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INHIBITION POTENTIAL OF RHAMNOLIPID BIOSURFACTANT AGAINST *CORYNESPORA CASSIICOLA* – A PHYTOPATOGEN OF KING CHILLI

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Background. The king chilli, *Capsicum chinense* Jacq., is a chilli variant well-known as one of the world's hottest chillies while *Corynespora cassiicola* is a fungal pathogen that causes severe rotting in different parts of the crop and affects the fruit yield and market values. This study was attempted for management of *C. cassiicola* by applying rhamnolipid biosurfactant extracted from bacterial strain *Pseudomonas aeruginosa* SR17.

Materials and Methods. The antifungal potential of crude and column-purified biosurfactant was studied against *C. cassiicola*; EC_{50} and IC_{50} values were calculated accordingly. The *in vivo* action of rhamnolipid on germination of king chilli seeds infected by the fungus was assayed by calculating the percentage of germination and vigour index of the seedling. The *in planta* antifungal efficacy of crude rhamnolipid was investigated by preventive and curative treatments. Management of post-harvest infection of ripe king chilli fruit was evaluated by measuring the percentage of disease index of rhamnolipid.

Results and Discussion. The EC_{50} values for crude and column purified biosurfactant were calculated as 489.39±1.58 mg/L and 438.18±2.55 mg/L, respectively, against the mycelia of *C. cassiicola.* The minimum inhibitory concentrations (MIC) for crude and column-purified biosurfactants were 400 mg/L and 200 mg/L, respectively, against the spores of *C. cassiicola.* The germination percentage of seedlings increased up to 55 % after biosurfactant treatment of *C. cassiicola* infected seeds. The disease percentages reduced up to 49±1 % and 51.3±1.55 % after performing curative and preventive *in planta* experiments in king chilli plants infected with *C. cassiicola*. The IC_{50} value of rhamnolipid was calculated as 895.74mg/L in post-harvest disease management of king chilli fruits.

Conclusions. The rhamnolipid extracted from *P. aeruginosa* SR17 exhibited *in vitro* and *in-planta* efficacy against *C. cassiicola*. The application of rhamnolipid was also found to be effective in reducing post-harvest loss of stored king chilli fruit.

Keywords: biocontrol, fungi, disease index, antifungal efficacy, post-harvest loss

INTRODUCTION

The king chilli (*Capsicum chinense* Jacq.) is an annual herbaceous shrub and important cash crop cultivated in many parts of the globe (Malakar *et al*., 2019). It is also known as ghost pepper or naga jolokia and is a naturally occurring interspecific hybrid between the *Capsicum chinense* Jacq. and *Capsicum frutescens* L. The *Capsicum chinense* Jacq. is native to South America. Later, it was introduced to Africa (western part) and North and South America while some of the hybrid cultivars of *C. chinense* are intensively cultivated in India. The ghost pepper is an annual herbaceous shrub and economically important plant, belonging to the family Solanaceae. The hybrid was once conferred the official title of one of the world's hottest chilli by the Guinness Book of World Records (Guinness World Records, 2006) measuring 1001304 Scoville Heat Unit (SHU). The ghost pepper contains high capsaicinoid compounds which have many applications in pharmaceutical, clinical, and defence sectors (Chanu & Singh, 2024; Malakar *et al.*, 2019). The value-added products, such as chilli flakes, dry or powdered chilli, and paste are in high demand in both domestic and international markets (Bora *et al.*, 2020). Nevertheless, the production of king chilli is frequently compromised due to pathogen invasion (Jamir & Jha, 2024). More specifically, the fungal pathogens that attack king chilli during both the cultivation and storage of ripe fruits cause severe impacts in production. It has been reported that several phytopathogens are responsible for 20–25 % reduction in the yield (Talukdar *et al*., 2015). For example, the pathogens like *Alternaria*, Colletotrichum, Corynespora, *Fusarium*, *Pencillium* etc. are the principal agents accounting for more than 40% loss in cash crop yield (Ling *et al.*, 2024). In king chilli, the anamorphic ascomycetous fungus, namely *Corynespora cassiicola* (Berk. & M. A. Curtis) C. T. Wei, causes leaf rot (Talukdar *et al*., 2015) and takes off stems, fruits, and roots (Anguiar *et al*., 2022). It belongs to the Family Corynesporasaceae, Order Pleosporales, Class Dothideomycetes, Phylum Ascomycota. This fungal pathogen invades 530 plant species of 380 genera, accordingly, the pathogen is distributed in East Asia, North and South Americas, Africa, and Europe including Austria, Bulgaria, Denmark, France, Germany, Hungary, Italy, Netherlands, Norway, Romania, Russia, the UK, and Ukraine (Dixon *et al*., 2009). *C. cassiicola* is a necrotrophic fungus that affects hosts under field conditions and during the post-harvesting stage (Ferreira-Saab *et al.,* 2018). Cell wall degrading enzymes, secreted by the pathogen, speed up the infection process (Zhang *et al*., 2023). Moreover, the fungus is also transmitted through airflow, rainfall, soil and seeds prevailing release of aerospores (Baiyee *et al*., 2019; Zhao *et al*., 2021). Therefore, proper management of *C. cassiicola* is of utmost importance to ensure the production of king chilli.

Various management practises for *C. cassiicola* include both chemical and biological mitigation strategies (Ling *et al*., 2024). However, the management strategies involving chemical fungicides such as benzimidazoles, quinone outside inhibitors and others confer limited success due to the emergence of resistant strains (Zhang *et al*., 2023). In addition, biocontrol agents involving microorganisms have limited success in field

trials (Baiyee *et al*., 2019) and accordingly, an approach is required for sustainable management of the pathogen. Biosurfactants are ecologically safe fungicides with low toxicity, biodegradability, and stability at extreme conditions that can alter the permeability of cell membranes due to a unique amphiphilic structure (Gayathiri *et al*., 2022; Jibrin *et al*., 2020). Amongst the biosurfactants, rhamnolipids have been used extensively in agriculture as an anti-fungal compound (Silva *et al*., 2024). Rhamnolipid biosurfactants secreted by *Pseudomonas*, *Burkholderia*, *Serratia,* and many other bacteria have been investigated for management of plant fungal pathogens (Adetunji *et al*., 2019; Gayathiri *et al*., 2022; Wittgens and Rosenau, 2020). However, application of rhamnolipid in the management of *C. cassiicola* in king chilli have been found to be scanty. Therefore, this study attempted to fill the gap in research. Thus, in this study, antifungal efficacy of rhamnolipid biosurfactant against the perilous phytopathogen of king chilli was investigated.

MATERIALS AND METHODS

Origin and maintenance of *Pseudomonas aeruginosa* and *Corynespora cassiicola culture.* The biosurfactant producing bacterial strain *Pseudomonas aeruginosa* SR17 (GenBank Accession Number KR028434) collected from Advanced Study in Science and Technology (IASST), Guwahati, India was stored at 4 °C in nutrient agar slants. Seed cultures were prepared in nutrient broth, incubated at 150 rpm (revolutions per minute) for 24 hrs at 35±1 °C.

The phytopathogen, *Corynespora cassiicola* ITCC 6748, sourced from Indian Type Culture Collection (ITCC) centre, Indian Agricultural Research Institute (IARI), New Delhi, India, was maintained on potato dextrose agar slant tubes at 4 °C, and was sub-cultured biweekly. Active fungal culture was prepared in potato dextrose agar and incubated at 28±1°C in the dark for 5–7 days.

Production of biosurfactant

Crude biosurfactant. Crude biosurfactant from *P. aeruginosa* SR17 was produced following a modified solvent extraction method described by C. Malakar *et al*. (2024). The strain was inoculated in mineral salt media with glycerol as the carbon source and incubated at 35±1 °C and 150 rpm for 48 hrs. The culture was centrifuged at 10000 rpm for 15 mins at 4 °C in a cooling centrifuge (Remi). The cell-free supernatant obtained was deproteinized at 110 °C for 15 mins, acidified with 6N HCl and stored overnight at 4 °C for biosurfactant precipitation. The precipitate was mixed thoroughly with ethyl acetate in a 1:1 ratio and the volatile organic phase was evaporated under vacuum at 60 °C in a rotary evaporator (Equitron 63R-D). Finally, the crude biosurfactant was collected and expressed as grams per litre.

Column purification of crude biosurfactant. Purification of the crude biosurfactant was carried out by silica gel column chromatography following the modified method of R. Patowary *et al.* (2016). One gram of crude biosurfactant dissolved in 5 mL of methanol was eluded through a silica-packed glass column (500 mm × 25 mm, mesh size 60–120). The solvent system comprised of methanol and chloroform of the gradient 3:50 v/v (300 ml), 5:50 v/v (200 mL) and 50:50 (100 mL) was used during elution at a flow rate of 1 mL/min. Column purified biosurfactant was obtained by evaporating the eluted solution in the rotary evaporator in vacuum at 40 °C.

Characterization of biosurfactant. The column-purified biosurfactant was chemically characterized by employing thin layer chromatography (TLC), Fourier transform

infrared spectroscopy (FTIR) and liquid chromatography mass spectroscopy (Dabaghi *et al*., 2023; Sen *et al*., 2017; Goswami *et al*., 2019). For TLC, 1 mg/1 mL sample dissolved in methanol was spotted on a 20×20 cm TLC silica gel plate (Merck KGaA, Silica gel 60 F254) using the solvent mixture of chloroform: methanol: water (in proportion 65: 15: 2). The chromatogram was dried and the spots were detected with iodine vapour. The FTIR analysis using KBr pellet (sample: spectral grade KBr = 5:100) pressed under 6,000 kg/cm^2 pressure for 120 s was carried out on a Nicolet 6700 FT-IR System, USA at 4 cm^{-1} resolution, 0.01 cm^{-1} wave number accuracy, and 32 scans with correlation for atmospheric CO_2. In LC-ESI-MS, a 2 µL aliquot of samples and standard R-95 dissolved in HPLC grade methanol were injected into Hypersil Gold C18 reverse phase HPLC column (2.1×150 mm, 1.9 µm). A 6410 Triple Quad MS coupled with a 1260 Infinity LC system (Agilent Technologies) was used with a mobile phase of acetonitrile/water (0.1% formic acid) 10:90 (v/v), scanning MS spectra in a range of 100–900 m/z at 135.0 V fragmentor voltage.

Experimental Design

Study of antifungal efficacy of biosurfactant against *Corynespora cassiicola* mycelium and spores *in vitro.* The antifungal efficacy of crude and column purified biosurfactant against the mycelium and spores of *Corynespora cassiicola* was evaluated using the plate (Sarma *et al*., 2024) and microtitre assay (Onlamool *et al.*, 2023), respectively. Biosurfactant dissolved separately in sterilized distilled water in a gradient concentration of 100, 200, 300, 400, 500, 600, 800 and 1000 mg/L was mixed with potato dextrose agar medium. A mycelial plug of 4 mm from an active culture of *C. cassiicola* was transferred on biosurfactant-amended borosil glass Petri dishes (90 mm × 18 mm) and incubated at 28±1 °C in dark mode for 7 days. Control was considered as fungal culture inoculated on media without amending biosurfactant. The anti-mycelial activity of biosurfactant was measured in terms of percentage inhibition and EC_{50} (effective concentration at which 50% inhibition of mycelia was observed) values were calculated. The percentage inhibition formula is

$$\%\,\text{Inhibition} = \left(1 - \frac{\text{Treated}}{\text{Control}}\right) \cdot 100,$$

where Treated – diameter of mycelia in media with biosurfactant; Control – diameter of mycelia in control.

For microtitre assay, crude and column purified biosurfactant dissolved in dimethyl sulfoxide was prepared following the concentrations of 25, 50, 100, 200, 300, 400, 500, 600, 800 and 1000 mg/L. Spores ($1 \cdot 10^5$ spore/mL) of *C. cassiicola* incubated in biosurfactant-amended potato dextrose broth in 96-well flat bottom microtitre plates were kept in the dark at 28±1 °C for 5 days. Control was considered as fungal spore inoculated in media with and without amending dimethyl sulfoxide. The percentage inhibition and IC_{50} (concentration that inhibits 50% of the pathogen) values were calculated. For MIC determination (minimum concentration completely inhibiting fungal growth), treated spores were transferred to a hole (0.5 mm^2 diameter) in the solidified media on Petri dishes. The crude biosurfactant was preferred for further experiments due to cost efficiency.

Influence of biosurfactant on king chilli seed germination under the plant-pathogen system. The *in vitro* influence of biosurfactant on pathogen-infected king chilli seeds was evaluated through a seed assay method as described by M. Goswami and S. Deka (2020). Seeds were surface sterilized (Younesikelaki *et al*., 2016), soaked

for 1 hour in biosurfactant solutions prepared in concentrations of 50, 100, 250, 500, 750 and 1000 mg/L. The seeds were inoculated with *C. cassiicola* spores, kept for 24 hrs and transferred to a sterile water agar plate (2% w/v) covered with sterile moistened filter paper and incubated at 25±1 °C in the dark for 14 days. Controls included untreated uninfected seeds (negative control), untreated infected seeds (positive control), while Bavistin (1000 mg/L) treated seeds were used as standard. The experiment was conducted in triplicate. The germination percentages (calculated after 14 days from day 1), root and shoot length (protrusion point of the seed to the root tip), shoot length (protrusion point of the seed to the leaf tip) of germinated seeds and vigour index were calculated following the method of M. Ananthi *et al.* (2014).

The percentage of germination of seeds and vigour Index were calculated as

$$\text{as \% Germination} = \left(\frac{\text{Numner of germinated seeds}}{\text{Total number of seeds sown}} \right) \times 100,$$

Vigour Index = Germination percentage · (Root length + Shoot length).

Assay of *in planta* antifungal efficacy of biosurfactant. *In planta* antifungal efficacy of crude biosurfactant on *C. cassiicola* – infected king chilli plants was tested under net house conditions following a modified method of J. Lahkar *et al*. (2018). The 28-day-old king chilli seedlings were planted in earthen pots (25 cm × 28 cm) filled with 3 kg of sterile sandy loam soil arranged in a complete randomized design and kept at 25–28 °C and 75–85 % relative humidity for six months of experimental trials (October–March). The experiment was conducted in 2 sets: preventive (Set 1) and curative (Set 2) using biosurfactant concentrations of 50, 500 and 1000 mg/L. In Set 1, biosurfactant was sprayed over the plant shoot prior to pathogen inoculation (20 mL spore suspension, 10^6 spores/mL) while in Set 2, biosurfactant was applied post-inoculation. The positive control plants were treated with sterile distilled water while the negative controls were untreated infected plants and Bavistin (1000 mg/L) was used as a standard. The experiment was conducted in triplicate; shoot length, root length and percentage of disease were assessed.

Post-harvest treatment of king chili fruits infected with *C. cassiicola.* To evaluate post-harvest disease mitigation, a fruit assay was conducted following a modified method of J. Lahkar *et al*. (2018). Surface sterilized fruits sterilized by above mentioned method were soaked for 20 mins in biosurfactant solutions in concentrations of 50, 100, 250, 500, 750 and 1000 mg/L. Fungal spore (10^6 spores/mL) was inoculated in the fruits through a wound of 2 mm. The fruits dipped in sterile water served as controls while those treated with Bavistin (1000 mg/L) served as the standard. All fruits were incubated at 25 °C in a sterile moist chamber in dark for 21 days, at relative humidity of 75–80 %. The experiment was performed in triplicate. The disease reduction percentage was calculated against the control (Ananthi *et al*., 2014) and percent disease index (PDI) was calculated by the following formula

$$\text{PDI} = \left(\frac{\text{Sum of all disease ratings}}{\text{Total number of chilli examined} \cdot \text{Maximum disease rating}} \right) \cdot 100,$$

0 = no symptom; 1 = 1–10 % fungal infection; 2 = 11–25 % fungal infection; 3 = 26–50 % fungal infection; 4 = 51–75 % fungal infection; 5 = > 75 % fungal infection.

RESULTS AND DISCUSSION

Characteristics of the biosurfactant. The biosurfactant sample obtained from *P. aeruginosa* SR17 yielded two spots in the TLC plate. The retention factor (R_f) values of the two spots detected on the TLC plate were recorded as 0.36 and 0.69. The appearance of the two spots in the plate indicates the presence of mono- and di-rhamnolipid in the biosurfactant sample. The R_f values displayed by the biosurfactant sample measuring 0.69 and 0.36 were comparable with the mono- and di-rhamnolipid components respectively found in the standard R-95 sample (Safari *et al*., 2023). In general, rhamnolipid biosurfactant is a mixture of mono or di-rhamnose sugar and a long fatty acyl chain containing C_8–C_{14} carbon atoms (Li *et al*., 2022).

The result of the FTIR spectra of the column purified biosurfactant and standard R-95 sample has been presented in **Table 1**. The FTIR spectra revealed the presence of the functional groups such as alcohols and phenols, alkanes, alkenes, carbonyls, amine and esters with stretching vibrations in the biosurfactant as well as standard samples. The –OH stretching vibrations for alcohol or phenol rendered a broad peak at 3418 cm^{-1} and 3417.62 cm^{-1}, respectively, in the biosurfactant and R-95 samples. The broad peaks at 3400 cm^{-1} in the biosurfactant sample were due to wagging of OH of rhamnose part (Dabaghi *et al*., 2023). Asymmetric stretching of C–H of aliphatic CH_2 and CH_3 groups rendered peaks in the biosurfactant sample at 2928 cm^{-1} and at 2929 cm^{-1} for R-95. Symmetrical C–H stretching of alkanes produced spectral bands observed at 2858 cm^{-1} in the biosurfactant sample and at 2859 cm^{-1} in R-95. Spectral bands that appeared at 1728 cm^{-1} both in the sample and standard might be due to the characteristic stretching of carbonyl group with vibration in –C = O at 1720–1740 cm^{-1} (Dabaghi *et al*., 2023). Another peak was recognised at 1573 cm^{-1} in R-95 which was absent in the sample. The presence of N–H bond was a characteristic feature of R-95 as it might be rendered by bending vibration of 1 ° amines which was absent in the biosurfactant

Table 1. **FTIR spectra showing bands and peaks representing different functional groups of biosurfactant sample and the standard**

Bands and peaks (cm^{-1})	Ranges	Type of vibration	Functional groups
3417*, 3418*	3400–3200	Stretching of O–H (H-bonded)	Alcohols and phenols
2928*, 2929**	2900–2800	Stretching vibration of (–CH_2 and –CH_3)	Asymmetric alkanes
2858*, 2859**	2900–2800	Stretching vibration of (–CH_2)	Symmetric alkanes
1728***	1705–1725	Stretching of (–C = O)	Aldehydes
1573**	1650–1550	Bending vibration showing NH_2 scissoring	1° amines
1386*	1390–1370 or 1430–1330	Bending vibration showing C–H_3 deformation in-plane O–H bending	Alkanes alcohols or ahenols
1172*	1210–1160	O = C–O–C stretching	Esters
1058**, 1069*	1050–1034 or 1085–1050	C–O stretching	1° alcohols

Notes: * – present in biosurfactant sample; ** – present in the standard; *** – present in both biosurfactant sample and the standard

sample (Onlamool *et al*., 2023) obtained from the P. aeruginosa SR17. The absorption peaks that appeared at 1386 cm^{-1} and 1172 cm^{-1} in the biosurfactant sample were probably due to CH_3 deformations of alkanes or in-plane O–H bending of alcohols and phenols while the latter might indicate the presence of O = C–O–C stretching of esters. Likewise, the peaks that appeared around the region 1100–1000 cm^{-1} were rendered by C–O stretching which detected the presence of the rhamnose ring in the biosurfactant. The absorption peaks that appeared in both the standard and the sample in the range 1100–1000 cm^{-1} might be because of C–O stretching of alcohols and phenols where carbon was bonded with oxygen atom of hydroxyl group from rhamnose ring or C–N of amines. The spectra that appeared in the corresponding ranges revealed that the lengthy hydrocarbon chains are connected to rhamnose rings which is consistent with data previously described in the publications (Hosseini *et al*., 2024).

The findings of LC–ESI–MS have been presented in **Table 2**. The spectra of the molecular ions for mono-rhamno-monolipidic congeners were observed in the mass ranges from 301–391, mono-rhamno-dilipidic congeners in the range 445–585, di-rhamno-monolipidic congeners in the range 453–479 and di-rhamno-dilipidic congeners in the range 667–749. The LC-ESI-MS revealed the presence of abduct ions like $[M + Na]^+$, $[M + K]^+$, $[M–H + Na_2]^+$, $[M–H + CH_3]^+$, $[M + H]^+$ and $[M–H]^-$ in the sample R-95. The calculated values of mono- and di-rhamnolipid were 68.75 % and 31.25 % in the sample and 66.7 % and 33.3 % in R-95, respectively. The higher percentage of mono-rhamnolipid over di-rhamnolipid made the biosurfactant a potent fungal inhibitor (Zhou *et al*., 2023). It is because the biosurfactant is more lipophilic in the presence of a single rhamnose sugar which further facilitates penetration of the biosurfactant in the cell wall. After entering the pathogenic cell, the mono-rhamnolipid containing biosurfactant destroys the cell and causes death of the pathogen (Zhao *et al*., 2022). The abundance of fatty acid components was also calculated in the sample. The active components (**Table 2**) detected from the sample in mono-rhamnolipid were Rha–(C_{10}–C_8) or Rha–(C_8–C_{10}), Rha–(C_{10}–C_{10}) and Rha–(C_{10}–$C_{12:1}$) and in di-rhamnolipid were Rha–Rha–(C_{10}–C_8), Rha–Rha–(C_{10}–C_{10}), Rha–Rha–(C_{12}–$C_{12:1}$) and Rha–Rha–(C_{12}–C_{12}). The length of fatty acid chain also imparts the anti-fungal strength to the biosurfactant. The fatty acid chains with carbon number C-10 confer better inhibition as stated by

Table 2. **Active congeners found in the rhamnolipid sample and the standard**

Sample	Mono-rhamno-mono-lipidic	Mono-rhamno-di-lipidic	Di-rhamno-mono-lipidic	Di-rhamno-di-lipidic
Biosurfactant	absent	[Rha–(C_{10}–C_8)] or [Rha–(C_8–C_{10})] [Rha–(C_{10}–C_{10})] [Rha–(C_{10}–$C_{12:1}$)] or [Rha–($C_{10:1}$–C_{12})]	absent	[Rha–Rha–(C_{10}–C_8)] or [Rha–Rha–(C_8–C_{10})] [Rha–Rha–(C_{10}–C_{10})]* [Rha–Rha–(C_{12}–$C_{12:1}$)] or [Rha–Rha–($C_{12:1}$–C_{12})] [Rha–Rha–(C_{12}–C_{12})]
R95	[Rha–$C_{8:2}$]	[Rha–(C_{10}–C_{10})]	absent	[Rha–Rha–(C_{10}–$_8$)] or [Rha–Rha–(C_8–C_{10})]

Notes: * – major active component in biosurfactant (Crouzet *et al*., 2020; Li *et al*., 2022)

D. Li *et al*. (2022). The presence of the mono-rhamnolipid congener Rha–C_{10} and the di-rhamnolipid congener Rha–Rha–C_{10}–C_{10} in the biosurfactant made it a suitable antifungal agent (Crouzet *et al*., 2020).

Antifungal efficacy of biosurfactants against *Corynespora cassiicola in vitro*

Effect on mycelium of the phytopathogen. The results of anti-mycelial inhibition efficacy of crude and column purified rhamnolipids against *C. cassiicola* have been presented in **Fig. 1**. Our results have revealed that the mycelial inhibition significantly increased after treatment with both the crude ($F_{7, 23}$ = 1118.14 p <0.05) and column purified ($F_{7, 23}$ = 356.31.65, p <0.05) rhamnolipids. Besides, it was found that the zone of inhibition increased significantly when concentration of the biosurfactant was enhanced during the treatments. The zone of mycelial inhibition against *C. cassiicola* ranged from 29.30±0.36 to 67.95±0.15 and 29.37±1.05 to 77.58±0.81 when treated with crude and column purified rhamnolipid biosurfactant respectively. As the concentration of biosurfactant is increased during treatments, rhamnolipid facilitates more micelle formations that mix with the lipids present in the cell membrane and solubilise the cell membrane causing a necrosis of cells (Li *et al*., 2023), which accounts for the enhanced inhibition of fungal mycelia at higher concentrations. When the crude and column purified rhamnolipid were applied in similar concentrations, the percentage of inhibition was found to be different. This may be due to the differences in composition of the crude and column purified biosurfactant samples. As a whole, the fungal inhibition conferred by column purified rhamnolipid was approximately 1.14-fold higher than that by the crude rhamnolipid. The calculated IC_{50} values against the mycelium of *C. cassiicola* were 490.69±2.19 mg/L (confidence interval 95%: 487.97–493.41) and 453.52±1.8 (confidence interval 95 %: 451.30–455.74) for crude and column purified biosurfactant respectively. The obtained data corroborates the previous findings of D. Li *et al.* (2022) where it was found that the rhamnolipid extracted from *Pseudomonas aeruginosa* was effective against different phytopathogens *Colletotrichum destructivum, C. sublineola* and *Fusarium oxysporum*. As a whole, it can be inferred that the infection mode of the pathogen was impacted by the signalling pathway of rhamnolipid biosurfactant sample. The fact behind this is the distortion of pathogen cell membrane structure by the bacterial metabolite. The destruction of plasma membrane can be caused by the formation of a transient pore due to direct intercalation of rhamnolipid with the plasma membrane of the target pathogen. This direct insertion is due to the complementary structure of dirhamnolipid with phosphatidylethanolamine of plasma membrane (Botcazon *et al*., 2022). Another important feature related to plasma membrane is dipole potential ΨD, which directs the macromolecule translocation and rate of translocation of ions through lipid bilayer. Rhamnolipid as a fungicide can modulate the ΨD leading to significant damage in fungal membrane (Rodríguez-Moraga *et al*., 2023).

Effect on the spores of the fungus. The result of inhibition of fungal spores of *C. assiicola* has been depicted in **Fig. 2** indicating a significant enhancement in inhibition of fungal spores when treated with both the crude ($F_{7, 23}$ = 7452.74 p <0.05) and column purified ($F_{7, 23}$ = 2373.08 p <0.05) rhamnolipids. Moreover, concentration dependent increment in spore inhibition and higher inhibition were observed at higher concentrations of rhamnolipid samples. The results also revealed a variation in the anti-spore efficacy between the crude and the column purified rhamnolipid samples. The percentage inhibition against spores of *C. cassiicola* ranged from 24.64±0.32 to 74.14±0.58 when treated with crude rhamnolipid biosurfactant. The zone of inhibition against the

fungal spore of *C. cassiicola* ranged from 25.95±0.14 to 76.83±0.45 when treated with column purified rhamnolipid biosurfactant. As a whole, the column purified rhamnolipid caused about 1.04-fold higher inhibition than the crude rhamnolipid on average against the spores of *C. cassiicola*. It has been reported that a higher concentration of rhamnolipid might interfere the spore germination as observed in case of *Alternaria alternata* and *Verticillium dahlia* (Thakur *et al.*, 2021). The IC_{50} values conferred by crude and column purified rhamnolipid against the spores of *C. cassiicola* were calculated as 380.81±1.59 mg/L (confidence interval 95 %: 378.84–382.78) and 342.77±2.41 mg/L (confidence interval 95 %: 339.77–345.77), respectively.

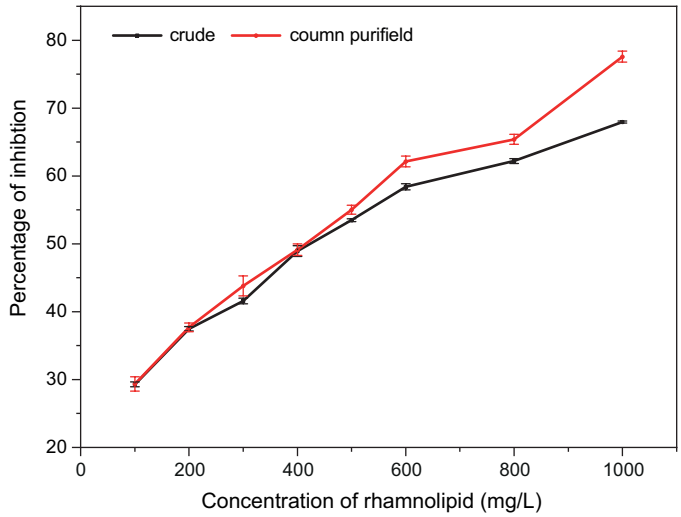

Fig. 1. Inhibition percentages of mycelia of *C. cassiicola* by applying gradient concentrations of crude and column purified rhamnolipids

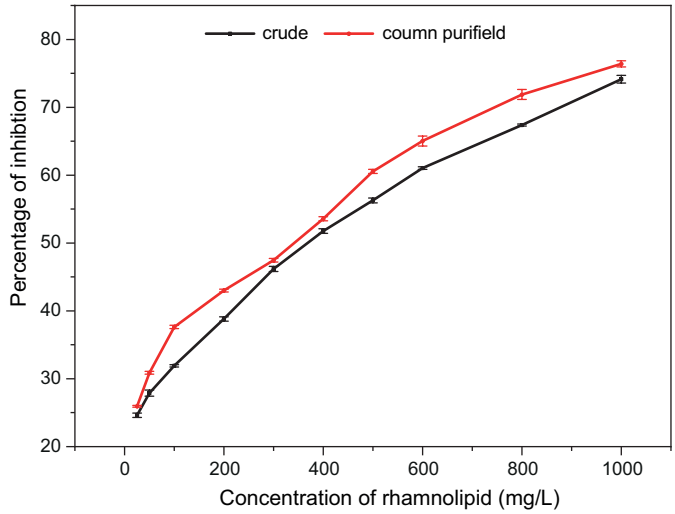

Fig. 2. Inhibition percentages of spores of *C. cassiicola* by applying gradient concentrations of crude and column purified rhamnolipids. Data is representation of the average of three replicates. Error bars represent standard error

The MIC values of crude and column purified rhamnolipid were found to be 400 mg/L and 200 mg/L, respectively. The inhibition percentages of fungal spores by employing crude and column purified rhamnolipids were significantly different in all the concentration treatments except for the inhibition conferred by 600 mg/L crude and 500 mg/L column purified rhamnolipids. Anti-spore action of rhamnolipid was found to be stronger than anti-mycelial activity at the same concentration which is supported by findings of D. Li *et al.* (2022). The difference in inhibition potential of rhamnolipid against the fungal mycelia and spores might be observed due to the difference in the cell wall of spores and mycelia (Karamchandani *et al*., 2022). The work of T. Onlamool *et al*. (2023) investigated the antifungal efficacy of rhamnolipid against pathogens like *Aspergillus* sp, *Cunninghamella* sp., and *Rhizopus* sp. F. A. Moo-Koh *et al.* (2022) examined the inhibitory effect on conidial germination of *C. cassiicola* by the root extract of *Croton chichenensis*, a member of the Euphorbiaceae family, and seven other plants.

Effect on king chilli seeds infected by *Corynespora cassiicola.* The action of rhamnolipid on *C. cassiicola*-infected seeds has been depicted in **Table 3**. The percentage of germination, shoot length ($F_{4, 14}$ = 191.74 p <0.05), root length ($F_{4, 14}$ = 101.60 p <0.05) and vigour index of fungus-infected chilli seedlings increased after treatment with crude rhamnolipids in the gradient concentrations of 50, 100, 250, 500 and 750 mg/L. However, no growth was observed in the seeds of negative control. The percentage of germination increased from 37% to 55% by applying higher doses of rhamnolipids. The commercial standard Bavistin caused a germination percentage of 54 % in fungus-infected seeds. The germination percentage was measured as 34 % in the positive control. The lowering of the germination index in seeds treated with rhamnolipid indicated a restrictive effect of rhamnolipid along with the increase in concentration. A study conducted by S. Li *et al.* (2023) showed that the germination index was higher when seeds were treated with higher doses of rhamnolipid. The application of increasing concentrations of rhamnolipid resulted in an increase in shoot length, ranging from 1.49±0.04 cm to 2.27±0.04. Notably, there was no significant difference in shoot length between pathogen-infected seeds treated with the commercial standard Bavistin (2.27±0.04 cm) and those treated with rhamnolipid at concentrations of 500 mg/L (2.28±0.06 cm) and 750 mg/L (2.28±0.04 cm). The shoot length of germinated seeds in the positive control was measured at 1.04 cm. The shoot lengths of the rhamnolipid-treated seeds at 500 mg/L and 750 mg/L are approximately 2.19 times greater than those of the positive control. The root lengths of pathogen-laden seeds increased from 0.57±0.04 to 1.14±0.06 cm after treatment with higher doses of crude rhamnolipids. The root length of germinated seeds treated with 750 mg/L rhamnolipid (1.14±0.06 cm) was not significantly different from that of seeds treated with Bavistin (1.10±0.03 cm). The root length of the germinated seeds in the positive control was measured to be 1.04 cm. The root length of rhamnolipid treated seeds at 750 mg/L was approximately 1.10 times greater than that of the positive control. The vigour index was calculated in the range of 73.6–182.0 by employing crude rhamnolipids of concentration gradient. Vigour indices in the positive control and the fungus infected seeds treated with the commercial fungicide bavistin were measured as 48.8 and 178.6, respectively (**Table 3**). This can be explained by the fact that rhamnolipid promotes micelle formation that mixes with the lipids present in the cell membrane which may solubilise cell membrane rhamnolipid. This may cause a death of the pathogen cells (Li *et al*., 2023). Another study conducted by A. Benson *et al.* (2022) on fungus-infected tomato seeds, glycolipid-type biosurfactant reduced the seed infection percentage by 87 %. This process is initiated when

water infiltrates the seed through the seed coat, resulting in the activation of metabolic processes and an increase in the size of the embryonic axis. This growth is contingent upon the permeability of the embryonic sheath tissues to water (Simões *et al*., 2023).

Table 3. **Germination percentage, root length, shoot length and vigour index of king chilli seeds treated by crude biosurfactant of different concentrations after infecting with spores of *C. cassiicola***

Treatments	Concentration (mgL^{-1})	Germination percentage	Shoot length (cm)*	Root length (cm)*	Vigour index*
Negative control	–	–	–	–	–
Positive control	–	34	1.04±0.02^{a}	0.45±0.04^{a}	48.8
Biosurfactant	50	37	1.49±0.04^{b}	0.57±0.04^{b}	73.6
	100	41	1.65±0.04^{c}	0.7±0.04^{c}	93.2
	250	47	1.88±0.04^{d}	0.92±0.03^{d}	128.5
	500	53	2.28±0.06^{e}	1.08±0.04^{e}	172.8
	750	55	2.28±0.04^{e}	1.14±0.06^{e}	182.0
Bavistin**	1000	54	2.27±0.04^{e}	1.10±0.03^{e}	178.6

Notes: * – values were expressed as mean ± SD of three replicates. In the same column indicating same parameter, different letters stand for significant differences (ANOVA, LSD test; p <0.05); ** – Bavistin was used as the standard during experimental trials

Assessment of the antifungal efficacy of biosurfactant *in planta.* The results of the curative and preventive pot trial by crude rhamnolipid on the *C. cassiicola*-infected king chilli plants have been deciphered in **Table 4**. The results indicate that the crude rhamnolipid extracted from *P. aeruginosa* SR17 exhibited antifungal effect on fungus-infected king chilli plants during both preventive ($F_{3, 11}$ = 147.17 p <0.05) and curative ($F_{3, 11}$ = 113.05 p <0.05) treatment. The preventive treatment by rhamnolipid reduced fungus spreading in king chilli plants from 71±1 % to 51.3±1.55 %. The disease percentage reduced from 69.3±1.53 % to 49±1 % during curative treatment. The commercial fungicide Bavistin reduced the disease percentage up to 52.3±1.52 % during curative treatment. On the other hand, the reduction in disease percentage by Bavistin during preventive treatment was found to be 53.7±2.08 %. The percentage of disease in negative control was 72±.1.73. However, disease reduction in king chilli plants treated with rhamnolipid in both preventive and curative manner was not significantly different from the negative control. Again, no significant difference was observed in curative and preventive treatment when the same concentrations of rhamnolipids were applied. The reason behind this is the amphiphilic properties possessed by biosurfactant, which demonstrates dual functionalities of the rhamnolipid biosurfactant with both curative and preventive effect, which makes it an efficient antifungal agent with dual role (Robineau *et al*., 2020). Rhamnolipids provide antimicrobial protection to plants, while also enhancing local and systemic immunity in the plants, resulting in comprehensive protective effects (Simões *et al*., 2023). The fact behind the boosting of plant immunity system by rhamnolipid is due to triggering of events like ROS accumulation, influx of calcium and phosphorylation cascade (Botcazon *et al*., 2022). The infection mode of

Table 4. **Percentage of disease of *C. cassiicola*-infected king chilli plants treated by crude biosurfactant of different concentrations**

Treatments	Concentration (mgL^{-1})	Percentage of disease		
		Control	Curative	Preventive
Positive control	–	–	–	–
Negative control	–	72.0±1.73	–	–
Biosurfactant	50	–	69.3±1.53[a]	71.0±1[a]
	500	–	61.7±1.53[b]	63.3±2.08[b]
	1000	–	49.0±1[c]	51.3±1.55[c]
Bavistin**	1000	–	52.3±1.52[d]	53.7±2.08[d]

Notes: * – values were expressed as mean ± SD of three replicates. In the same column indicating same parameter, different letters stand for significant differences (ANOVA, LSD test; p <0.05); ** – Bavistin was used as standard during experimental trials

the pathogen was impacted by the signalling pathway of rhamnolipid. It was postulated that rhamnolipid destabilizes the plasma membrane of plants resulting in the induction of defence in plants. The phosphatidylcholine and phosphatidylethanolamine layers get disrupted due to biosurfactant insertion which alters the permeability of the cell membrane (Crouzet *et al*., 2020). Another possible way of indirect resistance development caused by rhamnolipids is the series of events associated with the microbe-associated molecular patterns that trigger pattern-recognition receptors localized on the plant surface that signals cascades leading to early and late plant defence responses (Pierre *et al*., 2025). Further, the immune response in plants might be stimulated due to the entry of rhamnolipids in the plasma membrane rich in phosphatidylcholine on the outer leaflet which is promoted by sphingolipids (Rodríguez-Moraga *et al*., 2023).

Post-harvest disease management of pathogen infected king chilli fruits by biosurfactant. The findings of post-harvest management of pathogen-infected king chilli fruits have been included in **Table 5**. During post-harvest management of king chilli fruits, infection was reduced significantly ($F_{6, 20}$ = 554.33 p <0.05) over control. The reduction in disease percentage in *C. cassiicola*-infected king chilli plants was only 11.3 % when treated with 50 mg/L rhamnolipid while the percentage of disease was reduced up to 51.7 % while treated with 1000 mg/L rhamnolipid. The reduction in disease percentage by treatment with Bavistin at the recommended dose (1000 mg/L) was 49.3 %. The IC_{50} value of rhamnolipid was calculated as 895.74 mg/L. The percent disease indices (PDI) ranged from 91.6 to 46.7. The PDI values were 93.3 for the control and 44.4 for Bavistin with the concentration of 1000 mg/L (**Table 5**). A lowering in PDI is an indication of better inhibition potential as reported by E. A. Soliman *et al*. (2024). In a study conducted by C. O. Adetunji *et al.* (2019), it was found that the rhamnolipid extracted from *P. aeruginosa* C1501 prevented loss in firmness, total soluble solids and reduced decay in *Penicillium digitatum* NSP01-infected 'Agege sweet' orange. Antimicrobial activities of rhamnolipid prevented the spreading of diseases in fruits during post-harvest management. This might be due to the activation of antioxidant reactions or accumulation of antimicrobial metabolites and defence enzymes as was

Table 5. **Disease reduction over control (%) and PDI (percentage disease index) of infection caused by *C. cassiicola* on king chilli fruit after treating with crude biosurfactant of different concentrations**

Treatment	Concentration (mgL^{-1})	Disease reduction over control (%)	Percentage disease index (PDI)
Control	–	0	93.3
Biosurfactant	50	11.3[a]	91.6
	100	19.0[b]	83.3
	250	24.7[c]	75.0
	500	31.7[d]	66.7
	750	45.3[e]	55.6
	1000	51.7[f]	46.7
Bavistin**	1000	49.3[g]	44.4

Notes: * – values were expressed as mean ± SD of three replicates. In the same column indicating same parameter, different letters stand for significant differences (ANOVA, LSD test; p <0.05); ** – Bavistin was used as standard during experimental trials

observed in the fruits of tomatoes and grapevine (Crouzet *et al*., 2020). The efficacy of rhamnolipid is due to the distortion of pathogen cell membrane structure by the bacterial metabolite. It also induces local and systemic resistance in the host plant as it impacts the signalling pathways of the plant defence system (Simões *et al*., 2023).

CONCLUSIONS

The current research work found that the rhamnolipid biosurfactant extracted using glycerol as the carbon source from *P. aeruginosa* SR17 contained both mono- and di-rhamnolipid. The biosurfactant was found to be effective against mycelia and spores of *Corynespora cassiicola*, a destructive pathogen of king chilli. It inhibited fungal mycelia and spores in the ranges of 29.30±0.36 to 67.95±0.15 and 24.64±0.32 to 74.14±0.58, respectively. The rhamnolipid showed *in plant* antifungal effect on pathogen-infected king chilli plants. The rhamnolipid was also effective in post-harvest management of king chilli fruit. Further research on this line can incorporate rhamnolipid biosurfactant in integrated disease management as a green and safer disease controlling biological agent and immunity booster for plant hosts.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: the authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: this article does not contain any studies with human subjects performed by any of the authors

Animal Studies: this article does not contain any animal studies performed by any of the authors.

AUTHOR CONTRIBUTIONS

Conceptualization, [S.D.; N.S.]; methodology, [N.S.; S.D.]; validation, [S.D.; N.S.]; formal analysis, [S.D.; N.S.; H.D.].; investigation, [N.S.]; resources, [S.D.]; data curation, [S.D.; N.S.]; writing – original draft preparation, [N.S.]; writing – review and editing, [H.D.]; visualization, [S.D.; H.D.] supervision, [S.D.; H.D.]; project administration, [S.D.]; funding acquisition, [S.D.].

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ІНГІБУЮЧИЙ ПОТЕНЦІАЛ РАМНОЛІПІДНОГО БІОСУРФАКТАНТУ ПРОТИ *CORYNESPORA CASSIICOLA* – ФІТОПАТОГЕНА КОРОЛІВСЬКОГО ПЕРЦЮ

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Обґрунтування. Королівський перець (*Capsicum chinense* Jacq.), відомий як один із найгостріших перців у світі, часто втрачає врожайність і ринкову цінність через ураження грибковим патогеном *Corynespora cassiicola* (спричиняє плямистість листя) як у польових умовах, так і після збирання врожаю. Наше дослідження було проведено для боротьби з цим фітопатогеном через застосування рамноліпідного біосурфактанту, екстрагованого з бактеріального штаму *Pseudomonas aeruginosa* SR17.

Матеріали та методи. Було досліджено антиміцеліальну й антиспорову ефективність сирого та колонково очищеного біосурфактанту проти *C. cassiicola*, і, відповідно, розраховано значення EC_{50} та IC_{50}. *In vivo* активність рамноліпіду на проростання насіння королівського перцю, інфікованого патогеном, оцінювали, розраховуючи відсоток проростання й індекс енергії проростків. *In planta* ефективність сирого рамноліпіду було досліджено і порівняно з комерційним фунгіцидом Бавістином. Контроль інфекції рамноліпідом після збирання врожаю у дозрілих плодах королівського перцю оцінювали, вимірюючи відсоток індексу захворювання.

Результати й обговорення. Значення EC_{50} для сирого та колонково очищеного біосурфактанту становили 489,39±1,58 мг/л та 438,18±2,55 мг/л проти міцелію *C. cassiicola*. Концентрації MIC для сирих та очищених біосурфактантів становили 400 мг/л та 200 мг/л, відповідно, проти спор *C. cassiicola*. Відсоток проростання насіння збільшився до 55 % після обробки біосурфактантом насіння, інфікованого *C. cassiicola*. Відсоток захворюваності зменшився до 49±1 % та 51,3±1,55 % після лікувальних і профілактичних досліджень *in planta* на рослинах королівського перцю, інфікованих *C. cassiicola*. Значення IC_{50} становило 895,74 мг/л, якщо запобігали захворюванням плодів королівського перцю, уражених *C. cassiicola,* після збору врожаю.

Висновки. Рамноліпід, екстрагований із *P. aeruginosa* SR17, виявив ефективність *in vitro* та *in planta* проти двох тестованих патогенів. Завдяки обробці рамноліпідним біосурфактантом також вдалося зменшити втрати плодів після збору врожаю.

Ключові слова: біоконтроль, гриби, індекс захворюваності, протигрибкова ефективність, втрати після збору врожаю

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