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Concept of entomopathogenic fungi (EPF) and its exploration as a biopesticide for control of tick infestation in cattle

Jumade Pratibha and Gawande Priya

Abstract
Tick and tick borne diseases are one of the biggest public health and veterinary problems in the world. Control of ticks using chemical acaricides have developed resistance to wide range of acaricides and also producing environmental pollution. The demands of consumers for chemical free foods and the negative environmental effects of acaricides call for the development of alternative biological control strategies such as entomopathogenic fungi. An entomopathogenic fungus is a fungus that can act as a parasite of insects and kills or seriously disables them. Although entomogenous fungi have been used widely for the control of agricultural and forest pests, the applicability of bio-control potentials of entomogenous fungi against ticks has the promising results. The potential of entomopathogenic fungi as tick-control agents is due to ability to target different developmental stages of the host, relatively specific virulence, genetic variability, ability to penetrate through the cuticle and benefit of residual action. Entomopathogenic fungi such as *Beauveria bassiana, Metarhizium anisopliae Nomuraea rileyi, Paecilomyces farinosus* and *Paecilomyces fumosoroseus* are abundantly availed in soil as well as plant debris. The ready to use products are also available in market. Adhesion, germination and production of conidia are recognized as the main virulence factors of these fungi against ticks**.** Death of the insect is often due to a combination of the action of fungal toxins, physical obstruction of blood circulation, nutrient depletion and/or invasion of organs. Entomopathogenic fungi can be an alternative as a biopesticide to overcome the acaricidal resistance and environmental pollution.

Keywords: Cattle, ticks, acaricide, resistance, biological control, fungi

Introduction
Livestock health and animal diseases plays an important role in livestock production. The major constraint to livestock production are infectious and parasitic diseases which have great impact on livestock productivity by altering weight gain and reproduction, reduced production yield, mortality and altering quality of products such as milk and meat (Sharma, *et al*., 2016) [50].

Effects of tick parasitism in livestock
Among ecto-parasites, ticks are very important external parasites of mammals, birds and reptiles throughout the world (Furman and Loomis, 1984) [19]. Tick and tick borne diseases are one of the biggest public health and veterinary problems in the world. Ticks have an impact on the production and health of the animals either directly by the effect of their bites or by transmission of the infectious agents of viruses, bacteria, rickettsiae and protozoa (Eskezia and Desta, 2016) [16] such as Louping ill virus, *Theileria, Babesia, Anaplasma, Rickettsia, Borrelia* etc. The bites of ticks injure the tissues of animals at their feeding site, causing irritation, inflammation or hypersensitivity, various lesions which may predispose to localized dermatitis, secondary bacterial infections, or invasion by flies (miasis) that are attracted to bloody areas (Taylor *et al*., 2016) [53]. Massive tick infestations can cause anemia, as a result of blood loss. These bites of ticks to animals while feeding causes stress which weakens its immune response affecting its productivity results in the production losses of meat and milk, also increased morbidity mortality and indirect economic losses for producers related to the cost of prevention and control. Affected skin also loses its commercial value (Eskezia and Desta, 2016) [16]. Each engorged female tick on an average, is responsible for the loss of 1.37 g of body weight in *Bos taurus* cattle whereas 1.18 g in *B. indicus* (Roger *et al*., 2014) [46]. The total milk production of dairy cattle can reduce by approximately 90 l/lactation/cow by direct effect of ticks with reduction of 8.9 ml of milk by each fattened female tick (Daniel *et al*.,

2000) [12]. The average total financial losses (production losses plus control cost) per animal per year are USD $7.3 is reported by FAO.

Tick control methods and its hazzards

There are various methods to control ticks, but every method of tick control has certain shortcomings. The present technology to control ticks is based mainly on the use of chemical products; however, the ability of ticks to develop resistance to all currently used organophosphate-carbamates, synthetic pyrethroids and amidines (Martins *et al.*, 1995) [36]. The resistance and residue problems are the biggest concerns over the use of new acaricides. The use of acarcides on livestock is toxic and hazardous. They as artificial organic compounds can remain in the environment for many years and may be transported over a long distance (Kunz and Kemp, 1994) [32]. The residues of them in soil and water are considered as significant environment threats and even classified as carcinogenic pollutants in many countries (Dich *et al.*, 1997) [13]. Hence, the excessive application of these compounds over the past half-century has posed serious risks to human health (Kolpin *et al.*, 1998) [31]. The demands of consumers for chemical free foods and the negative environmental effects of acaricides call for the development of alternative strategies (Kay and Kemp, 1994). Although entomogenous fungi have been used widely for the control of agricultural and forest pests, the applicability of bio-control potentials of entomogenous fungi against ticks which are vectors of human and animal diseases has the promising results to overcome the acaricidal resistance, environmental pollution and to fulfill the demand of humans for chemical free food. The potential of entomopathogenic fungi as tick-control agents is due to several factors, namely ability to target different developmental stages of the host, relatively specific virulence, genetic variability, ability to penetrate through the cuticle and benefit of residual action. These characteristics support these fungi as promising agents in microbial control. (Alves, 1998; Samish *et al*., 2004) [1, 48].

Biological control of ticks

To overcome all these issues there is a call for the development of an alternate and absolute control method, such as biological control becoming an increasingly attractive approach and giving promising results to tick management because of increasing concerns about environmental safety and human health (Samish *et al.*, 2000) [47]. Biological control is the use of living organism to minimize the population density or impact of a specific pest organism, nontoxic to humans and to non target wildlife and should have no negative effect on the environment (Eilenberg *et al*., 2001; Gronvold *et al*., 1996) [15, 22]. Biological control of ticks using entomopathogenic fungus is proved to be most economical and safest method to overcome the risk of environmental pollution and acaricidal resistance. Entomopathogenic fungi are a major and integral component of integrated pest management techniques and are used as biological control weapon against various insect pests and other arthropods. They are being used as myco-insecticides in horticulture, forestry and agriculture sector.

Entomopathogenic fungi (EPF)

An entomopathogenic fungus is a fungus that can act as a parasite of insects. This fungus kills or seriously disables their host by their insecticidal toxicity. Entomopathogenic fungi (EPF) are bioinsecticides with an ability to infect and kill arthropods. Although they are mainly isolated from arthropod carcasses, their natural habitat is soil (Behie and Bidochka 2014) [2]. *Beauveria bassiana, Metarhizium anisopliae Nomuraea rileyi, Paecilomyces farinosus, Paecilomyces fumosoroseus, Verticillium lecanii* were found to infect various insect hosts species naturally (Thakur and Sandhu, 2010) [55]. The entomopathogenic fungi show minimal adverse effects on the animals and other non-target organisms. They can be used in integrated pest management replacing the conventional chemical insecticides (Pell *et al*., 2001) [41]. The application of EPF in biological control is increasing largely because of greater environmental awareness, food safety concerns and the failure of conventional chemicals due to an increasing number of insecticide resistant species. (Shahid *et al*., 2012) [49]. Numerous laboratory assays of both *M. anisopliae* and *B. bassiana* fungi have the lethal effects on several tick species of epidemiological and veterinary importance. (Ostfeld *et al*., 2006) [40]. Although entomogenous fungi have been used widely for the control of agricultural and forest pests, the applicability of bio-control potentials of entomogenous fungi against ticks which are vectors of human and animal diseases has the promising results. (Samish *et al*., 2004) [48].

Classification of EPF

These fungi are categorized in six classes: *Oomycetes*, *Chytridiomycota*, *Microsporidia*, *Entomophtoromy- cota*, *Basidiomycota*, and the most common *Ascomy- cota*. Among the known EPF, Entomophthorales (e.g., *Furia*, *Conidiobolus*, *Entomophaga*, or *Erynia*) show the highest insecticidal activity. (Mascarin and Jaronski 2016) [37]. Many common and/or important entomopathogenic fungi are in the order Hypocreales of the Ascomycota: the asexual (anamorph) phases Beauveria, Isaria (was Paecilomyces), Hirsutella, Metarhizium, Nomuraea and the sexual (teleomorph) state Cordyceps; others (Entomophthora, Zoophthora, Pandora, Entomophaga) belong in the order Entomophthorales of the Zygomycota. The most commonly used fungi in form of biopesticides based on species belonging belongs to the genera *Metarhizium*, *Beauveria*, *Paecilomyces*, *Isaria* and *Lecanicillium* have been used worldwide. These fungi have a wide spectrum of activity and can infect a wide variety of arthropod species (Khan *et al*. 2012; Castro *et al*. 2016) [29, 9].

Source and availability of EPF

Soil is considered an excellent environmental shelter for entomopathogenic fungi since it is protected from UV radiation and other adverse abiotic and biotic influences. (Domsch *et al*., 1980) [14]. The fungus survives in the soil as saprotrophic mycelia or as dormant propagules until its adhesion to an appropriate and compatible host in the surrounding micro-environment or associating with plants as endophytes.
(https://en.wikipedia.org/wiki/Entomopathogenic_fungus).

The fungal entomopathogens in the genera Beauveria, Conidiobolus, Metarhizium and Isaria (Paecilomyces) are commonly found in soil. These fungi can possibly also exist as saprophytes in soil. Entomopathogenic fungi are also distributed in a wide range of habitats including aquatic forest, agricultural, pasture, desert, and urban habitats. (Lacey *et al*., 1996 and Chandler *et al*., 1997) [33, 10]. *Metarhizium* and *Beauveria*, form complex relationships with plants described as endophytes of plant roots, stems, and leaves (Jaber and

Enkerli 2017) [26]. Fungal species *Beauveria bassiana, Isaria fumosorosea, Metarhizium anisopliae and Lecanicillium sp.* of which *M. anisopliae* is the most frequently detected one and more colony forming units in soils from organic fields, whereas *I. fumosorosea* in soils from the conventional ones. (Tkaczuk *et al*., 2014) [58]. The fungal species can also be isolated from insects inhabiting diverse habitats such as *Beauveria bassiana, Nomuraea rileyi, Paecilomyces farinosus and Paecilomyces fumosoroseus* were found to infect various insect hosts species naturally. (Thakur and Sandhu, 2010) [55]. Commercially manufactured pathogenic fungi based bio-insecticides are available in market. (Hafiza *et al*., 2014) [24]. Several commercial formulations of EPF have been developed for crop pest management. In recent times, about 90 genera and almost above 700 species were considered as insect infecting fungi that represent about all the major classes of fungi (Hajek and Leger, 1994) [25]. These species of entomopathogenic fungi which have a great potential as a biocontrol agents that when dispersed in the environment, provoke fungal infections in insect populations. (Pucheta *et al*., 2006, Ramanujam *et al*., 2014) [43, 44].

Growth and Production of EPF

Most entomopathogenic fungi can be grown on commercially available artificial media. Sabouraud's dextrose agar is a classic medium whereas Sabouraud's dextrose agar with chloramphenicol (inhibits bacterial growth). Potato-dextrose agar and Corn-meal agar used in slide cultures to induce spore formation, which aids in identification. (Maina *et al*., 2017) [35]. The mass quantity of fungi can be produced by cultivating on starch rich crushed maize or jowar grains by using pure culture by addition of antibiotic to prevent bacterial contamination. The appropriate temperature required for the growth of most of the fungi is 25-30 °C. Any fungus capable of growing at 37 °C should be considered potentially pathogenic. Full colony growth of EPF gets achieved on 9 to 10^{th} days after culture at 29 °C temperature and 75% relative humidity. (Ekesni *et al.,* 1999).

Mechanism of action of EPF

The mycosis begins with the adhering of fungal spore onto the outer cuticle layer of the susceptible host, followed by passive attachment of spores with the aid of water or wind. Hydrophobic forces of non-specific nature exerted by rod lets may be the possible reason for the attachment of dry spores to the cuticle. Carbohydrate binding glycoprotein lectin on the conidia plays the role in adhering of conidia to the insect cuticle. The fungi proceeds with rapid growth and germination, after adhering onto the surface of the host. The growth and germination of the pathogen on the host cell surface is influenced profoundly by the availability of nutrients, water, oxygen, pH, temperature and toxic compounds produced by the host on the surface. (Boucias *et al*., 1998) [5]. The entomopathogenic fungi enter via proximate penetration through the host cuticle. The cuticle consists of two distinct layers: the outer layer is epicuticle and the inner layer is the procuticle. Epicuticle is a thin layer with a very complex structure. Chitin is absent in the epicuticle instead phenol-stabilized proteins are present. This layer is covered by a waxy coating composed of fatty acids, sterols and lipids. (Neville *et al*., 1984) [38] stated that the procuticle layer consists of chitin fibrils, which are nested into a protein matrix along with quinines and lipids. (Hackman *et al*., 1984) [23]. The penetration is achieved by mechanical strain and enzymatic deterioration by diverse extracellular enzymes, including lipases, esterases, chitinases. The cuticle degrading enzymes are produced during the formation of appressorium are Endoproteases and aminopeptidases that are produced on the cuticle during the initial infection development steps. Biochemical investigations reveal the involvement of Ca2+ and cyclic AMP –secondary messengers in formation of this structure. (Latge *et al.,* 1987) [34]. N-Acetyl glucosaminidase is produced at a slower rate as compared to the proteolytic enzymes. (Singh *et al*., 2017) [51]. Fungi begin their infective process when spores are retained on the integument surface, where the formation of the germinative tube initiates, the fungi starting to excrete enzymes such as proteases, chitinases, quitobiases, Upases and lipoxygenases. (Goettel and Inglis, 1997) [21]. The mode of infection of these fungi includes adhesion, germination, appresorium formation by mechanical pressure, penetration of host cuticle, colonization of haemolymph, extrusion and sporulation (Kimberly and Seow, 2017) [30]. The fungal cells proliferate in the host body cavity, usually as walled hyphae or in the form of wall-less protoplasts (depending on the fungus involved). The insect after infection with fungi usually killed (sometimes by fungal toxins) and new propagules (spores) are formed in or on the insect if environmental conditions are again right (Fernandes *et al*., 2012) [17]. EPF *B. bassiana* produce considerable amount of toxins within the hosts like Beauverolides, Bassianolide, Beauvericin and Isarolides in infested hosts and *M. anisopliae* produce cytochalasins and Destruxins (DTXs) have been isolated from infected host. These toxins exert discrete effects on different tissues in the insect. (Elsworth and Grove, 1977); Bradfisch and Harmer, 1990) [6]. The effects are Partial or general paralysis, decreased irritability and sluggishness in infested insects, are certain behavioural symptoms that are persistent with the action of neuromuscular toxin. (Charnley *et al.,* 1984) [11]. Death of the insect is often due to a combination of the action of fungal toxins, physical obstruction of blood circulation, nutrient depletion and/or invasion of organs. After the host has died, hyphae usually emerge from the cadaver and under suitable abiotical conditions; conidia are produced on the exterior of the host. These are then dispersed by wind or water. (Goettel and Inglis, 1997) [21].

Mode of action of EPF against eggs of ticks

EPF are found effective against eggs of ticks The oxidation in the egg wax during and after laying of eggs causes reduction in the levels of unsaturated fatty acids which makes the wax harder after secretion, rise in its melting point and making wax stickier and better able to hold the egg mass. This process would thus improve the waterproofing properties of the wax and enhance the protection against various environmental hazards. However, as the age of the egg advances, oxidation of their surface wax proceeds and the amount of unsaturated fatty acid in their tegument declines resulting in weakening of anti–fungal properties which causes resistance As a result tick eggs appear to be more susceptible to the fungus compared to adult ticks. The mode of action of *Lecanicillium* and *Beauveria* fungi by forming colonies on the egg surface prevent the hatching of eggs but could not penetrate the chorion layer. (Booth, 1992 and Pirali-Kheirabadi *et al.,* 2007) [4, 42]. When female ticks lays eggs, Gene's organ secretes a waxy coating onto each egg. This Coating both renders the eggs- water proof and contains fungicidal components. The non-polar lipids are the main component of

40% of the tick egg wax. (Camish, *et al.*, 1977) [8].

Pathogenic effects of EPF on ticks

Research workers from all over the world reported their findings after conducting the in vivo and in vitro trials of entomopathogenic fingi at their provenances. Field trials of both *M. anisopliae* and *B. bassiana* fungal species in pastures and directly on cattle in Kenya and Brazil as the biocontrol agents have great results in reducing tick burdens on livestock. The mortality rates tend to be moderate to high for adult and immature livestock ticks from the genera *Boophilus*, *Rhipicephalus*, and *Amblyomma* exposed to entomopathogenic fungi in pastures or stables. Also the potential for replacing livestock dips using chemical insecticides with those employing fungal spores in solution seems quite high. No toxic effects of these fungal solutions on livestock or other terrestrial vertebrates have been reported. Fungal pathogenicity of *M. anisoplaie* in many species of ticks, such as *R.* (B.) *microplus, Rhipicephalus sanguineus, Rhipicephalus appendiculatus, Anocentor nittens, Ixodes scapularis, Amblyomma cajennense, Amblyomma variegatum, Amblyomma maculatum* and *Amblyomma americanum* caused 83% mortality and *Beauveria bassiana* caused 77% mortality ticks. The fungal species induced reductions in engorgement weights, fecundity, and egg hatchability in adult tested in Zebu cattle naturally infested with *R. appendiculatus* in the field. (Kaaya *et al.,* 1996; Bittencourt, 2000; Kaaya and Hassan, 2000; Rao and Narladkar, 2017) [28, 3, 27, 45]. Entomopathogenic fungi are the most promising of the currently available alternatives to chemical acaricides for tick control. The in *vitro* pathogenicity of two *Beauveria bassiana* and three *Metarhizium anisopliae* isolates on eggs and larva of *Amblyomma cajennense* found the significant reduction in the treatment group and the lower hatching rate in comparison with the control. *In-vitro* effects of *Metarhizium anisopliae* on engorged *Boophilus microplus* ticks was analyzed by Frazzon, *et al.* (2000) [18], found 100% death rate in ticks with treatment of 10^7 spores/ ml. The mortality of host observed at 4-16 days (depending mainly on the host species) after contamination. (Strasser *et al*., 2000) [52]. The pathogenicity of five species of entomopathogenic fungi (*Deuteromycetes, species: Beauveria bassiana, Metarhizium anisopliae, Metarhizium flavoviride, Paecilomyces fumosoroseus and Verticillium lecanii*) against the various developmental stages of *Boophilus annulatus* ticks under laboratory conditions showed that, *M. anisopliae* and *B. bassiana* strains are most virulent to engorged females with 85-100% mortality within 7-10 days post-inoculation (PI). All tested fungi prevented or reduced the egg laying capability of the ticks several days before their death. Females surviving after treatment with the most virulent *M. anisopliae* strain (Ma-7) reached only 7-8% of their egg laying capacity as compared with the control. Other fungi caused a reduction of the weight of laid eggs by 35.4-80.8% as compared with untreated females. Only *M. anisopliae and B. bassiana* strains caused 70-98% mortality of the treated eggs. The efficacy of these fungal species to *Boophilus annulatus* eggs, larvae and engorged female tick under laboratory conditions showed the larval hatchability from 2-10% of eggs treated with *B. bassiana* and from 10-30% of eggs treated with *M. anisopliae*, as compared with 85-90% from untreated control eggs. (Gindin *et al.*, 2001). The pathogenicity of four strains of entomopathogenic fungi *Metarhizium flavoviride var flavoviride* and *Metarhizium anisopliae var anisopliae in-vitro* against the bovine tick *Boophilus microplus,* found the highest level of effectiveness of biocontrol in groups treated with concentrations of 10^7 and 10^8 conidia/ml with *M. flavoviride* strain, CG-291 being the most effective. (Onofre *et al.*, 2001) [39]. Fernandes *et al.* (2012) [17], conducted the experiment on *in vitro* virulence of three isolates of *Metarhizium anisopliae var. anisopliae* to eggs and larvae of the tick *Boophilus microplus*, observed reduction in hatching percentage of treatment group as compared with control group. The effect of the *B. bassiana* strain tested on developmental stages of *R. sanguineus s.l* under laboratory conditions showed the significantly higher mortality on eggs, larvae, nymphs and adults than those of the control groups at 5 days post-infection. *B. bassiana* strain is highly virulent towards all life-cycle developmental stages of *R. sanguineus s.l.* and may be of potential interest as a biological control agent against these ticks. Cafarchia *et al.* (2015) [7].

Conclusion

The Entomopathogenic fungi are natural enemies of insects and arachnids and these fungi contribute to the regulation of their host populations. (Nicolai, 2007). Entomopathogens are microorganisms that are pathogenic to arthropods such as insects, mites, and ticks which can be a critical part of integrated pest management (IPM) against several pests. (Dara, 2017). The minimal effects of entomopathogenic fungi observed on the animals and other non-target organisms and can also be used in integrated pest management replacing the conventional chemical insecticides. (Pell *et al*., 2001) [41]. The percentage of ticks naturally infected by fungi varies considerably according to the stage and species of ticks and also the ecological conditions at the sample sites. Isolation of indigenous entomopathogenic fungi is important for developing isolates that avoid the introduction of new (exotic) fungal isolates for tick biological control in certain environments. The strains of commercially available entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, appear to be most promising for the control of some pests. Biological control is likely to play a substantial role in future IPM programmes for ticks because of the diversity of taxa that show high potential as tick BCAs. The considerable research is required to select appropriate strains to develop them as BCAs, establish their effectiveness, and devise production strategies to bring them to practical use. (Samish *et al*., 2004) [48]. EPF can be an alternative to chemical acaricides and can be replaced as a biological control agent to overcome the acaricidal resistance and environmental pollution.

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