Efficacy of genetically transformed *Metarhizium anisopliae* against *Spodoptera litura* and *Aphis craccivora*

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**Abstract**

To increase the insecticidal potency of the entomopathogen, *Metarhizium anisopliae* (Metsch.) Serok, the fungus was genetically modified with scorpion neuro-j-toxin LqqIT1a and two different insect specific heterologous toxic proteins viz., Cry1a and GNA. LqqIT1 is an anti-insect neurotoxin derived from yellow scorpion, *Leiurus quinquestriatus quinquestriatus* (Ehren.). The present study reports the bio-efficacy of genetically modified fungus, *M. anisopliae*, in which scorpion neurotoxin gene ‘LqqIT1’ is stacked in its genome, for improved efficacy against the tobacco caterpillar, *Spodoptera litura* (Fab.) and *Aphis craccivora* (Koch). All the transformed clones of *M. anisopliae* were found potent against *S. litura* and *A. craccivora* under laboratory conditions. The virulent clones viz., *Ma-2(2), Ma-2(7)* and *MaGKS-14* caused 40 to 90 per cent mortality at fourth day of treatment. Compared to untransformed parent strain, *Ma-C*, the median lethal time of transformed clones *Ma-2(2), Ma-2(7)* and *MaGKS-14* got reduced by 2, 3 and 3-folds, respectively. No significant differences were noted with respect to percent mortality of transformed clone, *MaGKS-13* in comparison to untransformed strain *Ma-C*. The results indicated that the incorporation of LqqIT1 toxin gene enhanced the potency of strain *Ma-C*, against immature stages of *S. litura* and *A. craccivora* by shortening the median lethal time without affecting conidial development. Therefore, LqqIT1 scorpion toxin gene showed the potential to improve efficacy of *M. anisopliae* against lepidopteran and hemipteran insects.

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1. Introduction

The tobacco caterpillar, *Spodoptera litura* (Fab.) is one of the famous noctuid insect pests with cosmopolitan presence and a vast host range of economically important crops including vegetables, oilseeds and many weeds (Sahayaraj and Paulraj, 1998; Ummidi et al., 2013). Due to broad host range, high reproductive rate and nocturnal feeding habits, the management of *S. litura* is challenging for plant protectionist. Cowpea aphid, *Aphis craccivora* Koch, is a well-known polyphagous, sap sucking and small sized insect pest. It is one of the major insect pest of cowpea reported to attack pulses from seedling stage to pod stage resulting in yield losses of about 80–100 per cent (Obopile, 2006; Mweke et al., 2018; Mweke et al., 2019). Severe infestation of cowpea aphid makes plants stunted and results in halted growth thus, reduction in yield (Blackman and Eastop, 2000). Currently, every-one is environment conscious and well aware about the adverse and ill-effects of synthetic pesticides. So, to overcome the ill-effects of synthetic pesticides, use of biological control agents such as Entomopathogenic Fungi (EPF), could be a better strategy of Insect Pest Management (IPM). EPFs offer a good option to replace deadly synthetic insecticides. Among EPFs, *Metarhizium anisopliae* (Metsch.) Sorok, *Lecanicillium lecanii* (Zimm.). *Beauveria bassiana* (Bals.) Vuill. are few species which are commercially used as biological controlling agents. These pathogens have developed mechanisms to overcome the host’s immune system. *M. anisopliae* is a well-known EPF with cosmopolitan presence and reported to infect several insect pests in general and member’s of noctuidae in particular (Robert and St Leger, 2004; De Faria and Wraight, 2007). However, these EPFs have some demerits also for example, it require high temperature,
and moist conditions for development, spore germination is also get badly affected by UV radiations in natural conditions. Fungal pathogenicity has heightened through incorporation and expression of certain insecticidal recombinant genes from spider, scorpion and other proteins using genetic engineering approaches (Deng et al., 2019; Fang et al., 2005; Qin et al., 2010; St Leger and Wang, 2010; St Leger et al., 1996). Heterologous toxin proteins such as lecins, toxin proteins from bacteria such as (Bt) from Bacillus thuringiensis (Ber.) are use to enhance the fungal efficacy. Soil born insect specific famous gram positive bacteria, B. thuringiensis has already been reported to secrete crystalline 6 endotoxins (Cry), cytotoxins (Cyt) and vegetative insecticidal proteins (Vip). It kills host by damaging the midgut (Yang et al., 2019). Other class plant lecins are considered to be defense-related proteins towards pathogens and several insect pests (Macedo et al., 2015). The mode of action of snowdrop lecins on insect is disrupting the midgut epithelial cells (Fitches et al., 2001, Fitches et al., 2012).

The anti-insect toxic gene of rLqqIT1 was synthesized on the basis of its amino acid sequence and expressed in Escherichia coli (Mig.). The synthesized recombinant product when injected (to check biotoxicity), showed enhanced insecticidal activity against S. litura and Helicoverpa armigera (Hub.) larvae, respectively (Murugan and Saini, 2019). The pathogenicity of the genetically modified four different fungal clones viz., Ma-2(2) and Ma-2(7) stack with recombinant toxic gene ‘LqqIT1a’ and other clone MaGKS-13 and MaGKS-14 respectively, expressed with heterologous fusion protein containing snowdrop lecmin, Galanthus nivalis agglutinin (Kunt.) (GNA) and insect specific toxin Cry protein from B. thuringiensis – ‘LqqIT1a/Cry1Ac-GNA’ against S. litura larvae and A. craccivora nymph was checked in comparison with the unretransformed strain ‘Ma-C’ in order to check whether LqqIT1 has any potential, to use as a candidate pathogenic factor to improve efficacy of bioagents.

2. Material and methods

The current study was performed in the Biological control laboratory, Division of Entomology, ICAR-IARI, Pusa, New Delhi. Virulence bioassays were carried out on the five day old, larvae of tropical armyworm S. litura and nymph of A. craccivora in laboratory culture. Insects were reared at 27 ± 1 °C and 65 ± 5 % RH with a 16 hrs. of photoperiod in artificial controlled environmental control chamber throughout the course of investigation. All the experiments were conducted in UV- radiated laminar air flow chamber.

2.1. Maintenance and rearing of test insects

Tobacco caterpillar- Last larval instars (5 and 6th) were collected from the cabbage field, Division of Floriculture, ICAR-IARI, Pusa, New Delhi, India in 2019, subsequent generations were used for the treatment. The collected larvae were reared on fresh castor (Ricinus communis, Linn.) leaves, individually in small sized, rearing dish. Pupae kept separate and emerged adults were shifted to mating jar in 1:1 (male:female) ratio and fed on 10 % honey solution fortified with vitamin E capsule. Newly hatched larvae < 24 h (newborns) were transferred to fresh tender castor leaf; briefly about 30–50 neonates from single egg patch transfer to each castor leaf. After 6–7th day of hatching, the survived larvae were individually placed in small rectangular vials (5x2.5x4.5 cm) singly and artificial diet block provided to feed. Artificial (chickpea flour based) diet was prepared as per the protocol reported by Gupta et al., 2005 with some modifications. Late fifth instar larvae were allowed to pupate inside the artificial diet. After sclerotization about 48 hrs pupae were separated from diet vial and shifted to new vial for adult emergence.

Cowpea aphid – Aphids were collected from the infested cowpea fields of ICAR-IARI, Pusa, New Delhi. Aphids were reared as per the Yeo et al., 2003 with few modifications. Adult females were transferred on to the, cowpea seedlings (Vigna unguiculata, (L.) Walp.) grown in the plastic jars (15.5 cm) using absorbant cotton, as per the Rakshith et al., 2018. This population was maintained as a stock culture throughout the study period.

2.2. Fungus culture

Transformed EPF, M. anisopliae clones including control (untransformed) were procured from Dr. Gurvinder Kaur Saini, Professor, Department of Biosciences and Bioengineering, IIT Guwahati, Assam, India in May 2019 and multiplied in the Biological control laboratory ICAR-IARI, Pusa, New Delhi for conducting bioassay test on selected insects.

2.2.1. Development of the recombinant strain

The M. anisopliae (MTCC 892) culture was procured from the Microbial Type Culture Collection and Gene Bank (MTCC, India), and it was kept on PDA (Potato Dextrose agar) medium at 28 ° C. After incubating for 7–10 days on SDA, spores were collected.

2.2.1.1. Gene synthesis

The LqqIT1a protein sequence was retrieved from the NCBI database (p19856.1) and the EMBL-EMBOSS Bank transeq software was used to reverse translate it (Rice et al., 2000). The reverse translated sequence was then codon-optimized for the expression in M. anisopliae. Along with LqqIT1a codon optimized sequence, 5'-untranslated region, Metarhizium collagen like promoter signal sequence (Mcl1-sp) was added and cloned in pUC57 vector harboring ampicillin resistant marker (Genscript, USA). Using the fungi expression vector pAL1, the LqqIT1 gene was chosen to be cloned into the M. anisopliae genome. Cloning was validated through the use of restriction enzyme digestion and PCR amplification of the LqqIT1a gene sequence.

2.2.1.2. Fungal transformation and screening

Agrobacterium mediated gene transfer strategy was followed for transformation into M. anisopliae. Agrobacterium tumefaciens (Beijerinck & van Delden) EHA105 (AtEHA 105) strain was used to electro-competent cell preparation (Shaw, 1995) and M. anisopliae transformation (Sevim et al., 2012). Engineered M. anisopliae enriched with pMC1-LqqIT1a was confirmed by genomic DNA isolation followed by PCR confirmation with gene specific primers. Agaroase gel electrophoresis and the NanoDrop technique were used for qualitative and quantitative analysis.

2.2.1.3. Sub-culturing of transformed fungus

The aqueous fungal suspension was initially subcultured using standard Potato Dextrose Agar (PDA). For mass production of M. anisopliae was grown on Sabouraud Dextrose medium (SDA) medium at 27 ± 2 ° C and 80 ± 5 % RH in complete darkness. Conidial suspension for the efficacy test was obtained from 25 to 30 days old fungal colonies. Conidia were harvested using 10 ml autoclaved distill water containing 0.02 % Tween 80 and filtered through sterile cheese cloth to remove mycelial mat (Chan-Cupul et al., 2010). Obtained filtrates were preserved at 4 °C for further bioassay purpose and before using the preserved aqueous fungal suspension was shaken vigorously. The conidial concentration of the resulting ‘stock’ suspension was estimated using an improved Neubauer hemocytometer (Marienfeld) under a Nikon ECLIPSE 80i microscope (400 x magnification) and number of spores present per ml was estimated using the formula by Aneja, 1996. A series of dilutions were made to get a range of concentrations based on bioassay requirements.
2.3. Bioassay procedure

2.3.1. Tobacco caterpillar

The virulence of the transformed fungal clones was examined by leaf treatment method (CIBRC, 2011). Prior to experimentation *S. litura* larvae were starved for four hours. The conidial suspension was applied both side of the castor leaves, (5 cm x 5 cm) by using UV radiated soft hair brush at concentrations of 1x10^6, 1x10^7, 1x10^8 and 1x10^9 spores/ml. The control batch was treated with 1 ml of sterile distil water containing 0.02 % Tween 80 only. The treated leaves were allowed to shade dry under laminar. The second instar of *S. litura* (10 larvae/leaf disc) was fed with fungal conidia applied leaves for 48 hrs following that they were fed with fresh and untreated artificial diet. Both the fungal treated and untreated larvae were maintained separately and the each larvae were measured daily for development, behaviour and morphological changes. Mortality was recorded at 24 hrs interval up to 7 days or adult emergence.

2.3.2. Cowpea aphid

To test the efficacy of each transformed fungal clones, five days old nymphs and 3–4 days old seedlings were used. Experiments were conducted in the laboratory by using the method by Yeo et al., 2003 and Saranya et al., 2010 with some modifications. Prior to fungal application, adult aperous aphids were released on the cowpea seedlings using a camel hair brush @ 1 aphid per seedling. In brief, totally thirty, five day old aphids were used for each replication. The respective concentrations of all the fungal conidial suspensions were sprayed on the seedlings + aphids using a pneumatic atomizer (1 ml spore suspension for 30 nymphs and 10 nymphs per seedling). After spraying, seedlings were maintained in small paper cups of size 7.5x7.5 cm (Fig. 2). Mortality, the mobility of treated insects, fungal growth observed and noted at each 24 h interval up to 7–10 days. Old seedling was changed by fresh and untreated cowpea leaves or seedling at each 24 h interval or on the basis of requirement.

Mortality symptoms were based on insect’s response to touch, body colouration and texture. Dead insects were placed in ethanol wiped Petri plate and line with damp sterile filter paper to facilitate mycosis and sporulation. Morphological features of the fungal clones (conidia shape and size) were studied by phenotypic methods only and fungal colonies were recorded for their identification as *M. anisopliae* (Tanada and Kaya, 1993). Each treatment was performed thrice independently. There were three different replicate dishes with thirty insects per concentration in each trial.

2.4. Statistical analysis

The experiment was conducted in a Completely Randomized Design with three replications for every treatment. Mortality data was subjected to probit analysis to determine the median lethal time values at 95 % fiducial limits (Finney, 1971) using IBM SPSS statistics version 21. The mortality data was analyzed using WASP (version 2.0) online statistical tool and the mean differences between the treatments were tested by ANOVA at 5 % level of significance. The percent reduction in population was calculated by using Henderson and Tilton’s formula (Abbott, 1925; Henderson and Tilton, 1955):

\[
\text{Percent reduction in population} = 100 \times 1 - \frac{[\text{Ta} \times \text{Cb}]}{\text{Tb} \times \text{Ca}}
\]

Where, Ta = Number of aphids after treatment, Tb = Number of aphids before treatment.
Ca = Number of aphids in control plots after treatment.
Cb = Number of aphids in control plots before treatment.

3. Results

All the tested transformed clones showed virulence against the model insects, but their intensity varied. Mortality was proportionally increased with conidial density. The documented data concerning the median lethal time, 95 % fiducial limit, P-value, percent mortality are presented in the Table No-1, 2, 3 and 4.

Cadavers infected with the clone Ma-2(2) and MaGKS-14 were covered with dusky white mycelia after 5 days of treatment and changed to dark green appearance by conidia from dusky white mycelia 7–10 days after treatment (Fig. 1). Survived, infected insects showed notable behavior changes. Infection symptoms were easily noticed such as pigmentation (browning) in body, more efforts for stretching the body and movement, enlarged instar duration, deformed pupae and adults with deformed wing in case of *S. litura*.

3.1. Pathogenicity of transformed *M. Anisopliae* clones against *S. Litura* larvae

The pathogenicity of transformed clones against *S. litura* larvae was compared with that of untransformed *M. anisopliae* (Ma-C) strain by leaf treatment method. The results demonstrated that the per cent mortality of *S. litura* larvae infected with transformed clones was significantly higher than that of those treated with untransformed Ma-C strain after 6 days of treatment (Table 3). Transgenic clone Ma-2(2), Ma-2(7) and MaGKS-14 produced > 50 per cent mortality at 96 hrs which was 61.05, 58.79 and 52.11% per cent, respectively which was 40 times higher than the time required to yield the same results in untransformed Ma-C strain (19.16 per cent at 120 hrs.) (\( F_{4,10} = 847.7, P = 0.000 \)) (Table 3 and Fig. 3). The LT50 after infection was 3.21 and 3.92 days by transformants, Ma-2(2) and MaGKS-14, respectively, which was 1.12 fold shorter compared with untransformed Ma-C strain (4.37 days) (\( P < 0.05 \)) (Table 1). Above results demonstrated that the enhanced virulence developed by scorpion toxin rLqPIT1 may arise after the fungus enters the host.

3.2. Pathogenicity of transformed *M. Anisopliae* clones against *A. Criccivora* nymph

The efficacy of the transformed clones Ma-2(2), Ma-2(7), MaGKS-13 and MaGKS-14 against nymphs of *A. craccivora* was screened based on the per cent mortality and LT50, and compared with that of untransformed Ma-C strain. In the topical spray experiment, nymphs treated with transformed clones had significantly higher per cent mortality than nymphs treated with the untransformed Ma-C strain (\( P < 0.05 \)) (Table 4 and Fig. 4) at 6 days of treatment.

Mortality for nymphs at lowest concentration (1×10^6 spores/ml) was 52.64, 69.42, 42.11, 61.58 per cent, Ma-2 (2), Ma-2(7), MaGKS-13 and MaGKS-14, respectively than those of nymphs treated with untransformed Ma-C strain (16.17 per cent) (\( F_{4,10} = 366, P = 0.000 \)). Similarly, the per cent survival of nymphs infected with transformed clones was significantly lower that that of those infected with untransformed Ma-C after 6 days of topical application at 1×10^8 spores/ml (\( F_{4,10} = 45.05, P = 0.000 \)) (Table 4). The LT50 after topical application was 2.49, 2.29 and 2.36 days by Ma-2(2), Ma-2(7) and MaGKS-14, which was 1.72 per cent shorter compared with untransformed Ma-C strain (4.051 days) (\( P < 0.05 \)) (Table 2). These results suggest that the rLqPIT1 gene, enhanced the pathogenicity of *M. anisopliae* during in vivo infection and thus ultimately leads to rapid death.
4. Discussion

Entomopathogenic fungi are one of the important biological controlling agents in use at the ground level (Ruelas-Ayala et al., 2013). Over 700 different fungal species belonging to about 90 genera have been reported to be pathogenic to insects (Khachatourians and Qazi, 2008). The mortality caused by anti-insect active substances is often related to the activity changes of some important enzymes in the host insects (Grewal et al., 2005). Secondary metabolites (active substances), are considered to accelerate oxidative stress and free radicals in the infected host and thus leading to host death (Sree and Padmaja, 2008). However, these naturally occurring mycoinsecticides also have some limitations. To address the paucity of naturally occurring EPFs, in few last year’s

Fig. 1. Symptoms of infection on treated *S. litura* larvae.

Fig. 2. Fungal treated cowpea aphids.
improving the fungal pathogenicity using genetic strategies has become a research hotspot.

Efficacy of EPFs could be enhanced by the expression of heterologous toxic proteins (Fan et al., 2011; Lu et al., 2008). Some cuticle degrading enzymes such as subtilisin like protease and chitinase enhance the pathogenicity of EPFs (Leger et al., 1993). The present study here reporting the potential insecticidal effects of genetically modified \textit{M. anisopliae} against immature stages of \textit{S. litura} and \textit{A. craccivora}. All tested transformed \textit{M. anisopliae} clones were virulent to immature stages of \textit{S. litura} and \textit{A. craccivora} under in vitro studies. The most virulent clones Ma-2(2) and MaGKS-13 caused 50–52.64 per cent mortality by Ma-2(2) and 61.68–76.34 per cent mortality by MaGKS-14 at lowest concentration (1x10^6 spores/ml) against \textit{S. litura} and \textit{A. craccivora}. Similar results were demonstrated by Peng and Xia (2015) who reported, same trend of per cent mortality by transformed \textit{M. anisopliae} strain \textit{M. anisopliae} strain Ma-2(2) requires least conidial concentration and time to cause 50 per cent mortality. Similarly, the median lethal concentration of genetically engineered \textit{M. anisopliae} (AaIT-Ma549) was reduced 22, 9.06 and 16 times against \textit{Manduca sexta} (Linn.), \textit{Aedes. Aegypti} (Linn.) and \textit{Hypothenemus hampei} (Fer.), respectively (Wang and St Leger, 2007; Pava-Ripoll et al., 2008 ). Likewise, \textit{M. acridium} expressing the LqhIT2 toxin gene, reported 22.6 fold increment in pathogenicity against \textit{L. migratoria}, where median lethal time value decreased with the increment in conidial concentration (Peng and Xia, 2014). Presence of LqqIT1 peptide sequence into \textit{M. anisopliae} could greatly improve the fungal virulence toward its host insect. According to Murugan and Saini (2019), rLqqIT1 is a sodium acti-

![Fig. 3. Mortality of \textit{S. litura} larvae infected with transformed \textit{M. anisopliae} clones @1 x 10^6 spores/ml.](image)

### Table 1

| Clone   | Conc. (spores/ml) | Slope ± SE | LT_{50} (days) | Fiducial (95 %) | \(\chi^2\) | P-value |
|---------|-------------------|------------|----------------|----------------|-----------|---------|
| Ma-2(2) | 10^6              | 2.75 ± 0.587 | 6.14           | 4.87–11.9      | 0.891     | 0.005   |
|         | 10^7              | 4.43 ± 0.67  | 3.86           | 2.58–7.09      | 0.095     | 0.001   |
|         | 10^8              | 4.32 ± 0.59  | 3.21           | 2.43–6.01      | 0.109     | 0.001   |
|         | 10^9              | 4.37 ± 0.588 | 2.68           | 1.87–4.94      | 0.401     | 0.001   |
| Ma-2(7) | 10^6              | 4.00 ± 0.66  | 4.41           | 2.78–8.69      | 0.335     | 0.002   |
|         | 10^7              | 3.31 ± 0.52  | 3.33           | 2.69–6.28      | 0.71      | 0.001   |
|         | 10^8              | 5.39 ± 0.786 | 3.83           | 2.31–6.27      | 0.061     | 0.001   |
|         | 10^9              | 5.80 ± 0.769 | 2.98           | 1.05–4.65      | 0.026     | 0.001   |
| MaGKS-13| 10^6              | 2.89 ± 0.54  | 5.5            | 3.18–9.23      | 0.867     | 0.003   |
|         | 10^7              | 4.14 ± 0.73  | 4.83           | 3.70–9.23      | 0.039     | 0.002   |
|         | 10^8              | 3.52 ± 0.56  | 3.92           | 2.95–7.64      | 0.332     | 0.002   |
| MaGKS-14| 10^6              | 4.20 ± 0.66  | 4.052          | 2.70–6.05      | 0.506     | 0.001   |
|         | 10^7              | 4.4 ± 0.64   | 3.61           | 2.51–4.85      | 0.561     | 0.001   |
|         | 10^8              | 4.29 ± 0.661 | 3.94           | 2.55–5.09      | 0.293     | 0.001   |
|         | 10^9              | 5.10 ± 0.68  | 3.06           | 2.47–4.13      | 0.667     | 0.001   |
| Ma-C    | 10^6              | 1.62 ± 0.42  | 6.28           | 4.55–9.13      | 0.175     | 0.013   |
|         | 10^7              | 3.98 ± 0.861 | 6.15           | 4.47–7.04      | 0       | 0.006   |
|         | 10^8              | 3.84 ± 0.59  | 4.37           | 2.97–6.34      | 0.711     | 0.002   |
|         | 10^9              | 5.10 ± 0.68  | 3.06           | 2.47–4.13      | 0.667     | 0.001   |
| Tween C | 10^6              | 0.695 ± 0.663| 28.98          | 25–43          | 0.984     | 0.343   |
|         | 10^7              | 0.713 ± 0.736| 38.78          | 34–89          | 0.972     | 0.37    |
|         | 10^8              | 1.10 ± 0.742 | 37.59          | 27–92          | 0.849     | 0.197   |
|         | 10^9              | 0.52 ± 0.74  | 39.20          | 36–72          | 0.438     | 0.513   |
Vibrated channel toxin, it causes quick paralysis and physiological dysfunctioning in *S. litura* and *H. armigera* larvae. Our study showed larval body liquification, pigmentation (dark brown to black) in fungal treated insects and this revealed that transformed clones may weaken host by halting the Voltage Gated Sodium Channel (VGSCs) of motor neurons. In the present report, no count of conidia produced on insect cadavers was made. Only the occurrence and absence was noted. The mere occurrence of mycosis

**Fig. 4.** Mortality of *A. craccivora* nymph infected with transformed *M. anisopliae* clones @1 × 10⁶ spores/ml.

**Table 2**
Bioefficacy of transformed *M. anisopliae* clones against first instar nymphs of *A. craccivora* via topical spray method.

| Clone | Conc. (spores/ml) | Slope ± SE | LT₅₀ (days) | Fiducial (95 %) | P-value |
|-------|-------------------|------------|-------------|----------------|---------|
| Ma-2(2) | 10⁶  | 5.25 ± 0.732 | 3.45 | 2.78–4.28 | 0.391 | 0.001 |
|        | 10⁷  | 3.064 ± 0.450 | 2.90 | 2.08–4.03 | 0.852 | 0.001 |
|        | 10⁸  | 3.92 ± 0.461 | 2.49 | 1.81–3.42 | 0.465 | 0.001 |
|        | 10⁹  | 2.382 ± 0.405 | 2.31 | 1.53–3.16 | 0.797 | 0.002 |
| Ma-2(7) | 10⁶  | 3.22 ± 0.504 | 3.59 | 2.99–4.91 | 0.607 | 0.001 |
|        | 10⁷  | 2.675 ± 0.430 | 2.85 | 1.97–4.13 | 0.33 | 0.002 |
|        | 10⁸  | 3.16 ± 0.448 | 2.29 | 1.64–3.19 | 0.208 | 0.001 |
|        | 10⁹  | 2.52 ± 0.413 | 1.81 | 1.20–2.72 | 0.383 | 0.002 |
| MaGKS-13 | 10⁶  | 2.80 ± 0.534 | 5.07 | 3.51–13.57 | 0.646 | 0.003 |
|        | 10⁷  | 2.10 ± 0.416 | 3.56 | 2.25–5.61 | 0.78 | 0.004 |
|        | 10⁸  | 3.391 ± 0.534 | 3.12 | 2.30–4.22 | 0.911 | 0.001 |
|        | 10⁹  | 3.54 ± 0.487 | 2.95 | 2.20–3.96 | 0.126 | 0.001 |
| MaGKS-14 | 10⁶  | 4.43 ± 0.654 | 3.64 | 2.85–4.65 | 0.030 | 0.001 |
|        | 10⁷  | 3.096 ± 0.447 | 2.36 | 1.69–3.29 | 0.050 | 0.001 |
|        | 10⁸  | 2.91 ± 0.430 | 2.38 | 1.67–3.36 | 0.325 | 0.001 |
|        | 10⁹  | 2.66 ± 0.418 | 1.97 | 1.34–2.90 | 0.209 | 0.001 |
| Ma-C  | 10⁶  | 2.03 ± 0.464 | 5.81 | 3.55–5.90 | 0.245 | 0.007 |
|        | 10⁷  | 1.83 ± 0.432 | 5.42 | 3.18–5.23 | 0.871 | 0.008 |
|        | 10⁸  | 2.45 ± 0.452 | 4.051 | 2.71–6.04 | 0.61 | 0.003 |
|        | 10⁹  | 4.304 ± 0.594 | 3.18 | 2.47–4.09 | 0.528 | 0.001 |
| Tween C  | 10⁶  | 2.196 ± 0.621 | 9.94 | 5.87–16.82 | 0.686 | 0.071 |
|        | 10⁷  | 1.25 ± 0.497 | 18.56 | 7.76–44.37 | 0.909 | 0.05 |
|        | 10⁸  | 1.87 ± 0.602 | 12.89 | 6.88–24.15 | 0.982 | 0.026 |
|        | 10⁹  | 1.84 ± 0.650 | 15.58 | 7.94–30.56 | 0.438 | 0.036 |

**Table 3**
Mean cumulative mortality of transformed *M. anisopliae* at various concentrations against *S. litura* larvae via leaf treatment method.

| Conc. (Spores/ml) | Larval Mortality (%) at 6 day after treatment |
|-------------------|---------------------------------------------|
| Ma-2(2)           | Ma-2(7) MaGKS-13 MaGKS-14 Ma-C F cal (df) CD ≥ 0.05 |
| 1 × 10⁶           | 50 ± 0b 74.8 ± 3.72a 41.9 ± 1.77c 76.34 ± 0.57a 15 ± 0.57d 541.8 (4,10) 1.050 |
| 1 × 10⁷           | 80 ± 0b 84.63 ± 1.77c 46.16 ± 0.66b 90 ± 0b 14.29 ± 0.58d 822.5 (4,10) 1.050 |
| 1 × 10⁸           | 90 ± 0ab 87.42 ± 0.67b 49.88 ± 0.34a 96.67 ± 1.76a 21.1 ± 0.89f 847.7 (4,10) 1.049 |
| 1 × 10⁹           | 96.67 ± 3.34a 96.34 ± 3.34a 69.99 ± 0.58b 100 ± 0a 36.65 ± 0.33c 25.84 (4,10) 4.63 |

Values are presented as Mean ± SEM, per cent mortality followed by the same superscripted letter in rows are not significantly different at 5 % level of significance by Duncan’s multiple range test (DMRT), df = 4,10 and P value equals to 0.000.
does not indicate infectivity, but rather indicates the efficiency of the invading fungal mycelia in the insect host to effectively cross the dermal barrier (cuticle) and germinate and spores on its host body (Devi and Rao, 2006).

Combining the heterologous toxins (LqqIT1a/Cry1Ac-GNA) the transformant clone MaGKS-14 caused 90 per cent and 69.10 per cent mortality in S. litura and A. crucivora, respectively after 6 day of treatment. It was obvious from the results that transformed clone Ma-2(2) and MaGKS-14 were more pathogenic to S. litura and A. crucivora. The integration of rLqqIT1 improved the potency of M. anisopliae suggesting that LqqIT1 neurotoxin has excellent ability for improving the pathogenicity of EPF.

5. Conclusion

Genetically transformed M. anisopliae clones enhanced virulence against model insects in terms of percent mortality, L150, and also exhibited mycelial and conidial growth on the cadavers. The overall pathogenicity of transformed clones was in the following order Ma-2(7) = MaGKS-14 > Ma-2(2) > MaGKS-13 > Ma-C. This was a preliminary study planned to screen the virulence of transformed M. anisopliae, but more detailed studies in the natural environment against different insect species are needed to ascertain the effectiveness and mass production of this transformed entomopathogen.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 4

| Conc. (Spores/ml) | Nymphal Mortality (%) at 6 day after treatment | Fa (df) | CD $\times$ 0.05 |
|-------------------|-----------------------------------------------|--------|-----------------|
| 1 $\times$ 10$^6$ | Ma-GKS-13: 52.64 ± 0.56$^a$ 69.42 ± 0.34$^a$ 42.11 ± 1.43$^a$ 61.58 ± 0.89$^a$ 16.18 ± 0.34$^a$ 366 (4,10) 1.050 |        |                 |
| 1 $\times$ 10$^7$ | Ma-2(2): 86.67 ± 0.89$^a$ 83.34 ± 0.33$^a$ 44.5 ± 0.66$^a$ 69.1 ± 0.32$^a$ 22.49 ± 0.59$^a$ 126.4 (4,10) 2.35 |        |                 |
| 1 $\times$ 10$^8$ | MaGKS-14: 100 ± 0$^a$ 86.33 ± 0.59$^a$ 46.34 ± 0.67$^a$ 98.1 ± 0.31$^a$ 26.67 ± 0.33$^a$ 45.05 (4,10) 4.33 |        |                 |
| 1 $\times$ 10$^9$ | Ma-2(2): 100 ± 0$^a$ 90 ± 0$^a$ 52.73 ± 0.35$^a$ 100 ± 0$^a$ 37.27 ± 0.88$^b$ 31.29 (4,10) 4.58 |        |                 |

Values are presented as Mean ± SEM, per cent mortality followed by the same superscripted letter in rows are not significantly different at 5 % level of significance by Duncan’s multiple range test (DMRT), df = 4,10 and P value equals to 0.000.

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