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Synergistic Effect of Imidacloprid and Fungus *Aspergillus flavus* Against Subterranean Termite (*Rhinotermitidae: Blattodea*) Under Laboratory Conditions

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ABSTRACT

A *termiticide imidacloprid* and a biocontrol agent *Aspergillus flavus* were applied against the subterranean termites, alone or in combination and their synergistic effect were investigated. *Aspergillus flavus* was combined with *imidacloprid, imidacloprid* improved the mortality caused by fungus strain, over the first 14 days, at both of the tested chemical concentration. They expressed the exceptional synergistic effect that raised the mortality of termites also decreased the sub-lethal time at a sub-lethal dose. *Imidacloprid* facilitates the *Aspergillus flavus* infection on termites, it caused stress which reduces the defence mechanism of termites and may weaken them. The percentage mortality were noted and assessed. The synergistic treatment showed the potential for integrated insecticide-fungus control method and need to be further investigated on termites.

Keywords: Termiticide, Biocontrol agent, Synergestic effect, Sub-lethal time, Sub-lethal dose, Defense mechanism, Insecticide-fungus control method

Introduction

Termites were reported to be nested within the *Blattaria* [1] It is well established that eusocial termites evolved from a sub-social ancestor [2,3]. Termites are hemimetabolous, social insects and major pests of different urban and agricultural objects, such as timber, paper and different crops, [4,5], and efficient decomposers of wood and leaves in natural systems [6-8]. Termites comprise four different castes; king, queen, soldiers and workers [9], and mature colonies may contain thousands of individuals [10], which are Termites are known to eat faeces, dead termites, cast-off skin, and debris, and process these waste materials for building nests [11].

There are approximately 3000 species of termites including 371 which are considered as pest species and comprise eight families, which can be split up into two categories build on habitat; 1. Wood dwelling: *Kalotermitidae, Stolotermitidae, Archotermopsidae 2. Subterranean: Hodotermitidae, Mastotermitidae, Rhinotermitidae, Stylotermitidae and Termitidae*. Four of these families are considered to be economically important: *Kalotermitidae, Hodotermitidae, Rhinotermitidae* and *Termitidae* . *Kalotermitidae* exclusively inhabit wood (dead, dying and living) and depend on cellulose, the main structural element in woody materials . Hodotermitidae attacks grasses , *Rhinotermitidae* are largely subterranean, but invade wood works in buildings and adjacent trees , and Termitidae is largest, and economically most important, both under the above ground dwellers .

Termites are known as systemic pests, as well as agricultural and forestry pests [12]. To cease the population of these pests in urban environment mainly subterranean termites (*Rhinotermitidae*), has been given considerable attention because they are capable for systemic destruction [13,14]. The progress of baits utilizing chitin synthesis obstruction has improved management mechanisms [15-17], even though significant amounts of termiticides in solution form are even now being used in non-rural pest control [13,18]. Observing the surrounding effects of these activities, nonchemical methods of control of termites need to be studied as a substitute to structural defense [19-21,4]. Once Lund [22] permitted fungal species as biotic control agents in opposition to subterranean termites, the use of entomopathogens for the management of termite's population was found 40 years earlier. The accumulation of facts in the laboratory testing for possible biotic control agents in opposition to different termite pest species indicates the degree of attentiveness in this area [21], especially with the utilization of entomopathogenic *fungi* but also with bacteria, nematodes and viruses. Ironically, several lab researches have indicated pronounced capability for field use of the biotic agents tested [21], yet little studies have recorded effective field experiments. Field victory were confined to populations of pile building or arboresque termites where significant quantities of fungal infectious agents expression were institute directly into the middle area of the nest [23-26] Numerous researchers have been investigating the possible utilization of entomo pathogens for insects as disease-causing agents in recent decades [27]. Grace [20] presented an analysis of termite's biological control and proposed that microbes, mainly entomo-pathogenic *fungi*, gave some advantage in blattodea 's biological control. A lot of laboratory data on the effectiveness of fungal pathogens for termite management are available, but minute field effective evidence now present. When huge amount of 1 strain of *Aspergillus flavus* conidia were applied directly into the nursery region of a mound building termite, it effectively reduces the populations of termites [19,28].

In subterranean termites, because of their composite burrowing patterns, the inundate method is theoretically limiting [29], to create an epizootic sign the scattering and repetition of the bio-control factor is required to enter the most of individuals. Thus an

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inundate technique using fungi as myco-insecticides is not practical for biological regulation of termites. For subterranean termites, the presence of epizootics is necessary to attain the mortality of colony [30]. *imidacloprid* is an insecticide which shows low toxicity to mammals. This works on the nervous system of insects by connecting nerve cells to the acetylcholine binding sites, or nicotinic acetylcholine receptors [31]. This formulation prevents information from being transmitted at these binding sites, contributing to a lasting dysfunction of the nervous system and ultimately insect death [32]. *imidacloprid* has great comprehensive effects and is successful in case of a wide variety of farm pests [33,34]. The *imidacloprid* success in the management of farm pests has elicit its utilization to control termites which are considered as urban pests [35,36] The idea of using a biochemical stressor to improve entomopathogens' effectiveness is not new, but on this pest management technique researches are insufficient. [37]. In bait, the application of *imidacloprid* greatly increased the vulnerability of Eastern Subterranean termites collected from the field [38].

Through use of fungus as an infectious agent for the biotic management of termites was built on the theory of classical biotic control method [39,40], using a toxic representative that can multiply itself in a termite nest and be spread through high social interaction from individual to individual to produce an outbreak and destroy the whole population. The effectiveness of this kind of a method was built on the given hypotheses: (1) Dampness and temperature factors in a termite nest and termites communal behavior permit the easy and fast spread of pathogens among entities of a population; (2) the geological environment typically provides perfect circumstances for surviving infection epizootics promotion; and (3) the self-replicating potential enables the production of epizootics in population of termites.

Ultimately, the causative agent must force the intended host to die. However, the degree of virulence could become crucial to an epizootic outbreak, and it focuses on following points that can be calculated in the research lab: the median lethal dose (LD50) and the median lethal period (LT50). A low LD50 means that to produce a diseased and dying host, a small dose of the causative agent is required. A low LT50 shows that it takes the etiological agent a less time to attack the host. If the infectious agents destroys the host too soon, however, it does not have the opportunity to outspread to the termite colony, and if the infectious agents destroys the host too steadily, infected individuals could be eliminated or withdrawn from the colony before the infectious agent may reproduce and transferred to other termites. Of all the infectious agents tested in opposition to different termite species in laboratory state, it has been indicated that numerous strains have sufficient LT50 and LD50 for field test implementations [28], however in subterranean termites; death rate recorded in research lab state is not mirrored by many in practical state [41].

Materials and Methods

Collection of termites

Termites were gathered from different sites of the area Johar town, Township, Punjab society and open fields. Through the baiting, some were also collected from the field that is in the buckets about 4-5 months buried wooden blocks. By using artificial baiting methods the collection were made viable. The synthetic baiting methods were wetted toilet rolls, bucket traps and cardboard in of polyester plastic bottles having small pits at sides also in the base which allows the entry of termites. The collected termites were firstly acclimatized and then used for experiment. The termites were acclimatized for 2-3 days/72 hrs under lab conditions.

Fungus and *imidacloprid*

Aspergillus flavus were grown on corn and then culture under laboratory conditions. PDA media was used for the streaking of fungus at 29°C -30°C in dark for 10-15 days. Media were prepared by dissolving 19 g of PDA in 500 ml of distilled water followed by autoclaving at 121°C at 15 psi pressure for 20 min. For the preparation of PDA plate's laminar flow was used. To check that the plates were free of contamination it was incubated at 37°C for overnight. The fungal stocks were collected and cultured on plates having media, incubated at 29°C -30°C for 4-6 days. *imidacloprid* were obtained from market. Different concentrations of *imidacloprid* and fungus were prepared and subsequently used in bioassays alone and in combinations.

Experimental design and bioassays

Different concentrations of *Aspergillus* (1×10^7, 1×10^6, 1×10^5, 1×104 and 1×10^3 conidia/ml) and *imidacloprid* (1000, 500, 250, 125, 62.5, 31.25 ppm) were separately applied on termites. Percentage mortality of termites was calculated. Then known concentrations of *imidacloprid* and *Aspergillus* (For example 31.25 ppm and 1.3×10^7 conidia/ml) were combined to apply and again percentage mortality of termites were examined and noted. The filter paper treated with water alone was used as a control treatment in each case (Figures 1-3).

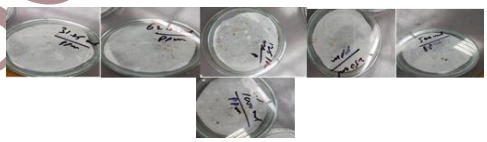

Figure 1: Different concentrations of *imidacloprid* against *Microtermes obesi*

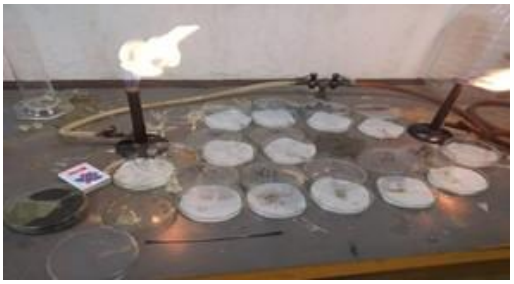

Figure 2: Different concentrations of *Aspergillus flavus* against *Microtermes obesi*

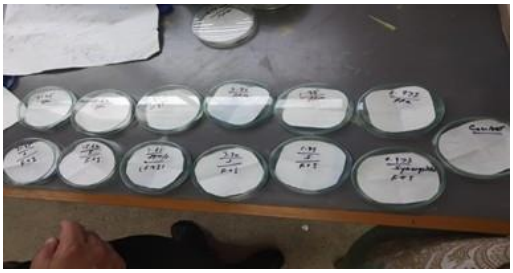

Figure 3: Different concentrations of *imidacloprid* and *Aspergillus flavus* synergistically under laboratory against *Microtermes obesi*.

Statistical analysis

Data were pooled before analysis. Two-way analysis of variance (ANOVA) was performed at P≤0.05.

Results

In this study termites were collected from different sites of the area Johar town, Township, Punjab society and open fields.

On selected media the culturing of *Aspergillus flavus*

Aspergillus flavus, effect insects including termites, is an entomopathogenic fungus. Fungus growth was checked on potato dextrose agar (PDA); it showed growth after five days. *Aspergillus flavus* is a fast growing fungus and at different levels of growth it changes colour. In first 4-5 days of growth white coloured colonies were obtained. Then changes to green colour followed by dark green colour having matured spores. It took two week to turn the fungus from white to dark green colour and on petri plates it gave velvety emergence. (Figure 4)

Figure 4: Growth of *Aspergillus flavus* on (PDA)

HRT different conentrations of *imidacloprid* against *microtermes obesi* after two week

Table 1 shows *imidacloprid*, after 2 week exposure, 88, 70, 65, 57, 44 and 30% mortality was observed at 1000, 500, 250, 125, 62.5, 31.25 ppm, respectively. Statistically two way ANOVA was applied against the termite mortality data and results as per concentrations used. Minimum mortality 30% was shown at 31.25 ppm and maximum 88% at 1000 ppm recorded. All means were statistically significant (P<0.05) (Tukey's test) (Table 1) (Figure 5).

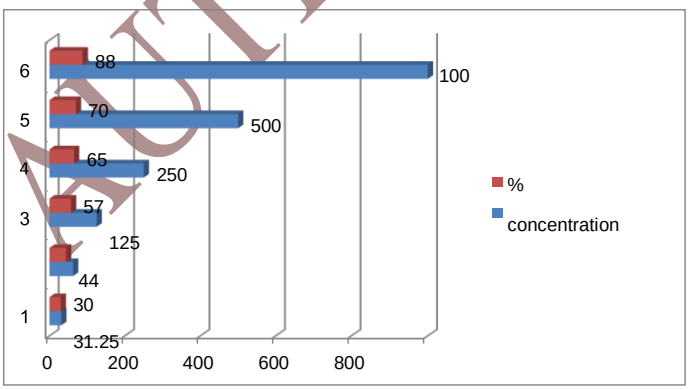

Figure 5: % age mortality of termites at different concentration of *imidacloprid*

Table 1: Different concentrations of *imidacloprid* against *Microtermes obesi* after two week

No. of days	%age mortality of *Microtermes obesi* at different concentrations of *imidacloprid* (ppm)						
		31.25	32.5	125	250	500	1000
1	0	5	12	15	21	25	43
2	0	9	15	17	24	27	48
3	0	12	18	22	27	32	51
4	0	13	21	25	32	35	54
5	0	15	25	27	36	38	57
6	0	17	26	30	39	44	60
7	0	19	28	34	44	46	65
8	0	20	30	36	47	49	69
9	0	21	33	38	49	52	71
10	0	22	36	41	51	55	74
11	0	23	37	46	55	57	78
12	0	25	39	49	58	61	81
13	0	27	43	53	62	67	85
14	0	30	44	57	65	70	88

Different concentrations of *aspergillus flavus* against *microtermes obesi* after two week

On constant size group of termites the consequences of different concentrations of *Aspergillus flavus* were checked. Different mortality rates of termites were observed at different concentrations of *Aspergillus flavus*. The rate of mortality of termites was raised with the increase of fungal spore concentration which indicates a direct relationship between mortality rate of termites and spore concentration of fungus. 95, 88, 70, 65 and 50% mortality was observed at the spore concentrations of 1×10^7, 1×10^6, 1×10^5, 1×10^4 and 1×10^3 respectively (Table 2).

Table 2: Analysis of variance for mortality

Source	DF	SS	MS	F	P
Replica-tions	1	24.5	24.47		
Days	13	23092.3	1776.33	7908.81*	0
Doses	5	37465.8	7493.17	33362*	0
Days×D-oses	65	1259.3	19.37	86.26*	0
Error	83	18.6	0.22		
Total	167	61860.5			

Statistically two way ANOVA was applied against the termite mortality data and results as per concentrations used. Minimum mortality 50% was shown at 1×10^3 and maximum 95% at 1×10^7 recorded. All means were statistically significant (P<0.05) (Tukey's test) (Table 3) (Figure 6).

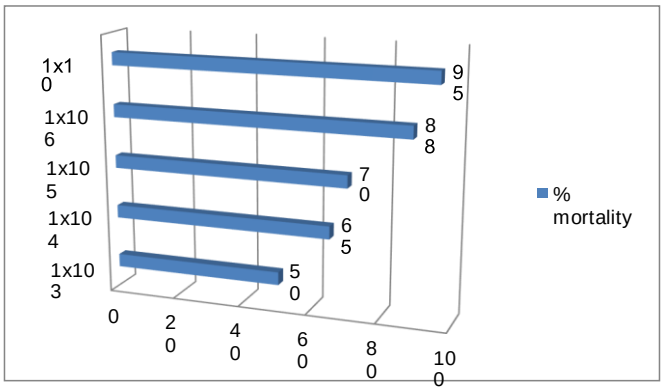

Figure 6: % age mortality of termites at different concentrations of *fungi* (conidia/ ml)

Table 3: Different concentrations of *Aspergillus flavus* against *Microtermes obesi* after two week

No. of days	%age mortality of *Microtermes obesi* at different concentrations of *Aspergillus flavus* (conidial/ ml					
	0	$1x10^3$	$1x10^4$	$1x10^5$	$1x10^6$	$1x10^7$
1	0	5	10	16	28	39
2	0	10	14	19	31	45
3	0	13	19	22	37	47
4	0	15	24	26	41	51
5	0	17	26	31	46	54
6	0	20	31	34	50	59
7	0	25	36	40	55	62
8	0	26	39	45	58	67
9	0	32	44	48	63	70
10	0	37	47	54	67	75
11	0	39	50	57	71	79
12	0	44	55	61	77	85
13	0	47	59	67	83	91
14	0	50	65	70	88	95

Different concentrations of *imidacloprid* at different concentrations of *aspergillus flavus* against *microtermes obesi* after two week

In the mortality of termites the combined treatment of *imidacloprid* and *Aspergillus flavus* played the major role. Basically *imidacloprid* raised the susceptibility of termite to *Aspergillus flavus*. From the combined treatment of *imidacloprid* and *Aspergillus flavus* the mortality rate of termites was higher than either treatment alone. After 14 days of continual experiment, mortality of termites revealed to the 1.3 × 107 conidia/ml of *Aspergillus flavus* and 31.25 ppm of *imidacloprid* was 100%. It was notably higher than the termites exposed to either *imidacloprid* or *Aspergillus flavus* alone. The relationship between the variables is significant (P<0.05) showed by two way ANOVA (Tables 4-9).

Table 4: Randomized Complete Block AOV Table for V003

Source	DF	SS	SS	MS	F	P
V001	1	2.4	2.4	2.401		
V002	13	5485.78	5485.78	421.983	5439.16	0
Error	13	1.01	1.01	0.078		
Total	27	5489.19	5489.19			
Grand Mean 26.850 CV 1.04						

Table 5: Randomized Complete Block AOV Table for V004

Source	DF	SS	MS	F	P
V001	1	2.89	2.893		
V002	13	7572.24	582.480	10861.8	0.0000
Error	13	0.70	0.054		
Total	27	7575.83			
Grand Mean 36.750 CV 0.63					

Table 6: Randomized Complete Block AOV Table for V005

Source	DF	SS	MS	F	P
V001	1	2.58	2.580		
V002	13	8431.07	648.544	13717.0	0.0000
Error	13	0.61	0.047		
Total	27	8434.27			
Grand Mean 41.839 CV 0.52					

Table 7: Randomized Complete Block AOV Table for V006

Source	DF	SS	MS	F	P
V001	1	2.89	2.893		
V002	13	9346.87	718.990	15145.4	0.0000
Error	13	0.62	0.047		
Total	27	9350.38			
Grand Mean 56.464 CV 0.39					

Table 8: Randomized Complete Block AOV Table for V007

Source	DF	SS	MS	F	P
V001	1	3.79	3.789		
V002	13	8109.70	623.823	19033.7	0.0000
Error	13	0.43	0.033		
Total	27	8113.91			
Grand Mean 65.275 CV 0.28					

Table 9: Different concentrations of imidacloprid at different concentrations of *Aspergillus flavus* against Microtermes obesi after two week

No. of days	Aspergillus flavus (conidia/ml)	%age Mortality (mean ± SE) at different concentrations of imidacloprid (ppm)					
		0.973	1.95	3.9	7.81	15.62	31.25
1	$9.0x10^3$	0.25 ± 0.05	1.85 ± 0.15	2.5 ± 0.5	4.75 ± 0.25	6.8 ± 0.2	8.5 ± 0.5
2	$2.9x10^4$	0.45 ± 0.05	2.5 ± 0.5	5.85 ± 0.15	7.8 ± 0.2	10.6 ± 0.4	11.8 ± 0.25
3	$5.4x10^4$	0.85 ± 0.05	3.8 ± 0.2	6.5 ± 0.5	9.6 ± 0.4	13.5 ± 0.5	16.9 ± 0.15
4	$8.7x10^4$	1.05 ± 0.05	6.7 ± 0.35	10.7 ± 0.3	12.8 ± 0.25	16.7 ± 0.3	19.6 ± 0.4
5	$1.9x10^5$	1.8 ± 0.2	9.85 ± 0.15	14.6 ± 0.4	18.8 ± 0.2	25.9 ± 0.15	26.8 ± 0.25
6	$4.6x10^5$	2.7 ± 0.3	12.5 ± 0.5	17.5 ± 0.5	22.5 ± 0.5	28.5 ± 0.5	33.9 ± 0.1
7	$7.3x10^5$	5.5 ± 0.5	15.7 ± 0.3	21.8 ± 0.3	26.9 ± 0.15	34.8 ± 0.2	38.5 ± 0.5
8	$9.8x10^5$	6.75 ± 0.25	18.6 ± 0.45	23.6 ± 0.4	35.5 ± 0.5	45.6 ± 0.4	50.8 ± 0.25
9	2.9x106	8.65 ± 0.35	20.5 ± 0.5	28.5 ± 0.5	38.5 ± 0.5	55.9 ± 0.15	58.7 ± 0.3
10	$6.5x10^6$	10.8 ± 0.2	23.9 ± 0.2	31.8 ± 0.2	47.7 ± 0.35	66.95 ± 0.1	74.5 ± 0.5
11	$9.2x10^6$	13.5 ± 0.5	27.7 ± 0.4	35.9 ± 0.1	50.9 ± 0.15	69.7 ± 0.3	87.6 ± 0.4
12	$1.0x10^7$	15.8 ± 0.25	30.5 ± 0.5	39.5 ± 0.5	53.7 ± 0.3	75.7 ± 0.3	91.7 ± 0.4
13	$1.2x10^7$	19.5 ± 0.5	33.9 ± 0.1	46.6 ± 0.4	61.9 ± 0.2	82.8 ± 0.2	96.5 ± 0.5
14	$1.3x10^7$	23.85 ± 0.15	35.8 ± 0.2	48.5 ± 0.5	64.5 ± 0.5	85.6 ± 0.45	99.5 ± 0.5

Note: All mean values in two way ANOVA test are significantly different from one another ($P<0.05$) (Two way ANOVA test and significance level at $\alpha = 0.05$).

Discussion

To limit the population of subterranean termites chemical control and biological control agents are two research methods that are widely used. Their isolated treatments have supermacy that are making them more effective against specific types of infections. For example, in an infested tree to fill the empty space a chemical that can be spume is well suited, and a fungus that has long-term shelf firmness can be transfer as a powder, but when introduced to a termite nest that has a moist environment it become active. So, with the single microbial agent it is hard to attain perfect control [42].

That's why possible synergistic effect can assist to overcome this problem. In this research work sub-lethal dosage of *imidacloprid* and fungus were firstly applied separately which causes death in termites and then they were put in fusion with each other. Established on their capability to generate mortality in subterranean termites in the preliminary studies, in this study *Aspergillus flavus* were selected. Strains of fungus were transfer among nest-mates which were determined when strains from cadavers were recovered and measured. The fungus was applied on 50% termites, so the rate of mortality higher than 50% showed that the fungus was moved between nest-mates.

Earlier to revealing termites with the combination of *imidacloprid* and *Aspergillus flavus*, the individual effect is checked and mortality rates were determined. Also we were able to confirm the transfer of *Aspergillus flavus* from termite to termite. The ability of any bio-control factor to develop between termites is the critical factor for its success. A member of a colony should be accomplished to transmit a microorganism that is encounter during foraging to the nest-mates that do not forage. A colony control will not be fortunate without the transfer between termites [43].

In each replicate only half of the termites were revealed directly to *Aspergillus flavus* so that the transport of strains of fungus was regulated. And a death rate more than 50% showed that strains are transferred from directly exposed termites to nest-mates. Termites usually prefer a surrounding that is favorable for the development of micro-organisms. Many micro-organisms have the capacity to produce death of termites when termites encounter these micro-organisms.

When soil treated with Permethrin both Microtemeres obesi and *Coptotermes heimi* species do not able to invade [44]. Compared to other insecticides the effectiveness of *imidacloprid* was slow and less. It was analyzed by [45] that if *imidacloprid* were not exposed to termites for an adequate amount of time then the effect of *imidacloprid* were minor and insignificant. They also observed that the toxicity of *imidacloprid* was less and it took many days for the death of termites by *imidacloprid*. Also it was analyzed by [46] that the complete death of termites requires 14 days when revealed to *imidacloprid* for 2 hours at the concentration of 500 ppm.

When termites were exposed to bacterial or fungal pathogens it develops a defensive immune system [47]. So against pathogens termites developed much effective protective system. It was observed by [28] that more than 90 isolates of *Metarhizium anisopliae* against *Coptotermes* species and *Nasutitermes exitiosus* workers and found that many isolates cause more than 80% mortality of termites. It was showed by [48] that *Metarhizium anisopliae* was more effective to *Odontotermes brunneus*. It was observed by [49] that 3 days of treatment of 21 isolates of *M. anisopliae* on Reticulitermes flavipes workers gave 100% mortality. [50] Found that at a concentration of 3×10^8 conidia/ml *M. anisopliae* were much effective against *Odontotermes formosanus* because after 3 days it caused 100%

mortality.

Termites were displayed to *imidacloprid* at a concentration of 0.973, 1.95, 3.90, 7.81, 15.62 or 31.25 ppm and the percentage mortality is calculated, the significant level was 0.0000. In the next experiment termites were exposed to different concentrations of *Aspergillus flavus* and again the percentage mortality is calculated, the significant level was 0.0000. So, the analysis of variance showed the significant level was $P \leq 0.05$ thus the results are significant.

The death rate of termites is faster when exposed to both *imidacloprid* and fungus than either trial alone. Boucias et al [35] reported that even at high dose after 2 weeks of continuous exposure fungus alone gave no significant mortality. When combined with *imidacloprid* it increases the susceptibility to the fungus. *imidacloprid* disturbs the capability of termites to defend them from pathogenic infections. It was explained that against many pathogenic infections termites social behaviors are their major defense. Also *imidacloprid* was not able to disrupt the cellular defense mechanism of termites. So, *imidacloprid* applied to soil containing 102 to 107 conidia/gram showed greater death of termites after 2 week of exposure [35].

To check the synergistic effect of *imidacloprid* and Aspergillus flavus on termites, the different concentrations of both chemical and fungus were applied against termites and the percentage mortality is calculated. Again analysis of variance showed the significant level that was 0.0000 so the results are significant ($P \leq 0.05$). The control contained termites that were not exposed to either the fungus or *imidacloprid* and none caused significant mortality.

Conclusion

In this research work the synergistic effect is checked when different concentrations of entomopathogen fungus and *imidacloprid* are used as combined treatment. This treatment was more effective than the alone treatment of fungus and chemical, and 100% mortality is obtained. The results showed that UASB reactors operated at ambient temperatures were highly effective in the treatment of wastewater at influent COD concentration 629 mg l-1 COD at HRT from 24 to 6 hours with the specific methane yield obtained was around 0.32 l CH_4 g^{-1} COD removed. COD removal efficiencies were high at 95% and total suspended solid removal was around 95%.

The UASB technology provides a low-cost system for the direct treatment of municipal wastewater and can be applied in small communities where the wastewater flow variation is high due to rainy season or population load increases during the tourist season or due to seasonally operated food industries.

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