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Entomopathogen mediated synthesis of Silver-Nanoparticles and it's efficacy on lepidopteran Larvae

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Abstract: The efficacy of Silver-Nanoparticles as a larvicide with the help of entomopathogenic fungi, *Trichoderma ressei* shows major larvicidal activity against the major Indian lepidopteran pest; *Spilosoma obliqua* . *T.ressei* synthesized silver nanoparticles (AgNps) were characterized structurally by UV vis Spectrophotometer and Fourier transform infrared (FTIR) spectra. The optimum pH (alkaline), temperature (28 – ^{o}30 C) and agitation (150 rpm) for AgNps synthesis and the stability of these AgNps were confirmed by change of colour. *S. obliqua* larvae (I – V instars) were found high susceptibility than any other lepidopteran pest. However, the rate of mortality was indirectly proportional to the larval instar and the concentration gradient. This is the first report for synthesis of AgNps from Entomopathogenic fungi i.e. *T. ressei* against *S. obliqua* (The Bihar Hairy Caterpillar) as biological pest control.

Keywords: *Trichoderma ressei*, Silver- Nanoparticles, *Spilosoma obliqua*, Biological Pest control, Larvicidal activity.

INTRODUCTION

The economy of India is basically dependent on agricultural productivity, with about two-third of population associated with and dependent on agriculture. Thus, increasing productivity and decreasing loses are of prime concern. There are various sources that result in crop loses but, insect pests are the agents that are responsible for major injuries to crops thus affecting agricultural productivity substantially. To reduce these losses, various chemical insecticides have been used. But, numerous studies have revealed the environmental hazards of these synthetic pesticides (e.g., Subashini *et al.*, 2004). This has prompted humans to seek for some safer alternatives and this pursuit has led to investigations into some eco-friendly and bio-rational alternatives.

Nanoparticles have been reported to play an important role in biotechnological, pharmaceuticals, food products, medical care, electronics, personal hygiene, etc. Silver nanoparticles, in particular, have been proven to be very effective because of their antibacterial, antifungal, larvicidal and antiplasmodial properties which have been studied by various authors like Elumalai *et al.*, 2010, Saxena *et al.*, 2010, etc. But, the synthesis of nanoparticles using physical and chemical methods uses rigorous methodologies including a very high temperature pressure, a lot of energy input, and a range of highly toxic chemicals. On the other hand, biosynthesis of nanoparticles using entomopathogenic agents has been found to be cheap and environment-friendly (Casida and Quistad, 2005).

Instead of using physical and chemical methods, nanopartin cles are made from biological method by using various plant extracts, microorganisms, enzymes, etc., which is much more eco-friendly method (Mohanpuria *et al.*, 2008). Nanoparticle synthesis by plants are often beneficial over different biological approaches, as a result it excludes the flowery process of maintenance of cell cultures and may even be appropriately scaled-up for significant NP amalgamation (Shankar *et al.*, 2004).

In central and north part of India, various pests affect the cotton crop, rice, wheat etc. but the major pest affecting these crops are *Spilosoma obliqua* (the Bihar hairy caterpillar). The variety of chemical pesticides and insecticides are used for the effective management *S. obliqua*; but, due to the harmful effects of these pesticides and insecticides, there is need for more eco-friendly alternative, which may be used to protect these crops from pests and insects. The *S. obliqua* , cotton bollworm, corn earworm are Lepidopteran moth, the larvae of which feed on a various plants, which include many important cultivated crops. It is a significant blighter in cotton and the foremost polyphagous and cosmopolitan blighter species, accounting for financial losses of about five crores in India (Manjunath *et al.*, 1985)

Therefore, in this study, I try to report the entomopathogenic synthesis of AgNPs and its efficacy on Lepidopteran larvae.

Entomopathogens in pest control: Many farmers have been using parasites and predators for biological pest control, but it is also possible to kill insects by using specific microorganisms. These entomopathogenic agents include fungi, bacteria, nematodes, and viruses. These entomopathogens are cosmopolitan within the nature and infect various pest species. Entomopathogens act as natural regulation method of controlling various insect populations. (Hajek, A. 2004) Most of the research in this direction is concerned with the finding of entomopathogenic agents and their use as biological pest control. Various entomopathogens analogous to chemical pesticides are formulated which are used to control various insect population. This, thus, includes nonpersistent remedial treatments that are released in the environment (Hajek, A.E., Siegel 2004, Evans, H.C. 2001).

Use of Entomopathogens is a traditional method. it act as a organic management agents of unfamiliar insect pests, and natural tormenter management with the help of entomopathogens has been increased by home ground management. (Hajek,*et al.* 2004).

Entomopathogenic fungi : There are supposed to be approx. 750 fungal species which cause toxicities to various insects or mites (Benserradj *et.al,* 2014). In a group, wide range of pests species are infected by them, but individual

species of fungi are also strain-specific. The fungi infect the host by producing spores that germinate on the host surface and then grow into its body. On the basis of type of spore and its quantity determine the death time, after death of host spores are producing continuously on it which dispersed in the environment and infect new host.

Traditional accepted organic control includes the general use of expected opponents against a range of hosts which are unusual in an area and have recognized without the full guild of its natural rivals. Various surveys are undertaken for the insect pest in their centre of origin for the identification of their suitable natural enemies which may be subsequently released in their newly expanded habitat range (Samways 1981). In short, successful classical organic control databases provide an efficient, suitable and a long-term insect pest control. In case of entomopathogenic control, only two explicit examples of classical organic control employing old encounter affinities have been reported, and both examples include entomophthogenic fungi.

Bio-Nanotechnology is a new and rapidly advancing field of research lies at the interface between biology and nanotechnology (Sahayaraj and Rajesh, 2011). The field of Nanotechnology is a science and technology of controlling and manipulating matter at the Nano scale level (Bala and Arya, 2013).

Nanotechnology and nanoscience's can be applied in all other major fields of science like physics, chemistry , biology, material sciences and engineering (Rajasekhar Reddy *et al.*,2009). The fabrication and application of materials at the nanometre scale in biology is a great concern in the field of nanotechnology (Safekordi *et al.*, 2011).

Nanotechnology involves the synthesis of NPs ranging in size from one to one hundred nm which might be appropriately manipulated for the desired/specific applications. Recently, much more impressive development should be done in the engineering field so with the help of new methodology nanoparticles of desirable shape and size should be prepared according to requirements.

Nanoparticles have significant value as controlled drug distribution or release formulations schemes once it appropriately encapsulated, a pharmaceutical will be delivered in appropriate size, the concentration should be maintained for extended time period and it should be protected from premature degradation. Due to its small size they should be injected into the vascular system directly (Manjuchandra prabha *et al.*, 2012). Insecticides synthesized from natural products such as gold , silver or silicon nanoparticles (Priya and Santhi, 2014).

Fungi and the products derived from them are non-toxic to non-target organisms but these products are extremely toxic to mosquitoes. Therefore the utility of endomophagous fungi and their products is deemed as a promising method for biological control of various insect pests, particularly mosquitoes (Kirschbaum, 1985). The application of entomopathogenic fungi against various mosquito larvae has been the topic of interest since long ago (Clark *et al.*, 1988; Alves *et al.*, 2002). A number of such studies have demonstrated that endomopathogenic fungi, under laboratory conditions, can be very effective at inducing mortality in mosquito larvae. Vector control is only reliance to control epidemic diseases like Dengue, malaria, filariasis, that are conveyed by different species of mosquitoes. With the long term use of insecticides to control vector transmission causes its harmful effects on a range of non-target organisms as well as on environment lead to the development of new eco-friendly harmless method of vector control.

Larvicidal activity: Entamopathogenic fungi are preferred because they are selective in their toxic action, are non-persistence in the environment, and they are not ingested by other microbes as reported by Maurya *et al.* (2011) and are non-toxic to non-target organisms. On a wide range, Emtomopathogenic fungi are used as biocontrol agents.

From last decades, a number of Entomopathogenic fungi have been employed for the effective control of Dipteran vector population as these fungi directly attack the insect species by penetrating its cuticle and not to be digested by insect itself to cause the disease. Entomopathogenic fungi are naturally pathogens of mosquito larvae, although no products based on fungi are currently available for the control of vector species. Entomopathogenic fungi have an advantage over other insect pathogens as they can infect all developmental stages of their hosts such as eggs, larvae, pupae and adults. Entomopathogenic fungi for the control of disease vectors such as *Culex, Anopheles* and *Aedes* (Laerciana *et al.*, 2013).

For larvicidal activity against mosquitoes various extracellular secondary metabolites from various fungi have been screened (Priyanka *et al.*, 2001; Prakash *et al.*, 2010).

Nanoparticle synthesis by fungi: The NP biosynthesis is receiving much attention due to the growing need to develop safer, cost-effective and environment-friendly technologies for Nanomaterials synthesis. In some studies, AgNPs have been synthesized by reduction of aqueous Ag+ ion from *Aspergillus terreus*, this reaction occur at a very low temperature in a few hours. These NPs were monitored by ltraviolet-visible spectroscopy and AgNPs bioreduction and characterization by transmission electron microscopy and X-

ray diffraction (Guangquan *et al.*, 2012). The utility of eukaryotic organisms for NP synthesis was first demonstrated by employing *Verticillium sp.* to synthesise gold nanoparticles. Intracellular silver NP synthesis using the fungus *Verticillium* was undertaken by *Mukherjee et al.,* 2001. The analysis of thin fungal cell sections by electron microscopy disclosed that these NPs were present below the surface of cell wall, and the possible reason of this observation, according to them, is the reduction of the associated metal ions by various enzymes present in the cell wall. Further, they speculated the surface trapping of AuC^{l4-} ions. The authors also speculated that the occurrence of fungal cells by their electrostatic interaction with some positively charged species like some essential amino acid residues of various enzymes existing in the mycelia cell wall (Mukherjee *et al.*, 2001).

When NP synthesis site is intracellular then downstream processing is usually rendered difficult. So, extracellular biosynthesis of silver NPs has been attempted by using the various filamentous fungi, e.g., *Aspergillus fumigatus* (Bhainsa and Souza, 2006). The majority of these filamentous fungi (e.g., *A. fumigatus*) which are used for extracellular biomass free synthesis of these NPs have been reported to be infective to plants as well as humans. Therefore, this makes biomass handling and disposal an apparently major inconvenience toward the successful commercialization of this process. So, there is a need to develop a novel technique to test the nonpathogenic fungus in order to achieve the successful synthesis as well as capping of nanosize particles.

The synthesis of green and highly stabilized Nano crystalline silver particles by employing agriculturally important and non-pathogenic fungus with the help of *Trichoderma asperellum* is reported by some authors, e.g., Mukherjee *et al.* (2008). The mechanism underlying the formation of silver NPs involves the process of growing NPs which consist of a series of steps. Firstly, $AgNO_3$ is bio-reduced to result in the production of AgNPs, which is followed by stabilization and/or encapsulation step, using a suitable capping agent.

The biosynthesis of silver NPs was also achieved, in some instances, by using an endophytic fungus, *Epicoccum nigrum,* isolated from *Phellodendron amurenso* cambium; AgNPs thus formed demonstrated the toxic activity against some pathogenic fungi. The synthesis of gold nanomaterial's (AuNPs) has also been achieved by the bio-reduction of chloroauric acid (HAuCl4) by employing the fungal culture filtrate (FCF) of *Alternaria alternata*. Synthesis of AgNPs using, *Trichoderma ressei*, an entomopathogenic fungus showed effective larvicidal as well as pupicidal activity against the dengue vector *Aedes aegypti*..

METHODS AND METHODOLOGY

Preparation of *Trichoderma ressei* culture: The fungus *T. ressei* was obtained from microbiological sources. *T. ressei* inoculates were prepared in potato dextrose agar (PDA) media at 28 C with 6 different concentrations in Petri plates for 120 hrs.

Preparation of cultural extract:

For the synthesis of NPs, the fungus was allowed to grow in 200 *mL* bottles, containing 100 *mL* of GC medium, which composed of glucose (0.5 %) and casein hydrolysate (0.4 %). The mixture was stirred continuously using a magnetic stirrer (rotary shaker IKA KS 260 basic) at 25–28 *C* (at 150 *rpm* for 72 hours). The reason to employ the GC medium was because a higher yield of *Trichoderma reesei* has been reported when reared in glucose-casein hydrolysate broth than in any other media. The Casein hydrolysate is a mixture of various amino acids and peptides produced by enzymatic or acid hydrolysis of casein.

The vegetative part of the fungus (mycelial part) mass was then separated from the culture broth by filtering it through a sterile filter paper. The settled mycelia were washed thrice using sterile distilled water. The mycelial mass thus harvested was subsequently used for the synthesis of AgNPs. The culture flask was incubated at 28 C (in an orbital shaker) and agitated at 150 rpm and the biomass was harvest after 72 h of growth by sieving through plastic sieve followed by extensive washing with sterile double distilled water to remove broth medium components from the biomass. *Trichoderma* biomass (20 g) was allowed to incubate in 100 mL sterile double distilled water in an Erlenmeyer flask for 48 h at 27 C. After incubation, the biomass was agitated and the filtrate obtained by passing it through Whatman filter paper no. 1.

Synthesis of silver nanoparticles: The synthesis of NPs was accomplished by stirring silver nitrate solution in mycelia free cell filtrate (MFCF). The formation of silver nanoparticles was observed by their colour change and further validated spectrophotometrically. Effect of concentration on synthesis of NPs was also observed. In MFCF appropriate amount of $AgNO_3$ was added to make the final concentration of 1 mM, 3 mM, 5 mM, 7 mM, 9 mM solution and the reaction was carried out in dark condition at 40 C. Simultaneously, a positive control (only the MFCF without AgNO3) and negative control (1 mM AgNO3 in de-ionized water) was also checked for comparison. The silver nanoparticles separate out by centrifugation (at 12,000 rpm for 10 min). The dry AgNPs were used for our further studies.

Characterization of silver nanoparticles (AgNPs):

The UV–vis spectra were recorded on double beam spectrophotometer (SL- 210 double beam) from 300 nm to 800 nm. The distilled water served as a blank, and the sample for analysis was prepared on carbon-coated copper grids.

UV–vis spectrum of silver nanoparticles: *T. ressei* culture was grown in culture specific media; and the culture was then harvested to get mycelia free cell filtrate (MFCF). After addition of $AgNO_3$ to MFCF, colour change was monitored by visual observation of the culture bottle containing AgNO3 solution. The colour of MFCF containing $AgNO_3$ changes from colourless to light brown after 5 minutes and eventually to dark brown. This colour change serves an indication for formation of AgNPs in the solution. MFCF without $AgNO_3$ did not show any visual colour change. The temperature effect on the speed of reaction will investigated by varying the temperature of the reaction from 10 ºC to 50 ºC and it was recorded on UV–vis spectra. It was observed that at lower temperatures; the speed of reaction was very slow. By increasing temperature from 10 ºC to 30 ºC, no significant increase in reaction rate was observed but as the temperature was increased to 40 ºC, the complete reduction of silver ions was observed within 4 h. Therefore, 40 ºC was shown found to be the optimum temperature for this reaction. After working out temperature optimization, the effect of $AgNO_3$ concentration on AgNPs synthesis was evaluated. For this, $AgNO_3$ solution concentration was varied from 1 mM to 9 mM and it ws observed that as the concentration of $AgNO_3$ was increased, the intensity of the colour also increased from yellow to deep brown. This reflected a significant effect of the $AgNO_3$ concentration on the NP synthesis. The increase in the colour intensity also depends upon size of AgNPs synthesize. The colour change during metallic nanoparticle synthesis is thought due to the collective oscillation of free conduction electrons induced by an interacting electromagnetic field, known as surface plasmon peak (Smitha *et al*., 2009)..

Culture and maintenance of Lepidopteran pest: Lepidopteran larvae (*Spilosoma obliqua*) were collected from various localities including urban, rural and semi urban location of Phagwara (Punjab) India, and reared in purified buckets and fed with Castor leaves. From these field-collected larvae, the culture was established in laboratory at optimum temperature (25±3 ºC) and humidity (65±5%). The larvae of *Spilosoma obliqua* were maintained as per the regular method. For the present research work, *Spilosoma obliqua* was considered because it is one of the major pests in India.

The observed life cycle of *S. obliqua* is as follows:

Egg : The freshly laid eggs were creamy white and turned pale yellow and are slightly compressed at one side. The egg measured about 0.25 ± 0.021 mm in diameter. When the eggs were about to hatch, it turns blackish which was developing-head of the larvae (Figure 4.1). One day earlier to hatching, the dusky head of the early larva was detected inside the egg shell. The period of incubation ranged from 5–6 days.

Figure..1 :- Eggs of *S.obliqua*

Larva: During its larval developmental, the caterpillar moults four times and consists of five larval instars. The total larval period ranged from 20-21days with an average of 20.55 days

Figure .2:- Larval period of *S. obliqua*

First instar: The newly hatched larvae were observed to be slaggy in nature which became active after some time. The young larvae were light yellow with dark head. The head is comparatively big and was bigger than any part of the body. The larval body was covered with number of long hairs arising from dark coloured tubercles (Figure 4.3). The first instar took 3 to 4 days (average of 3.3 days) to enter into next instar.

Figure..3:- 1 inster larvae

Second instar: As the insect enters into its second instar, the body grows quicker and as a result, the body turns broader than the head. The larva is shining light yellow in colour with prominent head and tubercles (Figure 4.4). During the second instar, the size of the head was 0.08 – 0.11 mm with an average of 0.9 mm. The second instar varied from 2 to 3 days (average of 2.25 days) to enter into next instar.

Third instar: The third larval instar was morphologically same to that of second instar with prominent setae and tubercles. The larvae were light yellowish in colour with

black patches on the anterior and posterior region of the body.

Figure.4. 2nd inster larvae

The spiracles were black in colour and are nine in number. The head capsule width of third instar larva was 0.17 to 0.19 mm with an average of 0.18 mm. The developmental duration of third instar larvae was 5 to 6 days (an average of 5.60 days).

Fourth instar*:* The 4th instar was yellowish in colour with yellow longitudinal stripes. Head and prothoracic shield were dark brown. Black patches were present on the anterior and posterior region (Figure 4.5). The size of fourth instar larva head capsule measured 0.21 to 0.28 mm (an average of 0.25 mm). The life period of fourth instar larvae was 3 to 4 days (an average of 3.80 days).

Figure. 5. 4th instar larvae

Fifth instar: The 5th instar larva was nearly similar to the 4th instar, with only difference observed in its size (Figureure 4.6). Black patches were present on the anterior and posterior region. The larvae had thick hairs on its body. The fully grown larva was stout and cylindrical, brown in colour, the head of the larva was dark brown and conspicuous dark anterior and posterior patches of the larva. The larvae had dense hairs on its body. The head capsule width of fifth instar larva was 0.40 to 0.54 mm (an average of 0.47). The duration of fifth instar larvae was 5 to 6 days.

Figure. 6. 5th instar larvae of *S. obliqua*

Pupa *:* Pupation occurred in the soil as sterilized soil was provided as medium. The pupa was elongated and oval in shape and it covered with fibrous shell called cocoon (Figure 4.7). The eyes and the antennal case were prominent in pupal stage. The freshly formed pupa was yellowish and gradually reached to dark brown (Figure 4.8). They undergone pupation inside the hairy cocoon, hence pupal sexing was not possible. The total pupal duration ranged between 8 to 9 days with an average of 8.60 days.

Figure .7. Pupa of *S. obliqua*

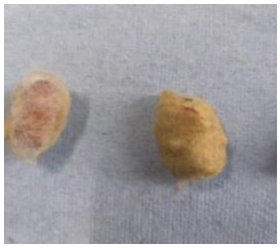


Figure.8. Cocoon of *S. obliqua*

Adult *:* The adults are medium sized brown moths and had pink abdomen. Wings pinkish with numerous black spots, with distinct head, thorax, and abdomen. The antennae and legs were light brown. Two long segmented filiform antennae are located dorsally on the head and close to the compound eyes (Figure 4.9). The male moth was almost similar to the female but it was smaller than female in size. The abdomen was sharply tapered compared to female. The average longevity of the adult male was 4.00 to 5.00 days (average 4.40 days). The female was relatively bigger than the male. The female abdomen was blunt while the abdomen of male was pointed and narrower. The average longevity of the adult female was 6 to 7 days (average 6.20 days).

Figure.9 Adult Moth of *S. obliqua*

Total life span: Total life duration of male and female ranged from 37 to 42 days with an average 39.80 days and 39 to 44 days with an average of 41.60 days, respectively.

Implementation of synthesized Silver nanoparticles on Lepidopteran larvae

The synthesized AgNPs was treated Castor leaves and feed these larvae. The different instars were feed separately in their favourable condition. Different concentrations were applied in different instars for different time period.

RESULTS

Result after enrichment of *Trichoderma ressei* culture:

T. ressei inoculates are enriched in Potato Dextrose Broth medium for 7 days in 28℃ after 7 days a good amount of fungus was grown in that broth flask. After observing those fungus under compound electronic microscope, these was confirmed that those fungus were *T.ressei* (filaments)

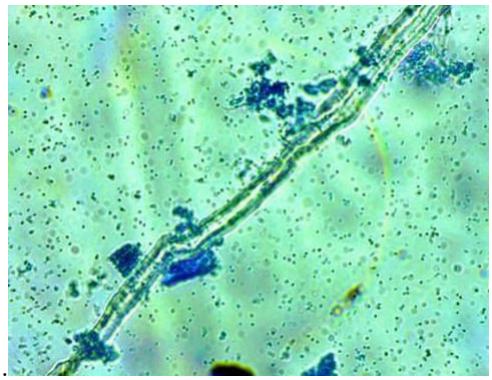


Figure.9: 10x view of *T.ressei* after enrichment.

Result after revive of *T.ressei* in culture plates:

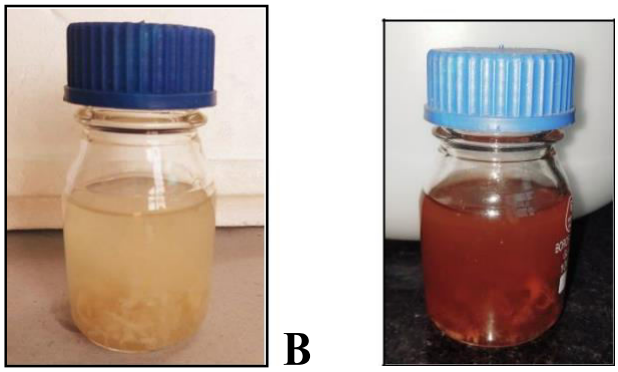
T.ressei strands are cultured for the colony formation in cultured plates for 14 days in 28C After 14 days it was observed that there is some grayish colony of filamentous fungi were observed. After observation of these fungi under microscope it was confirmed that cultured fungus was *Tricoderma ressei.*

Figure.10: 100x view of *Trichoderma ressei* after revive in PDA media in petri plate.

Figure.10: 40x view of *Trichoderma ressei* after revive in PDA media in petri plate.

Primary conformation of Silver-Nanoparticle:-

The primary conformation of AgNPs was done by its colour change. When the MFCF wet biomass of fungus were mixed with Silver Nitrate solution and incubated for 120 hour at 28 C then the colour of the solution changed from lime to deep brown. It confirms that bio reduction of $AgNO_3$ had taken place inside this bottle.



A B

Figure.11 The bottles containing *Trichoderma ressei* biomass before (A) and after (B) mixed to Ag+ ions for 120 hour.

Secondary conformation of Silver-Nanoparticles with the help of optical Spectroscopy measurements:

Ultra-violet visible spectroscopy: The secondary conformation of silver nanoparticle was done firstly by UV Vis spectroscopy. This bio-reaction was monitored by SL-210 double beam UV visible spectrophotometer at a range of 300nm to 800 nm. Silver nanoparticle synthesized from the *T. ressei* and $AgNo_3$ showed a maximum absorption at λ_{max} 390nm. So, from this peak value it can be elucidated that the synthesized solution contains Silver nanoparticles.

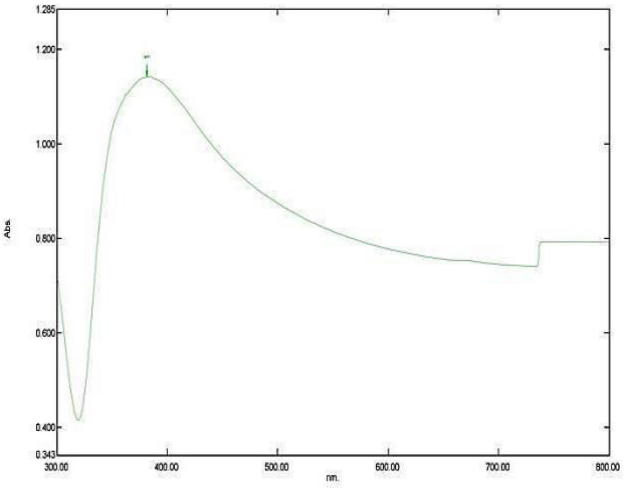


Figure 11: UV vis spectroscopy of AgNPs synthesized from *Trichoderma ressei*

Fourier-transform infrared spectroscopy (FTIR):

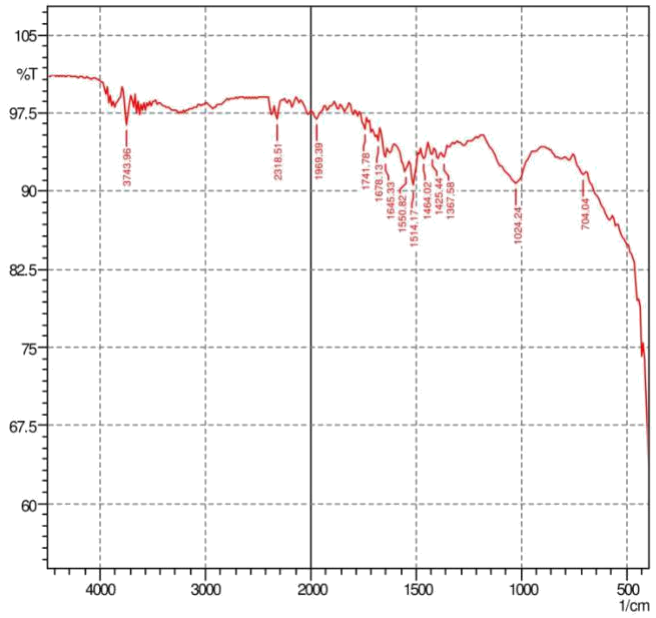
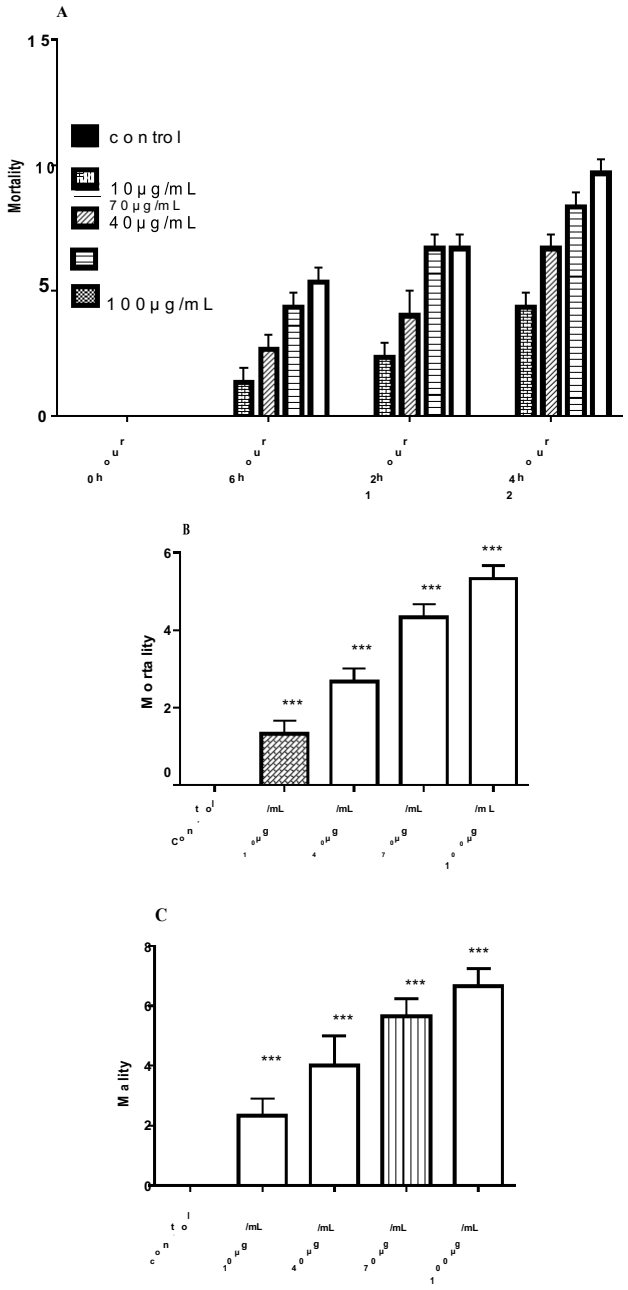


Figure 12. FTIR analysis for *T. ressei* synthesized AgNPs

After performing the UV Vis Spectroscopy of synthesized AgNPs, the Fourier-transform infrared spectroscopy was done for the high-spectral-resolution data over a wide spectral range. FTIR has been employed as a powerful tool to identify types of chemical bonds in a molecule by producing an infrared absorption spectrum that serves like a molecular "fingerprint". The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum. Because the strength of the absorption is proportional to the concentration, FTIR can be used for quantitative analyses. FTIR analysis of the *T. ressei* synthesized nanoparticles showed the major peak in both finger print region as well as functional group region. This sample showed the major peak at 1024.24, 1514, 2318.51, 3743.96 cm^{-1}. The strong broad absorption peak at 1024.24 due to CO-O-CO stretching of anhydride, strong absorption peak at 1514 due to N-O stretching, the strong and sharp peak at 2318.51 due to O=C=O stretching of carbon di-oxide and medium sharp at 3743.96 cm^{-1} for the O-H stretching of alcohol group.

Result after implementation of Silver- nanoparticles on *Spilosoma obliqua* larvae:

After implementation of different concentrations of synthesized AgNPs on these Lepidopteran larvae (pest), we observed the mortality rate of these species in different instars after various exposure times. Firstly, no mortality was observed in the larvae that were fed the normal diet, *i.e*, the castor leaves. Secondly, by using 10µg/ml concentration in 3^{rd} instar larvae, although the mortality was significant as compared to control, but only a few larvae were affected by AgNPs at 6hrs time duration. Therefore, it can be said that the efficacy is less in this concentration after 6 hrs. Further, it was observed that the application of higher concentrations like 40, 70 and 100 µg/ml on these larvae, the efficacy or mortality rate although increases but it is still not reasonable. Thereafter, we increased the exposure time and used the same concentrations for 12 hrs and then 24 hrs time duration (Figure 13 A). Increasing exposure time yielded better result in all the concentrations used. For third instar stage of this pest, it was observed that at 100 µg/ml concentration, the survival rate of these insects was very less after 12 hrs(Figure 5.7 C), and after 24 hrs (Figure 5.7 D), 99% mortality rate in this Lepidopteran species was recorded.



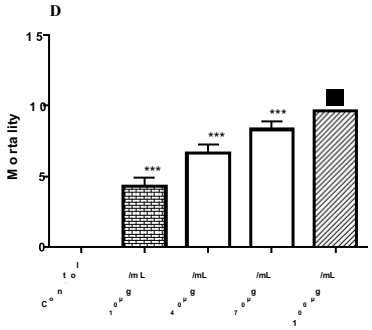
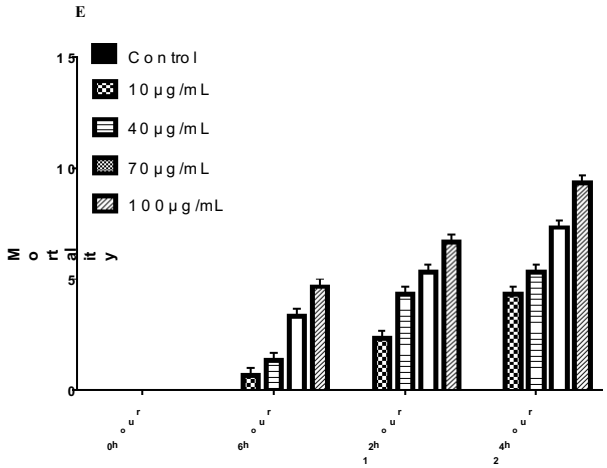


Figure 13 The graph (A) represents the larvicidal activity of silver nanoparticle against fifth instar larva after 0hr, 6hr, 12hr, 24hr. The data represent the mean mortality (n= 30) induced by the treatment, the stastical analysis of which was done by two way ANOVA test followed by Dunnett's comparison test , the graph (B) represents the effect after 6 hrs, the graph (C) represent the effect after 12 hrs, the graph (D) represent effect after24 hrs. *P≤0.5,**P≤0.01, ***P≤0.001 *vs* control.

Graph (5.7 A) indicates the comparison of mortality of 5th instar larvae, it was caused by different concentration of *T.ressei* synthesized AgNPs after different time intervals. There is no mortality found in controlled groups. In conc.10µg/ml, mortality rate increases with increase the time exposure. In 20µg/ml, high mortality was observed after 24 hrs as compared to 6 and 12 hrs. Same trend was followed by rest of all concentrations. Highest mortality was observed in 100µg/ml after 24 hrs. *i.e* 87%.

The graph (13 B) indicated the effect of AgNPs against lepidoptera larvae after 6 hrs. The mortality was observed in all concentration of AgNPs and it was also significant in all concentration. Similarly, The graph (13 C) indicates the larvacidal activity of AgNPs after 12 hrs of treatment. The mortality was significant in all concentration .The graph (13 D) represents the larvicidal activity of AgNPs after 24 hrs. The mortality was also significant in all concentration as compared to control.



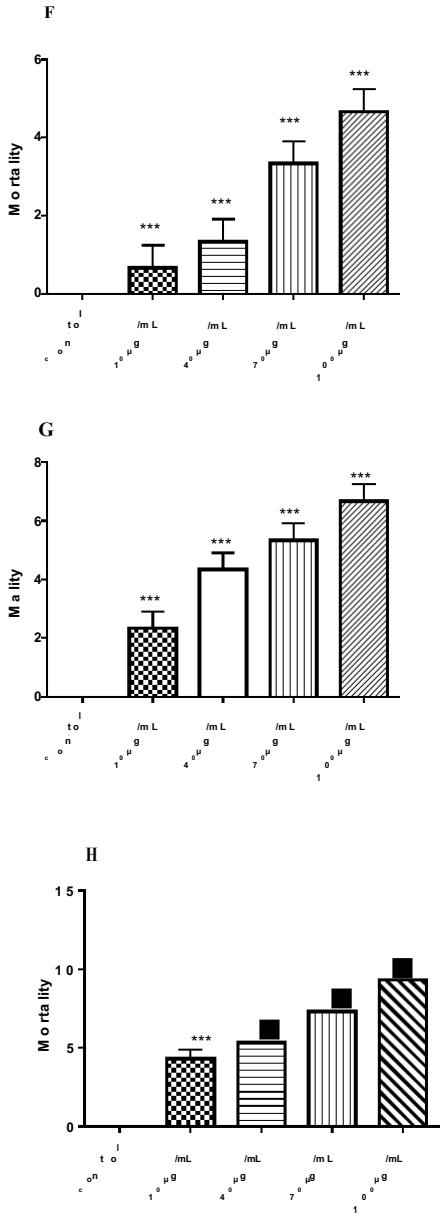
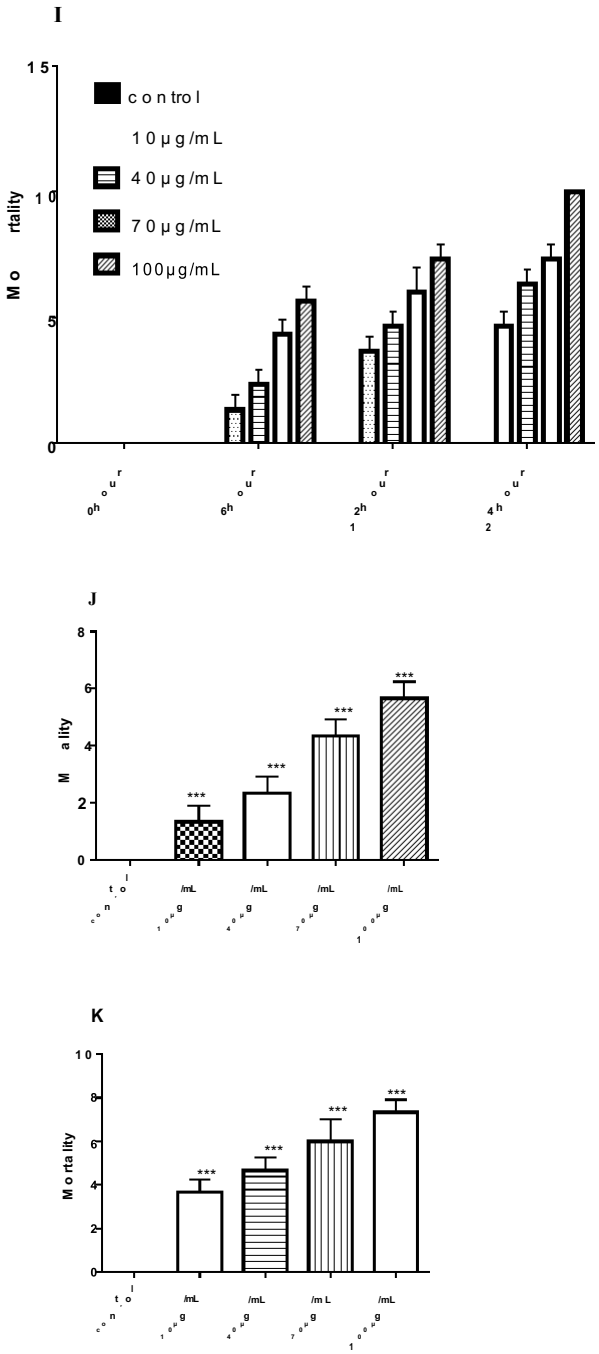


Fig (**13 E**)The graph (**E**) represents the larvicidal activity of silver nanoparticle against third instar larva after 0hr, 6hr, 12hr, 24hr. The data represent the mean mortality (n= 30) induced by the treatment, the stastical analysis of which was done by two way ANOVA test followed by Dunnett's comparison test , the graph (**F**) represents the effect after 6 hrs, the graph (**G**) represent the effect after 12 hrs, the graph (**H**) represent effect after24 hrs. *P≤0.5,**P≤0.01, ***P≤0.001 *vs* control.

Graph (**13 E**) indicates the comparison of mortality of 4th instar larvae, caused by different concentration of *T.ressei* synthesized AgNPs after different time intervals. There is no mortality found in controlled groups. In conc.10µg/ml, mortality rate increases with increased time exposure. In 20µg/ml, high mortality was observed after 24 hrs as compared to 6 and 12 hrs. Same trend was followed by rest of the concentrations. Higher mortality was observed in

100µg/ml after 24 hrs. *i.e*, 92% . The graph (**13 F**) indicated the effect of AgNPs against lepidoptera larvae after 6 hrs. The mortality was observed in all concentration of AgNPs and it was also significant in all concentration. Similarly, The graph (**13 G**) indicates the larvacidal activity of AgNPs after 12 hrs of treatment. The mortality was significant in all concentration .The graph (**13 H**) represents the larvicidal activity of AgNPs after 24 hrs. The mortality was also significant in all concentration.



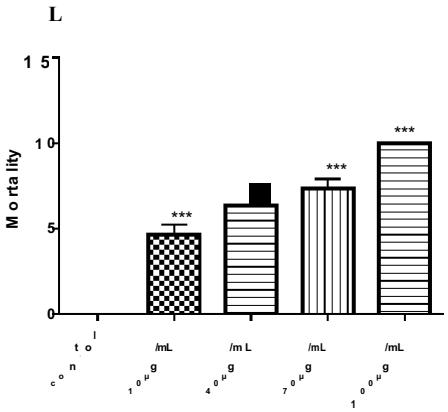


Fig (13 I) The graph **(I)** represents the larvicidal activity of silver nanoparticle against third instar larva after 0hr, 6hr, 12hr, 24hr. The data represent the mean mortality (n= 30) induced by the treatment, the stastical analysis of which was done by two way ANOVA test followed by Dunnett's comparison test , the graph **(J)** represents the effect after 6 hrs, the graph **(K)** represent the effect after 12 hrs, the graph **(L)** represent effect after24 hrs. *P≤0.5, **P≤0.01, ***P≤0.001 *vs* control.

Graph **(13 I)** indicated the comparison of mortality of 4th instar larvae, caused by different concentration of *T.ressei* synthesized AgNPs after different time intervals. There is no mortality found in controlled groups. In conc.10µg/ml, mortality rate increases with increase the time exposure. In 20µg/ml, high mortality was observed after 24 hrs as compared to 6 and 12 hrs. Same trend followed by rest of all concentrations. Higher mortality was observed in 100µg/ml after 24 hrs. *i.e* 97 %. Both time dependent and dose dependent responses were observed. The graph (**13 J**) indicated the effect of AgNPs against lepidoptera larvae after 6 hrs. The mortality was observed in all concentration of AgNPs and it was also significant in all concentration. Similarly, the graph (**13 K**) indicates the larvacidal activity of AgNPs after 12 hrs of treatment. The mortality was significant in all concentration .The graph (**13 L**) represents the larvicidal activity of AgNPs after 24 hrs. The mortality was highly significant in all concentration against control.

Figure 14. The larvicidal activity of synthesized AgNPs in 3rd instar stage at 100 concentrations for 24 hours (Stereo microscopic image)

Figure15. The larvicidal activity of *S. obliqua* (4th instar) after treatment of AgNPs treated castor leaves.

Similar observations were made in the case of fourth instar larvae of this pest. By using 10 µg/ml AgNPs concentrations in 4th instar larvae, only a few larvae were affected at 6hrs time duration (Figure 5.7 E), and using 40, 70 and 100 µg/ml on those larvae, the efficacy or mortality rate increased with increasing concentration. For this instar stage it was observed that efficacy at 100 µ g/ml concentration (Figure 5.7 H). The mortality was increased gradually with increasing the time and concentrations (Figure 5.7 F, G, H), and at 24 hrs time for100 µg/ml, it shows 98% mortality rate of 4th instar of this Lepidopteran pest (Figure 5.7 H).

We also performed the experiments on the 5th instar larvae, and it was observed that the mortality rate is a little bit less on this 5th instar as compared to 3rd and 4th instar(Figure 5.7 I). Before 12 hour and 70 µg/ml conc., the synthesized AgNPs was not much effective, but in 100 µg/ml conc. for 24 hrs. it was observed a high significant result on these larvae(Figure 5.7 L).

From the above mentioned results, it can be suggested that the synthesized AgNPs have the potential to act as a larvicide, and they specifically target the larval life stages.

Lastly, the synthesized AgNPs were directly applied (topical application) on these larvae. After 24 hrs, after the treatment it was observed a significant result. There are about 60% mortality occurred due to this topical application on these larvae (Figure 5.10).

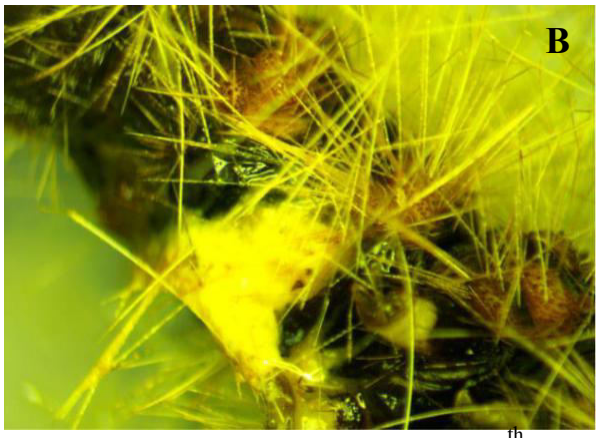


Figure 16. A shows the normal anterior portion of 4th instar before treatment and B shows the damage of anterior portion of 4th instar after the direct treatment of AgNPs. (Stereo mic. image)

Discussion: There are many farmers who have been using parasites and predators for biological pest control. Certain microorganisms also have the potential to kill insects, and can be thus used in various pest management programmes. These entomopathogenic agents include fungi, bacteria, nematodes, and viruses. Entomopathogens act as natural regulation method of controlling various insect populations. (Hajek, *et al,.*2004). Most of the research in this direction is concerned with the finding of entomopathogenic agents and their use as biological pest control. Various entomopathogens equivalent to chemical pesticides are used to control various insect inhabitants. (Dentrt *et al*., 2000; Hajek *et a.,* 2004). Use of Entomopathogens is a traditional process, and it acts as an organic controlling agent of unfamiliar insect pests (Hajek, *et al.,* 2004).

In the present study, we tried to select a perfect entomopathogen for the synthesis of silver nano-particle, one that is easy and cost-effective to synthesize. This led us to the synthesis of AgNPs from entomopathogenic fungus.

In green synthesis of AgNPs there are some parameters that should be maintained by a researcher. First of all, rate of production, secondly, it should be eco-friendly and lastly, it should be cheap, so that people can easily apply the product for pest control.

In the present research, we used entomopathogenic fungus, *Trichoderma ressei*. Different researchers have used different entomopathogenic fungus for the synthesis of different Nano metals. Some have used *Epicoccum nigrum,* isolated from *Phellodendron amurenso* to synthesize AgNPs, and it showed toxic activity against the pathogenic fungi. The synthesis of gold nanomaterial's (AuNPs) has also been achieved by the bio-reduction of Chloroauric acid (HAuCl4) using the fungal culture filtrate (FCF) of *Alternaria alternata*. Synthesis of AgNPs using, *Trichoderma asporellum*, an

entomopathogenic fungus showed effective larvicidal and pupicidal activity against the dengue vector, *Aedes aegypti* (Mukherjee *et al.,* 2008).

Trichoderma has an advantage over other fungi because it assists to defend plants against molds and bacteria. *T. ressei* creates a barricade that makes it impossible for harmful bacteria and pathogens to pass through it. *Trichoderma* work well with other microbes, binding up with anything and, thus, increases the health of the rhizosphere. It will exactly dissolve any pathogenic fungi without any adverse effects.

Trichoderma has a lifespan of about 28 days. It reproduces itself repeatedly all on its own but the quality of its enactment will fade over time. The basic significance is that, *Trichoderma* is absolutely natural and it doesn't hurt plants in the suggested dosages. (Sundaravadivelan and Padmanabhan, 2013)

After enrichment of *T. ressei*, we cultured it in PDA media for growing the colony. After growing the fungus successfully, the vegetative part was taken into Glucose casein hydrolase broth for further high amount of production. GC medium was employed because the growth of *Trichoderma reesei* is more in the GC hydrolase broth than any other culture media (Kabath *et al.,* 2011). 10g/L of *T. ressei* biomass was produced. In the current research, using this broth, approx. 8g/L of fungal biomass was obtained. This outcome may vary with the purity of GC broth and the purity of the mycelia used for the mass culture.

During this biological synthesis of Silver nanoparticles by a fungus, many enzymes are formed which decrease the salt to its Ag^+ solid nanoparticles through the catalysis. As compared to the other filamentous fungi, the *T. ressei* is measured to be the most operative extracellular enzyme manufacturer, and it has a long antiquity in the production of commercial enzymes (T.Oksanen *et al., 2000*).

On the basis of present study, it can be concluded that the biological synthesis of AgNPs by *T. reesei* is favoured for its safety, being inexpensive and its extensive production potential.

As discussed above, we can synthesize AgNPs biologically on a large scale by using *T. reesei*, which have the great profit over any other fungus culture method. Some studies reveal that *T. reesei* is not injurious to humans. From *T. reesei*, the production of extracellular enzymes and nanoparticles is more effective than other fungi also it is noticeable that *T. ressei* is much easier to handle and culture and for its high growth rate it is useful to both industrial and laboratory condition as well as having low cost in large scale production. There are different process for the production of AgNPs, out of them chemical vapour deposition, liquid solution reduction, irradiation usually produce large particles which are micrometres in size. These other methods have lower production and high cost value for the production of AgNPs when compared to the process that we have used to synthesis AgNPs (Baker *et al* 2000, Balaprashad *et al* 2005, Mukherjee *et.al*2008)

After the synthesis of AgNPs, it was observed that the solution turns from colourless to brown. This indicates the bio reduction of Ag ions into that solution. But it can't confirm that the formed solution contains Ag nanoparticle because the fungus contains different enzymes that may be extra cellular or intracellular. These enzymes may have reacted with the free Ag^+ ion present in $AgNO_3$ and thus resulted in the change in coloration.

For further confirmation, we used UV Vis spectroscopy, which confirmed λ_{max} at 390 nm. According to some previous studies, AgNPs have their absorbance between 300 to 800 nm. So, from this point of view it is clear that the synthesized nanoparticle were indeed AgNPs. The absorbance range may vary due to the clearance of test sample. So, before mixing $AgNO_3$ with *Trichoderma* fungal biomass, it should be made sure that no mycelia are present in the wet biomass.

After characterization of AgNPs, the synthesized AgNPs was applied on Lepidoptera larvae.

For our experiments, *Spilosoma obliqua*, commonly known as The Bihar Hairy Caterpillar were employed because, now a days *S. obliqua* has taken the top position for crop damage in India. This lepidopteran pest species gregariously feeds on the juvenile leaves of pea, wheat, maize, rice plants etc. This causes a high economical loss to the farmers. There are different lepidopteran pests like *Helicoverpra armigera*, *Spodoptera litura,* etc. But due to the high resistibility to other pesticides, nowadays chemical control of pest has become an environmental hazard. In the present research, it was repeatedly observed that these NPs have high efficacy on Lepidopteran species. Further, it was observed that these NPs cause time and dose dependent mortality.

Firstly, the efficacy was observed after the topical application of the synthesized NPs and then the insects were exposed to these NPs orally via their food. It is noteworthy to mention that the direct or the topical application on these NPs was not as efficient as when they were provided via food. This may be because these fungi directly act in the intestine of the larvae and damage their intestine (shown in Figure. 5.9)

Conclusion:

On the basis of present study, it can be concluded that the biological synthesis of AgNPs by *T. reesei* is favoured for its safety, being inexpensive and its extensive production potential.

Further, the results of this research make it clear that entomopathogen mediated synthesis of silver nanoparticle have ther ability to resist the Lepidopteran pests by showing their larvicidal activity. During this research we took 4 different concentrations of synthesized AgNPs. Out of them 100µg/ml conc. shows the most effective result. The mortality of lepidopteran pests was higher in 100µg/ml solution than other concentrations. In this study, we have reported the efficacy of AgNPs on a Lepidopteran pest species, but their efficacy also needs to be evaluated in other pests belonging to different order such as, Hymenoptera, Coleopteran, Hemiptera, etc. Therefore, there is a further scope of this study to see whether the green-synthesized entomopathogenic AgNPs are also efficient for the control of other pest species or they act only on a specific group of insects only.

REFERENCES:

Baker RA, Tatum JH (1998) Novel Anthraquinones from Stationary Cultures of Fusarium Oxysporum. J Ferment Bioeng, 85 359-361.

Balaprasad A, Chinmay D, Ahmad A, Sastry M (2005) Biosynthesis of Gold and Silver Nanoparticles Using Emblica Officinalis Fruit Extract, Their Phase Transfer and Transmetallation in an Organic Solution. J Nanosci Nanotechnol, 5(7) 1665-1671.

Benserradj, O.,and Mihoubi, I.2014.Larvicidal activity of entomopathogenic fungi *Metarhizium anisopliae* against mosquito larvae in Algeria. *International Journal of Current Microbiology and Applied Sciences.*ISSN 2319 – 7706 Volume 3 Number 1(2004) PP.54- 62

Bhainsa, K.C., and D'Souza, S.F., 2006. Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus colloids*. *Surf B Biointerfaces*, 47: 160-164.

Bhattacharyya, A., Bhaumik,A., Rani PU, Mandal,S., Epidi TT. 2010. Nano-particles—A recent approachto insect pest control. African J Biotech 9:3489–3493.

Bhuiyan, T.C. and Sardar, N. (1971). Studies on the phase variation in jute hairy caterpillar *Diacrisia oblique* (Walker) (Lepidoptera : Arctiidae). Pak. J. Zool., 3(1): 101-121.

Casida, J.E. and Quistad, G.B. 2005. Insecticide targets: learning to keep up with resistance and changing concepts of safety. *Agricultural Chemistry and Biotechnology,* **43**:185–191.

Djou, Y.W. (1938). A limabean leaf-hopper, *Diacrisia oblique* Walk. (Lepidoptera : Arctiidae). Lingran. J. Sci., 17(4) : 639- 645.

Fayaz, A.M., Balaji, K., Girilal, M., Yadav, R., Thangavelu, P. and Venketesan, K.R. 2010. Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. *Nanomed Nanotechnol Biol Med,* **6**:103–109.

Gaikwadsagar, Bhosale.A, 2012. Green synthesis of silver nanoparticles using *Aspergillus niger* and its efficacy against human pathogen. *European J. Exp. Bio.*, 2: 1654-1658.

Gotyal, B.S., Satpathy, S., Selvaraj, K. and Ramesh Babu, V. (2013). Comparative biology of Bihar hairy caterpillar, Spilosoma obliqua on wild and cultivated species of jute. Indian J. Entomol., 41(3): 219-221.

Govindarajan, M., Jebanesan, A., and Reetha, D., 2005. Larvicidal effect of extracellular secondary metabolites of different fungi against the mosquito, *Culex quinquefasciatus* say. *Tropical Biomedicine*, 22(1):1-3.

Hajek. P.,Verma P, Prakash S. Efficacy of Chrysosporium tropicum metabolite against mixed population of adult mosquito (Culex quinquefasciatus, Anopheles stephensi, and Aedes aegypti) after purification with flash chromatography. Parasitol Res 2010; 107: 163-166

Hooks CR, Kratochvil R. Agronomic Crop Production.Agronomy News University of Maryland Extension 501Court Lane, Room 208 Cambridge, MD 21613 410-228-8800, 2014.

Huang, J., Li, Q., Sun, D., Lu, Y., Su, Y., Yang, X., Wang, H., Wang, Y., Shao, W., He, N., Hong, J. and Chen, C. 2007. Biosynthesis of silver and gold nanoparticles by novel sun dried *Cinnamomum camphora* leaf. *Nanotechnology,* **18**:105104.

Hussain A, Tian MY, He YE, Ahmed S. "Entomopathogenic Fungi disturbed the larval growthand feeding performance of *Ocinara varians* Walker (Lepidoptera: Bombycidae) Larvae, *Insect Science*, 2009; 16(6):511-517.

Hussain A, Tian MY, He YE, Ahmed S.~ 1189 ~Journal of Entomology and Zoology Studies"Entomopathogenic Fungi disturbed the larval growth and feeding performance of *Ocinara varians* Walker (Lepidoptera: Bombycidae) Larvae", *Insect Science*, 2009; 16(6):511-517.

Hussain AMY, He Tian YR, Bland JM, Gu WX. Behavioral and electrophysiological responses of *C.formosanus* towards entomopathogenic fungal volatiles,Biological Control, 2010; 55:166-173.

Joseph R, Lakowicz Sf, Grattonnll E, Cherek H, Badri P, Maliwal S, Gabor Laczko SS 1984 Determination of Time-resolved Fluorescence Emission Spectra and Anisotropies of a Fluorophore-Protein Complex Using Frequency-Domain Phase-modulation Fluorometry. The *Journal of Biological* Chemistry, 259 (17) 10967-10972.

Kowshik M, Deshmukh N, Vogel W, Urban J, Kulkarni SK, Paknikar KM 2002 Microbial Synthesis of Semiconductor CdS Nanoparticles, their Characterization, and their Use in the Fabrication of an Ideal Diode. *Biotechnol. Bioengineer*, 78 583-587.

Kowshik M, Vogel W, Urban J, Kulkarni SK, Paknikar KM 2002 Extracellular Synthesis of Silver Nanoparticles by a Silver-Tolerant Yeast Strain MKY3. *Adv. Mater.*, 14 812-815.

Li G, He D, Qian Y, Guan B, Gao S, Cui Y, Yokoyama K, Wang L.Fungus mediated green synthesis of silver nanoparticles using *Aspergillus terreus*. In J Molecul Sci 2012; 13: 466-476.

Liang, H., Xing, Y., Chen, J., Zhang, D., Guo, S., and Wang,C., 2012. Antimicrobial activities of endophytic fungi isolated from *Ophiopogon japonicas* (Liliaceae). *BMC Complement Altern. Med.*, 12(1): 238.

Mansoori GA, SYNTHESIS OF NANOPARTICLES BY FUNGI, 2010, Patent application number: 20100055199, IPC8 Class: AA01N5916FI, USPC Class: 424618.

Milner RJ, Huppatz RJ, Swaris SC (1991) A new method for assessment of germination of *metarhizium* conidia. J Invertebr Pathol 57:121–123

Moore D, Bridge PD, Higgins PM, Bateman RP, Prior C (1993) Ultraviolet radiation damage to *Metarhizium flavoviride* conidia and the protection given by vegetable and mineral oils and chemical sunscreens. Ann Appl Biol 122:605–616

Morley Davies J, Moore D, Prior C (1996) Screening of *Metarhizium* and *Beauveria* spp. conidia with exposure to simulated sunlight and a range of temperatures. Mycol Res 100:31–38

Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Ramani R, Parischa R, Ajaykumar PV, Alam M, Sastry M, Kumar R 2001 Bioreduction of AuCl4-ions by the Fungus, *Verticillium sp.* And Surface Trapping of the Gold Nanoparticles formed. *Angew Chem Int Edu*, 40 3585-3588

Mukherjee P, Senapati S, Mandal D, Ahmad A, Khan MI, Kumar R, Sastry M 2002 Extracellular Synthesis of Gold Nanoparticles by the Fungus *Fusarium Oxysporum*. *Chem Biochem*, 3 461-463.

Mukherjee, P., Ahmad, A., Mandal, D., Senapati, S., Sainkar, S.R., Khan, M.I., *et al*., 2001. Fungus mediated synthesis of silver nanoparticles and their immobilization in the mycelial matrix: a novel biological approach to nanoparticles synthesis. *Nano Lett.*, 1: 515-519.

Mukherjee, P., Ray, M., Mandal Dey, G., Mukherjee, P.,Ghatak, J. *et al*., 2008. Green synthesis of highly stabilized nano crystalline silver particles by a non-pathogenic and agriculturally important fungus. *T.* Asperellum Nanotechnology, 19: 75103-75110.

Mukherjee.P, Ahmad.A, Mandal.D Fungus-Mediated Synthesis of Silver Nanoparticles and Their Immobilization in the Mycelial Matrix: A Novel Biological Approach to Nanoparticle Synthesis 2008

Okasnan.T, Pere.J, Treatment of recycled kraft pulps with Trichoderma reesei hemicellulases and cellulases,Mar 2000, Journal of Biotechnol.

Oksanen T, Pere J, Paavilainen L, Buchert J, Viikari L 2000 Treatment of Recycled Kraft Pulps with *Trichoderma Reesei* Hemicellulases and Cellulases. *J Biotechnol*, 78(1) 39–44.

Prakash, S., Singh, G., Soni, N., and Sharma, S., 2010. Pathogenicity of *Fusarium oxysporium* against thelarvae of *Culex quinquefasciatus* (Say) and *Anopheles stephens*i (Liston) in laboratory. *Parasitol. Res*., 107: 651-655. doi. 10.1007/ s00436-010-1911

Priya, S., and Santhi, S., 2014. A review on nanoparticles in mosquito control – A Green Revolution in Future. *International Journal of Research in Applied Science and Engineering Technology* (IJRASET).

Priyanka, J., Srivastava, N., and Soam Prakash, (2001).*Chrysosporium tripicum* efficacy against *Anophelesstephensi* larvae in the laboratory. *Journal of theAmerican Mosquito Control Association*, 17(2): 127-130.

Saxena, A., Tripathi, R.M. and Singh, R.P. 2010. Biological synthesis of silver nanoparticles using onion (Allium cepa) extract and their antibacterial activity. *Digital Journal of Nanomater Bioscience,* **5**(2):427–432.

Shankar, S.S., Rai, A., Ankamwar, B., Singh, A., Ahmad, A. and Sastry, M. 2004. Nat Mater **3**:482. Sharma, H. C. 2001. Cotton bollworm/legume pod borer, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera): biology and management. *Crop protection compendium*. International Crops Research Institute for the Semi-Arid Tropics.

Slansky, F. Jr. 1985. Food utilization by insects. Interpretation of observed differences between dry weight and every efficiencies. *Entomology of Experimental Application*, **39**: 47-60.

Slansky, F. Jr. and Scriber, J.M. 1985. Food consumption and utilization. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology (Ed. By Kerkut, G.A. and Gilbert, L.I.) **4** : 87-163. Pergamon Press, New York.

Smith PR, Holmes JD, Richardson DJ, Russell DA, Sodeau JR 1998 Photophysical and Photochemical Characterization of Bacterial Semiconductor Cadmium Sulfide Particles. *J. Chem. Soc., Faraday Trans*, 94 1235-1241

Subashini, H.D., Malarvannan, S. and Pillai, R.R. 2004. *Dodonaea angustifolia* – a potential biopesticide against *Helicoverpa armigera*. *Current Science,* **86**(1), 26-28.

Suganthi and Nagapasupathi, 2005. On *Achaea Janata*. Consumption and utilization of food by castor semi looper *Achaea Janata*. *Journal of Ecotoxicological and Environmental Moniter*, **15**(3):201-205.

Thomas, T.R.A., Kavlekar, D.P., Loka Bharathi, P.A., 2010. Marine drugs from sponge – Microbe Association – A Review. Mar. *Drugs*, 8: 1417-1468.

Watson JHP, Cressey BA, Roberts AP, Ellwood DC, Charnock JM 2000 Structural and Magnetic Studies on Heavy-Metal-Adsorbing Iron Sulphide Nanoparticles Produced by Sulphate-Reducing Bacteria *J. Magn. Mater*, 214 13-30.

Wen-Ru, L., Xiao-Bao, X., Qing-Shan, S., Hai-Yan, Z., You-Sheng, OU-Y. and Yi-Ben, C. 2010. Antibacterial activity and mechanism of silver nanoparticles on *Escherichia coli*.

Wraight SP, Inglis GD, Goettel MS. Fungi, In: Field manual of techniques in invertebrate pathology, L.A. Lacey and H.K. Kaya, (Eds.), 223-248, 2nd edition, Springer, Dordrecht, ISBN 978-1-4020-5931-5.

Yang, X., Zhang, L., Guo, B., and Guo, S., 2004. Preliminary study of vincristine-producing endophytic fungus isolates from leaves of *Catharanthus roseus*. *Zhong Caoyao*, 35: 79-81.

Yasur, J.,Pathipati,U.R., Lepidopteran insect susceptibility to silver nanoparticles and measurement of changes in their growth, development and physiology 2015.