Biocontrol strategies and their mechanistic approach to control hazardous pest of plants

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ABSTRACT:

Infestation of whiteflies on the crops, medicinal plants, fruits and on weeds are serious problem of the 21st century. To control insects many pesticides were used but its hazardous effects were much more on the plants and humans then their potential to control insects. The major objective of the work was to isolate the fungal strain from the soil which can be used to control the whiteflies which was adapting every climatic condition. The fungal strain was isolated from the rhizospheric region of plant grown in Agricultural field of lovely professional university by the serial dilution method. Fungal strain also identified with the help of morphological, physiological and biochemical tests. Then fungal strain formulated in the form of 4% sodium alginate beads and transferred to the rhizosphere region of plants to check the control of flies. Significant reduction observed in the appearance of Whiteflies. Aflatoxin extracted from the formulated beads with the help of chloroform evaporation and found absence of aflatoxin in the plant A. beads.

INTRODUCTION:

In our country, crops are very important which are provides food all overall the years to us, as well as, maintain economic status. But with increasing population of our country, crop yield decreasing due to existence of high variability, environmentally adapted insects.

An insect whitefly (Bemisia tabaci) which damaging crops every year in state of Punjab. 1.36 lakh hectares out of total 4.50 lakh hectares of cotton average attacked by the whitefly last season and had dropped by 40% (Pattanayak.S 2016). Whitefly is devastating insect which cause damage to many host plants like 20 crop fields, 13 medicinal plant,17 vegetables, 4 fruits and 35 weeds. In different season, whitefly change its host and infest with same manner (S.C. Kedar, R.K. Saini and K.M. Kumarag, 2018). This insect shows different life stages. Its life cycle varies from 14 days to 107 days (Dr. B. M. Khadi, 2007). In summer life cycle complete within 18 to 28 days and in winter 30 to 48 days. Adult fly (0.8 to 1.2 mm in size) lays nearly 150 eggs underneath the leaf during its life

cycle. Eggs are (0.2mm in size) hatched in 8 to 10 days. Egg hatched into first nymph instar (0.3mm in size) which is active and move inside the plant. Then moulting occur and first nymph change into the 2nd and 2nd into 3rd nymph instar. 2nd and 3rd nymph instar which not movable and 0.4 to 0.6mm in size. After that 3rd instar change into pupa which is completely inactive of the insect. Pupa is 0.6 to 0.8mm in size. Its nymph period varies from 9 to 14 days and 17 to 19 days in winter. Pupa period vary from 2 to 8 days (PVL Barathi (2015). When insect infested plant, that time plant show defence mechanism against insect by increasing the production of acid. This acid attack the insect and make feeding conditions difficult for him. But insect overcome this enzyme level and fluctuation observed that Jasmonic acid level decreased and another enzyme salicylic acid (SA) increased (Science and Technology**,**2019).Their flying speed increased with the increasing temperature (Filiz Ertunc).

After that when insect not killed by plant defence mechanism, then insect use that high amount of salicyclic acid to form sugary or sticky secretions called honeydew (Vndroon .A, 2015). Sticky substance spread by insect on the crop plant due to leaves covered by a white layer which act as a barrier for sunlight due to photosynthesis stop and plant become die.

To kill this insect many insecticides are present like neonicotinoid etc but these chemicals having very short life and insect get resistance from them very easily (Jun Xia et al, 2013). So, many recent studies found a biocontrol for this insect was fungi. Fungi used in higher manner due to of its merit like high reproductive capabilities, target specific activity, short generation time and it having longer survival then their host (Sandhu S. S et al, 2011).

Then fungus which can kill or stop the activity of insect is known as Entomopathogenic fungi. *Aspergillus niger* can form anywhere around us. It is mostly found in mesophilic environment. It mostly grown in temperature 6 to 47^0 C and pH (1.4 to 9.8).Whitefly is an exopterygota so its only joints and arthrodial membrane are non- sclereotized. Form these body parts, fungus can easily enter in the host. Ingested fungus cannot develop mature spores in the gut and come out with faeces (Ajay K. Gautam, et al 2011).But to enter in the host body fungus need to make adhesion with cuticle layer of the host[9]. Fugal spore sustained on the upper layer of the insect which is a conidium and after its microfold growth its modified into appressorium. Outer epicuticle is breached by the mechanical force which was developed by the tips of the appressorium in the form of pegs structure. Inner epicuticle was breached only by the enzymatic degradation due to its composition (polymerize lipoprotein and quinones). Further Penetration plates formed and breached the procuticle and then blastopores develop and dispersed through hemocoel and disable the digestive system of the insect. (A.E. Hajek and R. J. St. Leger, 1994). After accomplishing the pathogen progress, fungal spore leaves the insect body and moved to the soil which will sustain in soil till next infection stage. (Tingyan Dong et al, 2016). High humidity also helped the fungal spore to come out to dead body wall and transmit in the vertical and horizontal direction and spread disease to another insect (A. Keith Charnley,2013).

Aspergillus niger shown this same activity in the mosquito. This fungi release peptide and proteolytic enzyme which help them to pass or penetrate through the outer layer or cuticle and reach the hemo-lymph and kill the host. (Gavendra Singh and Som Prakash, 2012).

MATERIAL AND METHODS:

CULTURE MEDIA, MICROBIAL STRAINS AND THEIR CONDITION:

Agar, dextrose, yeast extract, peptone etc. were used from HI- MEDIA and chemicals of AR and GR grades were used from SIGMA.

TOMATO PLANTS: 2 tomato Plants NS-4266 collected from the agriculture field of lovely professional university and potted in the pots. Plant which infested with whiteflies mentioned as Plant A. and another which is not infested with whiteflies mentioned as a plant B.

WHITEFLIES COLLECTION:

Collect the whiteflies from the plant which grown in the green house of lovely professional university. Aspirator, pipette tips and yellow traps were used for the collection of flies method was used.

ASPERGILLUS STRAIN: *Aspergillus niger* strain no. MTCC871 were obtained from IMTECH Chandigarh, India and then grown on potato dextrose agar plates.

ISOLATON OF FUNGAL STRAIN FROM SOIL: The control of the whiteflies was isolated from the rhizosphere part of the plant which grown in Agricultural field of lovely professional university by the serial dilution method. Firstly, soil from the rhizospheric region of the plant and then dry and crush it. Then 1 g of soil was taken into 15 ml of centrifuge tube with the addition of 10 ml of distilled water. Shake the sample properly and then pipette out 100µ of soil suspension and add into the 2.5 ml of Eppendorf tube with addition of 900µ of distilled water. Repeat this dilution 5 times and the spread the 10µ suspension into the Potato dextrose agar plates which incubated for 3 days at $28^{\circ C}$. Then streak the fungal growth on another PDA plates.

MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACHTERIZATION OF ISOLATED FUNGAL STRAIN:

MORPHOLOGICAL CHARACHTERIZATION:

The fungal strain which is isolated from the soil was compared with the *Aspergillus niger* MTCC871 on the basis of morphology and method was used (Lisa Nathalie, 2011). PDA and Czapek Dox agar plates were formed and inoculated with the isolated fungal strain and standard culture of *Aspergillus niger* and incubated at 28^0 for 3 days.

PHYSIOLOGICAL CHARACHTERIZATION:

Isolated fungal strain and standard culture *Aspergillus niger* both activities checked on the different PH and different temperature method was used (Lisa Nathalie, 2011). PDA plates were formed and inoculated at temperature 4^0, 15^0, 30^0 and 40^0 for 3 days. Another PDA plates were formed at different pH 5,7 and 9 and incubated at 28^0 for 3 days.

BIOCHEMICAL CHARACHTERIZATION:

To check the biochemical character of the isolated fungal strain,4 different solid media was prepared using the method of Lisa Nathalie,2011

AMYLOYTIC ACTIVITIY:

To check an ability of isolated fungal strain to degrade the starch by releasing amylase enzyme. This media was prepared with different composition like NaNO3, 1g; K2HPO4, 1g; MgSO4.7H20,1g; FeSO4, 0.04g; soluble starch 20 g; agar 25 g and 1liter distilled water were used and inoculated with isolated fungal strain and standard fungal strain. Then incubated at 28 ^{0} C for 3 days.

PROTEOLYTIC ACTIVITY:

To check an ability of isolated fungal strain to degrade protein by protease enzyme. Media was prepared with NaNO3, 2g; K2HPO4, 1g; MgSO4.7H20, 0.5g KCL,0.5g; FeSO4.7H2O , 0.01g ; sucrose 30 g; Skim milk powder 10g ; agar 20 g and 1 liter distilled water inoculated with isolated fungal strain and standard fungal strain which incubated at 28$^{\circ C}$ for 3 days.

LIPOLYTIC ACTIVITY:

To check an ability of isolated fungal strain to degrade lipid by lipase enzyme. Media was prepared with Tween 20, 10 ml, agar 20g, peptone 10g; NaCl 5g; CaCl2.2H20 and distilled water 1liter was inoculated with isolated fungal strain and standard fungal strain incubated at 28^{0}C for 3 days.

PECTOLYTIC ACTIVITY:

To check an ability of isolated fungal strain to degrade pectin. Media was prepared with pectin ,10 g; yeast extract, 2g; K2HPO4, 1.5 g; MgSO4.7H20, 1.5 g; agar 25g and distilled water 1 liter and inoculated with isolated fungal strain and standard fungal strain which incubated at 28^{0} for 3 days.

SPORE COUNT OF FUNGAL STRAIN FOR BEADS FORMATION:

Fungal strain was grown on PDA plates that inoculate in 100µ distilled water in 2.5 ml of Eppendorf tube and shake it properly. Then transferred 20µ solution into the haemocytometer and count the number of spores under the compound microscope on 100 X.

FUNGAL STRAIN FORMULATED WITHIN SODIUM ALGINATE BEADS:

For beads formation 4% sodium alginate and 0.2 M Calcium Chloride was used. 4% sodium alginate dissolved in 50 ml of distilled in 100 ml of flask and autoclaved at 121^{0}C and 15 psi for 30 minutes. Then centrifuge the fungal

media at 2000 rpm for 10 minutes which was grown in 250 ml PD broth in 500 ml of flask and only pellets was added to the 4% sodium alginate solution and mix it well with the help of sterilized glass rod for 1 hour in laminar flow. Further used 200 ml of Chilled 0.2 M Calcium Chloride as a solvent for the formation of beads. 5 ml of plastic syringe was used for the formation of beads.

SCREENING OF BEADS:

To check the stability of the beads, 100 ml of PD broth were used in the 250 ml of flask and its inoculated with the 30 beads in the laminar flow. Then placed the flask in the incubator shaker at 28^0C and check the stability after each hour. Then incubate the media for a week.

FORMULATED BEADS TRASNFERED TO THE RHIZOSPHERE REGION OF THE PLANTS:

Firstly, filter the beads and then weight them. Then 50 % of the beads transferred to the rhizosphere region of the tomato plant A which is infested with the whiteflies and another 50% transferred to the plant B which is free from whiteflies. After 7 days, number of flies was same.

ANALYSIS OF INOCULATED BEADS IN THE RHIZOSPHERE REGION OF THE PLANTS:

To check the condition of formulated beads which was inoculated in rhizosphere region of the plant A. and plant B. After 2 weeks, soil was digged from the pots upto 6 cm depth and collect some soil and beads which was competeley in dry form. Then formed four 100 ml of PD media which was inoculated by the the spores and the soil suspenion.Incoulated beads of both the pots washed with autoclaved distilled water and then inoculated in the 100 ml of PD broth and incubated at 28^0C for 3 days to check the stabilty of beads . Then rhizosphere region of soil was used to form soil suspension and tarsnfered to the 100 ml PD broth and incubated at 28^0 for 3 days to check the abilty to growth of isolated fungal strain in soil which was formualted in the beads.

AFLATOXIN EXTRACTION FROM THE FORMULATED BEADS:

Incubated formulated beads in 100 ml PD broth was used to extract aflatoxin method was used. Firstly, centrifuge the media and then its supernatant was used. Further 10 ml of media was added to the 30 ml oakridge tube with equal amount of chloroform. Then shake the tube with the help of vortex for 20 minutes. Separate the upper layer of chloroform with the help of pipette and add into another tube with the same amount of chloroform. Then again repeat this procedure for 2 times. Then transfer the aqueous layer into petri plate and evaporate the chloroform with the help of heat. Wash the plate with the chloroform and liquid collected to the tube.

ESTIMATION OF AFLATOXIN WITH THE HELP OF THIN LAYER CHROMATOGRAPHY:

Slurry of silica gel was used to estimate the aflatoxin as a solid phase method was used. For mobile phase Toluene: Iso-amyl alcohol: methanol (90:32:3) ratio was used. Then observed the aflatoxin under UV chamber.

RESULT:

Isolated fungal strain was used against whiteflies in the formulated form of 4% sodium alginate beads applied in the rhizosphere region of the plants.

TOMATO PLANTS PLANTATION:

Tomato plant was taken from the Agricultural field of lovely professional university and potted in the lab. area of the university. The Ph of the soil of agricultural field and the pots both was acidic in nature.

Fig.1 Showing Tomato plant planted in the pot.

WHITEFLIES COLLECTION:

Whiteflies was collected from the green house of the lovely professional university during lower temperature. Due to lower temperature, their activation and flying speed was low due to their collection was easy.

Fig. 2 and Fig. 3 showing Collection of whiteflies in the drosohilla culture tubes from the green house .

TRANSFER OF WHITEFLIES TO THE POTTED PLANTS:

After 10 days of plantation , whiteflies was transferred to the plant A. Drosophilla tubes directly opened on the potted plants and then coverd the plant with the help of muslin cloth.

Fig. 4 and Fig 5. Showing Plant A. infested with whiteflies and covered with muslin cloth.

ADAPTAION OF WHITEFLIES ON THE POTTED PLANT:

After transfering the whiteflies on the plant, In first attemept 10 flies was transferred on the plant A. but no flies was found on the plant A. within next 24 hours. In 2nd attempt 25 flies was transferred to the plant A. but some honey dew formation was shown on the leaves but no flies found on the plants A. In 3rd attempt 50 flies was transferred to the plant but only 10 to 12 flies was sustained on the plants.

Fig. 6 and Fig. 7 showing adaptation of whiteflies on the tomato plants.

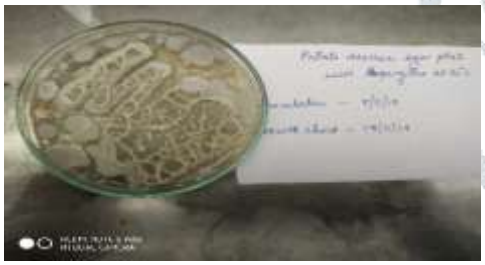
MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF ISOLATED FUNGAL STRAIN:

Figure 8. and figure 9. are the potato dextrose agar plate showing morphological charachterization. Figure 8. show *Aspergillus niger* growth and figure 9. showing isolated fungal starin growth on PDA plates.

Figure 10. and figure 11. are the Czapek Dox agar media plate showing morphological characterization. Figure 10. showing isolated fungal stain growth and figure 11. showing *Aspergillus niger* stock culture growth

Figure 12. and Figure 13. PDA plates showing physiological characterization. Figure 12. showing no growth at 4 ^{0}C of isolated fungal strain sample and figure 13. showing no growth at 4 ^{0}C of *Aspergillus niger*

Figure. 14 and Figure. 15 are the PDA plates showing physiological charachterization. Figure. H showing isolated fingal strain growth at 15 °C and figure. H showing *Aspergillus niger* showing at 15



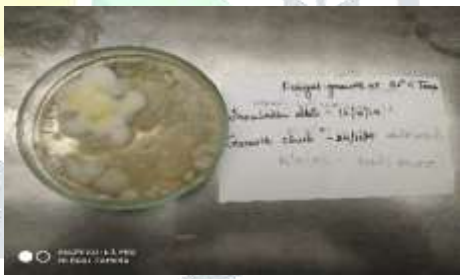


Figure. 16 and figure. 17 are the PDA plates showing physiological charachterization. Figure .16 showing *Aspergillus* growth at 30 ^{0}C and figure. 17 showing isolated fungal strain growth at 30^{0}C.

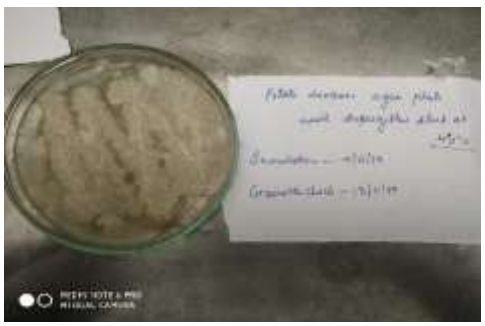


Figure. 18 and Figure. 19 showing PDA plates. Figure. 18 showing *Aspergillus niger* growth at 40 ^{0}C and figure. 19 showing no growth of isolated fungal strain at 40^{0}C.

Figure. 20 and Figure. 21 show amylolytic activity. Figure . 20 showing no activity of *Aspergillus niger* and Figure. 21 showing isolated fungal strain activity growth

Figure. 22 and figure. 23 shown proteolytic activity. Figure.22 Showing *Aspergillus niger* growth on proteolytic activity and figure 23. Showing soil sample growth in proteolytic activity

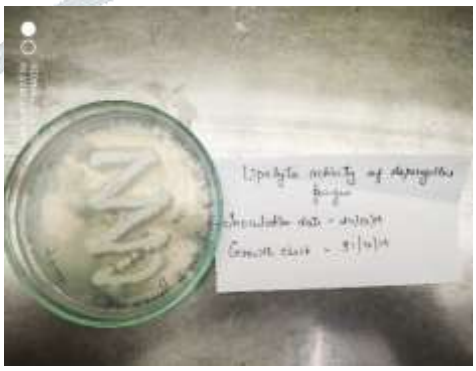


Figure .24 and figure 25. Shown lipolytic activity. Figure 24. showing isolated fungal strain growth on lipolyitc activity and figure 25. showing *Aspergillus niger* gowth on lipolyitc activity.

Figure. 26 and figure 27 Shown pectolyitc activity. Figure. 26 showing *Aspergillus niger* growth on pectolytic activity and figure. 27 showing isolated fungal strain growth on pectinolytic activity.

Characterization of fungal strain with different tests	Aspergillus strain activities	Isolated fungal strain activities
Morphological test	Positive (+)	Positive (+)
Physiological test		
At temeperature 4°C	Negative (-)	Negative (-)
At temperature 15°C	Positive (+)	Positive (+)
At temperature 30°C	Positive (+)	Positive (+)
At temperature 40°C	Positive (+)	Negative (-)

Biochemical test	Aspergillus strain activities	Isolated fungal strain activities
Amylolytic activity	Negative (-)	Positive (+)
Proteolytic activity	Positive (+)	Double positive (+)
Lipolytic activity	Positive (+)	Positive (+)
Pectinolytic activity	Positive (+)	Positive (+)

FORMATION OF FORMULATED BEADS WITH 4 % SODIUM ALGINATE:

For the beads formation, o.5% to 4% of sodium alginate was used in the 0.2 M Calcium Chloride.

Fig. 28:Sodium Alginate Beads prepared using 4% Sodium Alginate and 0.5 M Calcium Chloride.

STABILITY SCREENING OF THE BEADS:

To check their stabilty , 30 beads was inoculated in the 100 ml of PD broth. Incubated in shaker at 28^0C and checked their stabilty after each hour. After 5 to 6 hr observation, there was no dispersion of beads. After 1 week ,media was flitered and counted the number of beads which was same as earlier.

Fig. 29. Inoculation of formualted beads in the 100ml PD Broth in 250 ml of flask to check the stabillity of each hour used incubated shaker at 28^0C.

INOCULATION OF FORMUALTED BEADS IN THE RHIZOSPHERE REGION OF THE PLANTS:

To inoculate the formualated beads in the rhizosphere region of the plant, soil was digged from the pot in the circular shape around 6 cm depth. Depth was measured with the help of measurment scale.

Fig.30. Inocualtion of formualted beads in the rhizosphere region of the plant (plant A) which was infested with the whiteflies.

Fig.31. Inoculation of formulated beads in the rhizosphere region of the plant (plant B) which was not infested with whiteflies.

ANALYSIS OF INOCULATED BEADS IN THE RHIZOSPHERE REGION OF THE PLANTS

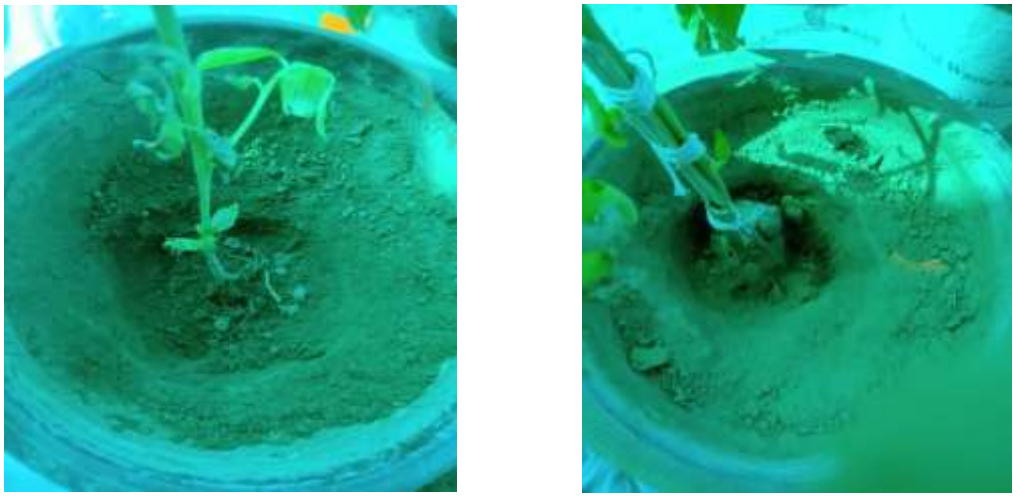


Fig.32. Analysis of Inoculated Beads in Rhizospheric region of Tomato plant.

AFLATOXIN EXTARCTION FROM FORMULATED BEADS :

After the extraction and estimation of the aflatoxin with the help of TLC method we found that plant A. that was infested with the whitefly having no content of aflatoxin with in the beads but plant B. which is free from whiteflies their beads having aflatoxin.

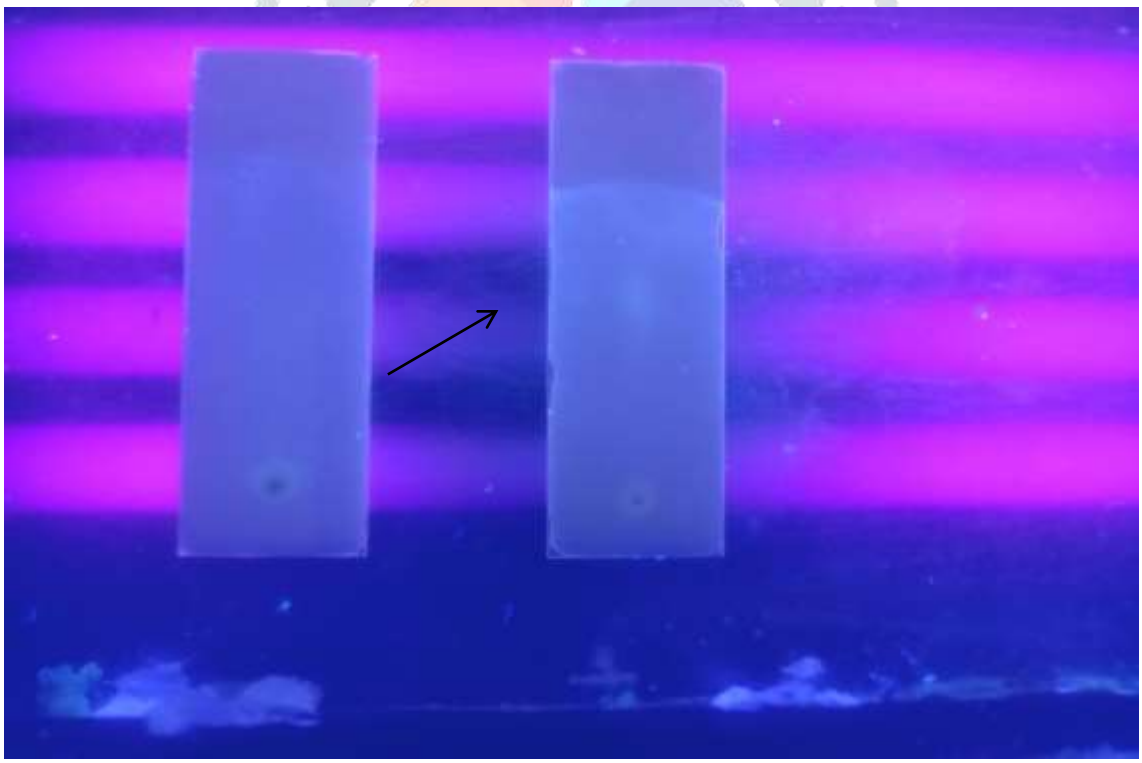


FIG 35. TLC plates in UV chamber after complete run of loaded aflatoxin extract (shown with the help of arrow).Plate A shows no aflatoxin however plate B displays Aflatoxin which simply clarifies that interaction

of whiteflies reduced the effect of fungus Aspergillus.However fungus also inhibits the further growth of Whiteflies.

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