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A Review on Entomopathogenic Facet of *Fusarium verticillioides*

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ABSTRACT

Entomopathogenic fungi are a group of fungi infecting insects by penetrating their cuticle, growing inside their bodies and feeding on them which eventually kill insects. Insects become more important competitors of human food damaging or even destroying crops. Fortunately, most insect pests have some pathogenic microorganisms associated with them. Control of large-scale insect pest infestations without harming the environment can be achieved by developing entomopathogens and incorporating them into integrated pest management strategies as they can be an effective biological control tool. The fungi *Fusarium verticillioides* has been established as phytopathogenic but now it is proven entomopathogenic. It proliferates throughout the insect's body, producing toxins like beauvericin and fumonisin. It effectively manages coleopteran and hemipteran pests under laboratory and field conditions. It can be effectively mass produced using specific media with suitable temperature and relative humidity. It is also compatible with insecticides. Thus, this review shades the light on merely explored EPF *Fusarium verticillioides.*

Keywords Entomopathogen, Biological, Fungi, *Fusarium verticillioides,* Environment.

INTRODUCTION

Naturally, pathogenic microorganisms have been regulators of insect pest population. Over last 50 years, insect pathologists expressed strong optimism towards potential of fungal entomopathogens. So, more recently, entomopathogenic fungi (EPFs) has been exploited to achieve biological control of crop pests sustainably. Unlike other biopesticides i.e., bacteria, viruses. Entomopathogenic fungi doesn't require to be ingested to cause infection which makes unique mode of action. Entomopathogen word is derived from two greek words i.e., "entomon" means 'insects' and 'genes' means 'arising in'. Etymological meaning is 'microorganisms which arise in insects', (Rai *et al*. 2014). It is defined as a group of fungi infecting insects by penetrating their cuticle, growing inside their bodies, feeding on them and eventually kills them (Dara 2017).

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The earliest study of insect mycosis was carried out in 1834-35, by Bassi with *Beauveria bassiana* infecting silkworms (Gul *et al.* 2014), his study gave the idea of using insect-infecting fungi to manage insect population. Then afterwards, potential of EPFs has been exploited by scientists. Today, our knowledge of EPFs midpoints around handful of fungal species which have been studied intensively and majority of them are hyphomycetes (Teetor-Barsch and Roberts 1983). Amongst them, genus fusarium is a well-known plant pathogen but on the other side it comprises definite species that are exceptionally effective in managing certain arthropod pests. During last decade, general investigations have been carried out to manage arthropod pests by utilizing this genus (Patel 2019).

Many *Fusarium* spp. causing rots and vascular wilt diseases in plants, while, some of them cause infection in insects, nematodes, spiders and mammals (Majumdar *et al.* 2008). Being a trans kingdom pathogen, one of the *Fusarium* sp. has also been isolated from the cornea of human eye (Cooke 1977). Among the animal kingdom, they are abundantly associated with insects majorly as fungal parasites or pathogens (Wollenweber and Reinking 1935). While numerous *Fusarium* spp. thrive and reproduce entirely as saprobes, many other species selectively parasitize specific insect hosts. The interchangeability between parasitic and saprophytic phases is observed in EPF but this phenomenon is distinct in *Fusarium* spp. which proves its ubiquitous nature (Wollenweber and Reinking 1935b). More than 13 *Fusarium* spp. are found pathogenic to insects with host range comprising Coleoptera, Diptera, Hemiptera, Hymenoptera and Lepidoptera orders of Insecta (Teetor-Barsch and Roberts 1983, Humber 1992) and Acarina in the class of Arachnida, Orthoptera and Isoptera (Wollenweber and Reinking 1935b).

Among different *Fusarium* spp. *Fusarium verticillioides* (Saccardo) Nirenberg earlier named *Fusarium moniliforme / Fusarium fujikuroi* is common fungal pathogen found in infected corn kernels. Surprisingly, in recent years, it is reported being entomopathogenic on different insects (Pelizza *et al.* 2011, Ronel *et al.* 2017). So, the question arises whether *Fusarium verticillioides* offers potential of being an effective insect bio control agent. It will be addressed in this review and along with that, literature also introduces a reader to merely explored fungal entomopathogen *Fusarium verticillioides*, it's biology, pathogenesis, epizootiology, some proof of concept studies of *in vivo* and *in vitro* efficacy and lastly ending with brief discussion on its mass multiplication for better commercialization and what future it holds for biological control of insects.

History of *Fusarium verticillioides* as an entomopathogen

An earliest record of *F. verticillioides* being entomopathogenic was found against European corn borer, *Ostrinia nubilalis*. It was isolated from and tested against *O. nubilalis* larvae showing a medium pathogenicity (Huger and Zimmermann, unpublished, (Cross ref) Butt *et al.* 2016). In France, *F. moniliforme* was isolated from naturally mycosed *O. nubilalis* (Vago 1958) and silkworm moth, *Bombyx mori* (Vago and Nicot 1954). Thereafter, it was recorded from Coleoptera, Lepidoptera, Diptera and Hemiptera orders (Teetor-Barsch and Roberts 1983, Humber 1992). Recently, it was isolated for the first time from naturally mycosed violet-winged grasshopper (*Tropidacris collaris*) in Argentina and its pathogenicity against the harmful grasshopper (*Ronderosia bergi*) was also proven (Pelizza *et al.* 2011).

Taxonomic position of filamentous *Fusarium verticillioides*

Domain: *Eukaryota*
Kingdom: Fungi
Phylum: *Ascomycota*
Subphylum: *Pezizomycotina*
Class: *Sordariomycetes*
Subclass: *Hypocreaomycetidae*
Family: *Nectriaceae*
Genus: *Fusarium*
Species: *Fusarium verticillioides*

Biology

The genus was established by Link (1809), for comprising species showing canoe-shaped conidia, characterized by a foot-shaped basal cell in mycelia.

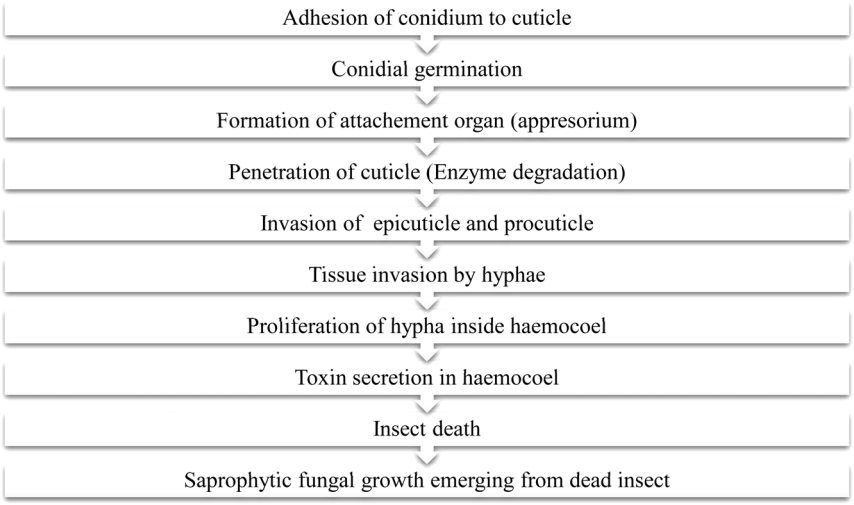

Fig. 1. Infection process of entomopathogenic fungi (Sinha *et al*. 2016).

It is a hyaline, filamentous fungus showing fluffy, aerial mycelium with white to pale peach-colored colony initially, which became pinkish brown in the middle at later stage on potato sucrose agar (PSA). In microscopic observation, 2-4 µm long, slightly falcate shaped (curved like a sickle), tapered ended, thin walled and septate macroconidia formed in pale orange sporodochia can be seen. While, microconidia formed in chain or clusters on monophialides, are hyaline, aseptate, 1.5-2.5µm short, with oval to club-shape. The teleomorphic stage of *F. verticillioides* is *Gibberella fujikuroi* (Pelizza *et al.* 2011, Gagkaeva and Yli-Mattila 2020).

Pathogenesis

In order to exploit EPFs as biological control agents, understanding fundamental behavioral process between insects and their pathogens is required. The spores of EPFs are present in the environment and when spore encounter their host, a series of events happen which is briefly explained here (Fig.1) (Sinha *et al.* 2016). All most all EPFs show a such similar type of infection process.

Adhesion and attachment of the insect cuticle

The first step is single conidium adhesion to insect cuticle among the numerous conidia (Butt and Goettel 2000). An aerial conidial surface of most hypocrealean EPF is covered with a rodlet layer with hydrophobin proteins conferring a hydrophobic charge which facilitates passive conidial attachment to hydrophobic surfaces i.e., insect cuticle (Holder and Keyhani 2005). Such hydrophobic layer is only seen in conidial phase. Currently, five hydrophobin genes have been identified in the *F. verticillioides* among them, HYD1, HYD2, and HYD3 encode class I hydrophobins. HYD4, HYD5 encodes class II hydrophobins and with their mutant genes, they form a higher conidial chain which indirectly enhances its pathogenicity (Fuchs *et al*. 2004). These hydrophobin genes regulate the surface attachment of spore to waxy epicuticle.

Coming to the epicuticle, an outermost layer of insect cuticle with procuticle layer beneath. It is concealed by a waxy layer with lipids, sterols, and fatty acids (Andersen 2010, Bamisile *et al.* 2018). Procuticle has sublayers of endo-, meso- and exo-cuticle. It majorly constitutes protein (70%) and chitin fibrils fixed in a protein matrix along with quinones and lipids (Xing and Yang 2020). Endocuticle, third innermost layer of the epidermis, surrounds and protects insect's internal structures (Andersen 1979). Fungi penetrate the insect cuticle by germ tube formation (directly) or by establishing an attachment

organ appressorium, (indirectly) which attaches to the cuticle followed by producing a thin penetrating peg (Ortiz and Keyhani 2013, Gabarty *et al.* 2014). Additionally, temperature, pH, sunlight and humidity also play important role in adhesion, growth and penetration of EPF into insect cuticle (Nickerl *et al.* 2014). Natural openings of the insect's body, e.g., spiracles, sensory pores or wounds are also entry gate for EPF (Sinha *et al*. 2016).

Penetration

Fungi need to penetrate host surface to reach the inner structure for absorption of nutrients. It requires both mechanical pressures and enzymatic breakdown and for that, appressorium aids in concentrating chemical and physical energy over a tiny part of mycelium to achieve efficient entrance (Frisvad *et al.* 2007, Zhang *et al.* 2010). A variety of enzymes involved in synergistic way to penetrate complex matrix of insect cuticle (Mondal *et al.* 2016). It is directly penetrated by extracellular enzymatic activity of fungal spore and then reaching the hemocoel or entering through mouthparts also (Sanchez-Perez *et al.* 2014). After fungal invasion, infected host outer layer shows dark melanotic lesions on the penetrated part because of melanin accumulation (Parle *et al.* 2017). Multiple cuticle-degrading proteases, chitinase and lipases are produced during the penetration. (Samuels and Paterson 1995, Dias *et al.* 2008, Butt *et al.* 2016, Sanchez-Perez *et al.* 2014). A wide range of proteases i.e., trypsin, chymotrypsin, esterase, chymoelastase and collagenase are produced (Motyan *et al.* 2013) hindering N-acetyl glucosaminidase production enhancing fast cuticle degradation.

Tissue invasion and hyphal proliferation inside hemocoel

After penetration, fungi reach to insect's respiratory system to get maximum nutrition for their vegetative and reproductive growth. For that, they produce blastospores and vegetative hyphae which further develops mycelia that disseminate through host haemocoel and invade diverse muscle tissues, fatty bodies, Malpighian tubes, mitochondria and haemocytes, leading to death of the insect (Mejia *et al.* 2008, Brivio and Mastore 2020) also they release a wide variety of secondary metabolic compounds (toxins) inside host body, specifically in haemocoel to accelerate killing process (Rai *et al*. 2014).

Toxin production

Several mycotoxins produced during pathogenesis by EPFs which act as a poison for hosts. *F. verticillioides* also produces various toxins i.e., beauvericins, fumonisins and fusarins (Majumdar *et al.* 2008, Stepien and Waskiewicz 2013, Liuzzi *et al.* 2017, Perincherry *et al.* 2019). Fumonisins a group of mycotoxins are derived from polyketides. It disrupts plasma membrane by accumulating toxic sphingolipid intermediates (Abbas *et al*. 1993). These intermediates disrupt de-novo sphingolipid biosynthesis, inhibit the enzyme ceramide synthase and hence, disrupt cell signalling and functions, alters apoptosis and replication. Ultimately, damages insects cell membrane and disrupts its functions (Merrill *et al.* 2001). Fusarins are also polyketides compounds inducing mutation in organisms whereas, beauvericin, a cyclodepsipeptide toxin, has previously been isolated from entomopathogenic deuteromycetous fungi, *Beauveria bassiana, Paecilomyces fumoso-roseus* (Fanelli *et al.* 2004, Reverberi *et al.* 2005) and the plant pathogenic basidiomycetous fungus *Polyporus sulphureus* (Proctor *et al.*1995). Beauvericin probably acts as an ionophore capable of making complexes with divalent cations (Voigt *et al.* 2007, Pusztahelyi *et al.* 2015). Thus, it is also cytotoxic and damage cell cytoplasm (Bakker *et al.* 2018). Such toxins aids in killing of hosts and allows rapid fungal growth inside host.

Insect death

After fungal infection, almost 3 to 14 days are required for the insect's death. Insect mortality is usually caused by nutritional deficiency, destruction of tissues and by effect of toxins on cytoplasm (Inglis *et al.* 2001, Kachhawa 2017, Rai *et al.* 2014).

Spore production on host

The hypocrealean fungi are hemi-biotrophic in nature so it switches from a biotrophic (parasitism) to a saprophytic phase in the host hemocoel, colonizing the body after death (Lovett and Leger 2017). After the infection process when host is dead, the fungus

switches back to its hyphal mode and under relatively humid conditions, it subsequently grows out of the cadaver surface to produce new, external, infective conidial saprophytic growth (Jianzhong *et al.* 2003, Mitsuaki 2004). However, under very dry conditions, it may persist in the hyphal stage inside the cadaver where the conidia are produced inside the body itself (Hong *et al.* 1997).

Transmission and dispersal

A fungus uses different strategies to enhance the chances of encountering a new host. For abundant spore-producing hyphomycetes, rain, wind and invertebrates are major transmission channels. Along with that, grown hypha out of insect cadaver is also a major source of conidial dispersion. Although, conidial germination and sporulation are dependent on high humidity and moisture but the fungal ability to infect multiple stages in the life of an insect, helps to spread disease faster within a population. For example, winged insects can spread spores and ultimately the disease spreads rapidly in insect populations (Steinkraus *et al.*1999, Dromph 2001).

Spore dispersal epizootiology

Epizootiology refers to a study of an animal disease at the population level including environment, host and pathogen population, so it is heavily allied with ecology. In simpler terms, epizootics means natural fungal outbreak. Fungi can create epizootic so it can be effectively used as biopesticides around the world to manage numerous pests (Fuxa 1998). The cryptic nature of soil-living insects promotes natural fungal epizootics. It is seen that soil is major inoculum source of many hyphomycetes (Steenberg *et al.* 1995) and most of the epizootics have been noted in the stages of insects that live in the soil because soil act as a storage/survival place for spores. Which provides primary and secondary sources of infection through spore dispersion in surrounding air and from the conidia on leaves to insects feeding on leaves. Secondary spread through, infected insects to non-infected ones. Major occurrences of epizootics mostly had been found in aphid species (Hatting *et al.* 2000). This occurrence could reach to 90% according to (Steinkraus *et al.*1999) hence, epizootics can reduce pest population.

Proof of concept studies

The scientific evidence of *F. verticillioides* being entomopathogenic are required to establish it as a novel biocontrol agent. So, in this direction, Srinivas and Pasalu (1990) at Hyderabad conducted a case study on the occurrence of fungal disease on brown plant hopper highlighting an increased microbial infection of *F. verticillioides* on BPH (*Fusarium moniliforme*) they noted higher humidity induced high natural mortality (4.80 to 15.80%) and also proved, pathogenicity on rice plant infected by BPH and inoculated with *F. verticillioides* spores giving BPH mortality after a week of spray. Further, re-isolation and identification proved the presence of *F. verticillioides.*

Torres-Barragan *et al*. (2004), at ecological reserve of Mexico, detected presence of *F. verticillioides (Fusarium moniliforme)* on *Acanophora femoralis, Dalbulus maydis, Spodoptera frugiperda* infecting papaya and maize crop. Thus, such studies evidencing natural occurrence of insectivorous fusaria. Further, using the same strain of *F. verticillioides,* Torres-Barragan *et al.* (2004), confirmed, high mortality (96%) of *Trialeurodes vaporariorum* (3rd instar nymphs) under green house in bean crop.

In India, Mehetre *et al*. (2008) presented a detailed field condition study, revealing the entomopathogenic effect of *Gibberella fujikuroi (*telemorph of *F. verticillioides*) against sugarcane woolly aphids with 60% population suppression by applying two sprays (1×10^8 cfu/g) at a weekly interval.

Toxins are EPFs key weapons causing very detrimental effect on their host metabolism. In that direction, Zhang *et al.* (2017) conducted a detailed study and detected toxin fumonisin B_1 (obtained from *F. verticillioides*) inhibits cell proliferation in ovarian cell culture of *Spodoptera frugiperda* (sf9). A gradual upsurge in the inhibition rate of cell proliferation, cell swelling and increased proportion of vacuoles was recorded after 48h with increasing concentrations of fumonisin B_1, leading to a reduction in the cell membrane potential of Sf9 cells.

Abdel-Galil *et al.* (2019) during their *in vitro* testing of indigenous isolates of *F. verticillioides* against aphids, reported 60% and 70% mortality of bean aphids (*Aphis fabae)* and wheat aphids (Diuraphis noxia), respectively after 72 hrs of EPF powder application. A similar study was conducted by Patel (2019) suggesting that the *F. verticillioides* was effective against okra mite inducing 75% mortality at 10 days after spraying conidial suspension (1×10^{10} cfu/g) and with its increased concentration, time after application, *F. verticillioides* gradually increased mite mortality.

F. verticillioides can also infect different stages of insects and in that context, Bakaze and his co-workers (2020) conducted comprehensive study on effect of *F. verticillioides* on different life-stages of *Cosmopolites sordidus* (Banana rhizome weevil). They detected the conidial suspension of *F. verticillioides* (10^7 cfu/ml) inhibits process of egg hatching in *C. sordidus*, along with inducing mortality in 2nd instar larva and adult weevil under *in vitro* conditions. Another recent study by Barbosa and his co-workers (2021) evidenced that *F. verticillioides* effectively suppresses nymphal population of citrus black fly (*Alerocanthus woglumi*) and induced considerable amount of mortality.

Being a novel entomopathogen, how *F. verticillioides* is performing in comparison to existing entomopathogens must be studied for recognising its commercial potential. *Sain et al.* (2021) conducted an in-depth study of comparative effect of selected EPFs on cotton whitefly (*Bemisia tabaci*) under field conditions and surprisingly, *F. verticillioides* was as effective as *Cordyceps javanica, M. anisopliae* and *B. bassiana* inducing 71 and 78% whitefly mortality in 2017–18 and 2018–19 years respectively.

Mass production

In order to further utilize EPF potential, developing mass production technology is very crucial.

In soil, around insect corpses, EPFs are heterogeneously distributed (Bara and Laing 2020). Therefore, pooling the samples may improve the likelihood of fungal isolation. Typically, from the rhizosphere or organic soil zone, soil samples are taken at a depth of 10-15 cm (Mason 1983). Before collecting soil, the collection instruments should be surface sterilized to prevent contamination of any kind. The samples are then stored for future experiments in a refrigerator for 4 °C. The EPF is purified to enable identification and mass culture using a dilution plate (Harris and Sommers 1968) or a direct plating approach (Golden *et al.* 1988).

The greater wax moth larvae (*Galleria mellonella* L.), in a same manner, can also be utilized as a bait to isolate EPF from soil (Zimmermann 1986). Before employing in the test, the soil utilized for this purpose should be kept at 4°C. Four *G. mellonella* larvae are placed in plastic containers having 275g of soil and sealed with perforated lids and kept at room temperature. Some larvae (3–5) of G. mellonella are kept in different container having sterile soil for negative control (Vilcinskas, *et al.*1997). Containers are examined daily and dead larvae are collected. The carcasses are surface sterilized with 1% sodium hypochlorite solution for 3 min, then rinsed with sterile dH_2O, are plated and incubated in a humidity chamber at 27 °C with 100% relative humidity (RH) to encourage fungus growth (Brownbridge *et al.*1993). The total number of fungi-infected plates is recorded, along with the number of EPF colonies, which are counted and morphologically identified (Islam *et al.* 2021).

A simplified mass production protocol for *F. verticillioides* has been developed by Patel (2019) as mentioned here (Fig.2).

Besides these sporulation, RH and shelf-life affect mass production of *F. verticillioides*. The maximum (6.64×10^9cfu/ml) sporulation of *F. verticillioides* found at 30ºC followed by 25ºC (6.28×10^9cfu/ml) along with 80% relative humidity. It can grow excellently with higher biomass in sabouraud's dextrose agar (7.01×10^9cfu/ml) and potato dextrose agar media (6.57×10^9cfu/ml). In case of liquid media higher sporulation (7.20×10^9cfu/ml) observed in sabaurad's dextrose broth. Grains are also a good medium for mass multiplication, *F. verticillioides* can flourish well in sorghum (6.82×109cfu/g) and wheat grain (6.19×10^9cfu/g). Coming to the shelf life, it largely depends on type of carrier material used for packing. A spore viability of *F. verticillioides*

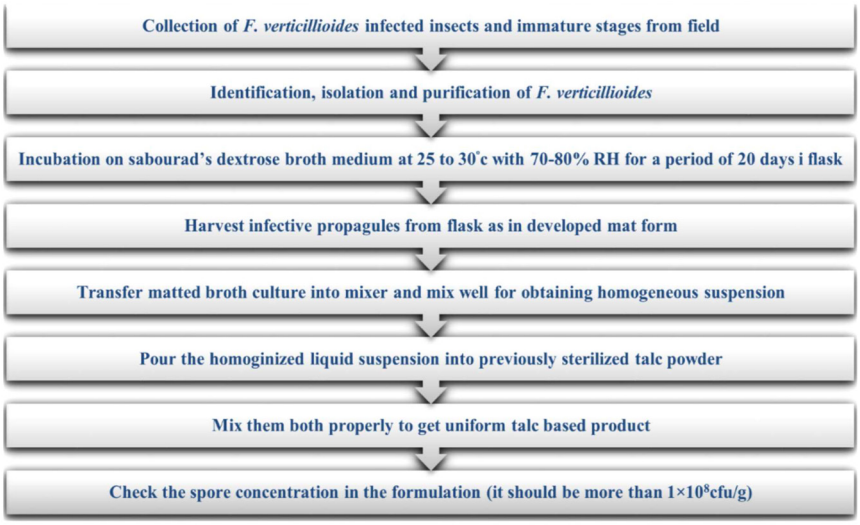

Fig. 2. A brief protocol for the mass production of *F. verticillioides* (Patel 2019).

is higher in talc as well as in combination of talc + diatomaceous earth (1.67×10^8cfu/g). An *in-vitro* study on compatibility of *F. verticillioides* revealed that it is compatible with various insecticides i.e., buprofezin 25 SC, chlorantraniliprole 18.5 SC, imidacloprid 17.8 SL and thiamethoxam 25 WG (Patel 2019).

Islam *et al.* (2021) noted EPFs have certain benefits over conventional synthetic pesticides which are as follows:

a) Environment-friendly and no residual effects
b) Host-specific
c) Compatible with synthetic chemical insecticides, creating synergistic effect
d) Self-perpetuating under conducive environmental conditions
e) Safer to biodiversity and reduce reliance on chemical insecticides
f) No or less pest resistance development

Indeed, the biological mechanism of EPF in insect pest control has immense potential, but it has some limitations as well according to Islam *et al.* (2021), which are as follows:

a) Need a specific fungicide free period and favorable environmental conditions for germination and infection
b) Secondary metabolites produced by EPF dangerous for living entities
c) Shorter shelf life
d) Requires 2–3 weeks for insect control
e) Needs accurate scouting information about pest prevalence before its application
f) Persistence and infection rate limited in challenging environmental conditions

CONCLUSION

Apart from being plant pathogenic, *Fusarium verticillioides* in recent years, reported being entomopathogenic on different insects, a hypocrealean fungi producing macro and microconidia infecting insects using variety of mechanisms. There are five hydrophobin genes have been identified in the *Fusarium verticillioides* majorly responsible for its virulence against insects. Other than that production of insect cuticle degrading enzymes and insecticidal toxins has also been recorded. Further, it facilitates epizootics. It mainly infects soft bodied insects, grass hoppers and many lepidopteran pests. So, with increasing evidence of *in-vitro* and in-field insect mycoses by *F. verticillioides,* it's time to consider its insecticidal property and further exploring its potential as an effective biocontrol agent for agricultural crop pests.

Future prospects

Biopesticides are expanding at a 10% annual rate (Patrick and Kaskey 2012) with mycoinsecticide coming second (27%) on the worldwide biopesticide market (Kabaluk *et al.* 2010) This expansion indicates mycoinsecticides will soon play a significant part in managing insect pests as EPF usage is unavoidable because it's a vital part of IPM strategies. Hence, obstacles must be solved and EPF effectiveness should be achieved.

Commercial firms seek EPFs strains showing quick knockdown effect, wide host range and higher environmental adaptability. Several genes of EPFs could be used to boost virulence, specificity, and ecological fitness. Therefore, advancement in these areas is still being made. The time it takes to kill pest and the application rate can be decreased by over-expressing virulence/venom genes, which benefits end users financially. Additionally, stress control in EPF could enhance field persistence or shelf life. A formulation selection has a significant impact on how efficacious entomopathogens will perform. New soaps, oils and diatomaceous earth have been created that reduces fungistasis thereby improving infection even under circumstances that are typically thought to be unfavorable to EPF. An efficacy-enhancing agents i.e., botanicals, various microbes (*Bacillus thuringiensis*), entomopathogenic nematodes (EPN) and low-dose insecticides enhancing EPF activity (Ansari *et al.* 2006, Ansari, *et al.* 2010, Kryukov *et al.* 2009, Shapiro *et al.* 2004).

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