

COMPATIBILITY AND SYNERGISTIC EFFECTS OF INSECTICIDES WITH *Metarhizium anisopliae* FOR SUSTAINABLE MANAGEMENT OF *Nephotettix virescens* IN NORTH-EASTERN HIMALAYAS

B. K. Sahoo^{1,*}, M. Pathak², S. Patra³, M. A. Waseem⁴, K. Ningthoujam², P. Dutta⁵, R. K. Patidar², H. Kalita⁶, S. Haritha², A. Raj⁷, S. Boruah¹, K. Sarmah¹, M. Ikram¹, M. Hnialum¹ and A.R. Ramya¹

¹Department of Agricultural Entomology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, 641003, India

² School of Crop Protection, College of Post Graduate Studies in Agricultural Sciences, Central Agricultural University (Imphal), Umiam, Meghalaya, 793103, India

³ ICAR Research Complex for NEH Region, Umiam, Meghalaya, 793103, India

⁴Department of Natural Resource Management and Conservation, Forest College and Research Institute, Mulugu, 502279, India

⁵ College of Agriculture, Kyrdemkulai, Central Agricultural University (Imphal), Umiam, Meghalaya, 793104, India

⁶Department of Molecular, Cellular and Developmental Biology, IOWA State University, IOWA, 50014, USA

⁷Department of Entomology, ICAR-National Research Centre on Litchi, Muzaffarpur, Bihar, 842002, India

*Corresponding author's email: bimalsahoo.1996@gmail.com

ABSTRACT

Rice, a staple food crop cultivated globally, sustains the livelihoods of millions of people. However, its productivity is significantly compromised by the infestation of various insect pests including green leafhopper (*Nephotettix virescens*). The overreliance on chemical insecticides has resulted in the development of resistance in green leafhopper, increasing the challenges for its effective management. So, this study aims to investigate the integration of microbial agents with chemical insecticides as a sustainable approach for the management of *N. virescens*. Both individual and combined applications of the entomopathogen *Metarhizium anisopliae* and chemical insecticides against *N. virescens* under laboratory and field conditions were assessed. Initially, effects of six insecticides (Pymetrozine, Deltamethrin, Thiamethoxam, Acephate, Imidacloprid and Diafenthiuron) on the vegetative growth of *M. anisopliae* were tested. Out of these, three insecticides (Imidacloprid, Pymetrozine, and Thiamethoxam) were found to be compatible with the fungus, promoting increased vegetative growth, and were selected for further evaluation. Bioassay study against adults of *N. virescens* revealed that the LC₅₀ and LC₉₀ values for the combined treatments were lower than those for the insecticides alone, indicating synergistic effects, except for thiamethoxam. Pooled results revealed that the combination of *M. anisopliae* and imidacloprid proved to be effective against *N. virescens*, with 63.68% and 67.06% mortality in the first and second sprays, respectively, compared with 56.23% and 60.44% with imidacloprid alone under field condition. Scanning electron microscopy confirmed that the highest degree of conidial attachment occurred in the softer intersegmental regions near the legs, whereas fewer conidia were found in the head. These results suggest that *M. anisopliae* can be safely administered in the integrated pest management programme without hampering its efficacy for the management of rice leaf hoppers. Farmers and policymakers are encouraged to adopt integrated pest management strategies combining compatible microbial agents and insecticides to enhance sustainable management of green leafhoppers.

Keywords: Green leafhopper, *Metarhizium anisopliae*, compatibility, synergistic effect, scanning electron microscopy

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INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for over 3.5 billion people worldwide, making it a crucial crop for global food security (Alam *et al.*, 2024). Most of its production and consumption is concentrated in Asia, with China and India together contributing approximately 55%

(Kong *et al.*, 2015). Global rice production in the year 2024-25 was reported to total 532.67 million metric tons (FAS, 2024). However, the crop faces significant challenges, including attacks from over 100 insect species, of which 20 are considered major pests, causing annual yield losses of nearly 90 million metric tons (He *et al.*, 2023). Pathogens and insect pests threaten rice

throughout its growth cycle, leading to yield losses of 10 to 30% annually (Chintalapati *et al.*, 2023). Among these insect pests, leafhoppers are common rice pests in Asia, often leading to outbreaks that result in significant crop damage known as "hopper burn" (Backus *et al.*, 2005). Among various insect pests that damage rice, the green leafhopper (*Nephotettix virescens*) stands out as a significant threat, resulting in a considerable 15–20% decrease in rice yield (Prakash and Rao, 1998).

Rice leafhoppers have developed resistance to insecticides, from organochlorines, organophosphates, carbamates, and pyrethroids. Hoppers may also exhibit resistance to combinations of chemicals from two or more groups. The extensive use of insecticides can accelerate resistance and result in the resurgence of herbivorous pests (Yan *et al.*, 2023). Numerous research investigations have suggested that, although insecticide usage initially leads to effective pest control, it is insufficient to maintain the desired level of pest management over the course of several years (Matsumura and Sanada-Morimura, 2010). To prevent damage of rice plants, various methods, such as cultural, biological, and chemical control have been implemented (Fahad *et al.*, 2015). Among these methods, chemical control is the primary approach for managing rice leafhoppers (Ko *et al.*, 2015). However, concerns about their impact on natural predators, pollinators, environmental pollution, and human health have led to increased regulations on insecticide usage and a push to reduce their application.

Microbial control is a highly effective and safe approach used in integrated pest management programs and has shown considerable potential for the management of insect pests (Singh *et al.*, 2019). Entomopathogenic fungi play a crucial role as biological control agents with minimal environmental risks (Zimmermann, 2007). Importantly, insect pests exhibit low susceptibility to developing resistance against entomopathogenic fungi (Knols *et al.*, 2010), and the persistent efficacy of these fungi can be enhanced through their diffusion by insects, water, or wind (Islam *et al.*, 2021). In practical applications, the simultaneous use of both fungal and chemical agents to target insects has shown significantly higher mortality rates than the use of each control strategy independently (Tang *et al.*, 2019). Furthermore, this combined approach is promising for tackling insecticide-resistant pests (Knols *et al.*, 2010). Specifically, the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin has been recognized as an effective biocontrol agent against sucking pests (Ibrahim *et al.*, 2021; Kawpet *et al.*, 2022).

Entomopathogenic fungi are often considered slow-acting and require more time than conventional methods to achieve sufficient insect mortality. To address this issue, integrating entomopathogenic fungi (EPF) into a management strategy with faster-acting materials may offer a solution. The combination of mycoinsecticides

with chemical insecticides has been found to exhibit synergistic action, increasing insect mortality and reducing the time until death (Sahoo *et al.*, 2024). Moreover, incorporating synergists into insecticides can significantly improve cost-effectiveness and eco-friendliness by reducing the required quantity and extending residual activity.

However, there is limited data regarding the compatibility of EPF with insecticides, and evaluation of synthetic insecticide combinations with mycoinsecticides against leafhoppers is scarce. Therefore, the present study assessed both individual and combined applications of *M. anisopliae* and chemical insecticides against *N. virescens* under both laboratory and field conditions besides examining the mode of infection through scanning electron microscopy (SEM).

MATERIALS AND METHODS

Culturing of *M. anisopliae*: The fungus inoculum of *M. anisopliae* (Accession no: OL375172) was procured from the Biopesticide Production Unit, College of Post Graduate Studies in Agricultural Sciences (CPGS-AS), Central Agricultural University (Imphal), Umiam, Meghalaya. The fungus was multiplied on potato dextrose agar broth by inoculating the optimum quantity of fungal culture and was transferred to a shaking incubator at 180 rpm for 48 h at 28±1 °C. An appropriate dilution of 10⁶ cfu was maintained before the compatibility of *M. anisopliae* with the different insecticides was tested.

Evaluation of compatibility of different insecticides with *M. anisopliae*: Commercial formulations of six insecticides, pymetrozine 50% WG (Pymate), deltamethrin 2.8% EC (Decis), thiamethoxam 25% WG (Eco champ), acephate 75% SP (Chetak), imidacloprid 17.8% SL (Imidacel), and diafenthiuron 50% WP (Jimmy Deluxe), were procured from local pesticide shops. A series of dilutions was prepared for each chemical, and the recommended dose of each insecticide was incorporated into potato dextrose agar medium in an Erlenmeyer flask before solidification, ensuring thorough mixing. The medium was transferred to Petri dishes and allowed to solidify. A 5 mm disc of the previously cultured fungus was kept at the center of the Petri dish using a sterile inoculation needle. The Petri dishes were sealed properly and kept in an incubator at 25±1 °C and 80±5% relative humidity. Media without insecticides were maintained as control plates. The compatibility of *M. anisopliae* with the above-mentioned insecticides was assessed by calculating the radial growth of the fungus recorded 3, 6 and 9 days after inoculation (DAI) via Vernier calipers (Nawaz *et al.*, 2022).

Laboratory evaluation of toxicity of different insecticides combined with *M. anisopliae* against *N.*

virescens: Of the six tested chemicals for the compatibility study, the three most compatible chemicals were selected for the toxicity evaluation combined with *M. anisopliae* against *N. virescens* under laboratory conditions. Adult male and female *N. virescens* were collected from the experimental field of CPGS-AS, Umiam, Meghalaya, and transferred into a plastic box covered with a metal mesh containing fresh rice seedlings. Adult males and females were left for 48 h inside a plastic box for oviposition. Newly emerged *N. virescens* were used in the laboratory bioassay. Serial dilutions of *M. anisopliae* were prepared to determine LC₅₀ and LC₉₀ values. The LC₅₀ of *M. anisopliae* was added to different serial dilution concentrations of insecticides to evaluate the synergistic effect under

laboratory bioassays. *N. virescens* (30 per treatment) was transferred to a column bucket with fresh rice seedlings and left for 24 h for starvation. Three replicates were used for each experiment. A 500 µl suspension of each insecticide combined with *M. anisopliae* was sprayed using a Potter Precision Sprayer. All treated *N. virescens* were transferred to a climatic chamber, and the rice seedlings were watered daily. Mortality data were collected daily for up to 72 h. The toxicity (LC₅₀ and LC₉₀) of the sole insecticides and combination treatments with *M. anisopliae* was calculated using probit analysis. The synergy factor (Kalyanasundaram *et al.*, 1985) and co-efficient of toxicity (Sarup *et al.*, 1980) were calculated using the LC₅₀ and LC₉₀ values obtained for each treatment.

$$\text{Synergy factor (SF)} = \frac{\text{Toxicity of insecticide (alone)}}{\text{Toxicity of insecticide with } M. \text{anisopliae}}$$

$$\text{Co - toxicity coefficient (CTC)} = \frac{\text{Toxicity of insecticide (alone)}}{\text{Toxicity of insecticide with } M. \text{anisopliae}} \times 100$$

From the obtained result, a SF value > 1 indicates synergism and an SF value < 1 indicates antagonism (Bhan *et al.*, 2015; Chenniappa and Kadarkarai, 2008).

Field evaluation of combined use of insecticide and *M. anisopliae* against *N. virescens*: The experiment was conducted at the instructional farm of CPGS-AS, CAU (Imphal), Umiam (25°40'52.32' N, 91°54'41.04' E), Meghalaya, India, spanning two consecutive kharif seasons in 2021 and 2022. The rice variety CAUS 107 was chosen for this trial, and each plot size was maintained at 30 m², with a 2 m² buffer zone between the experimental plots withno sprays. All plots were separated from each other by nylon net cages to avoid trespassing insects from one plot to another. A total of eight treatments with four replications were performed when there was moderate infestation of *N. virescens*. Among all treatments, three treatments were the sole application of the three chemicals, and the other three treatments were the combination of *M. anisopliae* with

these three chemicals. The control was maintained by spraying it with water. The treatments were applied twice, at 14-day intervals, using a knapsack sprayer in the evening. Ten rice plants were randomly selected from each treatment to count the green leafhopper adults. The adult population of green leafhoppers was recorded one day before the first spray and continued for one month, with counts taken at 3, 7, 14, 21, and 28 days after the first spray. The number of adult insects per hill was averaged and expressed as the pest population.

Both nymphs and adults were considered in the counting process to assess the treatment efficacy. The relative efficacy of each treatment was evaluated using the Henderson and Tilton formula (Henderson and Tilton, 1955).

Efficacy (%)

$$= \left(1 - \frac{\text{Number of insects in control before spray} - \text{Number of insects in treated plot after spray}}{\text{Number of insects in control plot after spray} - \text{Number of insects in treated plot before spray}} \right) \times 100$$

Studies on infection by *M. anisopliae* on *N. virescens* through scanning electron microscopy:

Ultramicroscopic examination of *N. virescens* adults was conducted using Scanning Electron Microscopy (SEM), in which healthy adults were compared with adults treated with a combination of insecticides and *M. anisopliae* to investigate fungal development within the insect body. The adults were sprayed with a 1×10⁸ conidia/ml spore suspension, and samples were collected for preparation. The samples were first fixed in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and rinsed three times in the same buffer. Dehydration was

achieved by sequential immersion in 30%, 50%, and 70% ethanol at 30-minute intervals, followed by 100% ethanol for 45 min to complete critical point drying (Ganassi *et al.*, 2010). The dried samples were then mounted on pin stubs in various orientations using double-sided tape, sputter-coated with gold, and examined under the SEM FEI Quanta 250 (Netherlands), equipped with an Everhart-Thornley detector and a tungsten electron source.

Statistical analysis: Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 22.0. The compatibility of chemical insecticides

with *M. anisopliae* was assessed with six treatments and one control (only *M. anisopliae*), replicated three times. Statistical analysis of the laboratory compatibility test was conducted using a Completely Randomized Design (CRD). The percentage mortality of green leafhoppers was calculated using Abbott's formula for laboratory bioassays (Abbott, 1925). The relationship between insect mortality and concentration was analyzed using probit analysis (Finney, 1971). The LC₅₀ and LC₉₀ values were calculated with 95% confidence intervals. Statistical analysis of the field experiments involved a two-way analysis of variance (ANOVA) with a Randomized Complete Block Design (RCBD), which included seven treatments and one control, replicated four times. To compare the means and determine significant differences, Tukey's Honestly Significant Difference (HSD) test was employed at a significance level of P<0.05.

RESULTS

Compatibility of different insecticides with *M. anisopliae*: Vegetative growth of *M. anisopliae* was significantly affected by the presence of chemical insecticides under laboratory conditions (Table 1). However, among all the tested insecticides, three insecticides (imidacloprid, Pymetrozine, and Thiamethoxam) did not inhibit the growth of *M. anisopliae* to some extent, which was compatible with *M. anisopliae*. The highest radial growth was observed in imidacloprid with a colony diameter of 53.67 mm on 9 DAI, which was significantly different from the control. This was followed by Pymetrozine recording a colony diameter of 51.33 mm and Thiamethoxam with 49.67 mm 9 DAI, which was significantly different from the control. Deltamethrin showed the highest inhibition of *M. anisopliae* with a colony diameter of 26.33 mm 9 DAI.

Table 1. Compatibility of *M. anisopliae* with different insecticides.

Treatments	Radial growth in mm		
	3 DAI	6 DAI	9 DAI
Control (<i>M. anisopliae</i>)	38.00±0.58 (6.16 ^a ±0.05)	56.33±2.96 (7.51 ^a ±0.20)	84.67±2.60 (9.20 ^a ±0.14)
Pymetrozine 50 % WG + <i>M. anisopliae</i>	24.33±1.43 (4.93 ^{bc} ±0.14)	34.67±1.65 (5.89 ^{bc} ±0.14)	51.33±2.78 (7.16 ^b ±0.19)
Deltamethrin 2.8 % EC + <i>M. anisopliae</i>	8.00±0.41 (2.83 ^f ±0.07)	14.00±0.82 (3.74 ^d ±0.11)	26.33±2.87 (5.13 ^d ±0.28)
Thiamethoxam 25 % WG + <i>M. anisopliae</i>	19.00±0.82 (4.36 ^{cd} ±0.09)	28.67±1.03 (5.35 ^{bc} ±0.10)	49.67±3.68 (7.05 ^{bc} ±0.26)
Acephate 75 % SP + <i>M. anisopliae</i>	9.33±0.62 (3.06 ^{ef} ±0.10)	18.33±1.18 (4.28 ^d ±0.14)	34.00±1.47 (5.83 ^{cd} ±0.13)
Imidacloprid 17.8 % SL + <i>M. anisopliae</i>	29.33±2.25 (5.42 ^b ±0.21)	40.67±1.03 (6.38 ^b ±0.08)	53.67±0.62 (7.33 ^b ±0.04)
Diafenthiuron 50 % WP + <i>M. anisopliae</i>	14.67±0.94 (3.83 ^{de} ±0.13)	28.33±2.87 (5.32 ^c ±0.27)	42.33±2.01 (6.51 ^{bc} ±0.15)
SEm (±)	0.17	0.21	0.26
CD	0.53	0.65	0.79

Figures in parentheses are square root transformed values. Means followed by the same alphabet do not differ significantly by Tukey's HSD test (P < 0.05)

DAI = Days after inoculation, CD = Critical Difference, SEm = Standard Error of Mean

Synergistic effect of different insecticides with *M. anisopliae* against *N. virescens* under laboratory conditions: The LC₅₀ and LC₉₀ values for each insecticide and their combination with *M. anisopliae* are shown in Table 2. LC₅₀ and LC₉₀ for the combined application of insecticides with *M. anisopliae* were lower than those for the sole application of insecticides. LC₅₀ and LC₉₀ values for the Imidacloprid 17.8% SL against *N. virescens* were 0.11 ppm and 0.39 ppm, respectively. After the combination of imidacloprid with *M. anisopliae*, the LC₅₀ and LC₉₀ dropped to 0.09 and 0.33 ppm. Apart from addition, the synergistic factor (SF) for

imidacloprid in combination with *M. anisopliae* was found to be greater than one (1.22 and 1.18) for LC₅₀ and LC₉₀ respectively, indicating that *M. anisopliae* had a synergistic interaction with imidacloprid against *N. virescens*. The co-toxicity coefficient (CTC) for the combined application of *M. anisopliae* and imidacloprid was also recorded to be 122.22 and 118.18 for LC₅₀ and LC₉₀ respectively.

A similar effect was recorded for the combined application of Pymetrozine with *M. anisopliae* with LC₅₀ and LC₉₀ of 0.11 and 0.37 ppm, respectively lower than LC₅₀ (0.14 ppm) and LC₉₀ (0.49 ppm) values recorded for

sole application of Pymetrozine. The SF value (1.27) and CTC value (127.20) suggested a synergistic interaction between Pymetrozine and *M. anisopliae* against *N. virescens* when applied in combination.

For Thiamethoxam, the LC₅₀ values for both sole application and combined application with *M. anisopliae* were found to be the same (0.26 ppm), indicating that there was no interaction between thiamethoxam and *M. anisopliae* LC₅₀. A combination of *M. anisopliae* with Thiamethoxam increased the LC₉₀ value from 0.88 to 1.00 ppm suggesting that there was an antagonistic interaction between *M. anisopliae* and thiamethoxam with SF and CTC values of 0.88 and 88.00 respectively for LC₉₀.

Bioefficacy of insecticides combined with *M. anisopliae* against *N. virescens* under field conditions:

The relative efficacies of different insecticides and *M. anisopliae* alone and their 1:1 combination against the green leafhopper *N. virescens* as pooled results of both years (2021 and 2022) are presented in Table 3. The pooled results showed that a significant difference in relative efficacy was observed among the various treatments against *N. virescens* in the first spray ($F_{8,5} = 80.71$, $P < 0.01$) and the second spray ($F_{8,5} = 134.54$, $P < 0.01$).

Among all treatment combinations, the combination of imidacloprid and *M. anisopliae* was the most effective against *N. virescens*, achieving the highest relative efficacy of 75.34% at 21 DAS followed by imidacloprid alone, with 63.44% efficacy, both showing statistically significant differences ($F_{8,4} = 726$, $P < 0.01$) in first spray. A similar trend was observed after the second spray, where the combined application of imidacloprid and *M. anisopliae* again showed the highest efficacy at 14 DAS (73.44%), significantly outperforming imidacloprid alone (62.30%) ($F_{4,83} = 505.3$, $P < 0.01$).

The combination of pymetrozine and *M. anisopliae* was the second most effective treatment, with relative efficacies of 56.13 and 58.80 % in first and

second sprays, respectively. However, these were statistically similar to pymetrozine alone (56.22 and 56.85 %) at 21 DAS for first spray ($F_{8,4} = 726$, $P = 1$) and second spray ($F_{8,4} = 505.3$, $P = 0.97$). The lowest efficacy was observed in the combination of thiamethoxam and *M. anisopliae* (47.16 and 52.13 %), which also did not differ significantly from thiamethoxam alone in first ($F_{8,4} = 726$, $P = 0.91$) and second spray ($F_{8,4} = 726$, $P = 0.94$).

Overall, the combined application of *M. anisopliae* and imidacloprid showed the highest efficacy against *N. virescens*, with 63.68% in the first spray, followed by imidacloprid alone at 56.23%. A similar trend was observed after the second spray, with the combination achieving 67.06% efficacy, compared to 60.44% for imidacloprid alone.

Ultramicroscopic changes in *N. virescens* adult during *M. anisopliae* Infection:

SEM images of uninfected *N. virescens* (Figure 1A-E) were analyzed and compared to those of *M. anisopliae*-infected *N. virescens* adults, as illustrated in Figure 2. Conidial adherence (indicated by a yellow circle) was observed in different body parts, such as the head, thorax, mouth (Figure 2A), leg joints (2C), and abdomen (2D). Conidia germination (denoted by a yellow arrowhead) resulted in the formation of germ tubes and appressoria, which facilitated cuticular penetration (Figure 2A, B, C). The maximum number of conidia with germ tubes and appressoria was observed in the intersegmental regions adjacent to the leg (Figure 2C) compared to the other cuticular regions, with the lowest number of conidia in the head region. Extensive hyphal development (yellow arrow) was also observed in the thoracic region (especially the leg joints) (Figure 2C) and the terminal abdominal region (Figure 2D), suggesting extensive fungal proliferation inside the body. After utilizing the nutrients in the insect body, extrusion of hyphae occurred from the adult body parts, such as the head, thoracic legs, and abdomen (Figure 2E).

Table 2. Toxicity of different insecticides with and without *Metarhizium anisopliae* against green leaf hopper under laboratory condition.

Treatments	Ratio	Regression equation	Chi-square (SE)	LC ₅₀ (fiducial limits) (ppm)	SF	CTC	Effect	LC ₉₀ (fiducial limits) (ppm)	SF	CTC	Effect
<i>Metarhizium anisopliae</i>	-	$y = 0.332x + 2.994$	0.929 (0.02)	1.04×10^7 cfu (2.5×10^6 - 4.3×10^8)	-	-	-	1.02×10^{12} cfu (2.7×10^{10} - 3.8×10^{13})	-	-	-
Imidacloprid 17.8% SL	-	$y = 2.422x + 7.301$	0.854 (0.20)	0.11 (0.09-0.14)	-	-	-	0.39 (0.24-0.62)	-	-	-
<i>M. anisopliae</i> + Imidacloprid 17.8% SL	1:1	$y = 2.371x + 7.482$	0.786 (0.22)	0.09 (0.07-0.11)	1.22	122.22	Synergistic	0.33 (0.21-0.51)	1.18	118.18	Synergistic
Pymetrozine 50% WG	-	$y = 2.409x + 7.054$	0.735 (0.26)	0.14 (0.12-0.17)	-	-	-	0.49 (0.31-0.79)	-	-	-
<i>M. anisopliae</i> + Pymetrozine 50% WG	1:1	$y = 2.422x + 7.392$	0.743 (0.27)	0.11 (0.09-0.13)	1.27	127.20	Synergistic	0.37 (0.24-0.56)	1.32	132.43	Synergistic
Thiamethoxam 25% WG	-	$y = 2.560x + 6.488$	0.860 (0.18)	0.26 (0.22-0.32)	-	-	-	0.88 (0.57-1.37)	-	-	-
<i>M. anisopliae</i> + Thiamethoxam 25% WG	1:1	$y = 2.354x + 6.346$	0.878 (0.20)	0.26 (0.21-0.32)	1.00	100.00	No effect	1.00 (0.60-1.68)	0.88	88.00	Antagonistic

SE = Standard error, LC50 = Lethal concentration 50, LC90 = Lethal concentration 90, SF = Synergistic factor, CTC = Co-toxicity coefficient

Table 3. Bioefficacy of different insecticides and their combination with *M. anisopliae* against green leaf hoppers in both seasons (pooled data).

Treatments	Conc. (ppm)	First spray (Pooled)						Second spray (Pooled)					
		3 DAS	7 DAS	14 DAS	21 DAS	28 DAS	Mean	3 DAS	7 DAS	14 DAS	21 DAS	28 DAS	Mean
Control	-	0.00±0.00 (0.00) e	0.00±0.00 (0.00) g	0.00±0.00 (0.00) f	0.00±0.00 (0.00) e	0.00±0.00 (0.00) f	0.00	0.00±0.00 (0.00) d	0.00±0.00 (0.00) f	0.00±0.00 (0.00) f	0.00±0.00 (0.00) f	0.00±0.00 (0.00) e	0.00
<i>Metarhizium anisopliae</i>	1.04 × 10 ⁷ cfu	17.04±2.43 (24.12) d	20.28±0.32 (26.74) f	34.70±0.29 (36.08) e	45.33±0.80 (42.32) d	41.69±0.66 (40.21) e	31.81	19.48±1.47 (26.11) c	23.24±0.79 (28.78) e	34.64±1.01 (36.02) e	43.89±0.57 (41.48) e	40.50±1.27 (39.49) d	32.35
Imidacloprid 17.8% SL	14 ppm	48.12±1.41 (43.91) a	51.55±0.97 (45.89) c	57.46±1.02 (49.30) b	63.44±1.02 (52.81) b	60.58±0.34 (51.11) b	56.23	53.20±3.74 (46.87) a	58.70±0.77 (50.03) b	62.30±0.76 (52.12) b	66.34±1.58 (54.56) b	61.67±0.56 (51.77) b	60.44
Pymetrozine50% WG	11 ppm	42.31±2.25 (40.54) ab	48.15±0.54 (43.94) d	51.92±1.41 (46.10) c	56.22±1.01 (48.58) c	51.50±0.40 (45.86) c	50.02	48.12±3.30 (43.91) ab	50.17±1.50 (45.10) c	53.66±0.52 (47.10) c	56.85±1.19 (48.94) cd	52.14±0.53 (46.23) c	52.19
Thiamethoxam 25% WG	26 ppm	30.84±0.91 (33.69) c	39.85±0.95 (39.11) e	44.62±0.69 (41.90) d	49.23±1.68 (44.56) d	46.62±1.66 (43.06) d	42.23	41.33±2.76 (39.96) ab	43.92±1.85 (41.49) d	49.44±1.21 (44.68) d	54.38±0.96 (47.52) cd	49.51±0.94 (44.72) c	47.72
<i>M. anisopliae</i> + Imidacloprid 17.8% SL	11 ppm + 1.04 × 10 ⁷ cfu	40.34±1.95 (39.39) ab	63.18±0.37 (52.65) a	68.06±1.42 (55.61) a	75.34±1.56 (60.28) a	71.47±0.85 (57.73) a	63.68	50.38±4.20 (45.22) ab	66.34±1.56 (54.57) a	73.44±0.75 (59.01) a	73.02±2.76 (58.92) a	72.15±1.32 (58.23) a	67.06
<i>M. anisopliae</i> + Pymetrozine50% WG	9 ppm + 1.04 × 10 ⁷ cfu	38.17±1.40 (38.12) bc	54.63±0.29 (47.66) b	59.21±0.39 (50.31) b	56.13±0.93 (48.52) c	53.68±0.68 (47.11) c	52.36	43.65±2.42 (41.33) ab	56.14±1.04 (48.53) b	60.55±1.23 (51.11) b	58.80±1.06 (50.08) c	52.39±0.73 (46.37) c	54.31
<i>M. anisopliae</i> + Thiamethoxam 25% WG	26 ppm + 1.04 × 10 ⁷ cfu	30.21±1.89 (33.25) c	39.45±0.64 (38.89) e	44.49±1.33 (41.80) d	47.16±1.02 (43.37) d	44.05±0.75 (41.58) de	41.07	37.86±1.52 (37.94) b	43.05±1.16 (40.99) d	46.53±0.76 (43.01) d	52.13±0.88 (46.22) d	43.67±0.42 (41.36) d	44.65
F stat		161.70	2275.00	921.50	726.00	1358.00	-	93.83	595.60	1257.00	505.30	1179.00	-
CD @ 5%		3.05	1.06	1.68	2.01	1.31	-	2.38	1.62	1.53	2.56	1.27	-
SE(m) ±		1.04	0.36	0.57	0.68	0.44	-	0.81	0.55	0.52	0.87	0.43	-
CV		7.02	1.91	2.84	3.17	2.32	-	9.15	3.69	2.46	3.75	2.50	-

Figures in parentheses are arc sine transformed values; Means followed by the same alphabet do not differ significantly by Tukey's HSD test (P <0.05), DAS = Days after spray, CD = Critical difference, CV = Coefficient of variance, SE(m) = Standard error of mean

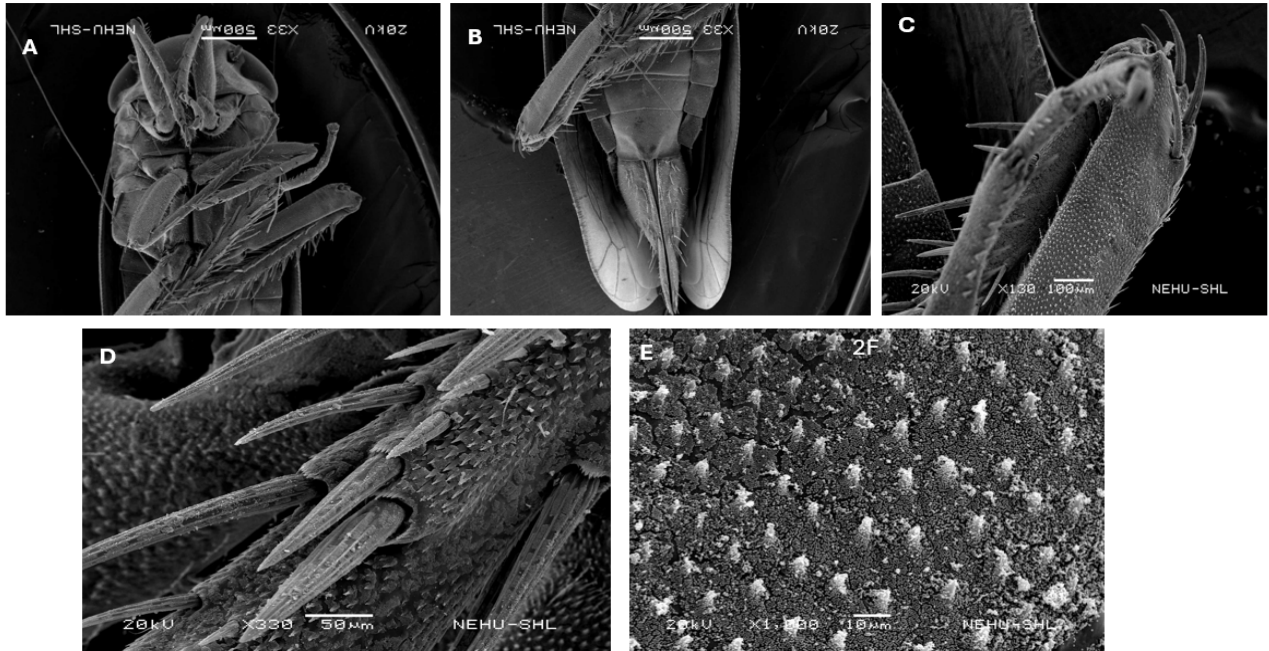


Figure 1. SEM images of a healthy adult *Nephotettix virescens*. A. Ventral view of the head showing the mouth and thorax region with legs. B. Ventral view of the abdomen C. Leg joint between femur and tibia. D. View of tibial spurs. E. View of the cuticular surface.

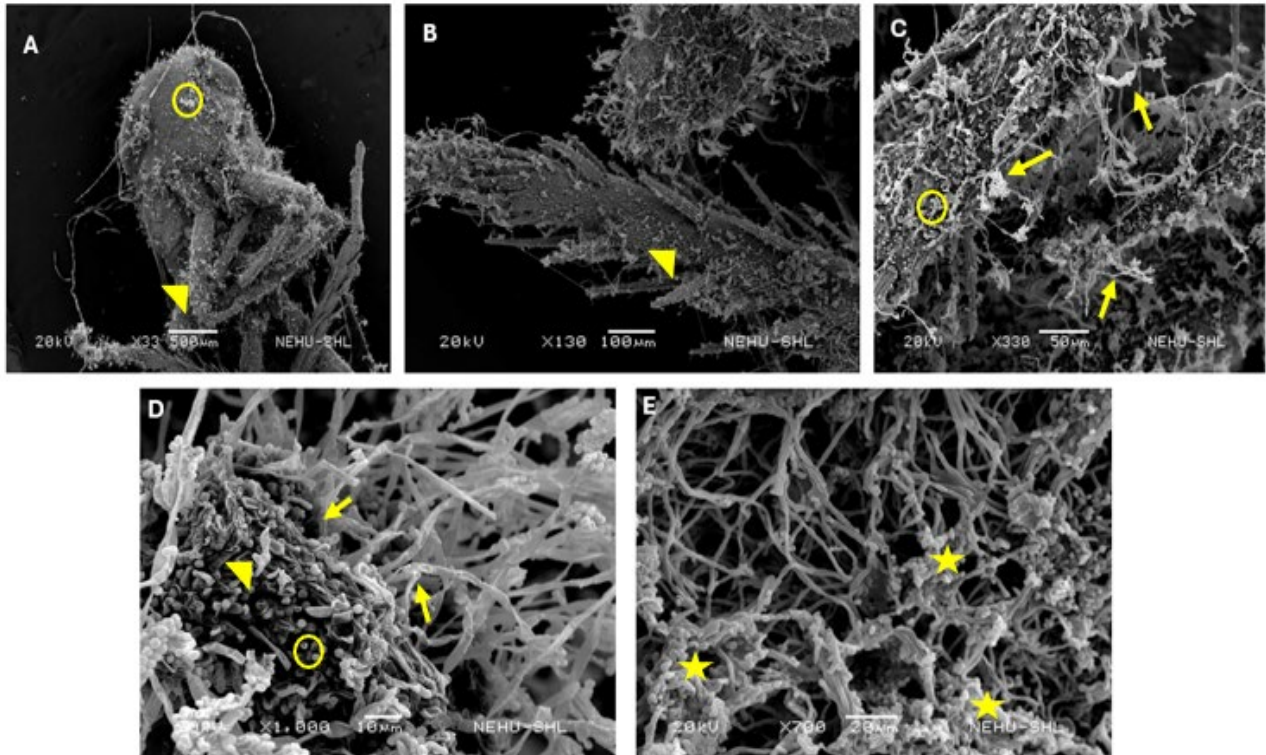


Figure 2. SEM images of an adult of *Nephotettix virescens*. A. Ventral view of head showing mouth parts and thorax region with legs adhered with conidia (yellow circle), germination of conidia (yellow arrowhead). B. Fungal mycelia on femur and tibia. C. Germination of conidia on leg joint between femur and tibia (yellow arrowhead). D. Conidia on abdomen (yellow circle), extensive hyphae development (yellow arrow mark) in the abdomen. E. extrusion of hyphae from insect body (yellow star mark).

DISCUSSION

Compatibility of different insecticides with *M. anisopliae*: Insecticides target various developmental forms of entomopathogenic fungi. The interference of insecticides on the conidial germination of fungi is one of the main factors which decide the compatibility between an insecticide and entomopathogenic fungus (Neves *et al.*, 2001). Our findings revealed that imidacloprid 17.8 % SL, Pymetrozine 50% WG, and Thiamethoxam 25 % WG were highly compatible with *M. anisopliae*, while deltamethrin 2.8 % EC, Acephate 75% SP and Diafenthiuron 50 % WP were highly toxic to the fungus used in this study. These findings are comparable to the previous work of Abidin *et al.* (2017), Johnson *et al.* (2020), Halder *et al.* (2021) and Nawaz *et al.* (2022), who reported a negligible impact of imidacloprid on vegetative growth and conidia production of *M. anisopliae*. In addition to imidacloprid, *M. brunneum* showed the highest conidial germination and mycelial growth at half the field-recommended concentrations (Yadav *et al.*, 2019). Similarly, several reports have been suggested that Thiamethoxam was also compatible with *M. anisopliae* (Khan *et al.*, 2012; Quintela *et al.*, 2013).

Fungi can metabolize insecticides, breaking them down into compounds that serve as secondary nutrients for their growth and development. In the insecticide-poisoned medium, fungus could develop defense capability by increasing conidial production. In addition, some insecticides may be directly used as carbon sources to support the growth of these fungi (Moino and Alves, 1998). Similarly, molecules analogous to the prosthetic group may diffuse into the cytoplasm, ultimately binding to specific receptors that affect membrane permeability, enzymatic synthesis, and metabolic processes (Oliveira *et al.*, 2003). The level of inhibition of the vegetative growth of fungi also depends on the nature of the chemical compound that blocks the conidia metabolic function, as well as the concentration of active ingredients (Antonio *et al.*, 2007). However, the inhibition of conidial germination in *M. anisopliae* by certain insecticides may result from interference with substrate recognition processes (Boucias *et al.*, 1988), suppression of germination initiation triggers (Leger *et al.*, 1994), or ion accumulation on the cellular membrane surface, leading to metabolic blockage (Ghini and Kimati, 2000).

Synergistic effect of different insecticides with *M. anisopliae* against *N. virescens* under laboratory conditions: *M. anisopliae* has been effectively employed to control several sucking pests, such as hoppers and variations in the interaction modalities (synergistic, antagonistic, or neutral) of EPF with insecticides have been documented in *M. anisopliae* (Jia *et al.*, 2016; Nawaz *et al.*, 2022). The results of the present

investigation revealed that the efficacy of *M. anisopliae* was enhanced by combining insecticides, such as imidacloprid and pymetrozine, against green leafhopper *N. virescens*, which might be due to weakening and disruption of insect defense and immune system by the chemical insecticides making them more susceptible to *M. anisopliae*. Jaramillo *et al.* (2005) revealed that the combined application of imidacloprid and *M. anisopliae* resulted in higher mortality of *Cyrtomenus bergi* compared to the individual application of fungus and insecticide. Similarly, Quintela and McCoy (1997) reported a synergistic interaction between sublethal doses of imidacloprid and *M. anisopliae*, leading to increased mortality in the citrus root weevil, *Diaprepes abbreviatus*. Halder *et al.* (2021) evaluated the efficacy of *M. anisopliae* and imidacloprid and thiamethoxam, both individually and in a 1:1 combination at half doses, against *Myzus persicae*. In contrast, Niassy *et al.* (2012) reported that combining *M. anisopliae* with imidacloprid or thiamethoxam did not produce synergistic or antagonistic effects in *Frankliniella occidentalis* larvae. However, using lower concentrations of the fungus with thiamethoxam resulted in a reduced lethal time (LT₅₀) compared to the individual treatments. The combined application of *M. anisopliae* with thiamethoxam or pymetrozine resulted in a shorter lethal time (LT₅₀) for *Nilaparvata lugens* and *Sogatella furcifera* than the use of *M. anisopliae* alone (Tang *et al.*, 2019). Similarly, 88.59% efficacy was reported in controlling *Aphis gossypii* through the combined application of imidacloprid and *M. anisopliae* (Nawaz *et al.*, 2022).

Lower doses of imidacloprid not only enhanced the efficacy of *M. anisopliae* but also reduced the quantity of inoculum required to achieve high mortality rates in *C. bergi* nymphs. The impaired mobility of insects exposed to insecticides may disrupt their normal molting process, preventing the shedding of fungal conidia from the cuticle. This could facilitate fungal penetration and invasion into the insect bodies (Jaramillo *et al.*, 2005). Insecticides can act as physiological stressors and behavioral modifiers, allowing fungal conidia to bypass the primary defense mechanisms of insects, thereby facilitating penetration into the cuticle (Inglis *et al.*, 2001). The synergistic effect of *M. anisopliae* and insecticides may be achieved by reducing the speed of killing and weakening the insect's immune system. For instance, phenoloxidase activity in insect hemolymph, a critical immune response, decreases over time following exposure to *M. anisopliae* (Quintela *et al.*, 2013; Yii *et al.*, 2016). In addition, the application of insecticides in combination with *M. anisopliae* may reduce enzymatic activities of glutathione S-transferase (GST), esterases (ESTs), phenol oxidase (PO), superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). This reduction may lead to

convulsions, a response commonly associated with *M. anisopliae* infections (Jia *et al.*, 2016).

In addition to the synergistic interactions observed in this study, an antagonistic interaction was detected between the fungus and thiamethoxam despite their compatibility. This outcome may be due to the combined low toxicity and limited conidial load, which are insufficient to overcome the insect defense mechanisms, rendering it less susceptible to infection. Similarly, Russell *et al.* (2010) found that low imidacloprid rates reduced fungal mycosis and conidial yield per cadaver in *Anoplophora glabripennis*. Similarly, Nawaz *et al.* (2022) showed an antagonistic interaction with *M. anisopliae* for LC₉₀ after 24 h for *A. gossypii*, however in the later days a synergistic interaction was observed when combined with fungus.

Bioefficacy of insecticides combined with *M. anisopliae* against *N. virescens* under field conditions:

There have been several reports on the management of plant hoppers and other sucking pests using the combined application of *M. anisopliae* and insecticides under field conditions (Jia *et al.*, 2016; Haider *et al.*, 2021). In the present study, *M. anisopliae* combined with insecticides (imidacloprid and pymetrozine) was effective against *N. virescens* under field conditions; however, *M. anisopliae* combined with thiamethoxam resulted in antagonistic effects. Jaramillo *et al.*, (2005) revealed that combined application of imidacloprid and *M. anisopliae* resulted in 88.1% mortality of *C. bergi* compared to 39.5% when the fungus was applied alone. Sahoo *et al.*, (2024) also found that the combined application of imidacloprid and *M. anisopliae* was more effective in controlling the rice gundhi bug, *Leptocorisa acuta*. Kassab *et al.* (2014) also found 82.1% and 78.6% mortality of spittlebug *Mahanarva fimbriolata* in sugarcane when *M. anisopliae* was combined with thiamethoxam and imidacloprid, respectively. Purwar and Sachan (Purwar and Sachan, 2004, 2006) reported a synergistic effect of combining *M. anisopliae* with imidacloprid against *Lipaphis erysimi* and *Spilosoma obliqua*.

Contradictory results have been reported by earlier researchers, such as Tang *et al.* (2019), who stated that *M. anisopliae* effectively suppressed populations of rice planthoppers *N. lugens* and *S. furcifera* by the combined application of *M. anisopliae* and thiamethoxam under field conditions. The median lethal time (LT₅₀) was reduced, and the overall mortality and percent mycosis increased significantly when *M. anisopliae* was combined with a sublethal dose of thiamethoxam to control of *Tibraca limbativentris* adults under field conditions (Quintela *et al.*, 2013). Similarly, Thiamethoxam alone achieved 65.0% mortality of *N. lugens*, but when combined with *M. anisopliae*, mortality increased to 81.25% under glasshouse conditions (Reddy *et al.*, 2021). This might be due to several factors influencing the

control efficacy of entomopathogenic fungi and chemical insecticides, such as weather conditions, the chemical's mode of action, and the specific *M. anisopliae* isolate used for targeting. Conversely, the genetic makeup of the pest population, along with the adaptive traits and mechanisms of insecticide resistance (Dubovskiy *et al.*, 2013), can significantly influence the effectiveness of control methods.

Ultramicroscopic changes in *N. virescens* adult during *M. anisopliae* Infection:

The analysis of ultramicroscopic imagery via scanning electron microscopy (SEM) revealed that the lipophilic characteristics of insect cuticles facilitate the adherence of the hydrophobic conidia of *M. anisopliae* across the body regions of *N. virescens* (Boucias *et al.*, 1996). Nonetheless, the conidial count was lower in the head region, attributable to the insect head being less susceptible to fungal attack due to the presence of heavily sclerotized cuticles compared to other body parts (Güerri-Agulló *et al.*, 2010). Similar observations were reported by Gabarty *et al.* (2014), Kiruthiga *et al.*, (2022), and Bitencourt *et al.* (2023) in studies on *Aedes aegypti*, *Agrotis ipsilon*, *Peregrinus maidis* and *Spodoptera frugiperda*, respectively. However, conidia germination can be inhibited by the presence of cuticular inhibitory compounds, such as phenols and quinones, as well as by the host antibiosis mechanism (Lord and Howard, 2004). Conidial attachment is followed by germination of conidia forming to the penetration peg and appressorium, which penetrates the insect cuticle either by the action of cuticle-degrading enzymes or by mechanical pressure exerted by the developing hyphae (Schränk and Vainstein, 2010). Our findings indicated that the maximum number of conidia with germ tubes and appressoria was observed in the intersegmental regions near the leg. This might be due to the softer composition of the intersegmental region compared to other body parts; therefore, fungi use fewer enzymes for cuticle degradation (Kiruthiga *et al.*, 2022). Proteolytic enzymes combined with chitinase secreted by the fungal mycelium may assist in digestion and subsequent penetration of insect cuticles (Lemos *et al.*, 2018). After penetrating the insect body, fungal hyphae proliferate by forming a dense network inside the host, deriving nutrients from the hemolymph, and secreting toxins to disrupt the insect's immune defense (Aw and Hue, 2017). The highest concentration of hyphae was observed in the terminal segments of the abdomen, suggesting the extensive proliferation of *M. anisopliae* hyphal bodies within the host.

Conclusions: The present study revealed that the combination of *M. anisopliae* and imidacloprid provided the highest efficacy against *N. virescens* under field conditions. Examination by SEM showed that infection of host insects by *M. anisopliae* involves a complex

sequence of events, including conidial attachment and germination, penetration peg and appressorium formation, host penetration, colonization, hyphal extrusion, and conidiogenesis. The experimental findings suggest that *M. anisopliae* can be safely administered in the integrated pest management programme without hampering its efficacy for the management of rice leafhopper. However, environmental factors such as temperature, humidity, and soil conditions could influence the field efficacy of *M. anisopliae*, necessitating further exploration of these variables in real-world settings. Additionally, the potential impacts on non-target organisms, such as beneficial insects, need to be evaluated to ensure the environmental safety of this combined approach. Long-term field trials are recommended to assess the sustainability and effectiveness of these integrated treatments under varying field conditions.

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