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# Synergistic Effect of Some Entomopathogenic Fungi and Synthetic Pesticides, Against Two Spotted Spider Mite, *Tetranychus urticae* Koch (Acari: Tetranychidae)

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**Abstract.-** Spider mites are emerging as major pests in many field crops including cotton in Pakistan. The present study was carried out to evaluate the effect of some strains of entomopathogenic fungi *viz.*, *Metarhizium anisopliae*, *Paecilomycis fumosoroseus (Pf*) and *Verticillium lecanii* on two spotted spider mite, *Tetranychus urticae* Koch, a pest on cotton during summer 2010. Three concentrations *i.e.*, 1x $10^6$, 1x $10^7$ and 1x $10^8$ conidia $mL^{-1}$ of each fungal strain were used against eggs and adult females of mites. All tested fungal isolates were pathogenic to both the eggs and adult females. The mortality in adult females increased with increase in conidial concentration while the % hatching in eggs decreased with increase in conidial concentration. The most virulent isolate against adult females was *Pf* (n32) with lowest $LC_{50}$ value ($9.1 \times 10^4$ conidia/ml on $8^{th}$ day post treatment) and $LT_{50}$ value (4.58 days at $1 \times 10^8$ conidia $mL^{-1}$) followed by V17 isolate with $LC_{50}$ value ($1.7 \times 10^6$ conidia $mL^{-1}$) and $LT_{50}$ value (5.45 days at $1 \times 10^8$ conidia $mL^{-1}$). Combined treatment of cotton with Pf (n32) and chlorfenapyr, an insecticide, proved best combination causing more than 90% mortality.The results indicated that *P. fumosoroseus* strains have potential as microbial control agent against *T. urticae* in Pakistan.

**Keywords:** Two spotted spider mites, *Metarhizium anisopliae*, *Paecilomycis fumosoroseus*, *Verticillium lecanii*, microbial control.

## INTRODUCTION

The two spotted spider mite (*Tetranychus urticae* Koch*)* is a cosmopolitan pest in cotton crop. They suck cell sap of the plants resulting in reduced production. They are capable of dramatically affecting growth, yield and fiber quality (Reddall *et al*., 2004). Insecticides have been used extensively to control these spider mites. The indiscriminate use of insecticides in the previous years has created not only the environmental issues but also caused the resistance problem in mites (Tsagkarakou *et al*., 1996; Ay and Gurkan, 2005; Gerson and Weintraub, 2012). In the recent years, due to the increasing trend of cultivation of transgenic cotton (Bt cotton) varieties by the farmers, the insecticide use has been reduced drastically which has resulted in out breaks of secondary pests including mites which are becoming more dominant and severe problem (Zhao *et al*., 2011). Mite control therefore, is becoming a challenge and interests for developing some microbial control agents against these pests is increasing (Chandler *et al*., 2000; Mietkiewski *et al*., 2000; Van der Geest *et al*., 2000).

Mites are smaller in size and hence receive much less attention for studying their pathogenicity despite their large numbers. On the other hand, due to their small size it is also difficult to diagnose their diseases. However, these can be reared easily in large quantities for studying epizootics on them (Van der Geest *et al*., 2000).

Various researchers in different parts of the world conducted bioassays on different crops and found one or two isolates of entomopathogenic fungi (EPF) to be highly effective against mites like *P. fumosoroseus* and *B. bassiana* etc (Nugroho and Ibrahim, 2004; Draganova and Simova, 2010).

EPF are just like parasite of insects which kill or seriously disable them. Unlike viruses and bacteria which need to be ingested, these pathogens require contact with cuticle under favorable

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conditions of temperature and high moisture (Dhaliwal and Koul, 2007). Since these are regarded as natural control agents and also safer for our environment, there is a great interest all over the world for utilizing and manipulating the EPF for biological control of insects and mite- pests. Moreover, the use of biological control agents is economically feasible, environmentally safer and sustainable. It is therefore, need of the time to find some alternate control methods which can be incorporated in the pest management program. A recent study of the author has revealed that the strains evaluated in the current study can be used in combination with some new pesticides (Amjad *et al*., 2012).

The present studies were therefore, aimed at evaluating pathogenicity of three fungal strains *viz*., *Metarhizium* (M440), *Paecilomyces* (*Pf*-n32) and *Verticilium* (V17) in the laboratory against two spotted spider mites and under semi-natural conditions for potentially compatible pesticides and fungal strains for their utilization in an integrated pest management program.

## MATERIALS AND METHODS

*Host plant*

Cotton, *Gossypium hirsutum* L. (cv. CIM-496) were grown individually in earthen pots (30 cm diameter) placed inside insect rearing cages (47.5 × 47.5 × 47.5 cm) made up of fine mesh transparent cloth with a zipper hole at the front. Water and fertilizer was supplied to the plants frequently. The plants were used for culturing of two spotted spider mite.

*Mite culture*

Mites (*T. urticae* Koch) were collected from infested leaves of cotton plants (*G. hirsutum*) from non-infective and non-insecticidal area at University and transferred on to caged cotton plants for subsequent culturing. The mites were identified in the Acarology Lab as *T. urticae* by following the detailed descriptions mentioned by Zhang (2003).

*Fungal culture and preparation of conidial suspensions*

The different strains of EPF used for bioassay studies were *Paecilomyces fumosoroseus* (Wize) Brown and Smith *Pf* (n32), *Metarhizium anisopliae* (Metschnikoff) Sorokin (M440) and *Verticillium lecanii* (Zimmerman) Viegas (V17). The strains were collected from College of Natural Resources and Environment, South China Agricultural University, Guangzhou, China.

All isolates were maintained on SDA (Saboraud dextrose-agar) at 25±2°C, 80±5% R.H. and 16:8 h (L:D) in the Acarology Laboratory for two weeks. Conidia in the plates were harvested with a sterile needle into 50 ml aq. Tween 80 (0.03%) followed by determination of conidia in the suspensions by haemocytometer (Neubaur®, China). The desired concentration was made by adding appropriate amount of distilled water.

*Bioassay procedure*

The viability of spores was determined by standard method (Goettel and Inglis, 1997) by plate counts of spores on SDA after 18 hrs of incubation of inoculated spores in the dark. The spore germination was observed and scored. In all tested strains >95% of the spores were germinated. The tested suspensions were $1\times10^6$, $1\times10^7$ and $1\times10^8$ conidia $mL^{-1}$.

Ten gravid female mites per leaf in different Petri plates were left for 24 h for getting homogenous eggs of similar age for subsequent fungal tests. The mites started ovipositing within 24 h under same temperature regimes at 65-75% R.H. The females were removed subsequently leaving 40 eggs/leaf disc (3 cm.), which were transferred to different fungal treatment arenas. The whole experiment was run under CRD in the laboratory with six replications.

For each fungal isolate, 40 adult females ($\leq$ 2 days after last ecdysis) and 40 eggs of *T. urticae* on 6 individual detached leaves (replicates) containing a thin layer of 1.5% water agar at the base in open Petri plates placed on a large tray were exposed to the spray of each conidial suspension simultaneously from a hand held atomiser. The leaves on water agar remain green for more than 10 days supporting the mites for normal development. The sprayer was operated at a constant working pressure and held at 30 cm above the tray. A single stroke of the hand atomizer produces 0.5 ml volume

and hence 1ml of the conidial suspension of each fungal strain was used per treatment while the same quantity on control leaves with 0.03% Tween 80 alone was sprayed. After application of fungal sprays, all the Petri plates harbouring eggs and females on detached leaves were covered with lids and put into the incubators at 25±2°C and 16:8 L:D. The relative humidity within the Petri plates was likely to exceed 90% due to water agar source. The adult female mortality in all treatments was recorded daily for 8 days until no more mortality could be observed. Cadavers were transferred into moist Petri plates and considered as 'mycotised' if fungal out growths were visible after 3 days of incubation at same laboratory conditions. Similarly, hatched eggs were observed daily at each conidial concentration until no more eggs were hatched in all treatments. The un-hatched eggs were transferred to moist Petri plates for observing fungal outgrowths. Those with outgrowths were recorded as died due to fungal pathogen. The percent mortality was calculated in all the treatments and the whole bioassay was repeated twice.

*Combined application of EPF and acaricides*

The adult females were taken from the cultured source and gently transferred onto caged cotton plants with the help of a fine camel hair brush. The population of mites was counted on individual cotton plants (having 4-5 leaves) before doing sequential sprays of chemical and fungus. The chlorfenapyr, fenpyroximate and pyridaben were used in the experiment. The plants were divided into eight groups, each containing five plants. Ten plants received a treatment of chlorfenapyr, 10, a treatment of fenpyroximate while 10, a treatment of pyridaben and 10 plants a treatment of water only (control). After 24 hours, five of the plants in each acaricide treated group were sprayed with *Pf* (n32) fungus. Five of the water treated plants also received fungus application. A combination of the two sprays in a sequence were carried out following the protocol of Cuthbertson *et al*. (2005) for compatibility studies in such a way that 1st spray was done of acaricide at recommended doses or water control and after 24 hours second spray of fungus or Tween 80 @ 0.03% as a control was applied by foliar application with hand atomizers on potted plants. The plants were sprayed to run off and the control plants received same volume of water or Tween 80. The fungal treatment consisted of each fungus applied @ $1\times10^8$ conidia/ml. Following the 2nd treatment the plants (each treatment group) were kept randomly at ambient temperature at natural photoperiods. The mortality was recorded (by the presence of fungal hyphae in case of fungal treatments) 3 and 5 days after the second treatment. The whole experiment was repeated 3 times. The procedure was repeated by using other entomopathogenic fungus (V17) as well. The% mortality was expressed and statistical analysis was done afterwards.

*Statistical analysis*

The mortality percentage of each treatment was analyzed by analysis of variance (ANOVA) and means compared by Tukey's HSD test (SPSS® 16.0). The $LC_{50}$ and $LT_{50}$ values and their 95% fiducial limits were calculated from probit analysis (Finney, 1971) using Minitab® 15 computer software.

## RESULTS

*Virulence of EPF against adult females of* T. urticae

The pathogenicity of tested isolates of entomopathogenic fungi against adult females at three different conidial concentrations is shown in Figure 1. All the three tested strains of EPF showed mortality at the tested conidial concentrations and virulence against adult females of *T. urticae*. Among the tested isolates, *Pf* (n32) showed the highest mortality percentages (79.16±1.65%) at the concentration of $1 \times 10^8$ conidia /ml. On comparing the mortality induced by the fungi after 8 days of fungal application at $1 \times 10^6$ conidia /ml, it is observed that there is a significant difference ($F= 99.21$, df; 3, 23 $P<0.001$) between the tested isolates. However, only *Pf* (n32) strain showed more than 60% mortality (61.25%). Similarly, at the median lethal concentration there is a significant difference ($F= 225.02$, df; 3, 23 $P<0.001$) between the isolates with V17 and *Pf* (n32) both the isolates showing more than 65% mortality (65.41% and 69.16%, respectively). While on the highest lethal concentration, there also existed a significant difference ($F= 595.82$, df; 3, 23 $P<0.001$) between

the isolates with V17 and *Pf* (n32) both the isolates showing more than 75% mortality (76.25% and 79.16%, respectively). The *M. anisopliae* isolate M440 remained least effective.

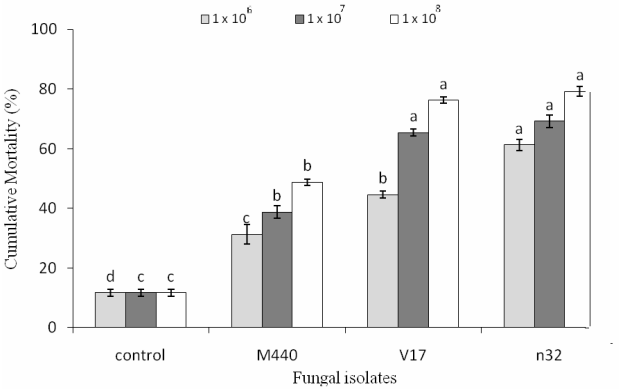


Fig. 1. The Cumulative Mortality (%) of *T. urticae* Koch adult females after exposure to fungal sprays (conidial suspensions $ml^{-1}$) at $8^{th}$ day after spray. Similar letters at the same bars are not significantly different (Tuky's HSD test $P<0.05$).

The virulence studies (Table I) showed that there is a significant difference between $LC_{50}$ and $LT_{50}$ values of the three fungal isolates. The isolate, *Pf* (n32) has the lowest $LC_{50}$ ($9.1 \times 10^4$ conidia $mL^{-1}$ on $8^{th}$ day ) and $LT_{50}$ (4.58 days at $1 \times 10^8$ conidia $mL^{-1}$) showing the most virulent strain among the all tested ones. The isolate, M440 recorded the highest $LC_{50}$ ($1.4 \times 10^8$ conidia $mL^{-1}$ on $8^{th}$ day) and $LT_{50}$ (7.55 days at $1 \times 10^8$ conidia $mL^{-1}$) have shown least virulent among all tested isolates.

*Virulence of EPF against eggs of* T. urticae

The eggs hatching results (Fig 2a, b and c) revealed that all the tested fungal isolates were lethal to *T. urticae* eggs despite variability in their infectivity among the tested isolates. The eggs hatching data showed that *T. urticae* eggs could not hatch out until day 3 post inoculations and then increased gradually reaching maximum on day 7 and maintained with no change in hatching after $8^{th}$ day. Hence the data for $8^{th}$ day was considered for further comparison among the tested isolates for virulence studies. Hatching in the control was high in all bioassay experiment. For each fungal isolate tested the % egg hatching at different conidial

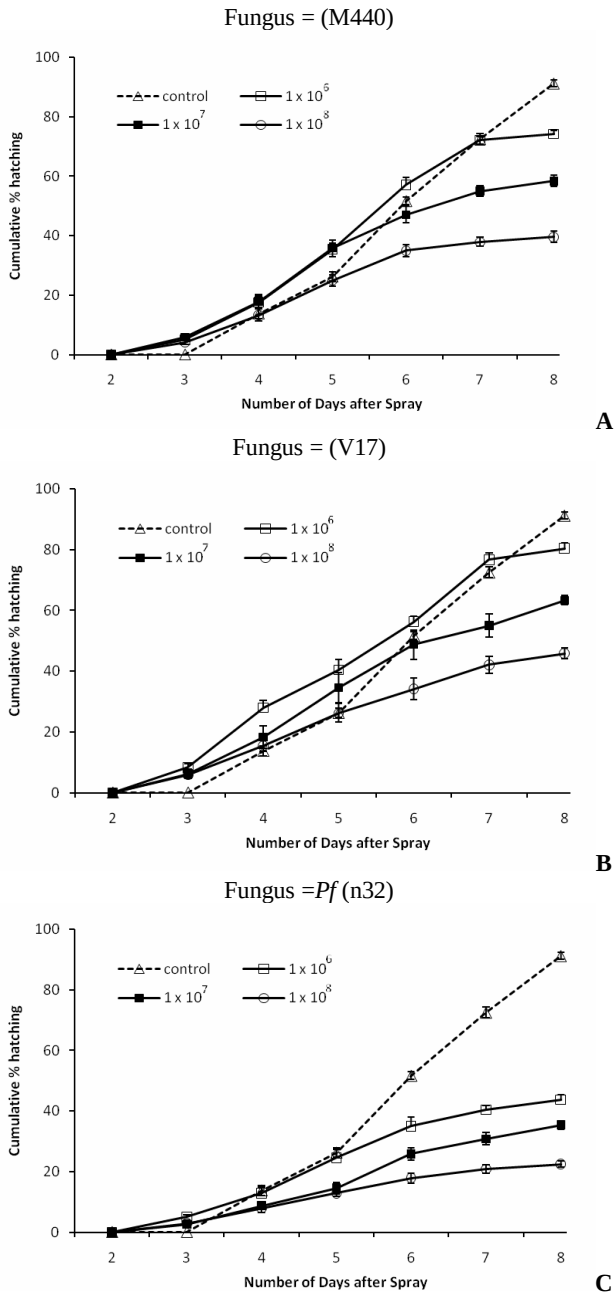


Fig. 2. The cumulative hatching (%) of *T. urticae* Koch eggs after exposure to three concentrations of different fungal isolates (No. of conidia $ml^{-1}$). Error bars = S.E.

concentrations has been shown by individual trends. At the higher conidial concentration, all isolates caused significantly less hatching and vice versa showing that the mortality in eggs increases with increase in the conidial concentration of the fungal

**Table I.- (a) $LC_{50}$ with 95% fiducial limits on 8th day and (b) $LT_{50}$ at $1 \times 10^8$ conidia $mL^{-1}$ after exposure to fungal isolates against adult females and eggs of two spotted spider mites in the laboratory.**

| Fungal isolates | stage | $LC_{50}$ (No. conidia/ml) | 95% FL+ | Slope±SE | $\chi^2$ | d.f | P |
|---|---|---|---|---|---|---|---|
| (a) | | | | | | | |
| M440 | female | $1.4 \times 10^8$ | $4.5 \times 10^7 – 2.8 \times 10^9$ | 0.099 ± 0.025 | 0.0653 | 1 | 0.798 |
| V17 | female | $1.7 \times 10^6$ | $7.4 \times 10^5 – 3.1 \times 10^6$ | 0.186 ± 0.026 | 1.092 | 1 | 0.296 |
| *Pf* (n32) | female | $9.1 \times 10^4$ | $1.6 \times 10^3 – 4.5 \times 10^5$ | 0.113 ± 0.026 | 0.214 | 1 | 0.643 |
| M440 | Egg | $2.7 \times 10^7$ | $1.6 \times 10^7 – 5.0 \times 10^7$ | 0.198 ± 0.026 | 0.032 | 1 | 0.857 |
| V17 | Egg | $5.7 \times 10^7$ | $3.3 \times 10^7 – 1.1 \times 10^8$ | 0.208 ± 0.026 | 0.117 | 1 | 0.732 |
| *Pf* (n32) | Egg | $3.5 \times 10^5$ | $3.5 \times 10^4 – 1.0 \times 10^6$ | 0.128 ± 0.026 | 0.642 | 1 | 0.423 |
| (b) | $LT_{50}$ (days) | | | | | | |
| M440 | female | 7.55 | 7.05 – 8.20 | 1.21 ± 0.077 | 4.69 | 6 | 0.584 |
| V17 | female | 5.45 | 5.26 – 5.66 | 1.89 ± 0.092 | 3.82 | 6 | 0.701 |
| *Pf* (n32) | female | 4.58 | 4.42 – 4.75 | 1.85 ± 0.083 | 11.62 | 6 | 0.071 |

+ FL= fiducial limits for the respective $LC_{50}$ values.

isolates. The overall hatching in the control remained to be 91.25%. At lower concentration on 8th day post inoculation, only *Pf* (n32) isolate caused less than 50% (43.75±1.76%) hatching in the eggs while the other two isolates M440 (74.17±1.94%) and V17 (80.42±2.03%) showed higher hatching. At the median lethal concentration on 8th day post inoculation, the same trend was observed that is only one isolate showing less than 50% hatching in eggs *i.e. Pf* (n32) (35.42±1.34%). At high concentrations, all the isolates showed significantly less hatching (50%) with M440 (39.58±1.12%), V17 (45.83±1.79%) and *Pf* (n32) (22.50±1.12%).

The virulence studies on eggs (Table I) showed a significant difference between $LC_{50}$ values of the three fungal isolates. The probit analysis was carried out and the goodness of fit test showed no significant heterogeneity in the linear relationship of all tested fungal isolates. The fungal spore suspension of *Pf* (n32) isolate has the lowest $LC_{50}$ ($3.5 \times 10^5$ conidia/ml) value on 8th day after inoculation showing the most virulent strain while the *V. lecanii* (V17) recorded the highest $LC_{50}$ ($5.7 \times 10^7$ conidia/ml) value being the least virulent among all tested isolates.

*Combined effect of EPF and selective pesticides*

A significant difference existed between all the treatments whether chemical alone and/or the chemical followed by the fungus ($F_{7, 23}$ =507.99, P<0.001) with the control for both of the tested fungi. On comparing the results obtained 3 days after fungal application with *Pf* (n32) (Fig. 3) it is revealed that the combined treatment of chlorfenapyr & the fungus showed highest mortality (72.33%±1.36) followed by chlorfenapyr alone (67.0%±1.42). Although, the combined treatment of *Pf* (n32) and chemical in a sequential manner showed higher mortality than the chemical alone but the results of fenpyroximate alone and combined with fungus and also pyridaben and fungus were statistically at par. Similarly, significantly different results were obtained with higher mortality after 5 days of fungal treatment ($F_{7, 23}$ =1642.22, P<0.001). Again the combined treatment of chlorfenapyr followed by fungus showed the highest mortality (90.33%±1.42) which was statistically at par with combined application of fenpyroximate and fungus (87.0%±1.20). Pyridaben showed least mortality among all.

Almost similar results were obtained when using the fungus (V17) but the mortality was slightly lower than that caused by *Pf* (n32) (Fig. 4). Significant differences were observed again 3 days after treatment ($F_{7, 23}$ = 616.84, P<0.001) and 5 days after treatment as well ($F_{7, 23}$ =807.54, P< 0.001). On 3 days after treatment, Chlorfenapyr and V17 showed the highest mortality (77.67%±0.78)

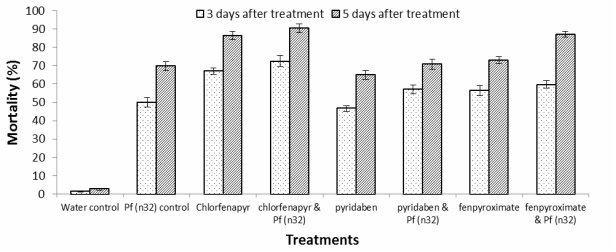


Fig. 3. Efficacy of *P. fumosoroseus* (@ 1× $10^8$ conidia/ml) alone and combined with selective acaricides against adult mites (TSM) on cotton at 25±2°C at natural photo phase. Error bars= SE.

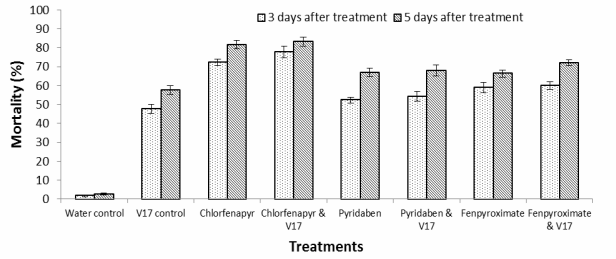


Fig. 4. Efficacy of *V. lecanii* (V17) (@ 1× $10^8$ conidia/ml) alone and combined with selective acaricides against adult mites (TSM) on cotton at 25±2°C at natural photo phase. Error bars= SE.

followed by chlorfenapyr alone (72.33%±0.78). Pyridaben and fenpyroximate alone and fungus combined treatment were statistically at par and none of them showed better control of nymphs compared to the chemical being used alone. On 5th day after treatment, the results of chlorfenapyr alone and combined with fungus were at par.

## DISCUSSION

The pathogenicity results of three EPF against adult females and eggs revealed that *P. fumosoroseus* (n32), *M. anisopliae* (M440) and *V. lecanii* (V17) can be employed for the control of *T. urticae*. A relatively longer period of incubation is required to achieve the desired control results. However, among the tested isolates, *P. fumosoroseus* (n32) yielded the highest mortality (79.16%) at a concentration of $1\times10^8$ conidia /ml which proved to be the best to be used for mite control. The mortality trend of *T. urticae* Koch females over different days after inoculation indicated that although the infection was developed and mortality started from the 3rd day but continued to increase up to 8th day. These results suggest that the experiments with some entomopathogenic fungi require longer period of incubation before the assessment of their outcomes. Thus these results were compared after 8 days of inoculation like Shi and Feng (2004) who evaluated some entomopathogenic fungi against *T. cinnabarinus* eggs and evaluated results on 9th day post inoculation.

Furthermore, the virulence studies suggested that *P. fumosoroseus* (n=32) isolate is efficient in controlling the adult female mites as having the lowest $LC_{50}$ ($9.1 \times 10^4$ conidia/ml) and $LT_{50}$ (4.58 days) values showing the most virulent strain among all tested isolates. These results are in conformity with those of Shi and Feng (2009) who used *B. bassiana, P. fumosoroseus* and *M. anisopliae* isolates and got 73.1, 75.4 and 67.9% mortality of mites after 10 days of spraying with 15.5% mortality in the control. The laboratory bioassays with different EPF are necessary. The efficacies in the field trials are usually supported to some extent by the laboratory bioassays e.g. some laboratory tested isolates of *M. anisopliae, B. bassiana.* and *P. fumosoroseus* are proved to be highly pathogenic to the adult females and eggs of spider mites, *T. cinnabarinus* (Shi and Feng, 2004; Shi *et al*., 2008 a, b) and *T. urticae* Koch (Alves *et al*., 2002). The bioassays with mite eggs were different from adult females as they don't move and it was difficult to observe and show their mortality status. Therefore, it was easy to determine the number of eggs hatched on a particular day after spray. Thus, the hatch rates were used to evaluate the egg mortalities at different concentrations of conidia. The $LT_{50}$ for eggs was not therefore possible as the mortalities in the eggs at a particular time interval post inoculation could not be observed and only $LC_{50}$ was assessed and evaluated. Wekesa *et al*. (2005) studied two isolates of *B. bassiana* and 17 isolates of *M. anisopliae* against *T. evansi* and observed that both the isolates caused more than 80% mortality at a higher concentration ($1 \times 10^8$ conidia $ml^{-1}$). The $LC_{50}$ estimates on eggs in our study suggests that the *P. fumosoroseus* (n32) isolate has the lowest value ($3.5\times10^5$ conidia/ml)

and hence most efficient in killing mites.

The application of fungus combined with other chemicals is an important tactics to be utilized in any IPM program and for its successful introduction in a program the information regarding its compatibility with chemicals is also important (Cuthbertson and Murchie, 2006). A lot of researchers like Quintela and McCoy (1998), Dayakar *et al.* (2000) and Rachapa *et al.* (2007) used commercial pesticides belonging to different groups with EPF and observed enhanced efficacy of EPF against insect pests. During current study in each combined treatment a higher mortality was observed as compared to the fungus or chemical alone. Although, pesticides or EPF used by other researchers were different but the current study coincides the basic idea that fungal pathogen makes the host sick enough to become susceptible to chemical pesticide and the latter in turn give more stress and weaken the pest sufficiently to make more susceptible for disease attack (Charnley and Collins, 2007). Chlorfenapyr showed the highest mortality combined with fungus but it is clear that the above mentioned two fungi can be applied sequentially with chlorfenapyr, pyridaben and fenpyroximate for controlling mites on cotton.

In conclusion, the results suggest that *P. fumosoroseus* has a potential to be used against spider mites alone and incorporated in any IPM program or combined with any control tactics that may enhance the fungal performance. In addition there needs to be studied the effects of environmental factors, temperature and humidity, as factors that might limit to the practical use of these or other isolates as microbial control agents.

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