD test at a significance level of $p > 0.05$.

prolonged exposure to BCF BC-SKRU-1, further confirming its disruptive effect on NKP4321.

4. Discussion

The green mold disease caused by *P. digitatum* results in significant yield loss in citrus worldwide (Cheng et al., 2020; Li et al., 2024). Therefore, this study was designed to investigate the biocontrol potential of *S. nematodiphila* BC-SKRU-1 against *P. digitatum* NKP4321 in tangerine fruits and to explore the potential mechanisms involved. Importantly, this is the first time that *S. nematodiphila* BC-SKRU-1 has been tested against the pathogen *P. digitatum*. The results are promising, showing that BC-SKRU-1 has the potential to inhibit the pathogen both *in vitro* and in tangerine fruit. Although the precise mechanisms of action are not completely understood, it appears that BCF BC-SKRU-1 obstructs the biosynthesis of ergosterol and significantly impairs both enzymatic and non-enzymatic antioxidant defense mechanisms in the fungal pathogen. Additionally, BCF BC-SKRU-1 has been shown to compromise the integrity of the plasma membrane.

Prior research has highlighted bacteria as promising antagonistic agents with broad-spectrum activity against plant pathogens (Shemshura et al., 2020; You et al., 2021). In line with these findings, our study focused on testing *S. nematodiphila* BC-SKRU-1, a bacterium exhibiting significant inhibitory effects against multiple plant pathogens. Specifically, we observed notable inhibitory effects of BC-SKRU-1 against seven pathogenic species, including *R. solani*, *Phomopsis* sp., *C. oryzae*, *A. flavus* PSRDC-4, *A. parasiticus* 3276, *C. gloeosporioides* PSU-03, and *P. digitatum* NKP4321. The reduced growth rates of these pathogens (61.67%–74.68%) when confronted with BC-SKRU-1 compared to the control indicate the biocontrol potential of this bacterial strain against pathogenic fungi. Significantly, BC-SKRU-1 demonstrated particularly robust antifungal activity against NKP4321 (74.68%), leading us to prioritize it for further investigation. These findings are consistent with previous studies demonstrating that the

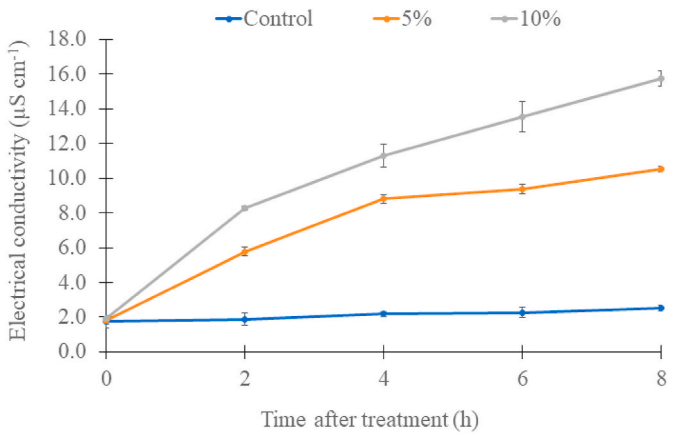


Fig. 4. The effect of *Serratia nematodiphila* BC-SKRU-1 culture filtrates (BCF BC-SKRU-1) concentrations (at 0% (control), 5%, and 10% (v/v)) on the cellular leakage of *Penicillium digitatum* NKP4321. Three-day-old mycelia were treated with BCF BC-SKRU-1 for 0, 2, 4, 6, and 8 h in distilled water. Subsequently, the mycelia were filtered, and the filtrate solutions were used to measure electrical conductivity. The presented data represent the mean of three replicates ± standard deviation (SD).

genus *Serratia* sp. has the potential ability to inhibit plant pathogens (de Senna & Lathrop, 2017; Firdu et al., 2022; Bellotti et al., 2023), further reinforcing its potential as a biocontrol agent.

In the ongoing battle against plant pathogenic fungi, antagonistic bacteria employ a diverse array of strategies aimed at curbing fungal growth and proliferation. This study delves into the potent antifungal activity of BCF BC-SKRU-1 against NKP4321, presenting compelling insights into its potential as a biocontrol agent against plant pathogenic fungi. Through rigorous investigation, we have uncovered the inhibitory effects of BCF BC-SKRU-1 on the mycelial growth and spore germination of NKP4321 across diverse conditions. Our experiments have shed light on the production of crucial metabolites by BC-SKRU-1, effectively countering the growth of NKP4321. These metabolites, released into the growth medium, have proven highly effective in halting the proliferation of these fungi. Significantly, BCF BC-SKRU-1 demonstrated substantial inhibition of the mycelial growth of NKP4321 in both solid and liquid cultures. Notably, even at concentrations as low as 5% (v/v), considerable inhibition was observed, with complete growth suppression achieved at 15% (v/v). Our findings align with those of Firdu et al., who reported effective inhibition of Botrytis fabae AAUBF-12 by *S. nematodiphila* AAUB146b, with inhibition rates of 21% at 5% and 79% at 20% culture filtrate concentrations (Firdu et al., 2022). Similarly, El Khaldi et al. (2015) demonstrated that the culture filtrates of *S. marcescens* suppressed the mycelial growth of *R. solani* by 65.6% in an agar-well diffusion test using 100 µL of culture filtrates (El Khaldi et al., 2015). Furthermore, Kamensky et al. (2003) suggested that the antifungal properties of *Serratia* could be attributed to antibiosis, alongside the production of siderophores and fungal cell-wall degrading enzymes such as chitinases. This broad-spectrum activity is likely mediated by various inhibitory metabolites produced by the organism. These findings corroborate our results, highlighting the superior inhibition performance of different concentrations of culture filtrates compared to direct strain application.

Moreover, a pivotal finding of our study is the superior efficacy of these metabolites in inhibiting mycelial NKP4321 growth compared to several conventional fungicides, including metalaxyl®, azoxystrobin®, and thiram®, which exhibited comparable effectiveness to propiconazole®, prochloraz®, and mancozeb®. This highlights the potential of BCF BC-SKRU-1 as a sustainable alternative for fungal disease management. Furthermore, our study showcased the ability of BCF BC-SKRU-1 to suppress spore germination of NKP4321 across diverse conditions, including dilution, autoclaving, and storage. Even under extreme conditions, such as high dilutions and heat treatment, BCF BC-SKRU-1 exhibited remarkable inhibitory effects on spore germination, underscoring its stability and efficacy as a biocontrol agent. Our findings align with various reports, indicating that the antifungal compounds in the culture filtrates of antagonistic bacteria effectively inhibit both mycelial growth and spore germination of various plant pathogenic fungi (Choub et al., 2021; Gorai et al., 2021; Wang et al., 2021; Wu et al., 2019; Xie et al., 2021). Collectively, these results contribute to the growing body of knowledge on the potential of BCF BC-SKRU-1 as an environmentally friendly and effective solution for managing plant fungal diseases.

Postharvest diseases of citrus present significant challenges, prompting our investigation into the biocontrol efficacy of BC-SKRU-1 against green mold disease caused by NKP4321 in tangerine fruit. Our results unveiled substantial reductions in disease severity, biocontrol efficacy, and disease symptoms across various concentrations of BC-SKRU-1, underscoring the effectiveness of this antagonistic strain in disease management. Comparison between BC-SKRU-1 bacterial cells, BCF BC-SKRU-1, and chemical fungicides revealed noteworthy differences in biocontrol efficacy. Notably, a concentration of 10^8 CFU mL^{-1} of BC-SKRU-1 exhibited superior efficacy in inhibiting green mold disease development, suggesting its potential as an alternative or supplement to chemical fungicides. Similar success has been reported with other antagonistic bacteria, including species within the genera *Bacillus* sp. (Deng et al., 2020; Liu et al., 2023; Tian et al., 2020), *Streptomyces* sp. (Tian et al., 2024), and *Pseudomonas* sp. (Qessaouia et al., 2022; Ferreira et al., 2023), further supporting the efficacy of biocontrol treatments in citrus disease management.

While BC-SKRU-1 suspension demonstrated comparable efficacy to propiconazole®, prochloraz®, and mancozeb®, BCF BC-SKRU-1 also exhibited notable inhibition, positioning BC-SKRU-1 as a promising option for biocontrol measures. Additionally, our evaluation of preventive and curative application methods provided insights into optimal treatment protocols, with the curative approach demonstrating higher efficacy, particularly when applied after pathogen inoculation. Notably, our findings differ from those reported by Deng et al. (2020), who demonstrated that *B. sonorensis* KLBC GS-3, when applied as a preventive treatment, was more effective than when used as a curative treatment in controlling decay caused by green mold. However, our results also indicated a decrease in efficacy when applying BC-SKRU-1 after 2 h for both preventive and curative applications, suggesting potential influences of complex environmental factors at the wound site. Further research is needed to understand the strain's persistence within fruits and its ability to elicit systemic resistance. Overall, our findings contribute to the growing body of knowledge on biocontrol strategies for postharvest diseases and highlight the potential of BC-SKRU-1 as an effective and sustainable alternative to chemical fungicides in citrus fruit protection.

A thorough understanding of the antimicrobial mechanisms employed by BC-SKRU-1 is important for an efficient and long-lasting biocontrol. Therefore, in this study we have presented the mechanisms that BCF BC-SKRU-1 use inhibiting NKP4321. Ergosterol is a vital component of fungal membranes, influencing a range of biological functions including membrane fluidity, the activity and distribution of integral proteins, and cell cycle regulation (Bard et al., 1993). Given the pivotal role of ergosterol and its biosynthetic pathway in fungal growth, understanding this pathway in NKP4321 is crucial for the development of new antifungal agents and enhancing biocontrol efficacy studies. In our investigation, the application of BCF BC-SKRU-1 significantly reduced ergosterol levels within the fungal cell walls of NKP4321 in a dose-dependent manner. This suggests that BCF BC-SKRU-1 impair cell wall integrity and disrupt normal cell membrane functions, underscoring the plasma membrane's vulnerability and its strategic importance as a target for antifungal strategies. Our findings support the hypothesis that BC-SKRU-1 may produce antifungal compounds, such as antibiotics or lytic enzymes (chitinases and β-1,3-glucanases), which

contribute to the degradation or disruption of the fungal cell wall. This action is similar to that observed in *S. plymuthica*, where Kamensky et al. (2003) reported that the production of the antibiotic pyrrolnitrin effectively combated *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Additionally, chitinases produced by *S. plymuthica* were found to inhibit spore germination and germ-tube elongation in *B. cinerea*, as demonstrated by Frankowski et al. (2001).

The proposed mechanism suggests that ergosterol depletion disrupts the function of essential membrane-bound enzymes, such as chitin synthase, which play a role in the detoxification of ROS. This disruption leads to enhanced oxidative stress responses (Shimokawa & Nakayama, 1992). Additionally, the fungal cell wall, a dynamically changing rigid structure, provides protection against osmotic pressure and helps maintain cellular shape. When this structure is invaded, it can lead to plasma membrane rupture and cell lysis, releasing cytoplasmic contents. Given its critical role in maintaining fungal cell integrity, the fungal cell wall is a promising target for new antifungal treatments. Cortes et al. (2019) emphasized that targeting the synthesis of cell wall components has been a traditional strategy in the design and discovery of fungicidal drugs.

The role of ROS in fungal biology, which includes defense mechanisms, signaling pathways, stress responses, and interactions with host organisms, significantly influences survival and pathogenicity (Gessler et al., 2007). However, the accumulation of intracellular ROS is also a primary biochemical trigger for apoptosis (Da et al., 2019). Additionally, many antibiotics and antifungal agents have been reported to induce cell death in bacteria and fungi by promoting the accumulation of intracellular ROS (Lee et al., 2020).

Additionally, ROS-related antifungal activities of microbial bioactive compounds have been documented (Gu et al., 2017; Millan et al., 2022). Despite the recognized importance of ROS, research specifically targeting the phytopathogen *P. digitatum* has been limited. Our study addresses this gap by demonstrating that BCF BC-SKRU-1 induce intracellular ROS production in NKP4321 in a concentration-dependent manner, leading to numerous detrimental cellular effects. These processes, such as nucleic acid fragmentation, tissue swelling, chromatin condensation, ATP depletion, inhibition of mitochondrial ATPase activity, and degradation of the phosphatidylserine lipid layer, together result in cellular death via apoptosis (Yoo et al., 2005).

Furthermore, our research explores the enzymatic antioxidant defenses within NKP4321, focusing on SOD and CAT. SOD catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide, a reaction critical for cellular protection against ROS (Fridovich, 1978). CAT plays a crucial role in detoxifying hydrogen peroxide, thus protecting the cell from oxidative damage (Halliwell, 2006). We observed that the activity of SOD and CAT increased in NKP4321 cells in response to varying concentrations of BCF BC-SKRU-1, suggesting an adaptive response to mitigate the oxidative stress induced by elevated ROS levels. Significant alterations were also noted in the concentrations of GSH, GSSG, and their ratio, which are vital for maintaining cellular redox balance and the overall capability to counter oxidative stress (Sies & Jones, 2020). These findings underscore the sophisticated mechanism of action of BCF BC-SKRU-1, which disrupt fungal cellular homeostasis by inducing oxidative stress and apoptosis. This demonstrates substantial potential as an effective strategy for controlling fungal infections, suggesting that leveraging the oxidative stress pathway could be a promising approach in developing new antifungal treatments for agricultural use.

The fungal membrane is crucial for maintaining cellular order and integrity, making it a common target for various antifungal agents (Avis, 2010). These agents often impact the membrane's permeability, leading to the leakage of intracellular components and loss of essential electrolytes, which can disrupt extracellular conductivity. Previous studies have shown that antifungal agents or bioactive compounds can kill fungi by specifically targeting the plasma membrane (Rautenbach et al., 2016; Sant et al., 2016). Therefore, it is vital to assess the impact of BCF BC-SKRU-1 on the membrane integrity of the fungi being investigated. Our findings indicate that changes in conductivity with BCF BC-SKRU-1 reflect alterations in the permeability of the NKP4321 cell membrane. These changes suggest that BCF BC-SKRU-1 may disrupt the membrane, leading to the leakage of cellular metal ions such as potassium (K^+), calcium (Ca^{2+}), and sodium (Na^+). Such leakage could have devastating effects on material transmembrane transport, energy metabolism, and membrane potential balance (Zhang et al., 2016). Supporting this, Elsherbiny et al. (2021) reported significant increases in membrane permeability and cellular leakage in *P. digitatum* after treatment with a filtrate from *Purpureocillium lilacinum*. Similarly, Chen et al. (2019) investigated the inhibitory effects of 7-demethoxytylophorine (DEM), an alkaloid derived from the rhizomes of *Cynanchum paniculatum*, on *P. italicum*. In another study, Chen et al. (2020) examined the antifungal activity of pinocembrin-7-glucoside (P7G), a flavanone glycoside from *Ficus hirta*, against *P. italicum*. Given these findings, it is not surprising that BCF BC-SKRU-1 affect the membrane integrity of fungi. The cell membrane is critical for retaining intracellular substances essential for cell survival and has been identified as a target site for several antifungal substances (Millan et al., 2022).

5. Conclusion

This study has established the isolate BC-SKRU-1, identified as *S. nematodiphila*, as a potent biocontrol agent against *P. digitatum*, demonstrating broad-spectrum antifungal activity with 74.68% inhibition of *P. digitatum* NKP4321. At a concentration of 15% (v/v), BCF BC-SKRU-1 completely inhibited (100% inhibition) the growth of NKP4321, with efficacy comparable to the chemical fungicides propiconazole®, prochloraz®, and mancozeb®. In addition, BCF BC-SKRU-1 retained its antifungal activity after dilution (1/1000), autoclaving (at 121 °C), and storage (at −20 °C). *In vivo* studies on tangerine fruit showed that treatments with BC-SKRU-1 at 10^8 CFU mL^{-1} significantly reduced green mold severity, with both preventive and curative applications proving effective. The antifungal mechanisms involved reducing intracellular ergosterol and decreasing antioxidant defenses (SOD, CAT, GSH, GSSG, and GSH/GSSG ratio), indicating disrupted plasma membrane integrity and impaired cellular defenses. These findings highlight BC-SKRU-1 and its metabolites as viable, effective alternatives to chemical fungicides, suggesting further research into its mechanisms and application methods to optimize its use in agriculture.

Funding

This research was financially supported by the Thailand Research Fund (TRF) under Research Grant No. RTA 6080010, the Research Fund Budget of Songkhla Rajabhat University (Contract No. 007/2022), and Sisaket Rajabhat University (Grant No. 002/2024).

Availability of data and materials

Not applicable.

Disclosure of potential conflicts of interest

The authors declare no competing interests regarding the publication of this study.

CRediT authorship contribution statement

Siriporn Yossan: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Jaturong Kumla:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Nakarin Suwannarach:** Writing – review & editing. **Wanida Petlamul:** Writing – review & editing, Funding

acquisition, Data curation. **Sirasit Srinuanpan:** Writing – review & editing. **Sawitree Dueramae:** Writing – review & editing. **Sawai Boukaew:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

Data availability

The authors do not have permission to share data.

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