Synergistic Effect of Commercial Pesticides and Entomopathogenic Fungi on *Coptotermes heimi* (Wasmann) (Blattodea: Rhinotermitidae)

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Synergistic Effect of Commercial Pesticides and Entomopathogenic Fungi on *Coptotermes heimi* (Wasmann) (Blattodea: Rhinotermitidae)

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DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

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Muhammad Shoaib

In the name of Allah, the most beneficent the most merciful

Dedication

With profound love & deep respect, this dissertation is dedicated to my sweet and loving parents

Whose affection, love, encouragement and prayers of day and night make me able to get this success and honor.

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LIST OF ABBREVIATIONS

Abbreviations	Full form
˚C	Degree Celsius
µl	Micro liter
APLC	Australian Plague Locust Commission
CPF	Chlorpyrifos
CTAB	Cetyl Trimethyl Ammonium Bromide
DDT	Dichlorodiphenyltrichloroethane
EC	Emulcifiable Concentrate
EPF	Entomopathogenic fungi
EPNs	Entomopathogenic nematodes
IPM	Integrated Pest Management
LPCB	Lactophenol Cotton Blue
M	Molar
PDA	Potato Dextrose Agar
Ppm	Parts per million
PVC	Polyvinyl chloride
PVP	Polyvinylpyrrolidone
RH	Relative humidity
Rpm	Revolutions per minute
SC	Suspension Concentrate
SD	Standard Deviation
UCCP	Union Carbide Corporation Plant

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ABSTRACT

The subterranean termite, *Coptotermes heimi* (Wasmann) (Blattodea: Rhinotermitidae), is recognized as a building infesting lower termite species in Pakistan, India, and Bangladesh. The irrational and extensive use of insecticides against *C. heimi* in urban environments has resulted in insecticide resistance and environmental contamination. This study was performed to control *C. heimi* using naturally occurring endogenous strains of entomopathogenic fungi *Fusarium falciforme*, *Aspergillus terreus*, *Penicillium citrinum,* and *Fusarium solani* in combination with sublethal concentrations of commercial insecticides fipronil, chlorpyrifos, and imidacloprid in a no-choice feeding assay for 12 days. The termite percent mortality rate for the combination of imidacloprid (25 ppm) with *F. solani* (1×10^9 conidia/ml) was 83%, higher than the combined percent mortality caused by these two variables in individual treatment at a continuous exposure of 12 days. Similarly, another combination of fipronil and *F. falciforme* showed an enhanced percent mortality (91%) compared to the individual percent mortality of these two variables in separate treatments. These results showed that sublethal concentrations of insecticides suppress the immune response and reduce the repellency of infected termites. Entomopathogenic fungi with a combination of sublethal concentrations can be used against insect pests and may reduce the insect resistance of targeted pests. Further detailed studies are necessary for developing and implanting this approach in successful field trials.

INTRODUCTION

1.1 Background

Termites are eusocial, wood-eating, polymorphic terrestrial soft insects with more than 3100 identified species belonging to 282 genera and 7 families worldwide (Chouvenc *et al.,* 2021; Rahman *et al*., 2022). Termites are frequently termed as "white ants", though they are quite different from ants as real ants belong to the order Hymenoptera while termites belong to the order Blattodea (Rasib *et al*., 2022). Termites first appeared as a monophyletic clade in the late Jurassic period of the Mesozoic era with the subsocial sister group of wood roach Cryptocercus: Cryptocercidae (Khan *et al.,* 2018). Termite's ancestors lived inside a single log that served as both food and a nest. Termites are considered eusocial cockroaches in the order Blattodea (formerly Isoptera), along with cockroaches (Nalepa, 2015).

Systematic entomology has shown that termites are nested within cockroaches, jointly forming the clade Blattodea, constituting 7500 identified species. Majority members of this group are wood feeders and globally recognized pests, hence, Blattodea are ecologically and economically important on a small as well as large scale, affecting the climate globally (Evangelista *et al.,* 2019). Both biotic and abiotic factors affect the physiology of termites. Abiotic factors like relative humidity, temperature, rainfall and pH significantly affect the survival, reproduction and development of termites (Pervez, 2018). Termites are primarily found in tropical and subtropical regions of the world, constituting 10% of all animal biomass (Ahmad *et al*., 2021). Except for Antarctica, termites are found on all other continents and a total of 1000 species have been identified in Africa, 435 in Asia, 400 in South America, 360 in Australia, 50 in North America and only 10 species in Europe (Govorushko, 2019).

Throughout Pakistan, approximately 52 termite species have been reported so far. Studies conducted in Lahore, Punjab have reported different species, among which *Odontotermes obesus* (Rambur), *Coptotermes heimi* (Wasmann), *Heterotermes indicola* (Wasmann) and *Microtermes obes*i (Holmgren), belonging to the Termitidae and Rhinotermitidae families, are reported (Afzal and Rasib, 2022). In Khyber Pakhtunkhwa,

districts Buner, Haripur and Swabi have major genera of termites: *Odontotermes*, *Angulitermes* and *Heterotermes*. In terms of species richness, the genus *Odontoteremes* is found the most (57%), followed by the genus *Heterotermes* (29%) and the genus *Angulitermes* (14%). Damages to the genera *Coptotermes*, *Microtermes*, *Heterotermes* and *Odontotermes* have been reported in Attock, Rawalpindi, Abbottabad, Islamabad, Murree Hills and Swat region (Zaman *et al*., 2022).

Termites act as decomposers that increase soil fertility, water infiltration, nutrient availability, and crop yield. They decompose plant lignocellulose, having a positive impact in soil-water dynamics and nutrient cycling as they build their nests and tunnels in the soil (Moreira *et al*., 2021). Tropical termitidae mounds significantly influence the growth patterns of vegetation as their mounds contain higher concentrations of micronutrients as compared to the topsoil surface. These mounds act as islands of nutrients for growth of vegetation (Jouquet *et al*., 2019). However certain species are serious threat to agriculture, forestry, and housing. Termites are estimated to cost the global economy more than US$ 40 billion per year, and much research has been conducted on their management (Ahmad *et al*., 2021).

1.2 Life Cycle of Termites

Termite colony is founded by the primary reproductives, the king and queen, after copulation during nuptial flight. In genus *Coptotermes* the primary limiting factor for successful colony establishment is the specified initial internal nutritional resources thar are carried by the alates during the nuptial flight (Chouvenc, 2022). In some species, colonies live out their entire lives as simple families led by the original founder king and queen. In other cases, the original founders of the colony are replaced by various neotenic (nymph or worker-derived) reproductive or, less commonly, by primary reproductives which are descendants of the original founding pair (Vargo, 2019). After the dispersal of alates for nuptial flight, wings are shed by the mating pair and the queen lays as many as 3000 yellowish eggs per day. Eggs hatch into nymphs. The caste differentiation is determined in post-embryonic development and larva becomes a worker, soldier and reproductive (Pervez, 2018).

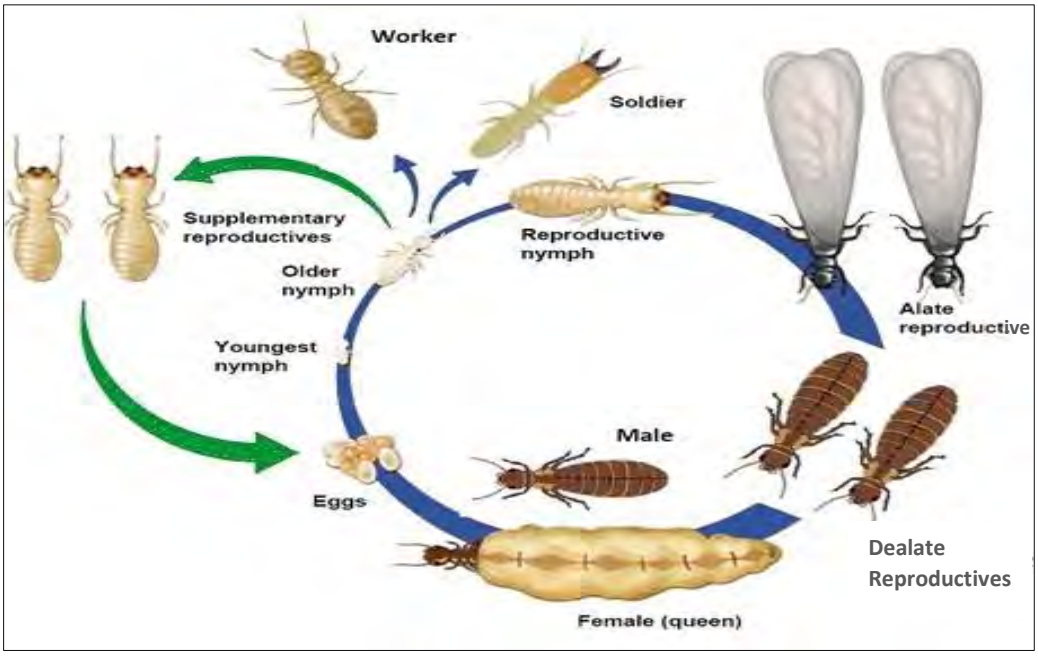

Figure 1.1: Life Cycle of Termites **(**Berlanga and Guerrero, 2016**)**

1.3 Different Castes of Termites

Termites make up a significant group of social insects and in their colonies, there are various types of individuals, referred as "castes" to which colony tasks are assigned and which engage in complex social behaviors (Prestwich, 1979). Caste differentiation is a complex phenomenon and is influenced by various factors like pheromones, light, temperature, food, endocrine system and genome (Henderson, 2019). The caste of each termite in a colony is predetermined from its embryonic stages and is also controlled by pheromones (Laine and Wright, 2003). There are two main types of castes in termites, fertile castes (reproductive) and sterile castes (neuters). Fertile castes include primary and secondary reproductives whereas sterile castes include workers, presoldiers and soldiers (Watanabe *et al*., 2014).

1.3.1 Fertile Castes

1.3.1.1 Primary Reproductives

king and queen are the primary reproductives which are derived from the alates. Their eyes are pigmented and fully developed. Queen lives up to 25 years (Pervez,

2018). They live deep inside their enclosed mounds or nest which ensure their safety from predators, thermal stress, and desiccation unlike the non reproductives that are mostly engaged in reproduction, colony formation and social labour (Tasaki *et al*., 2021).

1.3.1.2 Neotenic Reproductives

Termites exhibit the phenomena of neoteny. Neotenic reproductives are not alate derived but supplementary reproductives that appear when the founder king and queen die. Neotenics are wingless and also exhibit other larval features (Figure 1.2) like wing pads (Oguchi *et al*., 2022). Ergatoids are neotenics that develop from higher termite workers, whereas nymphoids are neotenics that develop from nymphs (Korb and Hartfelder, 2008).

1.3.1.3 Secondary Reproductives

Secondary reproductives may be either supplementary or replacement reproductive, depending upon their origin. They may arise in the presence of primary reproductive or in the absence of the primary reproductives and are called as supplementary or replacement reproductive, respectively (Roisin and Korb, 2011).

1.3.2 Sterile Castes

1.3.2.1 Workers

Workers develop through a premature, irreversible alteration from the imago pathway during the initial stages of lifecycle, usually after one or two larval instars, an apterous line produces the worker caste. In lower termites the workers are transparent, soft bodied without eyes and wings. Workers are sterile male and female possessing specialized mouth parts for chewing wood (Pervez, 2018; Roisin and Korb, 2011). They are wingless, have no developed eyes and are usually sterile but not necessarily (Roisin and Korb, 2011). Workers feed, groom, build nest and carry other young termites in the colony. Food processing in the colony is majorly performed by the major workers while minor workers feed on fungus comb and their major task is to feed the queen (Hinze *et*

al., 2002). In some species the workers excavate soil from underground surface for various functions.

The primary function is the protection of individuals from desiccation and predators while searching for food between foraging and nesting sites (Mizumoto, 2021).

1.3.2.1.1 True Workers and False Workers

True workers are individuals in the colony who deviate from their imagined development early and irreversibly (Lima *et al*., 2013). False workers are also known as Pseudergates. They are members of the colony who deviate the developmental imaginal line overdue during their initial stages of lifecycle (Noirot and Pasteels, 1987). False workers have broad developmental physiology of regressive, stationary and progressive moults and have the ability to become reproductives (Korb and Hartfelder, 2008).

1.3.2.2 Presoldiers

Presoldiers are a single transitional instar between worker and soldier, after post-embryonic developmental stages (Noirot and Pasteels, 1987). Formation of Presoldiers and soldiers is influenced by external and internal factors such as nutrition, age and hormones. Presoldiers formation are enhanced by the elimination of soldiers from the colony and also by better nutrition among the workers. Workers treated with Juvenile hormone after molting contribute to higher degree of pre-soldier formation (Park and Raina, 2003).

1.3.2.3 Soldiers

Soldier caste is considered to evolved prior to true workers and the ancestral in termites. The distinctive enlarged, sclerotized, capsulated head morphology and strong mandibles for defending the colony against various predators, is the characteristic of soldier caste (Noirot and Pasteels, 1987). Smaller soldiers are more efficient in attacking the enemies than larger soldiers. The larger soldiers are usually non-combative and flee at the time of predator attacks. Smaller soldiers fire their frontal gland secretion at the time of predator attack (Kriston *et al*., 1977). In some species of termites, soldier secrete chemical signals from frontal gland to recruit workers and other members of the colony

for forage (Leponce *et al*., 1999). In *Coptotermes formosanus* the soldiers are not able to collect food or construct tunnels physically, instead they are regularly engaged with the workers in foraging activities (McCarthy,2022). Factors like colony origin and feeding substrate can greatly affect the proportion of soldier caste formation (Du *et al*., 2019).

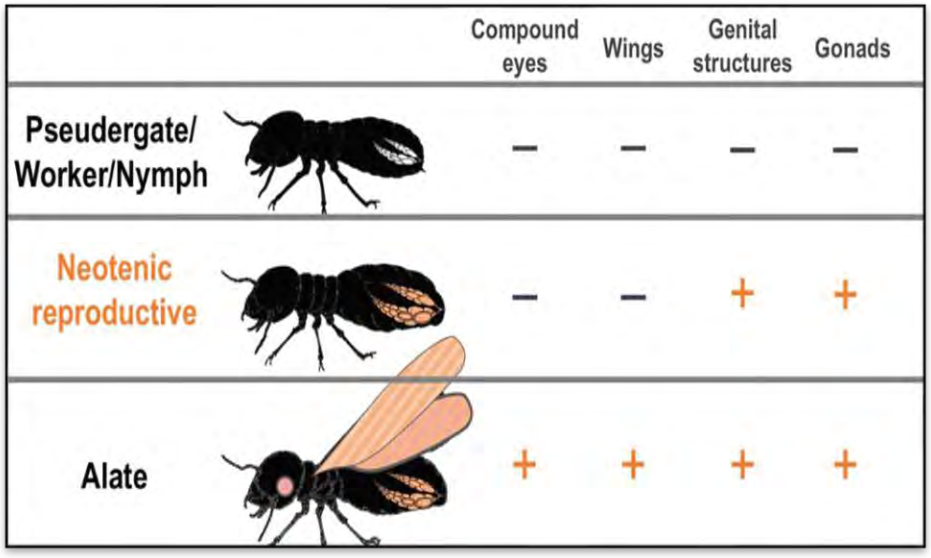

Figure 1.2: Schematic representation of Imaginal organs development in each caste of termites. "+" sign shows highly-developed imaginal organs while "–" shows suppressed or interrupted development of imaginal organs (Oguchi *et al*., 2021)

1.4 Termites Feeding Habits and Behaviors

Termites nests provide a place for feeding, reproduction and also shelter them against predators and environment conditions such as desiccation. Higher termites construct epigeal mounds made of aggregates of soil and their faecal materials, forming microclimate that control the porous structures of their mounds. *Cornitermes cumulans* being herbivorous, cut and transport grass and litter for construction of its mound. Different plant materials are stored in nodules made of saliva and faecal materials, and subsequently ingested by the workers (Moreira *et al*., 2021). Termites predominantly consume cellulose and lignocellulose as their major food source and account to process 50-100% recycling of biomass (Bignell and Eggleton, 2000).

Termites host symbionts such as protozoa, fungi, and flagellates. Lower termites feed on wood, preferring to do so if the wood is infected with fungi because fungi contain high portion of proteins. Higher termites being polyphagous feed on grasses, leaves, humus, and roots of plants (Radek, 1999). They are the best ecosystem engineers as their habitat vary from soil, grass, forest litter, dead woods to cultivated fungi. They can alter physical as well as chemical properties of the soil (Holt and Lepage, 2000). Some species like *Gnathamitermes tubiformans,* buffalo grass from May to August while in Spring and Autumn they prefer to feed on blue gram. Their diet significantly reduces during Spring as compared to Autumn. Drywood termite species *Cryptotermes brevis* prefer maple, western red cedar, balsa and poplar, but avoid other woods including pine (Pervez, 2018).

Termitidae comprises 38.3% of total soil feeding termites (Jones and Eggleton, 2011). Termitidae gut also contain arthropod body parts, plant roots and fungal hypae. Termites are classified as soil feeders, wood feeders, soil-wood interface feeders, grass feeders and plant litter feeders (Donovan *et al*., 2001). Some minor groups of termites feed on mounds of other termites, dung, vertebrate corpse, algae, fungi and lichens (Bignell and Eggleton, 2000).

1.5 Reproduction in Termites

In termites, mating is characterized by formation of pair and the establishment of colonies both during and after nuptial flight. Pair formation usually occurs just after the completion of nuptial flight by the alates (Calleri *et al*., 2007). Kings in termite colony produce viable sperm continuously to the queen in large number for decades. The queens store the sperm of their deceased mates for same duration (Keller and Genoud, 1997). Kings possess paired testes where sperms are produced after the process of spermatogenesis, initially at the nymphal stage (Ye *et al*., 2009).

Testes get fully matured when the male develops into an adult. Accessory glands and seminal vesicles in lower termites do not contain sperm, as these termites have expedited their growth in establishing the colony. Different vital secretions from these glands are

necessary to keep sperm viable and transferred into the female. These glands are either absent or very reduced in higher termites (King *et al*., 2011).

Morphology of sperm is variable in different species of termites. Multiflagellated arrowhead shaped, pin shaped non-motile, spheroidal without flagella sperms are mostly characterized in termites. Except for *Stolotermes ruficeps*, the external sclerotized genitalia are absent in kings and queens and erected small phallic lobes are present in male termites instead of sclerotized genitalia (Pervez, 2018).

Termite queens possess paired ovaries which grow substantially in size after swarming (Dean and Gold, 2004). After ejaculation of the sperm by the male, it is transferred to spermatheca, a recurved blind pouch for storage. Covering of spermatheca possess cells that are responsible for secretion of mucopolysaccharides (Raina *et al*., 2008; Ye *et al*., 2009). In termites physogastry occurs as a result of high demand of egg production, causing the abdomen of the queen to swell sometimes as many as 50 to 60 times of the initial size (Pervez, 2018).

1.6 Nuptial Flight

Swarming is an important episode in the life cycle of termites. After getting matured, the reproductive caste develop functional eyes as well as wings. These become darker and harder to help the swarmers in order to withstand less humid air and exposed light. Alates are winged and produce new kings and queens. Specific size of the colony is pre-requisite for alates production and hence several years are required for a colony to produce alates. Rainfall can act as a trigger for the very first nuptial flight of alates. The unrecognized kings and queens get into the mass swarming events that last for a few days, occurring once or twice a year. Both the mating partners fly away from the existing colony and form pairs, known as phase of dispersion and phase of pair formation respectively (Hanus *et al*., 2010).

Monogamy and polygamy, both are present in termites. Monogamous pairs are more potent in reproduction in terms of survival rate and quantity of progeny than polygamous pairs. In lower termites only one king and queen exist in the colony and other castes members if try to mate inside the same colony, are attacked and killed. However, in

certain higher termites multiple primary kings and queens exist in a single colony (Pervez, 2018). Nuptial flight does not end up in mating or copulation, though pair formation and mate selection are the features of nuptial flight. Copulation in mating pair occurs only after attaining security in the form of founding a colony. Courtship act gets start in the colony by grooming and mutual antennation whereas ends up by mating (Raina *et al*., 2003).

1.7 Classification of Termites

Generally, termites are classified into 7 famalies: Mastotermitidae, Kalotermitidae, Serritermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae and Termitidae. Mastotermitidae is the most primitive family of termites and is native to Australia. Serritermitidae and Rhinotermitidae are much alike and consume wooden structures in temperate regions. Rhinotermitidae is confined to South America. Termopsidae dwell inside the wood and wet dead rotten logs. Hodotermitidae decompose litter and are grass harvesters while Kalotermitidae dry wood feeders. Termitidae is the largest group, having more than 2000 species that accounts 75% of total identified termite species. Termitidae is further classified into 7 subfamilies: Foraminitermitinae, Nasutitermitinae, Apicotermitinae, Macrotermitinae, Syntermitinae, Sphaerotermitinae and Termitinae (Beccaloni and Eggllеton, 2013).

Termites are highly selective in feeding and hardness along with palatability of wood species. Hardness and palatability of plants are as defensive mechanisms by the plant (Evans *et al*., 2005). Wood feeding is one of the three basic characteristics found in all 28 invasive species of termites (Evans *et al*., 2013). Termites are classified as lower and higher based on various criteria, one of these criteria is their symbiotic associations with different types of bacteria such as *Enterobacter aerogenes*, *Enterobacter cloacae* and *Clavibacter agropyri*, isolated by Ramin and colleagues (2008) from hind gut of subterranean termite *Coptotermes curvignathus* and flagellates. Lower termites host protozoa, as well as bacteria and archaea in the hind gut while higher termites have bacteria and archaea as their symbionts (Scharf, 2020). These symbionts are attributed in large part to termites, the most successful eusocial group of insects (Poinar, 2009).

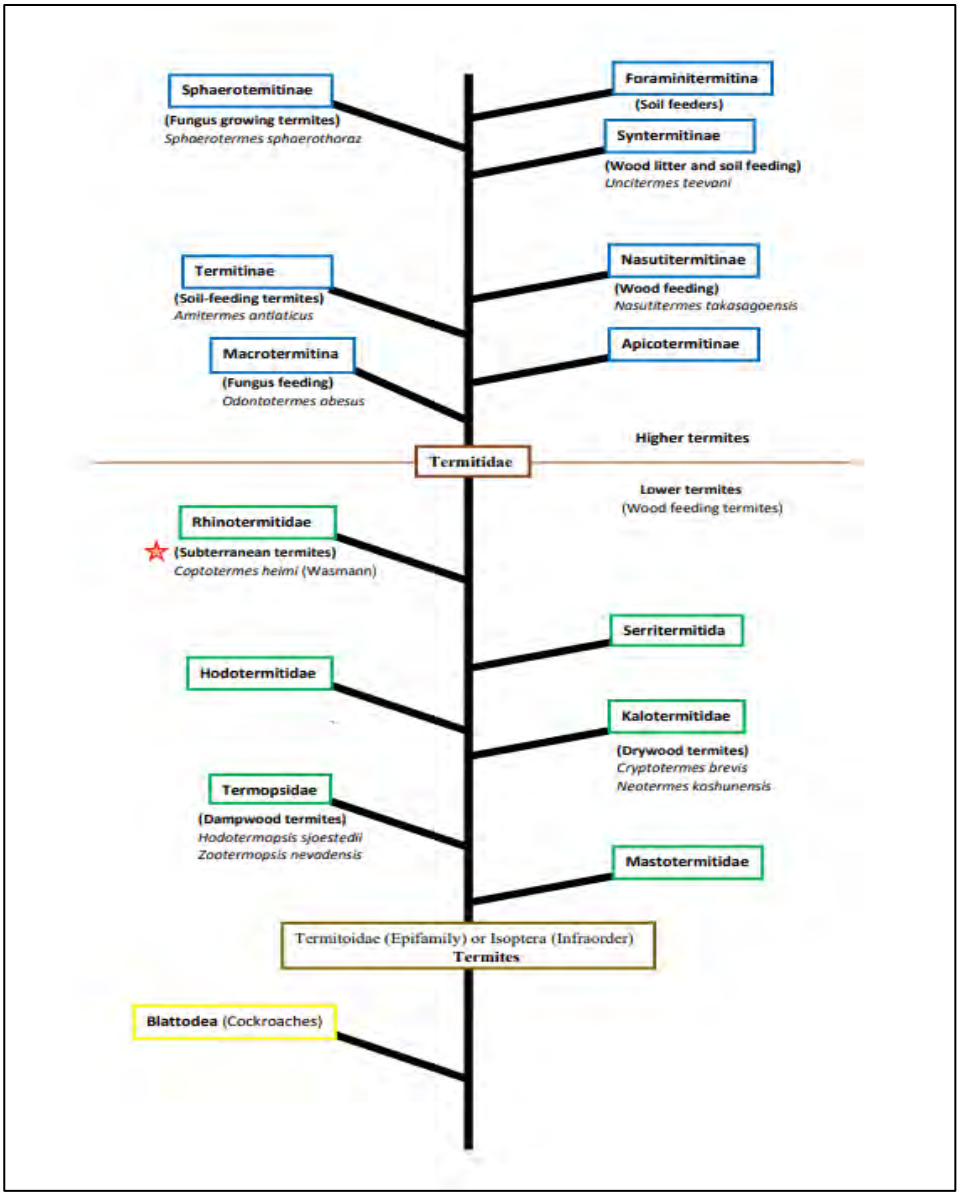

Figure 1.3: Phylogeny of lower and higher termites indicating their families and subfamilies. Asterisk indicates the species investigated in this study (Beccaloni and Eggleton, 2013)

Termites are classified based on their feeding, distribution, and requirement of moisture. They are classified as subterranean, dampwood and drywood termites. Drywood termites are efficient and well adapted to dry environment. Their cuticle is more impermeable than other termites that retain body moisture. and feed on good

drywood having little moisture content. Damp wood termites are mostly found in wood near in association to the ground though soil contact is not necessary to sustain themselves. They infest damp or decaying wood, dead trees, logs and stumps in association with decaying fungi (Pervez, 2018).

1.7.1 Subterranean Termites

Subterranean termites in the genus *Coptotermes*, family Rhinotermitidae are destructive pests which are distributed globally beyond their native range in Southeast Asia (Salunke *et al*., 2010). Subterranean termites require moisture for sustained feeding. They inhabit soil and infest moist wood (Sattar *et al*., 2013; Zukowski and Su, 2020). Subterranean termites need contact with the ground soil although Formosan subterranean termites can avoid ground contact when the moisture supply is accessible on ground (Gautam and Henderson, 2011). Subterranean termites build elongated tubes consisting of soil and their fecal materials. These elongated tubes are also called as exploratory tubes, which ensure their safety from desiccation and predatory ants. Suspended tubes contain more wood fibers in their structural composition and provide a channel from structural wood to the ground (Pervez, 2018).

Cellulose is the only nutrient source for subterranean termites and are completely dependent for its digestion on their gut symbionts. Workers directly feed on cellulose materials, accumulate food in their gut and return to other members in the colony through trophallaxis (Pervez, 2018). Wood feeding lower termites are completely dependent on their symbiotic gut microflora, composed of protozoa and bacteria for cellulose digestion (Raina *et al*., 2008). Subterranean termites are detrimental dilemma in both rural and urban areas, as these are responsible for massive destruction to plants, crops, and woods (Khanum and Javed, 2020).

Subterranean termites cause infestation in buildings, more commonly adapted to heated basements and central heating units as both these enhance their feeding activity. For establishing a new colony such termites often fly during winter. Least changes in architectural practices and use of specific materials for long time in construction

materials also increase the likelihood of termite infestations. Cellulose is the principal food of subterranean termites, obtained from plant tissues therefore wooden portion of buildings, fence posts and utility poles are highly favored sites of infestation. Various kinds of fabrics made of cotton, fiberboard and paper are also damaged by termites (Peterson, 2006).

Including building repair cost, termites impact the economy up to US$ 11 billion annually in United States. The estimated amount spent for the control of subterranean termites by the consumers were recorded US$ 1.5 billion in 1993 and increased to US$ 2.2 billion in 1999. These estimations did not include the repair cost of damaged buildings. Termite control sector in the United States is considered to account for 50% shares of the global market, the global economic impact by subterranean termites can cost US$ 22 billion annually (Su, 2002). Subterranean termites are serious pests of woods causing over US$ 2 billion in damage and control annually (Raina *et al*., 2003).

Coptotermes heimi (Wasmann) is one of the most common major structural pests of woods in Pakistan. *C. heimi* is recognized as the most destructive species of termites in Lahore city, Pakistan. It is also responsible for damaging clothes, paper and any cellulose containing substance. Detrimental infestations of *C. heimi* in standing trees have been recorded in Pakistan. *Populus deltoides* and *Mangifera indica* are the most whereas *Dalbergia sissoo* and *Cedrus deodara* are the least preferred wood species by *C. heimi* in Pakistan (Dugal *et al*., 2015). Termitidae is the largest and economically the most important family of termites. It grows fungus and is serious pest of agriculture and forestry in Pakistan. Genus *Odontotermes and Microtermes* of termitidae are reported as major pest of agriculture in Punjab, Pakistan (Ahmad and Qasim, 2011).Wood feeding preference of *C. heimi* has shown that *Populus euramericana* is the most susceptible and *Syzygium cumini* is the least susceptible to infestation in Pakistan. Other wood species that are usually damaged by *C. heimi* include *Ailanthus excelsa*, *Morus alba*, *Pinus roxburghii*, *Cassia fistula*, *Eucalyptus camaldulensis*, *Bambusa bamboo*, *Azadirachta indica* (Rasib and Ashraf, 2014). Subterranean termites are destructive pests of

sugarcane, wheat, maize, cotton, rice, sorghum, groundnut, and gram in Pakistan (Ahmad *et al*., 2011).

1.8 Control of Termites

1.8.1 Chemical Control

Different management practices are used for termite control that include physical barriers, baiting systems, cultural control, queen removal, dusting, natural products, and biological control. In recent past, termite control was mainly based on chemical approaches, especially on synthetic insecticides. Insecticides like triazophos, chlorpyrifos, fipronil, cypermethrin, imidacloprid, thiodan, heptachlor, carbosulfan are still commonly recommended (Ahmad *et al*., 2006). In 1998 the ban on chlordane has caused the pest control programmers to opt for pyrethroids, chlorpyrifos based products and organophosphates, as these are low in cost. In Malaysia, Arsenic trioxide is still used for dusting although it is a non-registered insecticide (Lee *et al*., 2018).

Most of synthetic insecticides kill the target pests by affecting four different target sites: nervous system, respiration, hormonal secretions, and cuticle formation (Khalid *et al*., 2022). Field trials conducted by the University of Hawaii for last 20 years have shown that the pyrethroid insecticide permethrin is long lasting soil insecticide. In recent years less repellent pyrethroid insecticides imidacloprid and fipronil are also introduced (Grace *et al*., 2002; Sapkota *et al*., 2020). Farmers in Pakistan most commonly use conventional commercially available synthetic insecticides such as organochlorines, organophosphates, pyrethroids and carbamates for eradication of subterranean termites (Akbar *et al*., 2019).

Fipronil is a phenylpyrazole insecticide that causes the blockage of gamma aminobutyric acid gated chloride channels of insect nervous system. It is very effective, slowly degrading broad spectrum insecticide with highly toxic and persistent end product than parent compound. It has been registered and in use by Australian Plague Locust Commission (APLC) since 2000 (Steinbauer and Peveling, 2011).

Imidacloprid is a neonicotinnodis (the most popular insecticides of today) insecticide which is frequently used for insect control especially carpenter ants and termite in preservation of wood. It emulates nicotine which is found naturally in many plants and acts as a neurotoxin for most of the insects. Currently it is licensed and safe for use in United States and many other countries for pest control (Hadi *et al*., 2020).

Chlorpyrifos (CPF) has been known to be effective against termites and earthworms (Silva *et al*., 2010). CPF has short term inhibitory effects on soil micro and macro fauna. Persistency of CPF is up to 2 months (Gilani *et al*., 2010).

1.8.2 Disadvantages of Chemical Control

Irrational, indiscriminate, and inadequate use of synthetic pesticides have negatively affected soil, groundwater quality, wildlife, human health, and agroecosystems (Khan *et al*., 2016). Lack of professional services and expert licensed workers in chemical control of termite is the primary problem (Lee, 2002). Residues of soil insecticides for many decades is highly toxic and hazardous to human health and environment, as has been observed in Dichloro-diphenyl-trichloro-ethane (DDT), chlordane and heptachlor treated soils (Sapkota *et al*., 2020).

Due to irrational and extensive use of non-biodegradable, synthetic and persistent insecticides many insects including termites have developed resistance against these insecticides. Although without chemical insecticides, it is almost impossible to achieve the desired eradication, yet sole dependency on these synthetic insecticides has been creating many issues including insect pest resistance, biodiversity eradication, environmental contamination, and secondary pest outbreaks (Akbar *et al*., 2019).

Carbaryl (Carbamate) synthesis is bridged to the biggest man-made disaster that took place in 1984, Bhopal, India. At this explosion the Union Carbide Corporation Plant (UCCP) released hazardous and highly toxic methyl isocyanate causing death of approximately 3800 people in one day. Neonicotinoids not only affect pest but also the pollinators specifically honeybees (Oberemok *et al*., 2015). Today numerous banned chlorinated hydrocarbon insecticides are still used for termite control, and it is need of the

day to shift towards alternative tools for termite control in natural habitats to reduce the use of chemical insecticides (Khanum and Javed, 2020).

1.8.3 Ecofriendly and Biorational Approaches for Termite Control

Phytophagous insects recognize their host plants using plant volatiles and use of essential oils and extracts are used as nonhost volatile emission to repel insects as alternative control approach (Abdullah *et al*., 2015). Ancient people first used botanical extracts as insecticides in China and then in Persia and Europe against mosquitoes, bedbugs, cockroaches and flies (Oberemok *et al*., 2015). Plants based insecticides can be recommended as ecofriendly and sustainable strategy in pest management. Plants extracts and essential oils are effective and efficient suitable alternatives of synthetic insecticides (Talukdar, 2006). Essential oils of *Allium sativum* and *Citrus aurantium* have shown effective suppression on subterranean termites under field conditions (Owusu *et al*., 2008).

Extracts of *Citrus aurantium* and *Allium sativum* have been proven effective against different insect pests including subterranean termites (Ajayi *et al*., 2020). Similar effectiveness of plant extracts (*Maesalan ceolata* and *Allium sativum*) and synthetic insecticide Diazinon 60 EC against termites have been observed (Ibrahim and Demisse, 2013). Entomologists are now formulating nucleic acid-based insecticides, particularly DNA insecticides. Species specific short single stranded DNA fragments are getting special attention as intellectual insecticides that can “think” prior to their action (Oberemok *et al*., 2015).

Many species of bacteria, fungi and nematodes occur naturally in soils and are have suppressive effect on termites. Entomopathogenic nematodes (EPNs) have high specificity in their host range and are compatible with many insecticides. EPNs, also known as beneficial nematodes, are environmentally safe biological control strategy. Many isolates of EPNs have been proven potential against termite pests management. Heterorhabditidae and Steinernematidae families of nematodes have been effectively used as biological control agents against termites found on soil surface and subterranean termites. (Khan *et al*., 2016).

1.8.4 Entomopathogenic Fungi

Entomopathogenic fungi are the most useful and effective against many species of termites. EPF can be applied either as direct exposure or in different methods of application such as in baits and as soil barrier. The fungal genera: *Metarhizium*, *Paecilomyces* and *Beauveria* act as pathogens of insects. Species belonging to these genera have shown promising results in commercial development of bioinsecticides (Ahmed *et al*., 2009). EPF have received more attention as potential biological control candidates against termites. *Metarhizium anisopliae* is distributed worldwide and has been isolated from approximately 200 species of insect including termites under 7 orders.

Metarhizium anisopliae is a semelparous parasite that possess an obligate killing mechanism at individual level. *M. anisopliae* first kill a termite, produce conidia, and then disperse among other members of the colony. This fungus exhibits multilevel virulence depending on the organizational level of the host (Chouvenc and Su, 2012). *Metarhizium anisopliae* has been utilized worldwide as biocontrol agent against insects and has shown promising results in field trails (Hao *et al*., 2021). *Beauveria bassiana* is environment friendly biopesticides used against a broad range of agricultural pests and insects of human and animal disease vectors, such as mosquitoes and ticks. *B. bassiana* has been studied as model system for investigation of host-pathogen interactions in biological control of insect pests (Zhang *et al*., 2011).

In the availability of an appropriate substrate and optimum environmental conditions, the spores of EPF get adhered to the surface of insect, followed by germination and formation of conidia. The appressorium penetrate the haemocoel and causes destruction of tissues leading to death of the host. The spores emerge on the surface of the host body and disperse into environment for further propagation (Bava *et al*., 2022).

Conditions like a restricted area and high relative humidity inside the subterranean termite colony enhance the survival and growth of entomopathogenic fungi. Factors like temperature required for optimal growth (usually above 30 ˚C), ease of cost in mass production, non-hazardous for humans and animals, spores longevity, robust spores

formation, easy formulation, and storage, are considered and probed before an isolate could be applied in the field (Khaenje, 2014).

Fusarium species are pathogenic to *Galleria mellonella* Linnaeus. Approximately 15 isolates of *Fusarium* have been reported from insects. *Fusarium solani* and *Fusarium oxysporum* have reported to caused 100% mortalities of insect larvae (Sharma and Marques, 2018). *F. solani*, *F. proliferatum*, *F. oxysporum*, *F. equiseti* and, *F. semitectum* are the most frequently reported entomopathogenic. *Fusarium* species are also associated with amphibians, reptiles, spiders, and most abundantly with insects (Santos *et al*., 2020).

Three endogenous strains of *M. anisopliae* were reported from Faisalabad, Murree and Gujranwala, Pakistan, collected from termites have shown significant and detrimental effects on the *C. heimi* workers (Ahmed *et al*., 2009). Presence of fungi, toxicity of their spores and degree of their decay are important factors affecting wood feeding by termites (Peralta *et al.,* 2004). Different combinations of sublethal concentrations of fipronil insecticide and *Fusarium solani* have shown suppressive effects on termites behavior and resulted in high mortality against building infesting termites (Afzal *et al*., 2018).

Naturally occurring entomopathogenic fungi in the soil and insect cadavers are used as biopesticides against many insect pests in combination with different commercial synthetic insecticides. Their synergistic effect not only increase their effectiveness, but also degrade in short period of time and are not persistent in the environment (Bakaruddin *et al*., 2018). Hence keeping in view, the synergism of naturally occurring entomopathogenic fungi and synthetic insecticides against different insect pests, the locally isolated endogenous stains of entomopathogenic fungi and synthetic insecticides in Islamabad, Pakistan were assessed in different combinations against the wood feeding termite species *Coptotermes heimi* (Wasmann).

Aim and Objectives

The aim of this study was to assess the synergistic effect of naturally occurring entomopathogenic fungi and commercially available insecticides on *Coptotermes heimi* (Wasmann) (Blattodea: Rhinotermitidae).

This study has been carried out with the following main objectives:

- Isolation of endogenous strains of entomopathogenic fungi by using microscopic and molecular identification techniques from insect cadavers and rhizospheric soil in Islamabad, Pakistan.
- Evaluation of individual potentials of commercially available insecticides and entomopathogenic fungi.
- Evaluation of synergistic effect of entomopathogenic fungi and commercially available insecticides against *Coptotermes heimi* (Wasmann).

MATERIALS AND METHODS

2.1 Study Area

This study was conducted in Islamabad, Pakistan's capital territory, located in the northwest of the country, close to the district Rawalpindi of Punjab province. It has latitudes 33° 49ˈand longitudes 72° 24ˈ east of Greenwich. The altitude of Islamabad ranges from 500 to 600 meters from sea level. The total area of Islamabad is 906.50 square kilometers. It is divided into urban area of 220.15 km^2, rural area of 466.20 km^2 and parks of 220.15 km^2. According to 2017 census the total population of Islamabad is 2003368 (CDA, Islamabad). It has humid subtropical climate with hot, humid summer followed by monsoon season and cool winter. The average minimum temperature is 2 ˚C in January while average maximum temperature is 46.1 ˚C in June. Average rainfall is 1143 millimeters yearly with 55% humidity (Pakistan Meteorological Department 2023).

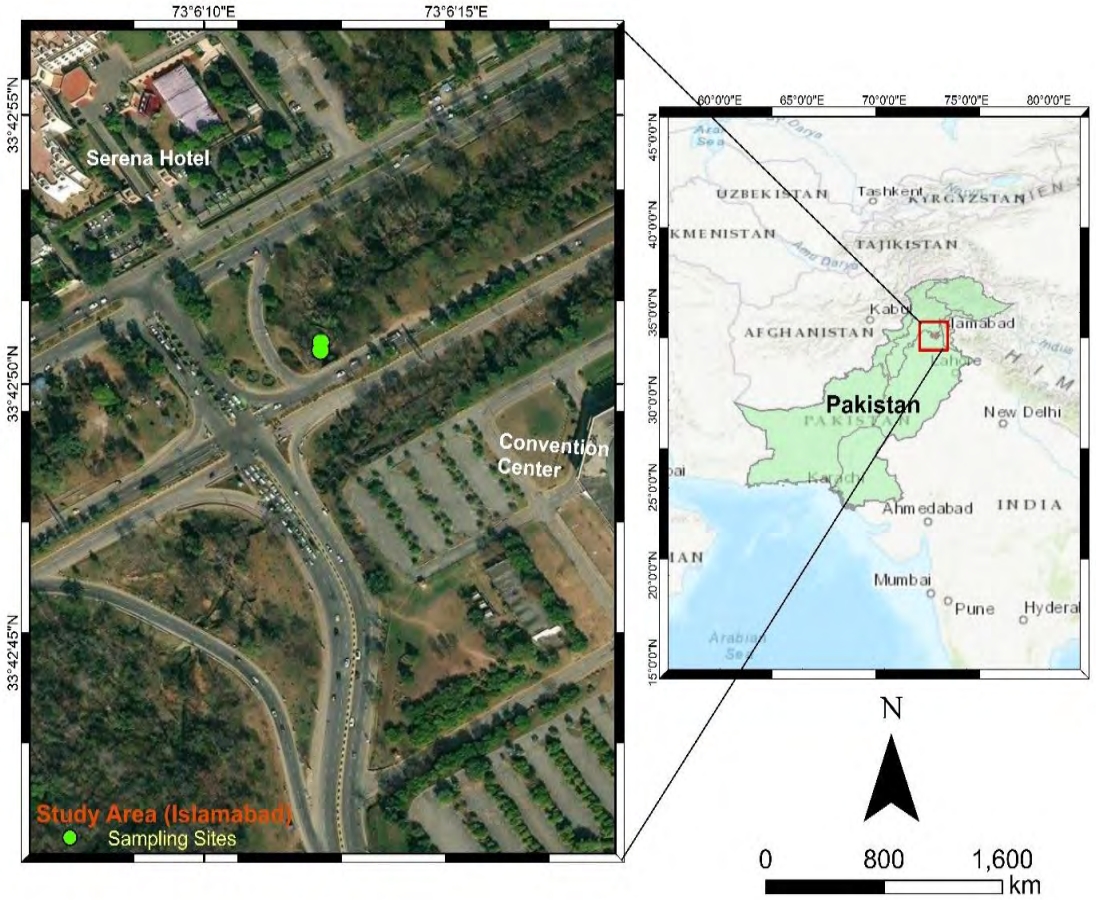

Figure 2.1: Map indicating sample collection sites of *Coptotermes heimi* (Islamabad)

2.2 Termites Collection

Termites were collected from infested Poplar trees (*Populus angustifolia*) located beside water channel in front of Serena hotel Islamabad during monsoon season (July to September) using bait methodology described by (Gautam and Henderson, 2011) with slight modifications. Plastic bottles (PVC) were used as baits with holes at base and lateral sides. Wet tissues and card boards were placed inside the bottles along with sugar cane molasses as phagostimulant to attract termites into the baits. These baits were regularly checked at interval of 14 days for termites. Workers and soldiers were collected into petri dishes having wet tissues and brought to the Parasitology and Entomology laboratory, department of Zoology Quaid-i-Azam University Islamabad Pakistan. Workers and soldiers of collected termites were identified microscopically as *Coptotermes heimi* using key of (Maiti, 2016).

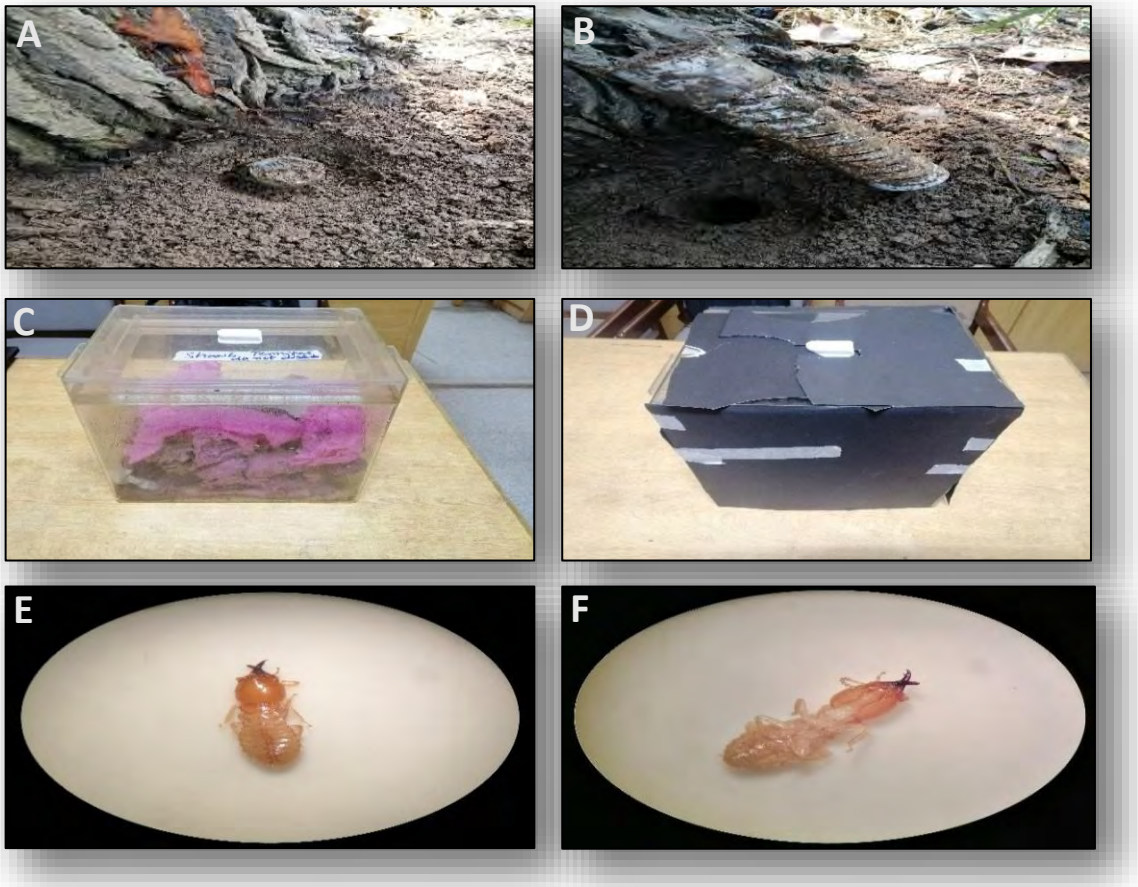

Figure 2.2: Termites collection from sampling sites. E and F show Soldier of *C. heimi*

As mentioned by Manzoor and Mir (2010) termites were kept in plastic container under favorable environmental conditions (70-80% relative humidity, dark area, 26-28 °C), as shown in the figure (Figure 2.2).

2.3 Insecticides

Three commercially formulated insecticides, Fipronil (Urgent; 5% SC W/V) ((RS)-5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)-1H- pyrazole-3-carbonitrile), Imidacloprid (Mirage; 5% SC) (1-(6-chloro-3- pyridyl methyl)-N-nitroimidazolidin-2-ylideneamine) and Chlorpyrifos (Duraflex; 48% W/V) (0,0-diethyl 0-(3,5,6-trichloro-2-pyridinyl)- phosphorothioate) were obtained from The Green Impex Corporation (Markaz F8; Islamabad, Pakistan) (Bonmatin *et al*., 2015).

2.3.1 Insecticides Stock Solutions and Sublethal Concentrations Preparation

For each insecticide, 100 ml stock solution of 100 ppm was prepared in distilled water using equation (A). Sublethal concentrations of 0, 10, 15 and 25 ppm were prepared in distilled water, from the stock solution for each insecticide using equation (B) (Zhu *et al.,* 2017).

Equation (A)

$$\text{As } 1\ ppm\ (V/V) = 1\ \mu l\ of\ solute\ /\ 1\ L\ of\ solvent$$

$$100\ ppm\ stock\ solution\ of\ toxicant = 100\ \mu l\ of\ insecticide\ /\ 1\ L\ dH_20$$

$$Or\ 10\ \mu l\ /\ 100\ ml\ dH_20$$

Equation (B)

$$\text{As } C_1V_1 = C_2V_2 \qquad V_1 = \frac{C_2V_2}{C_1}$$

Whereas:

C_1 is the concentration of the stock solution already prepared (100 ppm)

V_1 is the volume of stock solution, required to be dissolved in V_2

C_2 is the required concentration of sublethal concentration

V_2 is the required volume of sublethal concentration

Figure 2.3: Insecticides stock solutions and sub lethal concentrations

2.4 Isolation and Identification of Entomopathogenic Fungi

The entomopathogenic fungi were isolated from dead insect cadavers and soil using insect baiting methodology described by (Zimmermann, 1986; Barra *et al*., 2013) with few modifications.

2.4.1 Soil Sampling and Insect Collection

Because soil contains many species of entomopathogenic fungi in natural environment, soil samples were collected from Quaid-i-Azam University Islamabad, Pakistan, from rhizospheric areas associated with microbial diversity. All soil samples were thoroughly mixed and sieved. Out of 1kg soil stock, 10g of soil was dissolved in 100 ml of distilled water to make stock solution. Ten folds serial dilutions were prepared from the stock solutions. Whatmann No.1 filter papers were soaked in each diluted solution and placed in petri dishes. Different insects were collected in cyanide jar. After one day these killed insects were washed with ethanol and placed in petri dishes (Vivekanandhan *et al.,* 2020).

2.4.2 Culturing of Entomopathogenic Fungi

After two weeks spores were induced by fungi on dead insect cadavers in soil treated filter papers in petri dishes. The suspected EPF on insect cadavers were suspended in 1% aqueous solution of Tween 80 (Sigma-Aldrich Steinhein®, Germany) and vortexed

to avoid the clumping of spores and mycelia. Ten folds serial dilutions were performed with this stock solution. 5 µl of each dilution was inoculated on PDA medium and incubated at 26 ˚C for 3-4 days. Mix colonies were appeared on the medium. Each colony were picked and reinoculated on newly prepared PDA medium 3-4 times to get purified colonies of fungi (Liu *et al.,* 2022).

2.4.3 Morphological Identification of Pure Fungal Colonies

Morphology of each isolated fungi was studied under compound microscope and key of Lacey (2012) was used for identification. Hyphae structures (septate or non-septate), reproductive structures of sporangia and conidia were observed (James and Natalie, 2001). Microscopic slides (1.2 mm thick and 25 mm length×76 mm width) were prepared by dropping 2 drops of LPCB stain on the slide and then mixing young fungal mycelia from margins, with it (Njovu *et al*., 2021). A cover slip was carefully placed on it to avoid the formation of air bubbles. Each microscopic slide was observed at 40X and 100X with immersion oil under binocular microscope.

2.4.4 DNA Extraction and Molecular Identification of Pure Fungal Colonies

2.4.4.1 Solutions Preparation

1. CTAB Buffer

CTAB buffer was prepared by taking 7.44 gm sodium EDTA, 15.76 gm Tris-HCl, 81.82 gm NaCl and dissolved these in 1000 ml of distilled water. CTAB (2%) was then dissolved by heating up to 60 ˚C. Beta-mercaptoethanol (0.1%) and PVP (1%) were then added. And pH of final solution was adjusted to 8.0.

2. Chloroform: Isoamyl Alcohol 24:1 (V/V)

24 ml of chloroform were mixed with 1 ml of isoamyl alcohol.

3. Sodium Chloride

5 molar NaCl solution was prepared by adding 292.2 gm of NaCl to 1 liter of dH_2O.

4. TE Buffer

Tris (1 M) and EDTA (0.5 M) were dissolved in 100 ml distilled water. The pH of the solution adjusted to 8.0.

2.4.4.2 DNA Isolation Protocol

DNA of each fungal colony was extracted using protocol of (Iqbal *et al*.,2013) with slight modifications. Fungal mycelial biomass of each purified culture was collected in labelled 1.5 ml Eppendorf tubes and treated with liquid nitrogen for 15 seconds. Sterilized micro pestles were used for mechanical grinding of mycelium. Then 1000 µl of CTAB buffer, 10 µl of β-mercaptoethanol (0.1%), 20 µl of PVP (1%) and 2 µl Proteinase K were added to the digested mycelium in Eppendorf tube and vortexed carefully for 3 minutes, followed by incubation in water bath at 65 ˚C for 2 hours. After incubation samples were cooled down at room temperature for 5 minutes. 500 µl of CI (24:1) was added and mixed for 2-3 minutes. Centrifugation at 12000 rpm was performed for 10 minutes and supernatant was transferred to new Eppendorf tubes with same labels as previous one. Afterwards 500 µl chilled isopropanol followed by addition of 100 µl of NaCl (5 M) to the supernatant. This mixture was centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded, leaving a pellet at the bottom. 500 µl of ethanol (70%) was added to wash the pellet and centrifuged at 12000 rpm for 10 minutes. The pellet was allowed to air dried. After drying 50 µl of TE buffer was added to dissolve the DNA completely. The quantity and quality of DNA were better for further amplification.

2.4.4.2.1 Polymerase Chain Reaction (PCR)

PCR was performed using 20 µl of total PCR recipe containing 12.66 µl of sterile double distilled water, 4 µl Green Master mix, 0.67 µl of each forward ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse ITS4 (5′-TCCTCCGCTTATTGATATG C-3′) primer and 2 µl DNA sample. Gradient PCR thermal cycler (Kyratec®, Australia) were used for performing the reaction with following cyclic conditions, initial denaturation at 95 ˚C for 5 minutes, 35 cycles of denaturation at 95 ˚C for 30 seconds, annealing at 55 ˚C for 45 seconds, extension at 72 ˚C for 2 minutes, final extension at 72 ˚C for 10 minutes and 4 ˚C for hold on temperature (Bhatt *et al*., 2020).

2.4.4.2.2 Agarose Gel Electrophoresis

For quantification and purity of isolated DNA, agarose gel (1%) was prepared by dissolving 0.5 gm of agarose powder in 50 ml of TBE buffer. The mixture was heated at 100 ˚C for 2 minutes in microwave oven. Afterwards 3 µl of EtBr was added to the gel and casted in gel tray with comb. Gel was allowed to solidify for 15 minutes and then placed in gel tank, having 1X TBE. 2 µl DNA of each sample was mixed with 2 µl of loading dye and loaded into wells of the comb. Gel was run for 30 minutes at 80 volts. Gel was observed under UV in gel documentation system. DNA concentration was estimated by Nanodrop® and samples were sent for sequencing commercially. The phylogenetic tree was constructed by MEGA X and consensus of sequences were submitted to GenBank NCBI (Afzal *et al*., 2018).

2.5 Conidial Suspensions Preparation

Conidial suspensions of each fungus were prepared from 14 to 16 days old cultures of pure colony. Surface culture was scraped with a sterilized scapula and 1 gm of this culture was weighed on digital balance. After weighing this 1 gm hypae was collected in 5 ml (1%) stock solution of Tween-80. Smaller volume of suspension (5 µl) was taken from the stock solution and loaded on hemocytometer under the cover slip, using a pipette. Spores were counter in the four large corner squares and the central square, using the following equations:

A. $Percentage\ of\ viable\ spores\ = {}^{Number\ of\ viable\ spores}/_{Number\ of\ total\ spore} \times 100$

B. $Average\ number\ of\ spores/square\ =\ total\ number\ of\ viable\ spores/\ total\ number\ of\ squares$

C. $Dilution\ factor\ =\ final\ volume/\ volume\ of\ cells$

D. $Viable\ cells/ml\ =\ average\ number\ of\ cells/square\ \times\ dilution\ factor\ \times 104$

Conidial concentrations of 0, 1×10^3, 1×10^6 and 1×10^9 conidia/ml were prepared of each fungus. Viability of prepared concentrations was determined by plate count technique on PDA medium.

2.6 Compatibility Bioassay

Fungal spores germination, colony growth and spores formation were evaluated to investigate the fungistatic effect of insecticides. The conidial concentrations were inoculated on PDA media treated with insecticides concentrations of 10, 15 and 25 ppm of each insecticide and incubated at 26 ˚C for 24 hours. Spores germination was assessed by staining PDA medium with lactophenol cotton blue dye (LPCB) under microscope at 100X. Fungistatic effect on growth of the colony was measured by inoculating 5 to 10 mm mycelia on the center of PDA media treated with insecticidal concentrations in triplicates and relative increase in sizes were noted. Spores formation was determined by scrapping the cultures of fungi and suspended in 1% Tween-80. Solution were vortexed for 10 minutes, filtered and number of conidia were counted using hemocytometer under the microscope.

2.7 No-Choice Feeding Virulence Bioassay

In no-choice feeding virulence bioassay three different relative concentrations (10, 15, 25 ppm) with one control group (distilled water) were prepared from each of the three selected insecticides in triplicates (a, b, c). Similarly, three different conidial concentrations (1×10^3, 1×10^6, 1×10^9 conidia/ml 1% Tween-80) with one control group (distilled water) were prepared from each of four selected fungi in triplicates (a, b, c). Whatmann filter paper was used as substrate in petri dish, treated with one ml of each relative concentrations in insecticidal groups. In fungal treated groups, filter paper was treated with relative conidial concentrations (Ismayati *et al*., 2016).

For synergistic effect, highest concentrations of insecticides and conidia were selected in triplicates. Filter paper was 1st treated with 1 ml of 25 ppm of selected insecticide and allowed to air dry in flow hood for 30 minutes. The same filter paper was then treated with 1×10^9 conidia/ml of selected fungus. Each petri dish contained 30 termites and were sealed with parafilm. All petri dishes were stored in dark room at 26 ˚C, RH 80%. Distilled water were added to control and treated filter papers to maintain the required moisture contents. Average number of dead termites were recorded in each group of termiticide and fungi at 4th, 8^{th,} and 12th day of treatment. Average percentage

mortality and standard deviation were calculated for each group using the following equation (Ismayati *et al*., 2016). Percentage mortality = ODP/ TP ×100, Whereas. ODP is observed dead population of workers; TP is total population of workers. Feeding activity was observed by the reduction in weight of filter paper, using the following equation. Weight consumed by termites = Pre-weight of filter paper – post-weight of filter paper

2.8 Statistical Analysis

Experiments were performed in three replicates. The data was collected from triplicates of each group and pooled into MS-Excel (Microsoft 365) for calculation of percentage average mortality and standard deviation. As described by Gautam and Henderson (2011) a two-way ANOVA statistical test ($P \leq 0.05$) was performed to analyze the variance of percentage mortality among different concentrations of treatments for compatibility bioassay and synergistic effects using general linear model in Minitab® software (Version 21.3.1).

RESULTS

3.1 Isolation and Identification of Entomopathogenic fungi

3.1.1 Morphological and Microscopic Identification of Entomopathogenic Fungi

Abundant growth of fungal mycelia was observed after the inoculation of serial dilutions on PDA media. Pure colonies were isolated after repeated subculturing of the colonies and morphology was observed. Mycelia on the ariel side and back side of petri plates were observed. Colour of each pure colonies were noted, and morphological features like structure of hyphae and shape of conidia were observed as described by (Correll *et al*., 1986). These isolates were identified to be *Fusarium falciforme* (Figure. 3.1 A), *Fusarium solani* (Figure 3.1 B), *Aspergillus terreus* (Figure 3.1 C) and *Penicillium citrinum* (Figure 3.1 D).

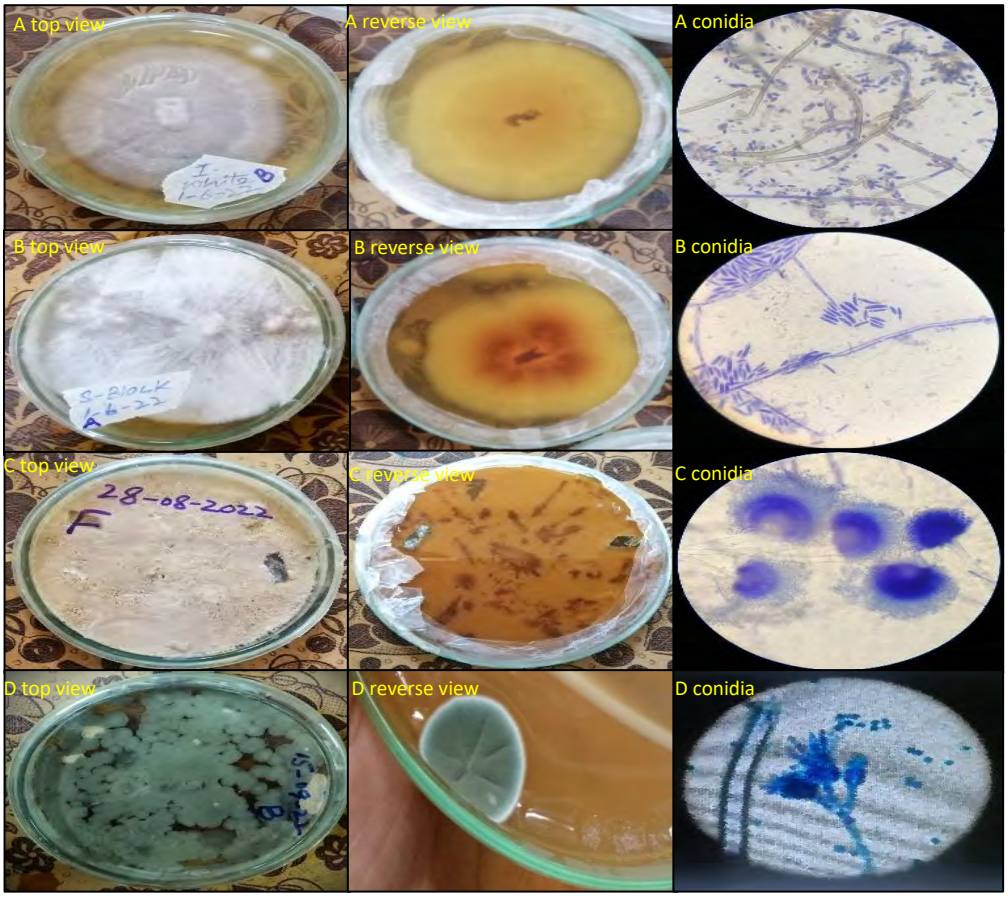

Figure 3.1: Fungal isolates on PDA media

3.1.2: Molecular Identification of Isolated Entomopathogenic Fungi

3.1.2.1: PCR Amplification of ITS-1 rRNA Gene of Fungal Isolates

The amplified PCR product of ITS-1 rRNA gene was confirmed by performing Agarose gel electrophoresis and observed under UV in Gel documentation system. The result showed fungal DNA isolated had a nucleotide sequence of 630 bp (Figure 3.2).

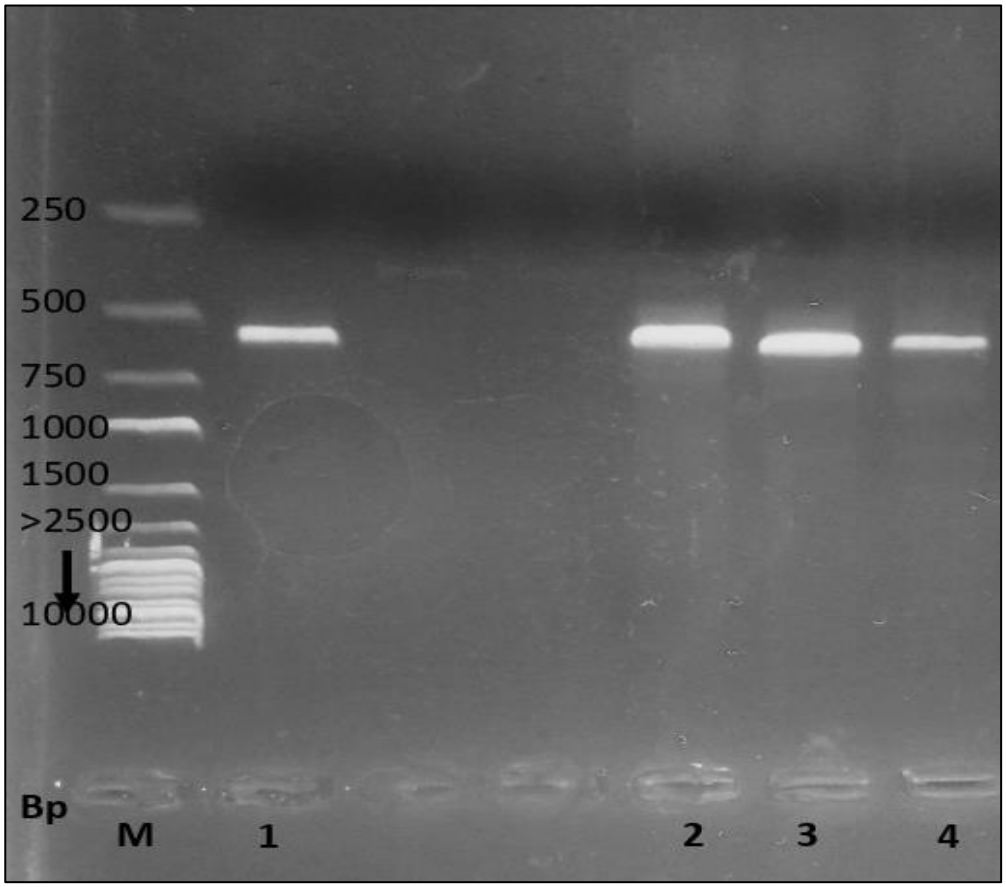

Figure 3.2: PCR amplification of the ITS-1 rRNA gene of fungi labeled as 1, 2, 3 and 4 samples (630 bp DNA bands). M is 10kbp DNA ladder marker

3.1.2.2: Phylogenetic Analysis

The PCR products were successfully sequenced commercially by Apha Genomics (Pvt) Ltd. PWD, Islamabad Pakistan using Sangar's sequencing method. The results were edited with BioEdit Sequence Alignment Editor (Version 7.2.5) software and irregular sequences were deleted at the beginning and end of sequence. The sequences were analyzed on BLAST for maximum similarities with different fungi and deposited in

NCBI GenBank database. Phylogenetic tree was constructed by neighbor-Joining method using MEGA 11 (Version 11.0.13) software. These phylogenetic analysis also confirmed the results of NCBI- BLAST for fungal isolates (Figure. 3.3).

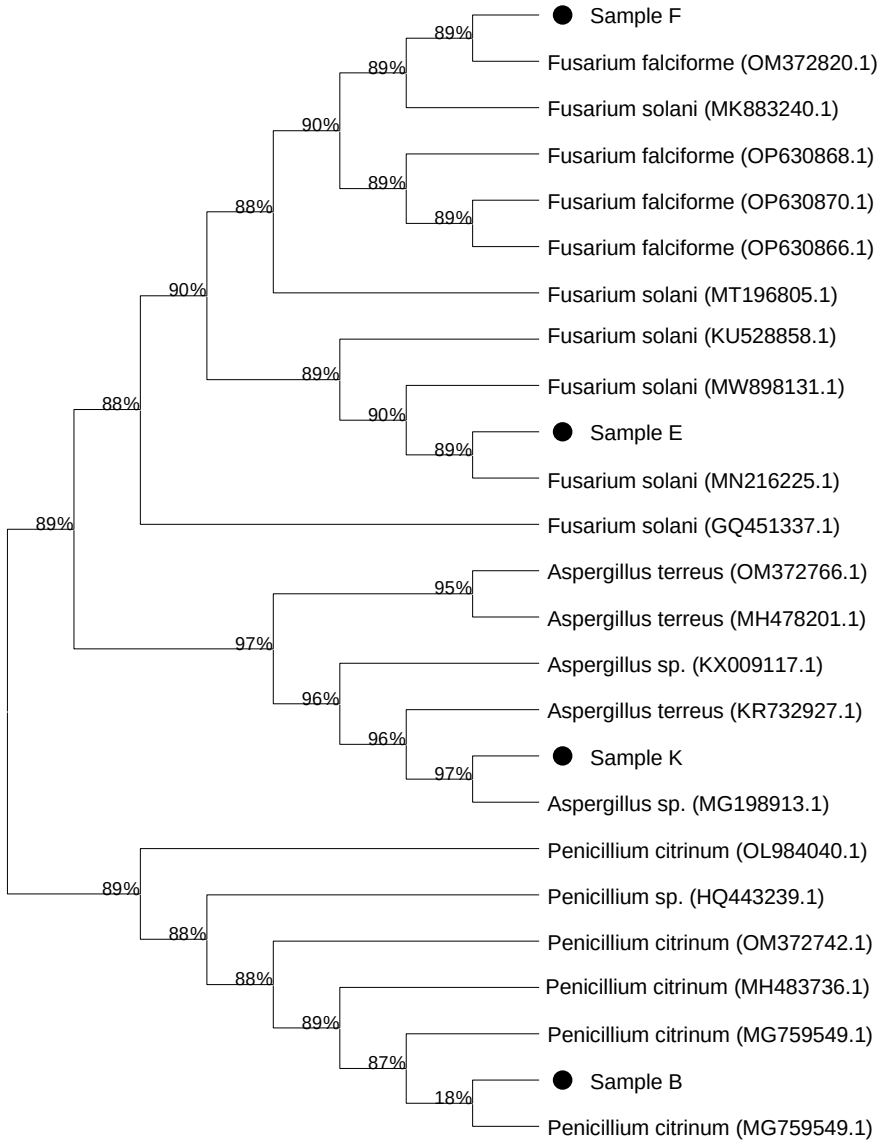

Figure 3.3: Molecular phylogenetic tree for fungal isolates (Sample F, E, K and B) by neighbor-Joining method using MEGA-11 software

3.2 Compatibility Bioassay

Spores germination was estimated after 24 hours by cutting PDA medium into small pieces and then stained with LPCB stain. The enlarged bloated conidia along with germ tubes were observed under the microscope at 100X confirming the germination of inoculated conidia. The increased relative size of colony observed for 12 days showed the growth of fungal colony (Table 3.1). Spores formations were observed by mixing mycelia of the newly grown fungal colonies in 1% Tween-80. Suspensions were observed in a hemocytometer under microscope and total number of spores were counted.

Table 3.1: Percent germination, vegetative growth, and sporulation of fungi on PDA media treated with different concentrations of insecticides. Values with different letters are significantly different (P≤0.5) according to the Tukey's LSD test.

		A Germination (%) (Mean±SD)	B Vegetative growth (mm) (Mean±SD)	C Sporulation 10^9 conidia/ml (Mean±SD)
Fipronil concentrations (ppm)	control	90.33±1.24 ^{a}	61.66±1.69^{a}	91±2.4^{a}
	10	71.66±2.05^{b}	30.33±1.24^{b}	82±0.81^{bc}
	15	65.33±1.24^{c}	28.66±1.69^{b}	77±1.24^{cd}
	25	61.33±0.94^{cd}	23.66±1.69^{cd}	68±0.94^{ef}
Imidacloprid concentrations (ppm)	control	91±2.16 ^{a}	58±0.18^{a}	89.33±0.47a
	10	70.66±0.47^{b}	29.33±2.05^{b}	85.66±2.86^{ab}
	15	65±1.41^{c}	28±0.81^{bc}	74±2.44^{de}
	25	60.66±1.24^{cd}	23.33±0.94^{d}	65.66±2.05^{f}
Chlorpyrifos concentrations (ppm)	control	92±2.16^{a}	58.33±1.24^{a}	89.33±1.24^{a}
	10	70.66±1.69^{b}	29.66±1.24^{b}	89±2.44^{a}
	15	62.66±0.47^{cd}	28.66±0.47^{b}	76.33±2.49^{cd}
	25	59.33±1.24^{d}	26±0.81^{bcd}	68±2.44^{ef}

3.2.1 Germination

Isolated fungi showed significant difference in percentage germinations of their conidia on PDA media treated with (0, 10, 15 and 25 ppm) concentrations of insecticides from control. The germinations were significantly different from each other in terms of concentrations of each insecticide whereas no significant differences were observed between the three different insecticides (Figure 3.4.). Highest percentage germination was observed at lowest concentration (10 ppm) and lowest germination at highest concentration in each insecticide treatment. In Fipronil treated group, the highest degree of percentage germination was observed in control group followed by that of lowest concentration (10 ppm). The lowest percentage of spores germination was observed at highest concentration of 25 ppm. Similarly, in the Imidacloprid treated group highest germination was observed in control, followed at 10 ppm and lowest at 25 ppm among the three concentrations. Overall lowest germination occurred at highest concentration (25 ppm) in Chlorpyrifos treated group (Table 3.1.A).

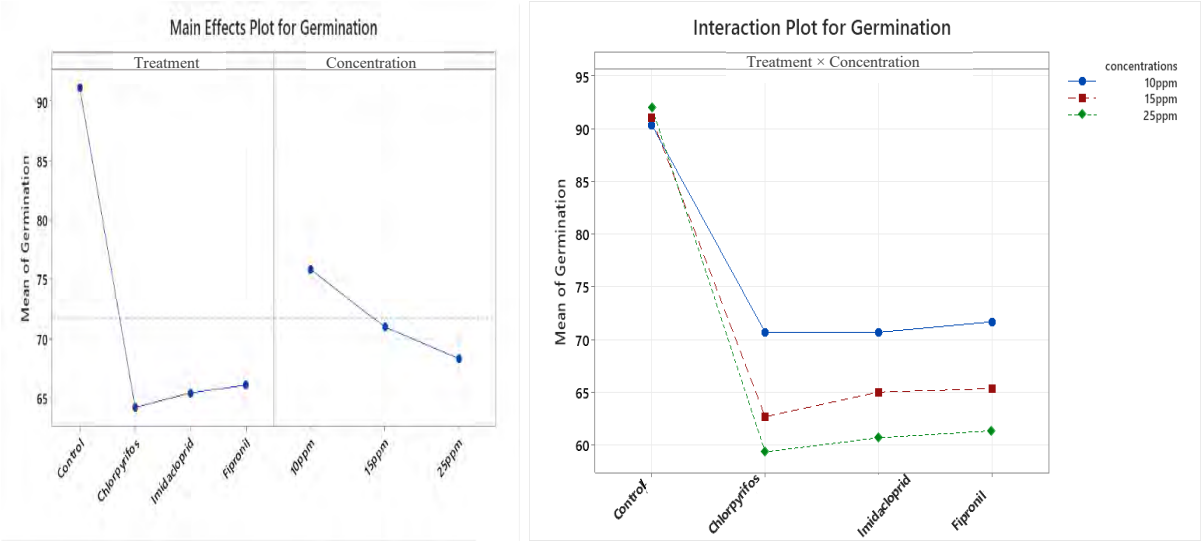

Figure 3.4: Main effect and interaction plot for Percent germination of conidia at different concentrations of insecticides

Vegetative Growth

The vegetative growth of colonies showed the same tendency. Colonies growths were significantly different for all three concentrations from the control (Figure 3.5). In Fipronil treated group highest growth (61.66±1.69 mm) occurred at control followed by minimum concentration of 10 ppm (30.33±1.24 mm). lowest colony growth was observed at maximum concentration of 25 ppm (23.66±1.69 mm). Same tendency was observed in Imidacloprid and Chlorpyrifos treated groups (Table 3.1.B).

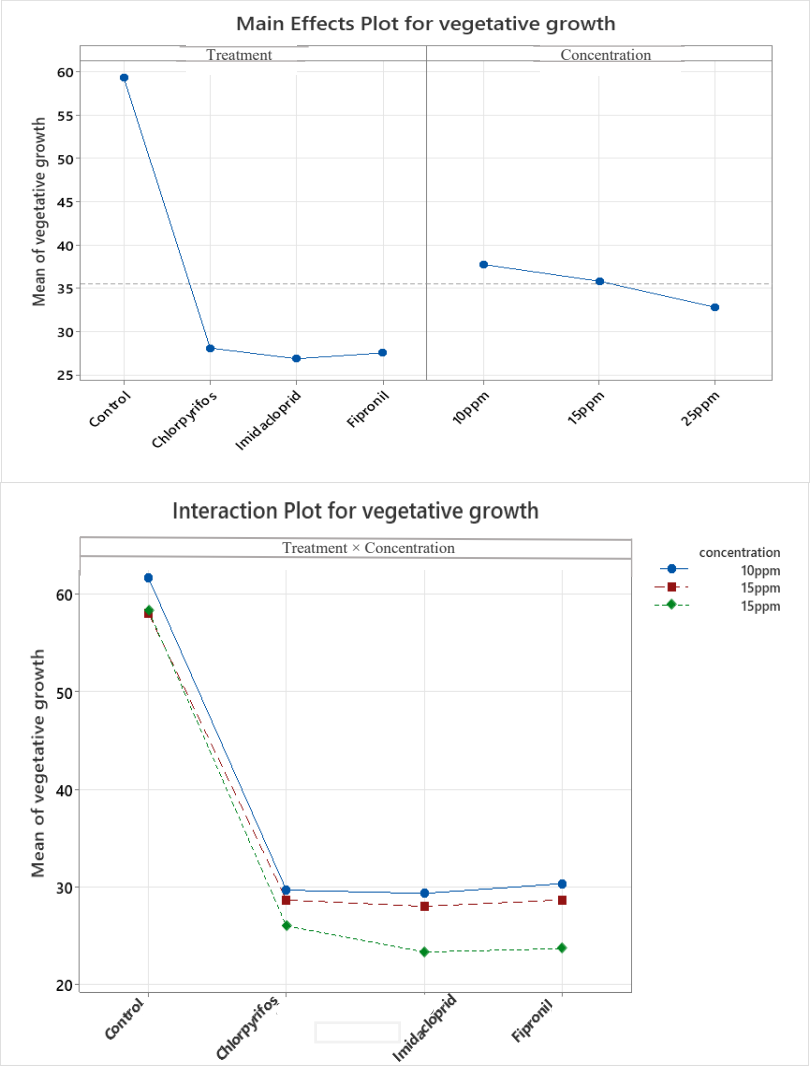

Figure 3.5: Main effect and interaction plot for Percent vegetative growth of fungal colonies at different concentrations of insecticides

Sporulation

Sporulation was not significantly affected by the insecticides. Maximum sporulation in each treated group was observed at control whereas in terms of different concentrations in each group, highest sporulation occurred at minimum dose of 10 ppm (Figure 3.6). Lowest sporulation occurred at the highest dose of 25 ppm in each insecticide group. (Table 1.3.C).

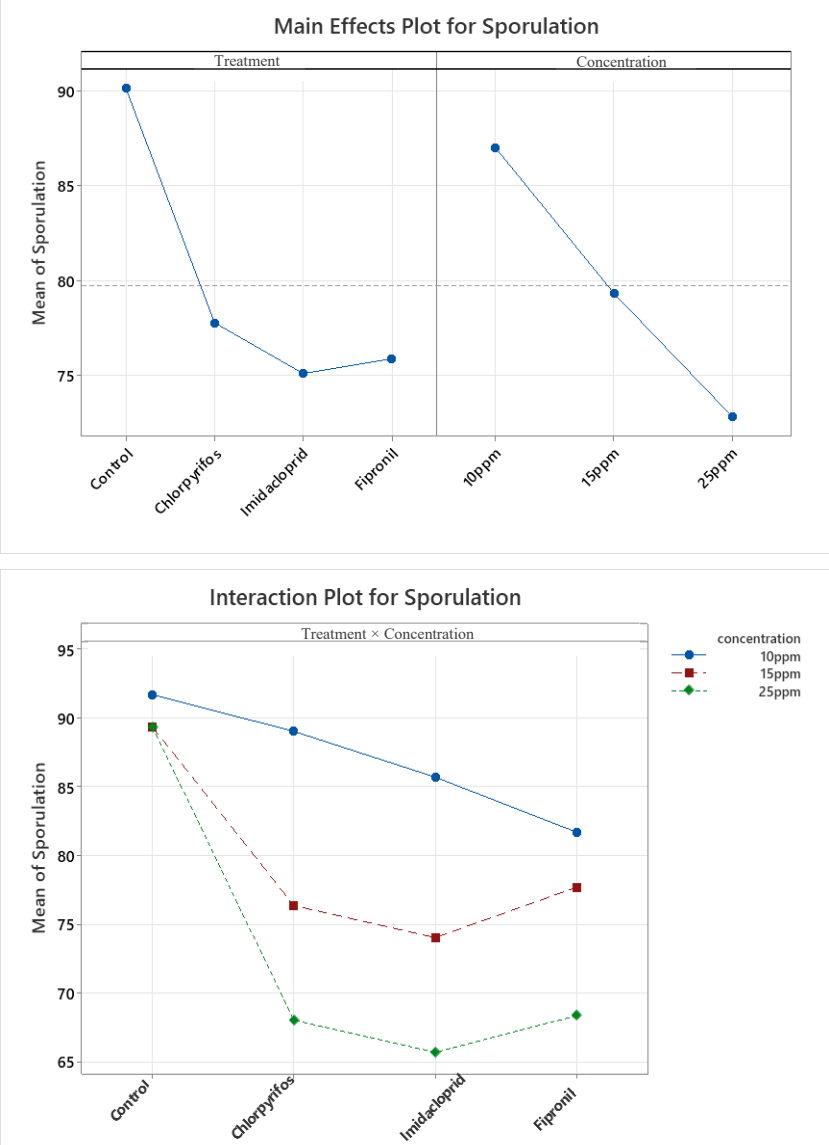

Figure 3.6: Main effect and interaction plot for Percent sporulation of conidia at different concentrations of insecticides

3.3 No-choice Feeding Virulence Bioassay.

3.3.1 Effect of Insecticides

In no choice feeding assay the effect of different concentrations of each group was significantly different from each other and also from the control. Highest mortality was caused by highest dose of 25 ppm, followed by 15 ppm and 10 ppm, different from mortality caused by the control (Table 3.7). The effect of time and treatment was significant in response to mortality. Highest concentration (25 ppm) of Fipronil caused highest mean mortality (32.22%) at 12th day. Imidacloprid followed the same tendency at highest concentration on 12th day resulting in mortality (30%) whereas Chlorpyrifos resulted in 26.66% mortality. Intermediate concentration (15 ppm) of each insecticide resulted in different percentage mortality that was increased with the passage of time from 4th day to 12th day. Lowest insecticidal concentrations were observed to cause lowest mortality (7.77, 6.66 and 5.55%) in each group that was not significantly different from each other (Table 3.2).

Table 3.2: Percent mortality (Mean±SD) of different concentrations of insecticides at different days (Tukey's LSD test with P value ≤ 0.05)

Percentage mortality±SD										
Days	**Control**	**Chlorpyrifos (ppm)**			**Imidacloprid (ppm)**			**Fipronil (ppm)**		
		10	**15**	**25**	**10**	**15**	**25**	**10**	**15**	**25**
4	5.55±1.57^{c}	7.77±1.57^{c}	11.11±1.57^{cb}	14.44±1.57^{ef}	6.66±2.72^{cb}	13.33±2.72^{cb}	17.77±1.57^{ef}	5.55±1.57^{c}	10±2.72^{cb}	13.33±2.72^{f}
8	8.88±1.57^{c}	10±2.72^{cb}	13.33±2.72^{cb}	18.88±1.57^{cd}	11.11±1.57^{cb}	14.44±4.15^{cb}	21.11±3.14^{de}	12.22±1.57^{cb}	16.66±2.72^{bc}	18.88±1.57^{def}
12	14.44±1.57^{b}	16.66±2.72^{bc}	21.11±3.14^{dc}	26.66±2.72^{bc}	17.77±3.14^{bc}	22.22±1.57^{dc}	30±5.44^{ac}	21.11±1.57^{dc}	27.77±4.15^{cd}	32.22±3.14^{a}

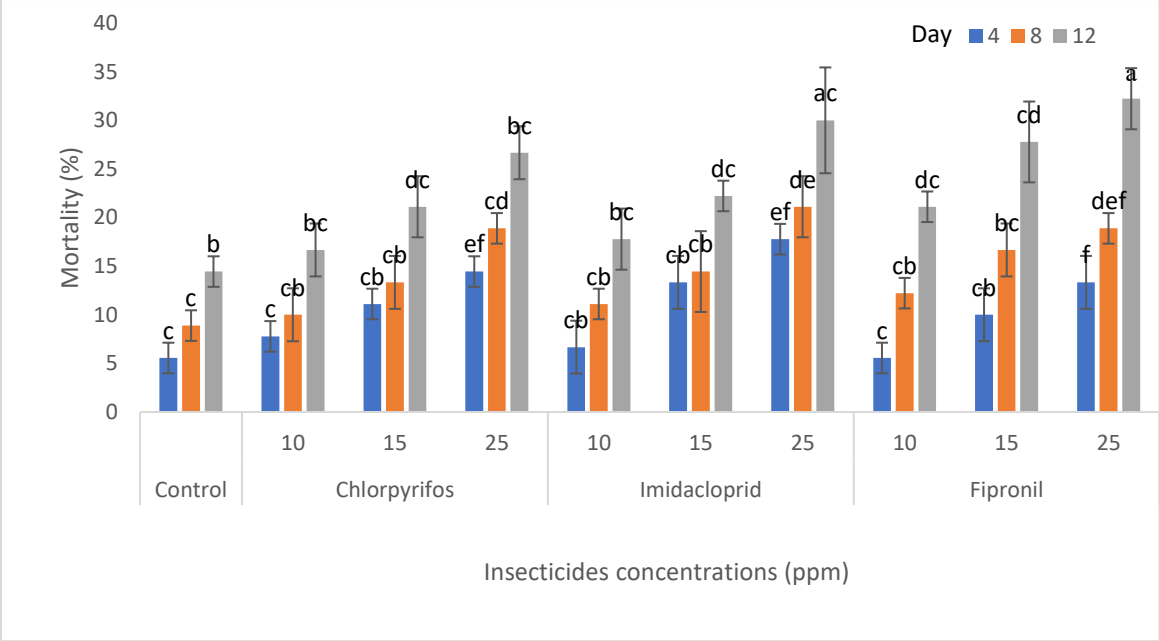

Figure 3.7: Percent mortality (Mean± SD) of different concentrations of insecticides at different days

3.3.2 Effect of Entomopathogenic Fungi

In each fungi treated group a positive increase in percentage mean mortality occurred with increase in number of conidia/ml/filter paper and number of days for treatment. Percentage mean mortality of *Fusarium solani* was highest (32.22%) at 10^9 conidia/ml/filter paper at 12^{th} day, followed by *F. falciforme* (28.88%) and significantly different from percentage mean mortality of Control (8.88%) (Figure.3.8). Highest mortality caused by *P. citrinum* (24.44%), and *A. terreus* (25.55%) were approximately the same. lowest percentage mortality (5.55%) were observed at *F. solani* and *A. terreus* conidial concentration 10^3 Conidia/ ml/filter paper at 3^{rd} day (Table 3.3).

Table 3.3: Percent mortality (Mean±SD) of different conidial concentrations of fungi at different days (Tukey's LSD test with P value ≤ 0.05).

Percentage mortality±SD													
Days	**Control**	***P. citrinum***			***A. terreus***			***F. solani***			***F. falciforme***		
		10^3 conidia/ml	**10^6 conidia/ml**	**10^9 conidia/ml**	**10^3 conidia/ml**	**10^6 conidia/ml**	**10^9 conidia/ml**	**10^3 conidia/ml**	**10^6 conidia/ml**	**10^9 conidia/ml**	**10^3 conidia/ml**	**10^6 conidia/ml**	**10^9 conidia/ml**
4	4.44 ± 1.57^{c}	5.55 ± 1.57^{c}	10 ± 2.72^{b}	15.55 ± 1.57^{f}	7.77 ± 1.57^{bc}	8.88 ± 1.57^{b}	12.2 ± 1.57^{b}	5.55 ± 1.57^{c}	11.11 ± 1.57^{b}	13.33 ± 2.72^{bf}	7.77 ± 1.57^{bc}	14.44 ± 1.57^{bf}	16.66 ± 2.72^{f}
8	6.66 ± 2.72^{c}	11.1 ± 1.57^{b}	17.77 ± 1.57^{f}	21.11 ± 1.57^{cd}	13.33 ± 2.72^{bf}	16.66 ± 2.72^{f}	18.8 ± 1.57^{f}	12.22 ± 3.14^{b}	16.66 ± 2.72^{f}	18.88 ± 4.15^{f}	15.55 ± 1.57^{f}	18.88 ± 1.57^{f}	20 ± 2.72^{cd}
12	8.88 ± 1.57^{b}	18.88 ± 1.57^{f}	22.22 ± 1.57^{cd}	24.44 ± 1.57^{ab}	17.77 ± 1.57^{f}	24.44 ± 4.15^{de}	25.55 ± 1.57^{bd}	14.44 ± 1.57^{ef}	21.11 ± 1.57^{bc}	32.22 ± 1.57^{a}	21.11 ± 1.57^{ef}	23.33 ± 2.72^{bcd}	28.88 ± 1.57^{a}

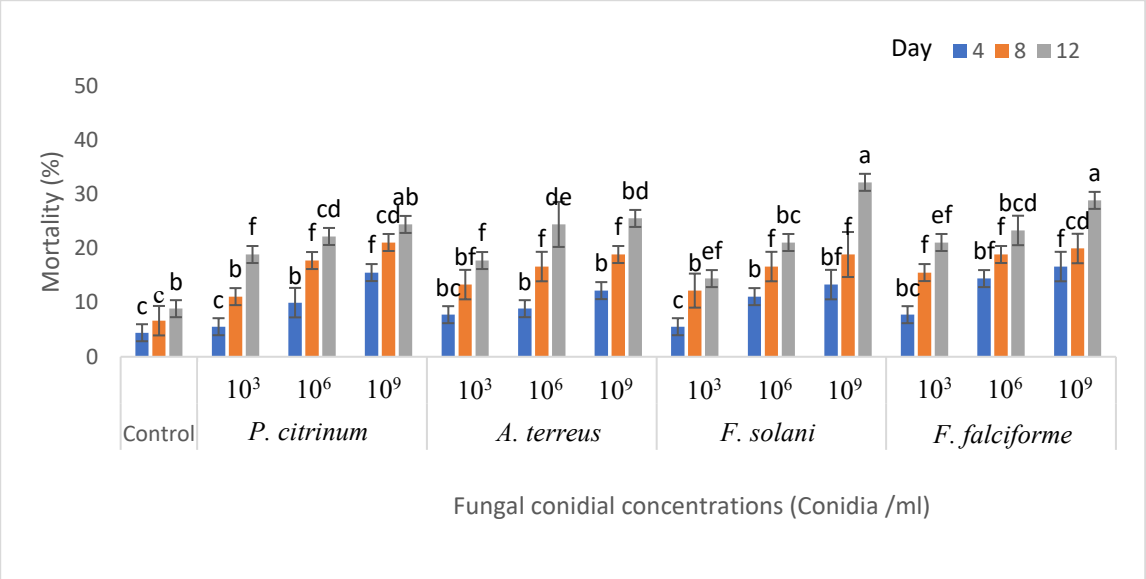

Figure 3.8: Percentage mortality (Mean±SD) of different conidial concentrations of fungi at different days

3.3.3 Synergistic Effect of Insecticides and Entomopathogenic Fungi

The synergistic effect of *F. solani* and Fipronil was analyzed using Tukey's test ($P \leq 0.05$) in general linear model. Mortality caused by 25 ppm concentration was highly significant from mortality of control. Variable time was observed to be significant (12^{th} day). Among the three different insecticidal combinations with *F. solani*, maximum mortality occurred at 12^{th} day (Figure.3.9). On the 3rd day lower mortality rates were observed that increased with increased in treatment time. The lowest mortality was observed for imidacloprid × *F. solani* on the 3rd day among the treated groups. The difference in termites mortality rate was more signified when exposed to 25 ppm concentration for 12 days continuously. Highest percentage of mortality occurred in Imidacloprid combined with *F. solani* which was comparatively greater than the combined effect of highest dose of Imidacloprid and highest conidial concentration of *F. solani* separately in previous treatments (Table. 3.4).

Table 3.4: Percent mortality (Mean±SD) of different insecticides in combination with *F. solani* at different days. (Tukey's LSD test with P value ≤ 0.05)

Days	Control	Fipronil 25 ppm × *F. solani* 10^9	Imidacloprid 25 ppm × *F. solani* 10^9	Chlorpyrifos 25 ppm × *F. solani* 10^9
4	8.88 ± 1.57^{c}	32.22 ± 4.15^{bcd}	12.22 ± 1.57^{d}	24.44 ± 1.57^{cd}
8	12.22 ± 1.57^{c}	43.33 ± 2.72^{bcd}	26.66 ± 2.72^{bcd}	26.66 ± 2.72^{bcd}
12	13.33 ± 2.72^{c}	64.44 ± 3.44^{ab}	83.33 ± 2.72^{a}	58.88 ± 2.15^{abc}

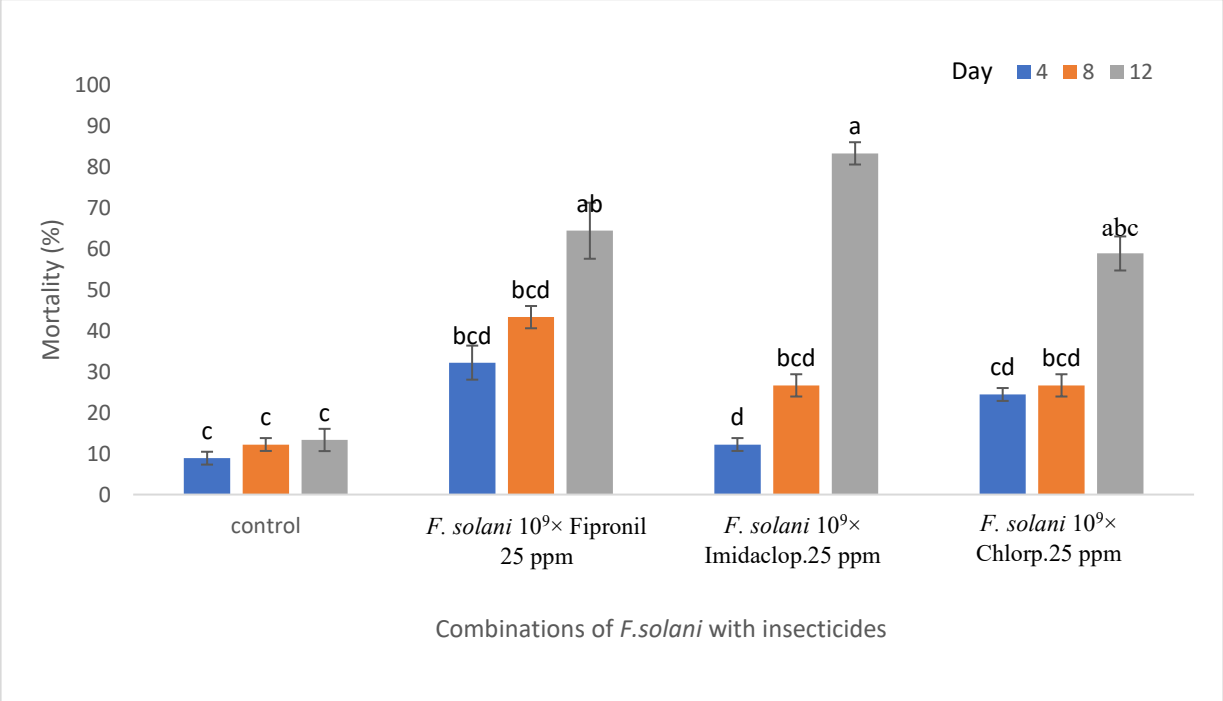

Figure 3.9: Percent mortality (Mean±SD) of different insecticides in combination with *F. solani* at different days

Combinations of highest concentrations of insecticides with *F. falciforme* resulted primarily in additive responses in termites mortality. Imidacloprid and Chlorpyrifos resulted in 67.77% and 64% mortality respectively, at day 12 (Figure 3.10). Both these

insecticides showed additive response on termites mortality. The combination of Fipronil was observed to cause highest mortality (91.11%) at day 12 and indicated synergistic effect that was greater than the individual effect of Fipronil and *F. falciforme*. No prominent indications of synergism were observed in all other combinations (Table.3.5).

Table 3.5: Percent mortality (Mean±SD) of different insecticides in combination with *F. falciforme* at different days. (Tukey's LSD test with P value ≤ 0.05)

Days	**Control**	**Fipronil 25ppm ×*F .falciforme* 10^9**	**Imidacloprid 25ppm ×*F. falciforme* 10^9**	**Chlorpyrifos 25ppm ×*F. falciforme* 10^9**
4	7.77 ± 1.15^{c}	15.55 ± 2.72^{c}	18.88 ± 1.14^{c}	20 ± 2.72^{c}
8	12.22 ± 1.57^{c}	36.66 ± 2.72^{bc}	25.55 ± 2.15^{bc}	33.33 ± 2.72^{bc}
12	13.33 ± 2.72^{c}	91.11 ± 1.57^{a}	67.77 ± 2.15^{ab}	64.44 ± 4.15^{ab}

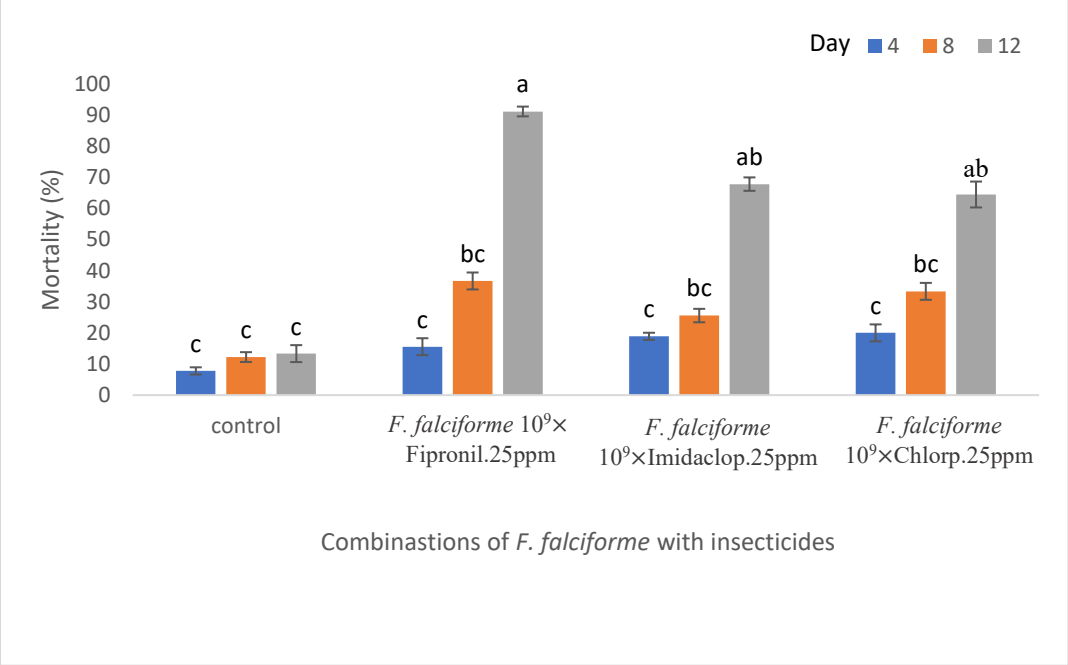

Figure 3.10: Percent mortality (Mean±SD) of different insecticides in combination with *F. falciforme* at different days

DISCUSSION

Entomopathogenic fungi are related to insects and contribute to ecofriendly biological control of arthropod pests of important crops worldwide. Using the potential of these fungi, several commercial insecticides have been formulated with entomopathogenic fungi for pest control. However, survival of conidia in the field environment is critical in the success of pest control program using entomopathogenic fungi, since the onset of epizootics is dependent on the capacity of conidia to germinate on the targeted pest body (Oliveira and Neves, 2004). Alizadeh and Colleagues (2007) reported that insect's immune responses, social behaviour and microbiota associated with the targeted pest may affect the viability of conidia that can render the success of entomopathogenic fungal infection.

In a disease susceptibility test, seven termite species from five families were tested against entomopathogenic fungi in laboratory conditions. Results showed a multilevel resistance of each species against fungi by various mechanisms including immune response against fungal infection (Chouvenc *et al*., 2009). Secretions of different glands which contain terpenoids, quinones and antifungal peptides are also responsible for antifungal properties. In subterranean termites allogrooming of nestmates has been observed as the primary fungal resistance mechanism which is evolved as adaptive defense mechanism despite of favorable environmental conditions for fungi growth (Bulmer and Crozier, 2004).

Fusarium isolates are highly specific to their hosts and have been reported to cause high mortality to insects. They are safe in use against crop pests. *Fusarium solani* strains have been used against Hemiptera, Lepidoptera, and Coleoptera orders. High mortality rates are reported against members of the orders Hemiptera and Coleoptera (Santos *et al.,* 2020).

The present study focused on the responsiveness of *C. heimi* to different conidial concentrations of entomopathogenic fungi in combination with sub lethal concentrations of commercial insecticides. In this study no significant association was observed at combinations of Imidacloprid and *F. solani* at the beginning (day 1-day 4). This is

probably due to the less compatibility and inability of the fungi and imidacloprid to encounter the defense mechanism of the *C. heimi*. Previous studies also suggested that combination of different concentrations and time of interaction can affect the outcomes. Biotic interactions and abiotic factors in the soil also affect the effectiveness of entomopathogenic fungi and sublethal concentrations of insecticides (Pallero *et al*., 2020).

Fusarium isolates have excellent survival ability in soil but sometimes these strains do not exhibit their pathogenicity against arthropods (Sharma and Marques, 2018). The interaction between imidacloprid and *F. solani* were increased from day 8 to day 12 of the assay. Both variables caused 83.33% mortality in termites. Similarly, Fipronil and *F. falciforme* showed enhanced interaction with the increase in time of assay. At the beginning of assay no significant increase in mortality rate were observed but after 12 day 90.11% of termites were dead which showed the enhanced combined effect of both variables.

Previous studies have indicated that *Beauveria bassiana* treated soil and filter paper with highest concentrations has caused less than 5% mortality after two weeks of treatment but concurrent exposure of subterranean termites to *B. bassiana* treated soil and Imidacloprid treated filter paper significantly enhanced the susceptibility to fungal pathogens after one week (Ramakrishnan *et al*., 1999). Similarly, the mortality rates of termites was significantly enhanced after two weeks up to 99% when treated with 1×10^5 conidia in combination with 0.001% Imidacloprid. Also, the synergistic effects of *Fusarium solani* and Fipronil against *Heterotermes indicola* Wasmann have been studied. Termite mortality after continuous exposure of 20 days was 10% exclusively for fungal treatment of 1×10^9 conidia/ml, whereas 100% mortality occurred just after 16 days when 5 ppm sublethal concentration of Fipronil was applied in combination of fungal treatment (Afzal *et al*., 2018).

Imidacloprid and fipronil enhance the susceptibility of termites to *F. solani* and *F. falciforme* by distracting the social and physical behaviour as a result of intoxication, as previously reported by Quintela and McCoy (1998). These insecticides act as

physiological and behaviour modifier in insect pest (Inglis *et al*., 2001). Hiromoni and Nishigaki (2001) reported that insecticidal sublethal concentrations result in fungal infections to termites by germinating their conidia and passing the primary defense mechanism of allogrooming. The sub lethal doses weaken the immune system of termites and hence unable to cause the oxidation of phenolic compounds of insecticides after continuous exposure to combination of insecticide and fungi.

The results of this study revealed that Imidacloprid and Fipronil induce their insecticidal stress in termites which suppress the immune system of termites. *Fusarium* species also produce different toxins like Enniatins that belong to enniatin antibiotic family. These toxins exhibit arthropods pathogenicity (Santos *et al.*, 2020). They also cause reduction in the repellency potential of termites against fungal spores by primary defensive mechanism of allogrooming. However, further detailed investigations are needed to highlight factors and mechanisms for increased susceptibility in termites against entomopathogenic fungal infection by combination of insecticides and entomopathogenic fungi.

CONCLUSION

Widespread and indiscriminate use of insecticides has resulted in insecticide resistance. The use of endogenous strains of entomopathogenic fungi in combination with commercial insecticides can provide effective and environmentally friendly alternative approach against insect pests control in IPM. Sublethal concentrations of imidacloprid and fipronil were comparatively more compatible with conidial concentrations of *Fusarium solani* and *Fusarium falciforme* respectively. Mortality rates were observed for synergism of insecticides and entomopathogenic fungi which revealed enhanced mortality rates for combination of imidacloprid and *F. solani* in one group whereas fipronil and *F. falciforme* in another group.

Results of this study recommends that insecticides-fungi combination can be use in IPM which can greatly reduce the use of conventional pesticides, detrimental to environment. However, for successful field trails further studies are needed to improve the viability of conidia and fungistatic effects in combination with sublethal concentrations of insecticides.

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