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The effects of Mixed function oxidase, Chitinase and Attacin in the resistance of *Locusta migratoria* against *Metarhizium anisopliae* and Mad1.

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ABSTRACT

The detoxifying enzymes and toxicity of attacin, a kind of anti-microbial proteins (AMPs) play pivotal role in immune response of insects. Little is known about the effects of *Metarhizium anisopliae* and Mitotic Acetic Division (Mad1) protein on detoxifying enzymes and attacin activity in migratory locust. The aim of this study is to evaluate detoxifying enzymes and attacin generation in migratory locusts when *Metarhizium anisopliae* and Mad1 protein mixed with bait formulation and feed to Migratory Locusts. We concluded that the activities of detoxifying enzymes such as Mixed-Function Oxidase (MFO), Chitinase (CHI) and AMPs significantly increased in the body of host at 72 hours feeding with *M. anisopliae* and Mad1 protein. The increased activities of MFO, CHI and AMPs subsequently activated the locust immune system with the application of *Metarhizium anisopliae* and Mad1.

KEYWORDS: *Metarhizium*, MFO, CHI, Attacin (AMPs), Migratory Locust, Immune system

INTRODUCTION

The migratory locust mostly found in Northern China and is considered as top listed major pests of crops and grass in China for years (Peng & Wang, 2008). The damage caused by the locusts during the period from 1995 to 2001 were estimated to be 8,000,000 hectares in 12 to 30 provinces and autonomous regions of China as reported by the Chinese Ministry of Agriculture (Dong 2001).

Application of toxic chemical pesticides has caused hazardous effects to human, livestock and animals and subsequently caused water and environmental contamination (Peveling 2001). *M. anisopliae* is one of the well-known environment friendly agent for the control of locusts and grasshoppers (Driver, Milner *et al.* 2000).

Two different adhesions of Mad1 and Mad2 belonging to *Metarhizium*, of which the Mad1 adheres to invader body and causing fungal penetrate peg while Mad2 adheres to plant surface and produces internal and external relationship (Wang and St Leger, 2007). Insect have well developed mechanism to combat fungal infection by EPF fungi (Gottar *et al.*, 2006). Insects release different enzymes, which play crucial role in its defense against foreign invaders (Serebrov *et al.*, 2001). Mixed-function oxidase (MFO) play pivotal role in resistance of all kinds of insecticides by degradation due to its genetic constitution wide substrate specificity and catalytic activities (Feyereisen 2005).

Cuticle of insects possesses strong defensive system due to its thickness and chemical composition to avoid spore adhesion, conidial germination and penetration by the fungal hyphae (Leger *et al.*, 1988). Entomopathogenic fungi is capable of producing chitinase (CHI) enzyme which play significant role in fungal infection stages (St Raymond, Cooper *et al.* 1987). Moreover, CHI is responsible in the molting process of insects and the decomposition of old cuticles as well and Dubovskiy *et al.*, 2012). Study suggests that the antifungal, antiviral and antibacterial peptides are abundantly found in insects (Mishra & Wang 2012).

Toxic chemicals are affecting the insect haemolymph and cellular immune system. First of all, haemolymph and immune response is activated when foreign invaders attack on host; initially some proteins identify the microbial agents and start the release of various anti-microbial proteins (AMPs) including cecropins, attacins and Dipterocins (Hetru 1998). AMPs attacin have great significance to combat bacterial and fungal infection and believed to be acting on outer membrane of microbial cells (Engström *et al.*, 1984).

Similarly, the melanin production can increase rapidly rather than anti-microbial peptides that result ultimately to the production of reactive oxygen species (ROS) and contribute to kill the pathogen as regulated by phenol oxidase (PO) cascade (Ragan *et al.*, 2009). In the present work, we have studied MFOs, CHI, and

AMPs (attacin) in migratory locust for their enhanced activities in response of *M. anisopliae* and Mad1.

MATERIALS AND METHODS

Insect Rearing: Migratory locust's eggs were kept in incubator at 27 °C and were maintained at 27±0.5 °C, 60±5% RH, and 12:12 L:D photoperiod for hatching purpose when the nymph hatched then shifted to the wooden rearing chambers (60 cm × 50 cm × 60 cm), after that they were fed with wheat seedlings twice a day.

Fungal and Mad1 Culture: *Metarhizium anisopliae* was cultivated on Potato Dextrose Agar (PDA) and incubated at 26 °C for seven consecutive days. Mad1 was spread on Yeast Peptone dextrose plates at 27 °C for 24 hours and then transferred to buffered glycerol complex medium containing yeast extract 20g, peptone 6g, KH₂PO₄ 22.16g, 10XYNB 20 mL, glycerol 2 mL and were then kept on rotatory shaker for 72 hours at 30 °C. The medium was centrifuged at 12235 g for 10 minutes and the obtained pellets were transferred to BMGY with the addition of 20 mL 10X YNB, 2 mL methanol and allowed to shake for additional 24 hours at 250 rpm under 30 °C. After that 2mL of methanol was added with an interval of 24-hours till 72 hours. Pellets were collected by centrifuge at 12235 g for 10 minutes adding 20 mM Tris pH 8.0 and cells were broken through ultrasonic. Protein concentration of Mad1 was checked and confirmed as 1.4 µg / µL on 8% SDS PAGE.

Bioassay: Third instar locusts were recognized by 3rd Instar and feed with bait formulation containing 5 mL Mad1, 5 g wheat bran 500 µL oil (Mad1 treatment), in addition 0.08 g spores of IMI330189 along with Mad1 to have mixed treatment of Mad1+IMI330189.

Whereas, the IMI330189 treatment was prepared with addition of 0.08g of IMI330189 powder in bait formulation and the final concentration was adjusted at 3×10¹⁰ spores mL⁻¹.

The control group was feed with adding Mad1 protein grown without methanol in bait formulation. After 24 hours of treatment application, the bait was removed and nymphs were released on fresh wheat seedling for feeding. Moreover, after 72 hours, the whole bodies of locust were collected for enzyme assay. Fifteen individuals of locust were used with three replications.

Enzyme Activity Assay: MFO, CHI and Attacin (AMP) activities were measured by given protocol of (Nanjing Jiancheng Biochemical Institute, Nanjing-China). The enzymatic activities were measured as U/g.

Third instar locust nymphs were homogenized using 2 mL of 0.1 mol L⁻¹ phosphate buffer solution for phenol oxidase (pH 7.0 phosphate buffer), and for superoxide dismutase and peroxidase (NaCl 0.7%); the homogenized samples were then left on ice for 3 min. The homogenized samples underwent centrifugation at

12,235×g for 10 min at 4 °C. The supernatant was moved into a fresh Eppendorf tube and centrifuged once more at 12,235×g for 15 min at 4 °C. Subsequently, the supernatant was employed for protein and enzyme detection

Data Analysis: The data was analyzed through One-way Analysis of variance (ANOVA) to measure the activities of MFO, CHI and attacin. Similarly, the activities in nymphs of locust, treated with *M. anisopliae* (IMI330189), Mad1 alone and Mad1+IMI330189 through SPSS (SPSS version 21.0) were carried out. The least significant difference test at P≤ 0.05 was settled.

RESULTS

Application of *Metarhizium anisopliae* and Mad1 causing virulence to Migratory Locust:

Virulence of migratory locust was recorded in terms of percent mortality. Response of locusts was absolutely different (P≤0.05) using the three formulated treatments (IMI330189, Mad1, Mad1+IMI330189). Maximum mortality (%) of Migratory Locusts was seen when the *M. anisopliae* (IMI330189) and Mad1 were applied in combination followed by independent treatment of IMI330189. However, the application of Mad1 alone had no effect on the mortality of locusts. Mortality ranging from 15 to 86 whereas, maximum mortality (86%) was noticed with the application of Mad1+IMI330189, followed by 85 % death rate with the independent treatment of *M. anisopliae*. Least mortality (15%) was observed under the Mad1 application (Figure. 1). There was no significant variation in the initial four days of application however, the sudden death rate was observed after 4th day of application of *M. anisopliae* and Mad1+IMI330189. A continuous trend of increased mortality was noticed till 7th day, which became stable afterwards with highest mortality on the 12th day. There were no significant variations for mortality rate under the Mad1 and control treatments.

The activity of Mixed-Function Oxidase (MFO) in Migratory Locust:

The present results revealed that the activity of MFO significantly (P≤0.01) increased in migratory locust when feed by IMI330189, Mad1 and Mad1+ IMI330189 (combined treatment) as compared to control treatment after 72 hours. Among the treatments, maximum MFO activity was noticed in combined treatment (Mad1+ IMI330189). Although the independent applications of Mad1 and IMI330189 were significantly not different with each other for the MFO production however, they were significantly different (P≤0.05) with that of control (Figure. 2).

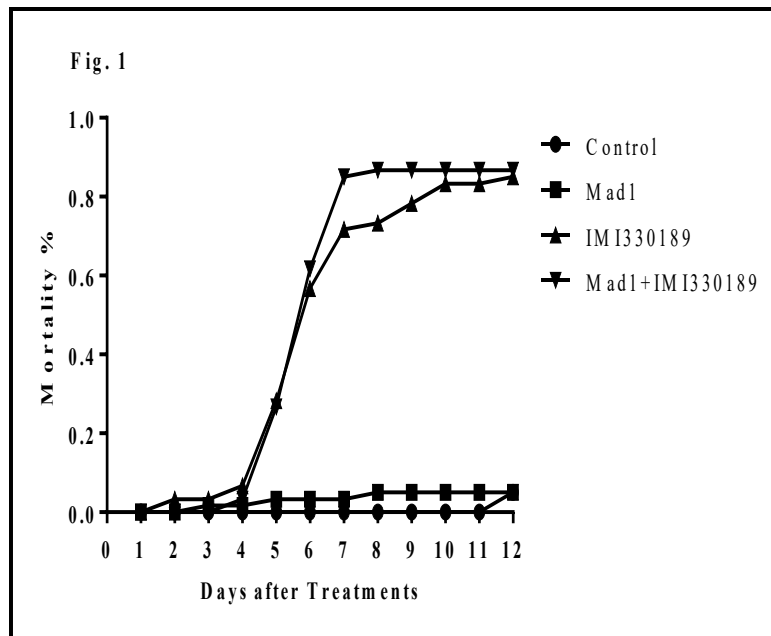


Figure 1 Mean mortality percentage (%) of migratory locusts

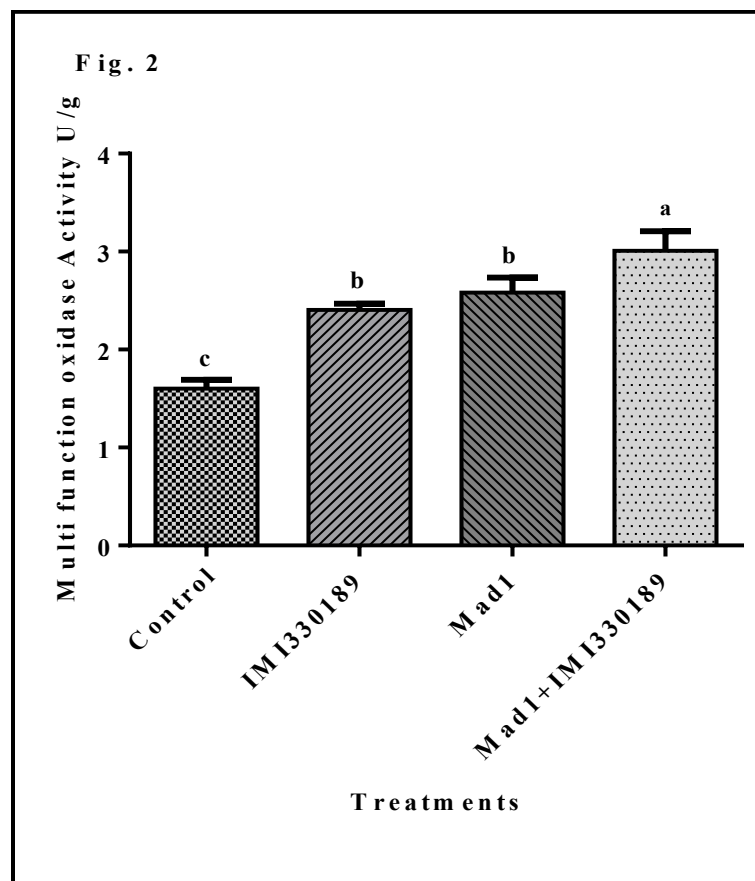


Figure 2 Mixed-Function Oxidase activity in migratory locust

The activity of Attacin (AMPs) in Migratory Locust: Feeding migratory locusts by IMI330189, Mad1 and Mad1+ IMI330189 (combined dose) significantly increased the level of attacin after 72 hours. In a similar manner to that of MFO activity, the maximum activity of attacin was recorded when the locust fed on combined treatment of Mad1+

IMI330189 followed by Mad1 protein bait as compared to the control. Although the attacin activity under the application of separate dose of *M.anisopliae* was comparatively lower than the Mad1 and Mad1+ IMI330189 however, it was still significant compared to the activity noticed in control (Figure. 3).

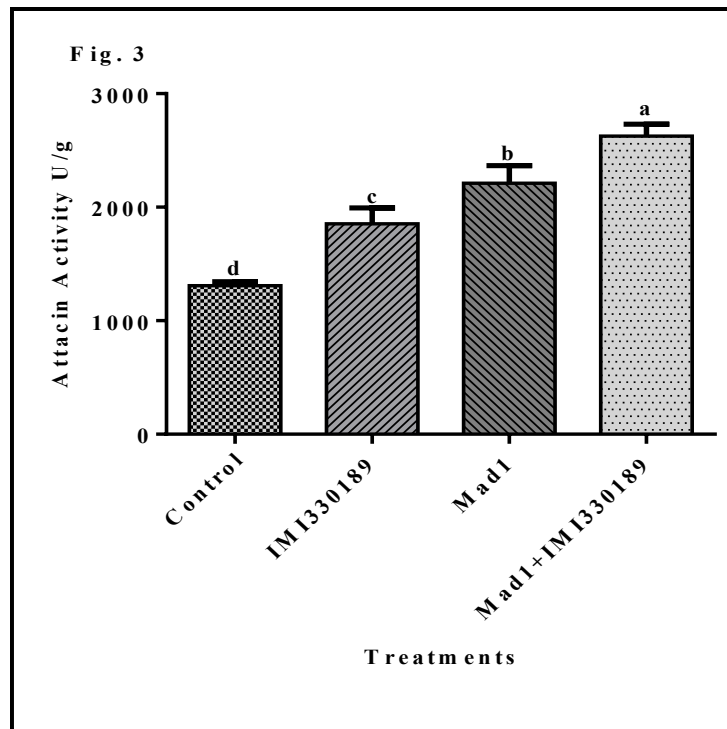


Figure 3 Attacin (AMPs) activity in migratory locust

The activity of Chitinase (CHI) in Migratory Locust: Highly significant differences were observed among the treatment applications of IMI330189, Mad1 and Mad1+IMI330189 on migratory locust through feeding method. Maximum CHI activity was observed when the locusts fed on combined dose of Mad1+IMI330189. Compared to control, the

independent application of Mad1 and IMI330189 significantly enhanced the CHI activity, however, no significant variation between the application of Mad1 and IMI330189 was recorded in migratory locusts. The expression of Mad1 and IMI330189 had the similar pattern as recorded for MFO activity in migratory locusts (Figure. 4).

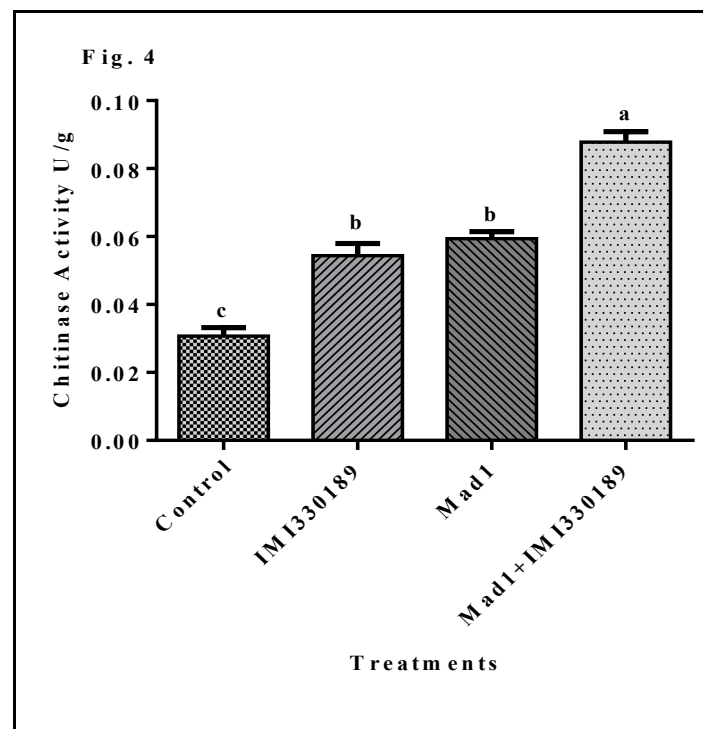


Figure 4 CHI activity in migratory locust

DISCUSSION

Mixed-function oxidase (MFO), estrases (ESTs) and glutathione-S-transferases (GSTs) are the three important families of enzymes, which are playing key role in detoxification of pesticides and insect resistance to various insecticides (Li *et al.*, 2007). The activities of MFO significantly decreased on 3rd and 5th day after the application of chlorantraniliprole (Cao *et al.*, 2016 & Jia *et al.*, 2016). However, no such significant effect on MFO and POD have also been reported using dsPTPN4 and dsPTK5 applications in *Oedaleus asiaticus* (Chang *et al.*, 2019).

Our results indicated that the Migratory Locust immune response was activated with increased MFO activities by using *Metarhizium anisopliae* and Mad1 protein after 72 hours of treatment. The usage of Mad1+IMI330189 increased the activity of MFO significantly as compared to the control. Moreover, the independent dose of IMI330189 and Mad1 also upregulated the MFO compared to the control, however there were no-significant difference among them. Our results are related with the findings of diamondback moth (Xing *et al.*, 2011) and were in contrast to that of *Oedaleus asiaticus* (Cao *et al.*, 2016 & Chang *et al.*, 2019).

The foreign invaders attack the haemocytes and the tracked by pattern recognition receptors which in turn inducing a more systemic response and up-regulation of AMPs in the fat body and haemolymph (Broderick *et al.*, 2009). Similarly, the majority of insect AMPs are ineffective against entomopathogenic fungi, while gram-positive bacteria and fungi mostly stimulate the Toll pathway, which induces the creation of AMPs associated to control particular microorganisms (Glinski & Buczek 2003).

Our results showed the increased level of AMPs after 72 hours of feeding migratory locusts by IMI330189, Mad1 and Mad1+IMI330189 (combined dose). Each treatment had a different response as compared to the control however; the highest level of AMPs was recorded for the combined dose. On average, the response of treatments was significant towards the AMPs activity, however the expression pattern of independent dose of IMI330189 and Mad1 was different from that of CHI activity. This suggests that the increasing activity of AMPs in the body of locust showed resistance against *Metarhizium anisopliae* and Mad1 protein resulting immune system activation.

Just like MFO, the chitinase (CHI) is also an important component of insect growth and development. CHI degrades the chitin found in insect cuticle and peritrophic membrane (Ali *et al.*, 2010). Study also suggests that CHI involved in insect defense against entomopathogenic fungi and parasites (Wang *et al.*, 1996).

Our results showed that chitinase activity was significantly increased in the body of Locust with the usage of *Metarhizium anisopliae* and Mad1 protein

after 72 hours of application. The expression of CHI was almost similar in nature to the MFO, where the mixed treatment of Mad1+IMI330189 increased the CHI as compared to the separate dose of Mad1 and IMI330189, which had significant role in comparison with the control but were non-significant with each other. This showed that the enhanced activity of CHI activated the immune system of locust against the application of *M. anisopliae* and Mad1 after 72 hours of infection. However, the variation (up and down) in CHI activity may be checked for an extended period of one week to verify the survival rate and its expression. Similar, findings and results have been reported in whitefly and *Spodoptera exigua* by applying the fungus *Lecanicillium muscarium* (Lai and Su 2011; Cao *et al.*, 2016; Ali *et al.*, 2017).

In migratory locust, the increased activities of chitinase and Arylamidase (AA) during the initial infection period have been reported with the application of combined dose of chlorantraniliprole and *M. anisopliae* (Cao *et al.*, 2016 & Jia *et al.*, 2016).

In future, necessary research needs to be undertaken to understand the *Metarhizium anisopliae* and Mad1 protein interaction with the immune response of migratory locust and other insect species.

CONCLUSION

From the present study it was concluded that migratory locust showed resistance to *Metarhizium anisopliae* and MAD1 during early infection period till 72 hours of treatment application by increasing the activities of Mixed-Function Oxidase (MFO), chitinase (CHI) and AMPs. In future, necessary research needs to be undertaken to understand the *M. anisopliae* and MAD1 interaction with the immune response of migratory locust and other insect species.

AUTHOR CONTRIBUTIONS

Conceptualization, Z.Z.; Data curation, N.A.A.; Formal analysis, N.A.A.; Funding acquisition, Z.Z.; Investigation, N.A.A. and Z.Z.; Methodology, N.A.A.; Project administration, Z.Z.; Resources, I.K., R.K., and N.A.A.; Supervision, Z.Z.; Validation, N.A.A.; Visualization, N.A.A. and R.K.; Writing - original draft, N.A.A. and R.K.; Writing - review and editing, I.K.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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