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RESEARCH ARTICLE

In vitro assessment of entomopathogenic potential of indigenous *Clonostachys rosea* CR 02 against the whitefly *Bemisia tabaci* in Potato

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Abstract

Bemisia tabaci Gennadius (Hemiptera: Aleyrodidae) are economically important polyphagous pest species causing severe yield losses in Potato. To develop a biopesticide in an environmentally safe manner, the virulence and pathogenecity of the indigenous *Clonostachys rosea* CR 02 (Ascomycota, Hypocreales: Bionectriaceae) was assessed against whitefly under laboratory condition. The mortality was assessed at varying conidial concentrations and exposure times. Both factors, along with their interaction had significantly influenced, where the higher concentrations and longer exposure times increasing the mortality rate. Maximum mortality of *B. tabaci* nymphs of 75.83% at 1.0 × 10^8 conidia/mL on day 10, with median LC_{50} values of 3.24 × 10^6 and LT_{50} values of 1.0 × 10^8 was 6.82 day. For adult *B. tabaci*, mortality increased exponentially, reaching 52.5% by day 10 at a concentration of 1.0 × 10^8 conidia/mL. However, *C. rosea* CR 02 showed lower virulence against adults compared to nymphs. Scanning electron microscopy (SEM) images of infected nymph *B. tabaci* revealed conidial adherence, hyphal growth, hyphal penetration, conidiophore formation and completely colonized the host's surface (24-96 hours post-inoculation). For adult *B. tabaci*, mycelial colonization observed 7 days after treatment. This study highlights the entomopathogenic potential of indigenous *C. rosea* CR 02 against *B. tabaci* and suggests their use for the environmentally sustainable pest management.

Keywords

biocontrol; *Bemisia tabaci*; *Clonostachys rosea* CR 02; entomopathogenic fungi; SEM

Introduction

Potato (*Solanum tuberosum* L.) is a highly nutritious food crop and the third most important globally in terms of human consumption, following rice and wheat (1). In 2023, global potato production was estimated at 375 million tonnes, with India as the second largest producer after China, contributing 59.74 million tonnes (2). However, the crop was infested by various insect pest which cause substantial losses in tuber quality and yield (3). India has a vast diversity of insect pests, poses varied and complex challenges to the potato farmers in managing the crop (4). One such major concern is the whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), which causes significant damage to potato crops through feeding by both nymphs and adults, as well

as indirectly transmission of plant viruses (5). Most notably, it transmits the begomovirus *Tomato leaf curl New Delhi virus-potato* (ToLCNDV- Potato), causing apical leaf curl disease in potato crops leading to huge yield losses and degeneration of seed stocks (3). For the management of whitefly populations, farmers have extensively applied broad-spectrum synthetic chemicals, leading to the development of resistance to major classes of insecticides (6). These chemicals also result in hazardous residues in the environment, potentially altering soil ecology and negatively impacting beneficial fauna (7). Growing awareness towards the detrimental consequences of pesticide residue on fruit, vegetable and agricultural produce poses a serious concern. To mitigate this issue, an imperatively emphasis to adopting an eco-friendly bio-pesticides as an alternative to the chemical pest control (8).

Entomopathogenic fungi (EPF) attaining notable interest due to their virulence and pathogenicity against broad range of insect host and their distinctive mode of action involves direct penetration of insect integument, which leads to mycosis (9). More than 700 fungal species from 90 genera have been identified as pathogenic efficacy against insects (10). Globally, a well-known entomopathogenic fungi belonging to the order Hypocreales, such as *Beauveria bassiana, Metarhizium anisopliae, Isaria fumosorosea, Hirsutella thompsonii, Nomuraea rileyi, Lecanicillium lecanii and Cordyceps sp.* have been widely utilized to control various crop insect pests (11). Among the entomopathogenic fungi, *Clonostachys rosea* (Link) Schroers, formerly *Gliocladium roseum* (Hypocreales: Bionectriaceae), as a prominent biocontrol agent against plant pathogenic fungi, insect pest and plant parasitic nematodes by underlying mechanism of mycoparasitism, antibiosis and production of secondary metabolites (12). It has been reported as potential entomopathogenic fungi to many insect pest such as *Thrips tabaci* (13), *Varroa destructor* (14), *Amritodus atkinsoni* (15), *Ommatissus lybicus* (16). Moreover, a EPF has high degree of genetic variation among their different biogeographic strains, because their virulence and pathogenicity of isolates can vary significantly, based on their unique enzymatic and molecular traits (17). There are no reports of indigenous *C. rosea* being used against *B. tabaci* in potato crops in India. This laboratory study examined the virulence, pathogenicity and ultramicroscopic interactions of the indigenous *C. rosea* CR 02 isolate with *B. tabaci*. These findings contribute to the development of sustainable pest management strategies.

Materials and Methods

Rearing of whitefly

Whiteflies were collected from the potato cultivar *Kufri jyothi* grown in a field in Thalavadi, Erode district, TamilNadu, India (11˚ 67′87.95" N, 76˚ 88′28.92"E, elevation - 809 m mean sea level). It was identified and confirmed using PCR with the primer pair of LCO 1490 and HCO 2198 (18) and deposited in NCBI GenBank with accession number of PQ721428. Whitefly colonies were maintained on brinjal plants in a glasshouse laboratory. For bioassay, the plants infested with eggs were transferred to another insect cage and allows 15 days to develop into the second instar nymph stage (19). Older plants were replaced with new ones to ensure the presence of healthy and active whiteflies.

Fungal culture

The biocontrol agent *Clonostachys rosea* CR 02 (Accession No. ON025052) was maintained in the laboratory for bioassay. This isolate was used to evaluate the pathogenecity and virulence against *B. tabaci.* The fungal isolate was grown and maintained on potato dextrose agar (PDA) slant and stored at 4 °C till further use.

Preparation of conidial suspension of the fungal isolate

For preparation of conidial suspension, a well sporulated 12-14 days old culture plate was submerged with 10 mL of sterilized 0.05 % Tween 80® and then gently scraped the mycelium with the help sterile scalpel blade. The mycelial suspension was transferred to the sterile vial and vortexed vigorously for 2 min, then filtered through sterile three-layer muslin cloth to remove the mycelium. The conidial concentration was determined using an improved Neubauer hemocytometer under a phase contrast microscope. Five concentrations (1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4 conidia/mL) were prepared by serial dilution from the stock suspension and stored at 4°C until the bioassay (8).

Laboratory bioassay for second instar B. tabaci nymphs

The experiment was conducted following the method (20), with minor modifications. Freshly excised potato leaves (cv. *K. Jyothi*) placed in an insect breeding dish with a 1 % plain agar layer. Twenty second instar nymphs were transferred to the abaxial side of the leaves using a camel hairbrush and then treated by topical spraying of five concentration of conidial suspension using glass atomizer and 0.05 % Tween 80® served as control. All the treatments were incubated 25± 2°C, with photoperiod of 14:10 h. The mortality was recorded on the 3rd, 5th, 7th and 10th days after treatment. The experiment was conducted twice with four replication and single leaf for each replication.

Laboratory bioassay against B. tabaci adult

This experiment followed the leaf dip bioassay method as described (19) with slight modifications. Potato leaves were immersed in each concentration of conidial suspension for 15s and for control, 0.05 % Tween 80® solution was used. The treated leaves were blotted on sterile filter paper and then transferred to dish lined 1 % plain agar. Adult whiteflies ≥ 5 days old were released onto the treated leaves and then closed with lids containing ventilated cap holes covered with nylon mesh. Mortality was assessed at 3rd, 5th, 7th and 10th days after treatment (DAT). The experiment was conducted twice with four replicates, each replicate consisting of a single leaf.

Scanning Electron Microscopic (SEM) observation of insect mycosis

SEM analysis used to investigate the ultramicroscopic observation of the colonization of *C. rosea* CR 02 in the insect body. A spore concentration of 1×10^8 conidia/mL was sprayed on second instar nymphs and adult *B. tabaci* and samples were collected at 24, 48, 72, 96 hours post infestation (hpi) for nymphs and at 7th day for adult *B. tabaci*. Insects

used as control were collected for comparison. The samples (about 10 nymphs) were initially immersed in 2.5 % v/v glutaraldehyde for 24 h and then rinsed three times with 0.1 M phosphate buffer. Then dehydrated by series of ethanol using 30 %, 50 %, 70 % and 100 % with each step lasting for 10 min (21). The dried sample mounted on aluminium stubs using double sided adhesive carbon tape and then sputter-coated using an EMITECH quorum sputter-coater. The SEM images were captured by FEI Quanta™ FEG- 250 model.

Statistical analysis

The experimental data from the bioassays were analyzed using R studio software, version 4.4.0. A two-way factorial analysis of variance (ANOVA) performed for the mortality data of insects, where conidial concentration and exposure time taken as a factor. Differences between the treatment means were compared by Duncan's multiple range test ($p \leq 0.05$) (22). Probit regression analysis, where the median lethal concentration (LC_{50}) and median lethal time (LT_{50}) were calculated with 95 % confidence limits (23).

Results

Mortality response of C. rosea CR 02 against second instar nymph of B. tabaci under laboratory condition

Concentration and Time Mortality response : A two-way factorial ANOVA of second instar *B. tabaci* nymph bioassay revealed that conidial concentration ($F_{4,60}$= 544.144, $p < 0.001$) and time ($F_{3,60}$ = 95.503, $p < 0.001$) and their interaction ($F_{12,60}$= 378.598, $p < 0.001$) all had a significant effect on the mortality of *B. tabaci nymphs* are represented in Table 1. Mean percent mortality of *B. tabaci* nymphs exhibited a concentration and time-dependent manner, with mortality rising in accordance with increasing conidial concentrations and exposure time. The mortality rate exhibited an increasing pattern for all conidial concentrations from days 3 to 10 (R^2 - 0.98 - 0.99).

Table 1. Mean percent mortality of 2nd instar nymphs of *B. tabaci* subjected to different concentrations of indigenous *C. rosea* CR 02 under laboratory conditions

Source	DF[a]	SS[b]	MS[c]	F-value	p-value
Concentration	4	9,682.230	2,420.557	544.114	< 0.001
Time	3	1,274.575	424.858	95.503	< 0.001
Concentration x Time	12	20,210.881	1,684.240	378.598	< 0.001
Error	60	266.917	4.449		
Total	79	31,434.603			

Two factorial ANOVA at α=0.05

[a]DF - Degrees of Freedom, [b]SS - Sum of Squares, [c]MS - Mean square

Mortality Pattern Over Time : A maximum mortality of second instar whitely nymphs was observed on the 10th day at a concentration of 1 ×10^8 conidia mL^{-1}, followed by 1 ×10^7, 1 ×10^6, 1 ×10^5 and 1 ×10^4 conidia mL^{-1}. At 10^8 conidia mL^{-1}, the mortality percentages on days 3, 5, 7 and 10 were 8.33 ± 0.16 %, 25.83 ± 0.11 %, 45.00 ± 0.61 % and 75.83 ± 1.54 %, respectively. For 10^7conidia mL^{-1}, the corresponding mortality percentages were 5 ± 0.11 %, 17.50 ± 0.29 %, 34.17 ± 0.08 % and 54.17 ± 0.07 %, while for 10^6 conidia mL^{-1}, they were 3.33 ± 0.02 %, 10.83 ± 0.12 %, 20.83 ± 0.15 % and 40.00 ± 0.43 %, respectively. For the lower concentrations of 10^5 and 10^5 conidia mL^{-1}, no mortality was observed on the 3rd day. However, mortality percentages on days 5, 7 and 10 for 1.0 × 10^5 conidia mL^{-1} was 5 ± 0.08 %, 15 ± 0.18 % and 27.5 ± 0.39 %, while for 1.0 × 10^4 conidia mL^{-1} was 3.33 ± 0.05 %, 8.33 ± 0.07 % and 16.67 ± 0.12 % are shown in Fig. 1.

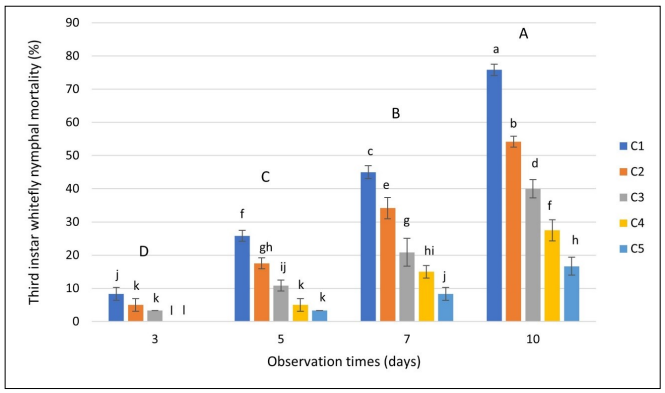

Fig. 1. Bioassay of second instar nymphs of *B. tabaci* exposed to five different concentrations of indigenous *C. rosea* CR 02 isolate (C1- 1.0 × 10^8, C2 - 1.0 × 10^7, C3 - 1.0 × 10^6, C4 - 1.0 × 10^5, C5 - 1.0 × 10^4 conidia ml^{-1}) under laboratory conditions to assessed mean percent mortality and standard error. In two-way ANOVA followed by Duncan's multiple range test ($p \leq 0.05$), a small letters indicated at the top of each bar showed the significant differences among the five concentration and the capital letters indicated the significant differences among the percent mortality at different time period (days).

Probit regression analysis : The mean LC_{50} and LT_{50} for the data of percent corrected mortality of second instar *B. tabaci* nymphs. The median LC_{50} values for 7th and 10th day was 2.5 ×10^8, 3.24×10^6 conidia mL^{-1} respectively were presented in Table 2 and median LT_{50} values of 6.822, 8.981, 11.599 days for the concentration 1.0 ×10^8, 1.0 ×10^7 and 1.0 ×10^6 was respectively. were presented in Table 3.

Table 2. Median lethal concentration (LC_{50}) values for the indigenous *C. rosea* CR 02 bioassay against 2nd instar nymphs of *B. tabaci* under laboratory conditions

Time (days)	LC_{50} (Conidia/ ml)	Lower and upper fiducial lim i (conidia/ml)	Slope	P - value
7	2.50X10^8	1.15 X 10^7 – 5.39 X10^9	Y= 2.361+0.3143X	< 0.001
10	3.24X10^6	8.98X10^5 – 1.17 X 10^7	Y=2.3739+0.4039X	< 0.001

[a]LT_{50} - Median lethal concentration

Table 3. Median lethal time (LT_{50}) values for the indigenous *C. rosea* CR 02 bioassay against 2nd instar nymphs of *B. tabaci* under laboratory conditions

Concentration (conidia/ml)	LT_{50}[a] (days)	Lower and upper fiducial limit (days)	Slope	*p* - value
1 x 10^8	6.822	5.814 - 8.003	Y=-2.108+3.4668X	< 0.001
1 x 10^7	8.981	7.032 - 11.469	Y=-2.0859+3.0075X	< 0.001
1 x 10^6	11.599	8.094 - 16.621	Y=-1.8356+2.879X	< 0.001

[a]LT_{50} - Median lethal time

Concentration and time mortality response of C. rosea CR 02 against B. tabaci adult under laboratory condition

A two-way factorial ANOVA for adult *B. tabaci* bioassay revealed that conidial concentration ($F_{2,36}$= 95.447, $p < 0.001$) and time ($F_{3,36}$ = 26.316, $p < 0.001$) and their interaction ($F_{6,36}$= 38.079, $p < 0.001$) all significantly affected the mortality of adult *B. tabaci* are given in Table 4. Mean percent mortality of *B. tabaci* adult exhibited exponential pattern for the three conidial concentrations from days 3 to 10. A maximum

mortality of whitefly adult was recorded on the 10th day at a concentration of 1.0 ×10^8 conidia mL^{-1}, followed by 1.0 ×10^7, 1.0 ×10^6 conidia mL^{-1}. No mortality was observed at any concentration on the 3rd day. At a concentration of 10^8 conidia mL^{-1}, the mortality percentages on days 5, 7 and 10 were 17.5 ± 0.35 %, 35 ± 0.06 % and 52.5 ± 0.64 %, respectively. For 10^7 conidia mL^{-1}, the mortality rates were 10 ± 0.14 %, 27.5 ± 0.25 % and 37.5 ± 0.56 %, while for 10^6 conidia mL^{-1}, the values were 2.5 ± 0.05 %, 10 ± 0.04 % and 17.5 ± 0.22 % were shown in Fig. 2.

Table 4. Mean percent mortality of adult *B. tabaci* subjected to different concentrations of indigenous *C. rosea* CR 02 under laboratory conditions

Source	DF[a]	SS[b]	MS[c]	F-value	p-value
Concentration	2	5,037.500	2,518.750	95.447	< 0.001
Time	3	2,083.333	694.444	26.316	< 0.001
Concentration x Time	6	6,029.167	1,004.861	38.079	< 0.001
Error	36	950.000	26.389		
Total	47	14,100.000			

Two factorial ANOVA at α=0.05

[a]DF - Degrees of Freedom, [b]SS - Sum of Squares, [c]MS - Mean Square

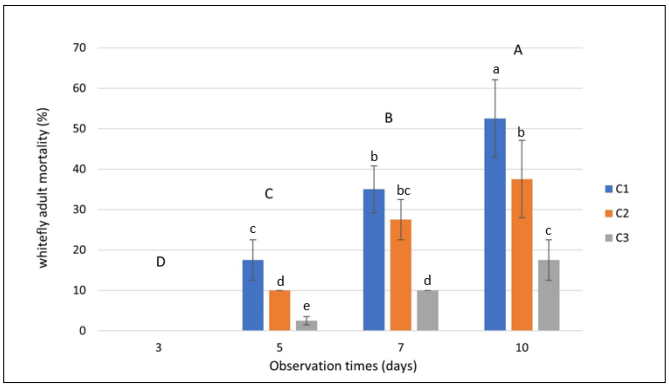

Fig. 2. Bioassay of *B. tabaci* adult exposed to three different concentrations of indigenous *C. rosea* CR 02 isolate (C1- 1.0 × 10^8, C2 - 1.0 × 10^7, C3 - 1.0 × 10^6conidia ml^{-1}) under laboratory conditions to assessed mean percent mortality and standard error. In two-way ANOVA followed by Duncan's multiple range test ($p \leq 0.05$), a small letters indicated at the top of each bar showed the significant differences among the three concentrations and the capital letters indicated the significant differences among the percent mortality at different time period (days).

Ultramicroscopic observation of infected cadavers

Scanning electron microscopic images of healthy second instar *B. tabaci* nymph showing no sign of infection in Fig. 3. A - C. At 24 hpi, conidia observed on the host surface, then at 48 hpi, conidial germination and emergence of hyphae and later, hyphal penetration and proliferation over the surface of the cuticle along with the emergence of conidiogenous structures followed by covered entire body surface are shown in Fig. 3. D - J. SEM images of healthy *B. tabaci* adult are shown in Fig. 4. A - C. In treated *B. tabaci* adult individuals, after 7 days post inoculation, hyphal colonization and emergence of conidiogenous structures was observed on the body surface and at later stage, mycelium completely covered the host surface are shown in Fig. 4. D - G.

Discussion

The extensive and frequent usage of highly persistent synthetic insecticides leads to environmental contamination and health risks, which highlighted to find the safer alternatives (24). Entomopathogenic fungi (EPF) constitute a diverse and systematically organized group with significant variation in the biological characteristic and mostly pathogenic to insects which help to reduce and regulate the harmful insect's population (25). However, many commercially available EPF products struggle to tussle, due to their lack of adaptability to varying agro ecological condition. This can be overcome by isolating EPF from the local environment, as isolates native to specific regions are more likely to thrive and be effective under those conditions (9). In this study, *C. rosea* CR 02 is reported for the first time as an entomopathogenic fungus against *B. tabaci* in India.

The pathogenicity and virulence bioassay are a crucial factor in assessing biocontrol agent, as it can vary between different isolates against target pest. This variation is influenced by the distinct enzymatic activities and molecular characteristics of each isolate (17). The bioassay with whiteflies revealed that *C. rosea* CR 02 was relatively more virulent against second instar nymphs than adult *B. tabaci*. The maximum nymphal mortality was 75.83 ± 1.54 % at a concentration of 1.0 × 10^8conidia/mL, with LT_{50} values of 6.82 days and LC_{50} of 3.24 × 10^6 conidia/mL. For adult *B. tabaci* the mortality reached 52.5 % of 10 days after treatment. Similarly, isolate of *Isaria javanica* (Cjc-03 and Cjw-01) and *Purpureocillium lilacinum* (TS-01) caused second instar nymphal mortality of 91.1 %, 76.5 % and 62.7 % at 1 × 10^8 conidia $mL^{-1}$of 8 DAT, respectively and for adult *B. tabaci* more than 50 % mortality of 7 DAT (20). Similarly, *I. fumosorosea* IF-1106 caused mortality of second instar nymph of 83 % within 7 days at 10^7 conidia mL^{-1} with LT_{50} ranged from 4.5 days (21). The entomopathogenic nature of *C. rosea* has also been reported against several other pests include 82.5 % mortality of *Oncometopia tucumana* of 14 DAT (26), 96.6 % mortality of *Amritodus atkinsoni* and 33 % and 85 % mortality in the nymph and adult populations of *Ommatissus lybicus* of 7 DAT, respectively (15, 16).

To gain a better understanding of their infection process, ultramicroscopic changes in *B. tabaci* during *C. rosea* CR 02 infection were examined through scanning electron microscopy (SEM). During the infection process of *C. rosea* CR 02 in *B. tabaci* nymph, the conidial adhesion and germination on cuticle surface at 24 hpi and proliferation and penetration of hyphae at 48 hpi. In later stage of 78 to 96 hpi, mycelium was covered the entire body and formed mummified appearance. Similarly, in adult *B. tabaci*, seven days after treatment, the mycelium was covered the entire body surface. Similarly, where second instar nymphs of *B. tabaci* treated with *I. fumosorosea* (IF-1106) exhibited conidia adherence and appressorium formation at 6-24 hours post-inoculation (hpi), followed by hyphal penetration at 48 hpi, with a visible color change in the nymphs during this period. At 72 hpi, conidiophores emerged with phialide production and by 120 hpi, conidiophores and hyphae covered the entire body surface (21). The ultramicroscopic observations of the infection process in *I. javanica B. tabaci* nymphs revealed conidia adhesion and germination, formation of a germ tube, hyphal penetration of the integument and the development of dense mycelium covering the cuticle surface (20). Likewise, the

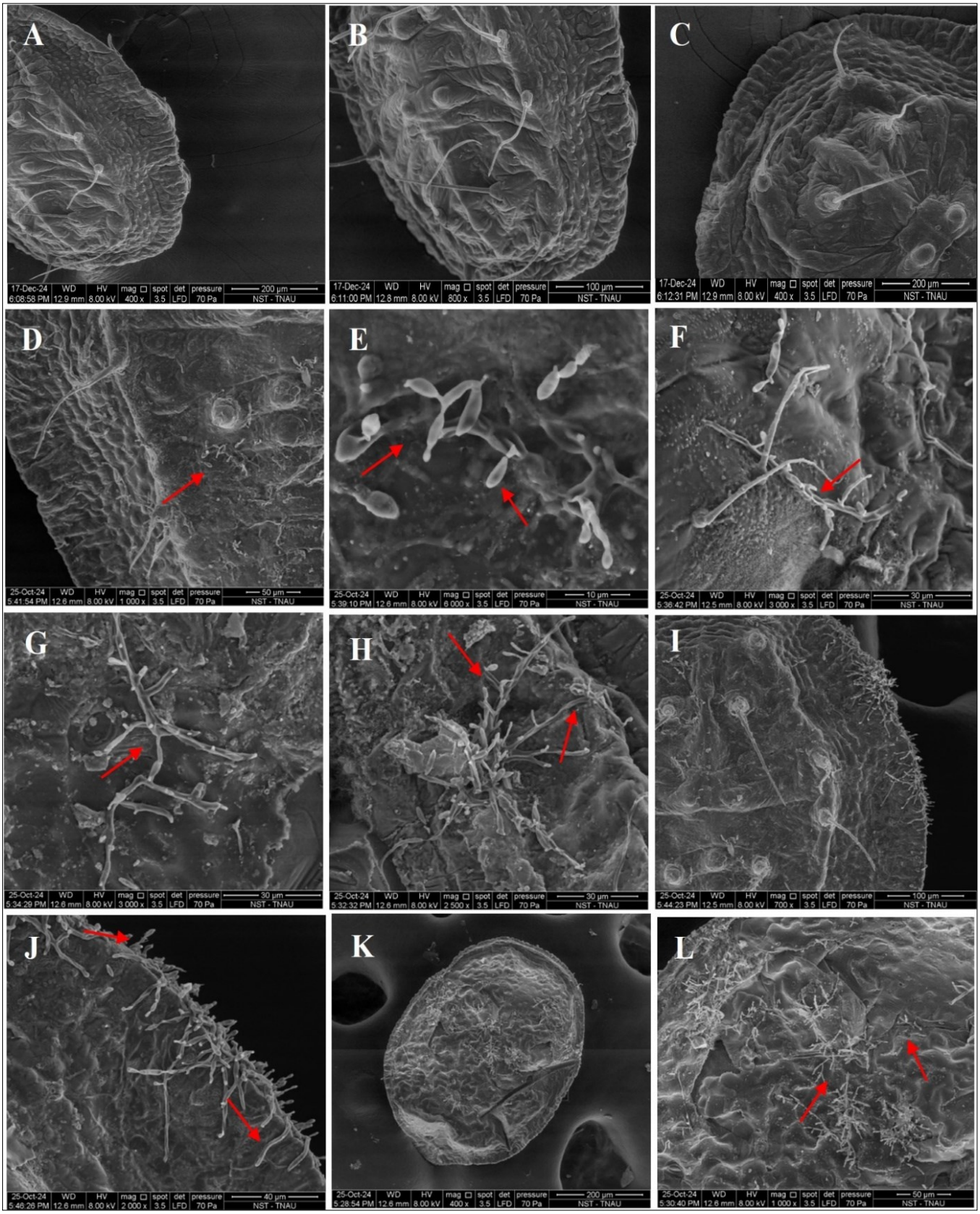

Fig. 3. Scanning electron microscopic (SEM) images showing the infection process of *Clonostachys rosea* CR 02 in second-instar nymphs of *Bemisia tabaci*. (A-C) Overview of a healthy nymph (uninfected control); At 24 (hpi) (D) Conidia observed on the host cuticle; (E) Magnified view showing a cluster of conidia (arrow); At 48 (hpi) (F, G) Hyphae emerging on the cuticle surface (arrow); (H) Hyphal penetration into the host (arrow); At 72 (hpi) (I) Hyphal proliferation; (J) Close-up view of proliferation of hyphae and emergence of conidiogenous structures (arrow); At 96 (hpi) (K) Nymph completely covered with mycelium; (L) Enlarged view of hyphae and conidia.

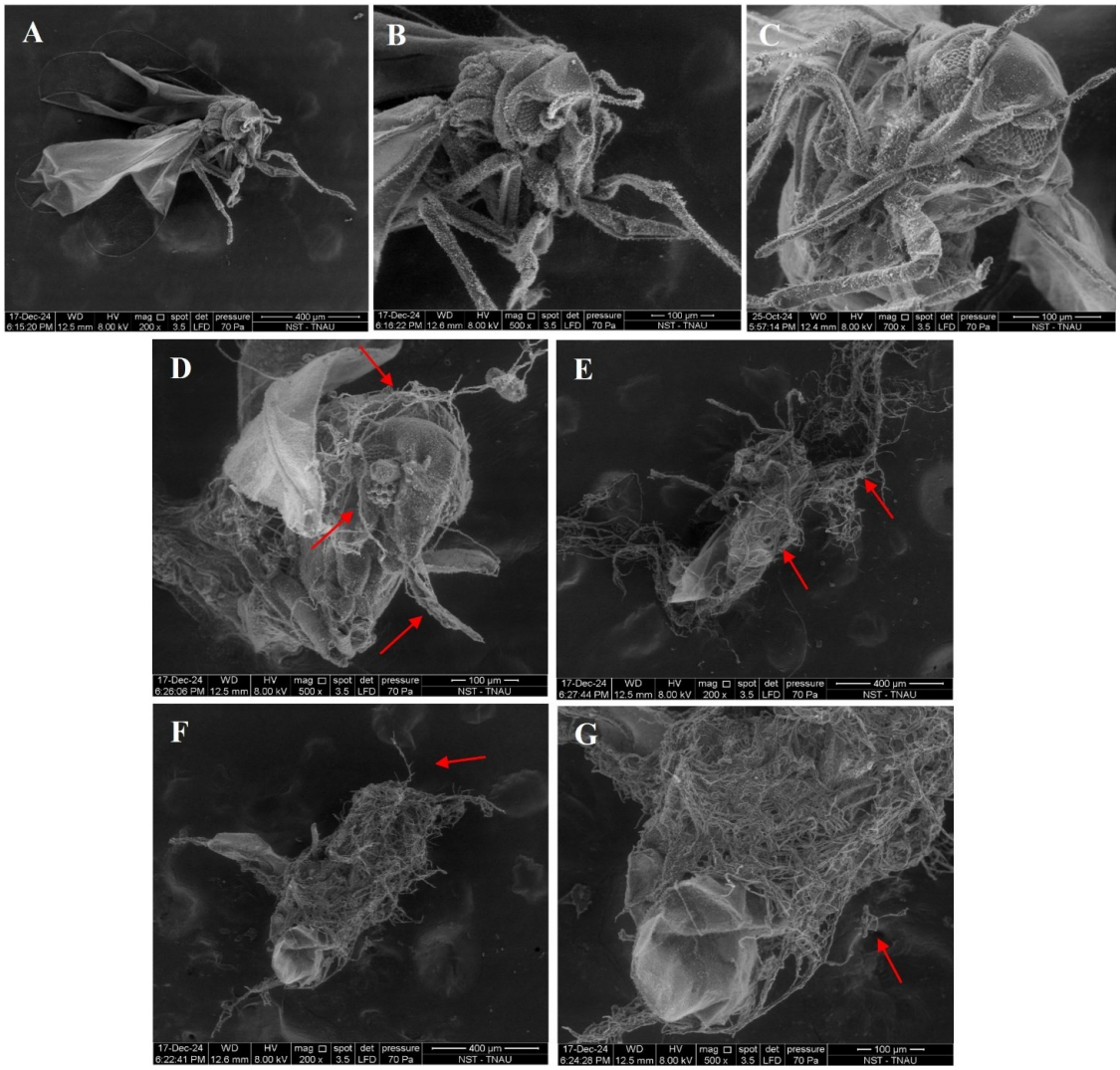

Fig. 4. Scanning electron microscopic (SEM) images showing the colonization of *Clonostachys rosea* CR 02 in adult *Bemisia tabaci*. (A, B) Overview of a healthy adult (uninfected control); (C) Close-up view of the head region, highlighting the compound eye and mouthparts of a healthy adult; After 7 days post-inoculation (dpi) (D) hyphal colonization observed on the head region, including the compound eye and mouthparts (indicated by an arrow); (E) Mycelium completely covering the host (arrow); (F) Lateral view of the insect cadaver completely covered by mycelium, with the emergence of conidiogenous structures (arrow); (G) Close-up view of the dense mycelial network coverage (arrow).

ultramicroscopic observations *C. rosea* against *O. lybicus* adults and nymphs showed the adhesion and penetration structures conidia and later formation of a dense mycelial network over the insect cadaver (16).

Conclusion

It is concluded that, the indigenous *C. rosea* CR 02 isolate performed well against *B. tabaci* in concentration-time dependent manner during laboratory bioassay. Probit regression analysis determined the LC_{50} and LT_{50} values for whitefly nymphs, confirming the pathogenic potential of *C. rosea*. Scanning electron microscopy revealed the ability of *C. rosea* CR 02 to infect and colonize host surfaces, providing insight into its pathogenic mechanisms. So, this isolate serves as an effective biocontrol agent, playing a vital role in the integrated pest management in potato. In future studies will focus on field evaluation of this fungal isolate against these pests, as well as examining its interactions with natural enemies.

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Authors' contributions

JSB wrote the original draft, performed formal analysis and data curation. VKP, IJ and MK reviewed the manuscript, conceptualized and supervised the research and performed data curation. TE and PHM reviewed the manuscript and formal analysis. RA, MJ reviewed the manuscript and data curation.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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