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Title: Articulating fragrant agarwood formation as an outcome of the interaction between the insect *Zeuzera conferta* and *Aquilaria* trees – A Review

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Abstract

Agarwood is the resinous infected wood formed by *Aquilaria* species, which is a highly priced product in the flavour and fragrance market. Its formation is a complex process of interaction between the plant, insect, and microorganisms. Multiple studies concerning the interaction of microorganisms with the *Aquilaria* tree have been reported. However, the significant interaction between the insect *Zeuzera conferta* Walker (Lepidoptera: Cossidae) with *Aquilaria* have been overlooked and only exiguous studies have been accomplished. Considering the dearth of available literature on this interesting phenomenon a review has been attempted. The taxonomy, morphological descriptions proffered by researchers and the insect life cycle is discussed. The review lays emphasis on the chemical ecology of the interaction between *Z. conferta*, *Aquilaria* and associating microorganisms as a possible continuum operating in the form of complex chemical signalling via release and sensing of Volatile Organic Compounds (VOCs), and Herbivore Induced Plant Volatiles (HIPVs) and Microbial Volatile Organic Compounds (MVOCs). The review also scrutinizes the future perspectives of understanding the interaction in devising suitable management strategies to prevent uncontrolled infestation and simultaneously develop artificial rearing technology for the insect *Z. conferta* as a strategy for ensuring sustainable livelihood of farmers dependent on agarwood production.

Keywords: Insecticides, Frass, Taxonomy, Artificial rearing, Interaction, Lepidopteran.

Declarations

Funding

1. ST Fellowship, Award No:201920-NFST-ASS-01039 from Ministry of Tribal Affairs, Govt. of India to AK.
2. Grant No CRG/2018/003308, Department of Science and Technology, Govt. of India to SS.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material

Not applicable.

Code availability

Not applicable.

Authors contribution

AK conceived, designed the study, reviewed the literature and drafted the manuscript. SS supervised and critically reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval

Not applicable.

Consent to participate

Not applicable

Consent for publication

Not applicable

INTRODUCTION

Zeuzera conferta Walker (Lepidoptera: Cossidae) is one of the principal insect pests that has been found to associate with the *Aquilaria* trees. It is also known as *Neurozera conferta* Walker (Syazwan *et al*., 2019). It belongs to the Class Insecta, Order Lepidoptera, and Family Cossidae in a systemic classification. It is widely distributed in South East Asian countries, Eastern Himalayas, Sri Lanka, Bangladesh, Taiwan, Andaman Islands and the Philippines. The insect is particularly prevalent in the agarwood plantations of the north-eastern part of India particularly in the state of Assam and is known to influence the formation of the rare resinous and fragrant agarwood by infesting *Aquilaria* trees. In fact, agarwood from Assam is regarded as a high-quality material in the global agarwood market. The borer insect is known locally as "Pukh" in Assamese and "Emphu" in Bodo languages, with both the terms meaning insect which is used as a general nomenclature by the cultivators, traders, and people conversant with the *Aquilaria* trees in Assam. The *Aquilaria* trees which belong to the Thymelaeaceae family are commonly known as Agarwood, Eaglewood, or Aloes wood besides various other regional names. Agarwood is the dark-coloured resinous fragrant wood that has a high commercial value. Further, the formation of agarwood involves a complex process of interaction between the plant, insect, and microorganisms. The process of formation of agarwood is a defense response that is connected to this response to injury, created through natural and artificial means. To prevent or to recover from the injury, the *Aquilaria* trees produce oleoresins at the site of the injury as a product of plant defense response (Zhang *et al*., 2012). The site of the injury is colonised further by microorganisms leading to the accumulation of the oleoresin which is called agarwood.

So far, a total of 19 insect pests have been recorded to associate with the *Aquilaria* trees which belong to 16 families and 5 orders of which the preponderance of the sap-sucker is found higher, followed by leaf defoliators and lastly wood borers (Syazwan *et al*., 2019). However, the wood borer and the leaf defoliator forms the major pest of the *Aquilaria* trees which causes a serious damage to the *Aquilaria* trees (Ong *et al*., 2014). The larvae of the wood borer *Z. conferta* Walker (Figure 1) infest the woody stem of the *Aquilaria* trees and facilitates subsequent microbial infections. The larvae make vertical tunnels inside the trunk of the *Aquilaria* trees as they feed and move up spreading the microbial infection where the oleoresin accumulates (Kalita *et al*., 2015). From brown streaks to dark brown and finally to black coloured wood are the changes that occur in the healthy wood where the initial infestations occur. Successively, these lead to stunted and poor development, formation of cankers on the trunk, swelling, symptoms of dieback on the top and outer branches of the trees (Saikia and Nath, 2001). Subsequently, the scenario of a visible wound, stem distortions, decayed branches, uneven and irregular trunk, and odoriferous dispenses evidence of agar formation inside the tree. The incidence of *Z. conferta* is however not observed in all the *Aquilaria* trees that are grown. Its selectiveness in infesting the *Aquilaria* trees is interesting, as significant differences are being observed in the infestation process among the *Aquilaria* trees that are grown separately at a distance of a few meters with one area being completely infested and the other area with none. These variations in the infestation process have led the traders to practice artificial process of injury and induce infections through physical, chemical and biological means or by their combinations as a method of treatment in the *Aquilaria* trees where insect incidence is not usually observed. However, the increase in commercial demand and slow natural process of agarwood formation have also pressurized the traders to execute the process of artificial infections. (Chippa and Kaushik, 2017). Despite its success in producing agarwood through the application of artificial techniques the quality of the agarwood remains an issue and is found incommensurate in comparison to naturally occurring ones (Kalita *et al*., 2015). Moreover, the price of the agarwood, the durability of the fragrance, long shelf life, and the extent of the microbial attack are all found to be higher in agarwood that is induced only after the insect (*Z. conferta*) infestation (Hoque *et al*., 2019).

Even with these evidence about the importance of the *Z. conferta* in the formation of agarwood, studies are yet to elucidate its actual role. There is a possibility of insect- microorganism relation which might have a role in the superior quality agarwood formation (Hoque *et al*., 2019). However, literature is deficient concerning *Z. conferta* and therefore, in the present review, efforts have been made to compile the information scattered in diverse domains and bring out the perspectives that require a closer study to improve agarwood production in future.

TAXONOMY, MORPHOLOGY AND DEVELOPMENT

Kingdom:	Animalia
Phylum:	Arthropoda
Class:	Insecta

Order:	Lepidoptera
Family:	Cossidae
Genus:	*Zeuzera*
Species:	*Zeuzera conferta*

The genus *Zeuzera* was classified based on the external characters only, for instance, the presence of the crossvein Subcosta (Sc)- Radius Sector (Rs), the shape of the humeral plate, and the length of the anal plate (Sutrisno, 2015). From a total of 52 species across the world, only 5 are reported to occur in the Indian Subcontinent (Arora, 1976). According to him, the original illustration given by Walker, (1856) on the *Z. conferta* reported the female to be whitish with black antennae. The thorax possessed two interrupted green stripes and three rows of green spots in the abdomen. Legs were reported to be mostly green and wings with innumerable tiny transverse green or aeneous streaks with green dots down the border. The fore wings were without streaks at the parts of the disk. The length of the body was 13 lines and that of wings 28 lines. The species was known from the Sylhet region of Bangladesh and Labuan and Luzon of the Philippines. It was reported to be close with *Zeuzera indica* Herr. -Sch regarding the origin of the vein in the fore wing and the evenly-rounded outer margin in the hind wing. However, phylogenetic studies based on the Cytochrome Oxidase subunit I gene (COI) sequence on the other hand revealed *Z. conferta* to be closely related to the *Z. lineate* (Sutrisno, 2015). The COI gene is regarded as highly conserved and felicitous in identifying a species due to its low variability (generally less than 1-2%). Even for closely associated species its value is found to be less than 1%. Furthermore, for Lepidoptera the COI gene is one of the most common to be used in inferring the relationship among the closely related species. Yakovlev, (2011) stated the species to be medium in size with males possessing cup-shaped antennae and female filiform (Figure 2a). Dorsally white thorax, with minute black dots on the lateral surface, rounded minute segment on the abdomen, and laterally minute pair of black dots on every segment. Elongated forewings, apically acute, white to coffee-coloured, dark bright dots on wings margins, with minute rows of black dots on veins and patternless hindwing with indistinct dark spots on the outer margin. The uncus of the male genitalia was long, with middling thickness, with a minute acute apex of the beak in shape. Separately, thick gnathos, a leaf-like smooth valve at margins of middling thickness, and juxta with a well-developed lateral process. The saccus was small and semi-circular and aedeagus short, thick, and slightly curved in its proximal third with no cornutus. Nevertheless, the genitalia of the female were not studied. The pupae and adults have been recorded by Ong *et al*., (2010) concerning *Rhizophora apiculata* plant. Furthermore, Senthilkumar and Murugesan, (2015) reported the male forewings to have black spots being strongest on the vein and opaque white zone, free of black spots, at the end of the cell and in female presence of typical transverse black striae and again black spots free zone at the end of the cell. Adult females are however found to be larger than males, possessing long ovipositor at the end of the abdomen, enabling them to position their egg in the bark crevices (Ong *et al*., 2010) (Figure 2b). However, the latest studies carried out by Borthakur *et al*., (2021) on the *Z. conferta* biology, revealed the adult to be of medium in size with a wing expanse of 27 to 35mm. The arrangement of the forewings was found to be flickering bluish to black in colour with asymmetrical striations or white pellucid background. The hindwings were also reported to be spotty and translucent. The abdomen was brownish in colour and beard black dots which was covered with fur (Figure 2c).

Overall, the genus *Zeuzera* has been actively studied by Roepke (1955; 1957), based on New Guinea and Malayan fauna and Holloway (1986), based on Bornean fauna. The latter suggested the genus to be similar and well-defined sections of *Xyleutes*, a genus of moths that belongs to the Cossidae family. Schoorl (1990), also made a detailed study on the morphological aspects of the genus *Zeuzera*. The genus was interpreted based on the presence of crossvein Sc-Rs, humeral plate triangular in shape and anal plate comparatively long to short. Based on his hand cladogram, various characteristics believed to be apomorphies of genus *Zeuzera* and its relationship within its genus were also presented. However, it was felt that his study needed further assessment due to the growing evolution of understanding in the field of study to testify its validity (Sutrisno, 2015).

The species of the *Zeuzera* mostly live as a larva in plants (Sutrisno, 2015). Eggs are laid by the female moth chiefly in groups directly in cracks, crevices of the stem and larger branches of the host plant (Moaty *et al*., 2019). A total of 180-250 eggs are laid by the single female *Z. conferta* in one batch with the size ranging from

0.2mm in length to 0.1mm in breath (Borthakur *et al*., 2021). The caterpillars emerge out from the egg after its development and are called first instars till it molts. It enters the second instars after the molt and increases in size. Every stage of molting distinguishes another instar. Typically, a caterpillar passes through a total of five instars as it eats and grows, wherein each instar its general appearance changes from one to the next. According to Borthakur *et al*., (2021), the size of the larvae increased from 0.03 cm to an average of 4.5±0.7 in length and from 0.02 cm to 0.06 cm in breath from first instars to fifth instars. The change was also observed in the colour pattern of the larvae form light reddish pink to light pinkish from first instars to fifth instars. Before entering the stage of the pupation, the matured larvae prepares the pupal tunnel and also the exit hole near the bark surface. The pupa measured 1.9 to 2.5 cm in length and and 0.05 cm in breath and weighted 0.46 gm and completed its pupal period within 14-30 days and emerged out as an adult moth. In conclusion they reported that *Z. conferta* have two generations in a year which was also earlier reported by Baksha and Islam (1999), with regards to *Sonneratia apetala* trees in Bangladesh.

ECOLOGY AND INTERACTIONS

1. Diversity of Hosts

Besides *Aquilaria* trees, the *Z. conferta* has a broad range of host of different families of which are, *Sonneratia apetala, S. alba, S. ovate* of family Lythraceae, *Aegiceras corniculatum* of Myrsinaceae, *Avicennia lanata, A. marina, A. officinalis* of Avicenniaceae, *Ochroma lagopus* of Bombacaceae, *Eucalyptus deglupta* of Myrtaceae, *Rhizophora apiculata, Rh. mucronata* of Rhizophoracaea, *Theobroma cacao* of Sterculiaceae, *Coffea* of Rubiaceae, *Erythroxylum* L. of Erythroxylaceae, *Elettaria cardamomum* of Zingiberaceae and *Tamarix indica* of Tamaricaceae family (Islam, 2004; Yakovlev, 2011). The preferential habitat of the majority of these host trees are coastal mangroves forest, moist, lowlands with few from the tropical forest (Ong *et al*., 2010; Senthilkumar and Murugesan, 2015). The larvae target the stems, trunks, twigs, and shoots of the host plant for the infestation. The association of *Z. conferta* with *S. apetala* is extensively studied in Bangladesh where the larva of *Z. conferta* is also termed as “bee hole borer”. The tree *S. apetala* is largely utilized as a plantation species to construct a shelter belt along with the coastal areas and offshore islands of Bangladesh. The larva is reported to bore in the barks and later make large, profuse, oval, and ramifying tunnels in the stem rendering the tree to wind breakage. Later, the larvae and pupae of the *Z. conferta* are found to be eaten by woodpeckers such as *Dinopium benghalense* and *Picoides canicapillus* and small black ants (Islam, 2004). In *Avicennia spp*, the infestation by the larvae *Z. conferta* is reported to occur at an interval of seven years in natural mixed forest of Brazil (Vannucci, 2002). Few other host plants of the *Z. conferta* includes Cocoa (*Theobroma cacao*), Balsa, Coca (*Erythroxylum* P. Br), and *Barringtonia* (Arora, 1976; Schoorl, 1990). It is however interesting to note that with regards to these hosts of *Z. conferta*, only in *Aquilaria* trees it is reported to have a productive role i.e., in the formation of resinous wood called as agarwood.

2. Interactions: Insect-plant-microorganism

Plant and its biotic interactions in natural environment has diverse manifestations. In nature, plant interacts with the insect by attracting pollinators for sexual reproduction on one side and on the other protecting itself from herbivores, pathogens and even other plants by synthesizing various chemical compounds (Schiestl, 2010). Plants, being sedentary organisms use volatile compounds as a vernacular to communicate and interact with the surrounding environment (Dudareva *et al*., 2006). Volatile organic compounds (VOCs) are released by the plants constitutively that herbivores utilize for host location (Penaflor and Bento, 2013). The chemical signals are perceived by the herbivores with the aid of olfactory system and commence the behaviours for communication with the host (Field *et al*., 2000; Fatouros *et al*., 2008; Leal, 2013). The attraction of the insect towards the host plant due to the volatile phytochemicals, are perceived by specialized chemoreceptor neurons on the antenna (Loon, 1996). Herbivores are recognized by the plants through damage-associated molecular patterns (DAMPs) and herbivore- associated molecular patterns (HAMPs), also called elicitors which include extracellular protein fragments, nucleotides, peptides, glucose oxidase, fatty acid-amino acid conjugates (FACs), β-glucosidase, inceptins, and caeliferins (Giron *et al*., 2018; Hogenhout and Bos, 2011). The herbivore attack on the host plant releases a large diversity and a greater amount of VOCs, called as Herbivore Induced Plant Volatiles (HIPV) (Paré and Tumlinson, 1999; Howe and Jander, 2008; Penaflor and Bento, 2013). HIPVs are important olfactory cues produced by the plants under herbivores attack, in a manner that they reveal indirect information about the presence of the herbivores (Aartsma *et al*., 2019). Parasitoids, specifically carnivorous insects use HIPVs in locating and regulating the herbivores as their natural enemies (Forbes *et al*., 2018; Kessler and Heil, 2011) resulting in tritrophic interaction. Terpenoids, aromatics, green leaf volatiles (GLVs—C6 aldehydes, alcohols, and their esters), and amino acid volatile derivatives are some of the volatiles emitted by herbivore-damaged plants (Dudareva *et al*., 2006). The recognition of the DAMP and HAMP by the plants activates the diverse defense mechanism in the plants aiming to reduce the damage caused by the herbivorous insect (Giron *et al*., 2018). Plants respond to the herbivores attack by two mechanisms known as, Direct and

Indirect defense. Direct defense includes all plant traits enhancing the resistivity of the plant and thereby changing the insect's behaviour or physiology. Indirect defense on the other hand includes all the plant traits but does not have a direct effect on attacking herbivores but it can attract the natural enemies of the herbivores which can be any carnivorous insects (Aljibory and Chen, 2018). The metabolites of lipoxygenase (LOX) pathway, the shikimic acid pathway, and product of the terpenoid pathway are the prevalent volatile signals involve in direct and indirect defense (Pichersky and Gershenzon, 2002). However, some herbivores have developed resistivity to such a response and alter plant metabolism by injecting effectors into the host plant and repress the plant defense system (Hogenhout and Bos, 2011; Kaloshian and Walling, 2016; Giron *et al*., 2018). Secondary metabolites of the plant also act as the defense system towards the insect herbivory and also several classes of secondary' products are produced through infection, wounding, or herbivory. Insect, however, becomes immune or develops an adaptation mechanism to such a defense mechanism of plants due to feeding on or infecting a particular plant (Bennett and Wallsgrove, 1994). Throughout the phase of feeding or during egg deposition, the herbivores alters the phenotype of the plants through changes in the production of central and specialized metabolites, morphological traits, and architecture (Dicke and Baldwin, 2010; Hilker and Meiners, 2010; Howe and Jander, 2008; Mithofer and Boland, 2012).

The oviposition of the insect has also been found as a threat to the plants. Similar to herbivory induced volatiles, the oviposition by the herbivores also activates the release of oviposition induced volatiles (Hilker *et al*., 2002; Fatouros *et al*., 2005b; Salerno *et al*., 2013). The female wound the trees prior to the egg deposition and the elicitors which is procured from the secretion attaching eggs to plants, forge contact with the inner plant tissues through this wound inflicted (Hilker *et al*., 2005). The female has the aptitude to acknowledge the finest plant or host quality for the fine growth of larvae. The mechanism of specific site preference for oviposition by the female is also a master plan to procure defense against predation on premature stages of development. The oviposition is a critical step, peculiarly in Lepidoptera owing to the relative immobility of the hatching larvae and thus depending on the judicious choice of food plant by the adult female (Fenny *et al*., 1983; Renwick, 1989). Various events leading to oviposition follows a sequence of searching, orientation, encounter, landing, surface evaluation, and acceptance (Renwick and Chew, 1994). All these stages of the sequence depend on the sensory cues of the insect, however, definitive experiments of the sequential mechanism are difficult to perform (Morris and Kareiva, 1991). After the insect gets descend on a plant, it determines its site suitability for oviposition through the physical and chemical contact perception on the various surface of plants. Tarsi, antennae, proboscis, and ovipositor of lepidopterans are the sensory receptors involved. The Central Nervous System (CNS) acts as the final processing of information provided by the various sensory inputs received by the insect acceptance or rejection of a site for oviposition (Renwick and Chew, 1994).

The microorganisms also interact with the insects through the production of microbial volatile organic compounds (MVOC). The MVOCs have been found to closely associate with the behaviour of the insects (Davis *et al*., 2013). Insects are sensitive to odors and highly responsive to microbial volatile emissions (Ezenwa *et al*., 2012; Price *et al*., 2011). MVOCs have ecological functions such as some MVOCs that can attract or repel insects, stimulate oviposition, inhibit the growth of microorganisms competing the associate insects, mimic plant hormones or induce defense resistance (Davis *et al*., 2011; Ryu *et al*., 2003, 2004). The microbial associates are also responsible for the important physiological functions of the insects (Haine *et al*., 2008; Rozen *et al*., 2008) and the interaction of the insect-microbes might even play a significant role in quorum sensing (Lowry *et al*., 2008; Ma *et al*., 2012; Tomberlin *et al*., 2012a). Symbiotic microorganisms associated with the insect also plays a role in finding a suitable host and food resources for the insect (Davis *et al*., 2013).

Till date there is no scientific evidence and no experimental studies have been executed to explain about the mechanism of interaction between the *Aquilaria* tree and *Z. conferta*. Moreover, meagre studies have been accomplished on the biology of *Z. conferta*. Based on the analogous strategy of insect-plant interaction, therefore the insect *Z. conferta*, and *Aquilaria* tree might also trail a similar pattern of interaction (Figure 3a- l). The *Aquilaria* tree (Labelled a) might produce aroma related volatiles such as Volatile Organic Compounds (VOCs) to attract the herbivore, *Z. conferta* (c) as the plant volatiles are the significant molecules in the insect-host recognition (Qiao *et al*., 2012). The adult moth, *Z. conferta* then possibly detect the signals of the various volatiles released by the *Aquilaria* trees through their olfactory system and receives through the chemoreceptors (e). As VOCs possess the potential to trigger the behaviour of the insects, it might stirred up the adult *Z. conferta* to look for the host plant for the oviposition. On finding the host plant, the adult female moth then undertakes various sequence of events preceding the oviposition in order to confirm the suitability of the oviposition at a favourable site in the host plant (f). On obtaining the suitable site the female moth lay eggs and completes its life cycle (g). The larvae which emerged from the eggs then initiates the phase of infestation in the *Aquilaria* trees by chewing the wood. Throughout the period of infestation, the larvae might inject various elicitors into the woody stem of the host plant. The host plant then activates its diverse defense mechanism to combat the herbivory by synthesizing disparate secondary metabolites and also releasing various HIPVs. The HIPVs are released by the *Aquilaria* trees to attract the natural enemies of the *Z. conferta* and accordingly maintain a mutualistic relationship with the trees (h). Hitherto, however there has been no literature available with reference to the natural enemies of the *Z. conferta*. The injection of the elicitors represses the defense mechanism of the *Aquilaria* trees and the larvae of *Z. conferta* might survive the phytochemicals released by the *Aquilaria* trees. The microorganisms present in the tree might also emit the Microbial Volatile Organic Compounds (MOVCs) triggering the behaviour of the insect *Z. conferta* (d). Fungi of the genera *Fusarium* and *Lasiodiplodia* have been reported to produce two compounds, **δ-**lactones and mullein which releases an odour that have the potential to act as an insect attractant (Nago and Matsumoto, 1994). The interesting finding is that both these fungi are associated with the *Aquilaria* trees (Mohamad *et al*. 2010; Chippa and Kaushik, 2017). Therefore, such microorganisms might also be releasing the volatile compounds and alluring the insect *Z. conferta* towards the *Aquilaria* trees. In a chemometric evaluation of interaction study carried out by Sen *et al*., (2017), between the agarwood and fungus *Fusarium* revealed the appearance of ecologically important semio-chemicals (e.g. Pheromones). Semio-chemicals are the organic compounds which have the potential to stimulate the activity of organisms used by insects and other animals for the purpose of biological communications. The role of semio-chemicals, however, in the fungal colonization in *Aquilaria* trees through the insect *Z. conferta* intervention needs further investigation. Consequently, after the infestation of the larvae in the *Aquilaria* tree, spiral, oval, or ring-shaped injury or wound (Figure 4a) is developed which served as the gateway for initiation of microbial infection (Kalita *et al*., 2015) and the tree response to it by the formation of resinous wood called agarwood along the zone of infestation as a mechanism of defense reaction (Figure 4c) (j-k). The size of the tunnel increases as the larvae grow and also the metamorphosis from larva to pupa takes place inside the tree. The pupa partially moves out from the exit hole before finally attaining its maturity as a moth (Ong *et al*., 2010). The larvae then complete its life cycle and emerges out as an adult moth through the exit hole leaving the exuvia intact (l). However, the release of the HIPVs by the *Aquilaria* trees to attract the natural enemies of the *Z. conferta* and to prey on it might debarred it from completing its life cycle. Research carried out on the antennal and behaviroal response of the *Heortia vitessoides*, one of the major leaf defoliator pest of the *Aquilaria* tree also showed that the female moth was attracted to the volatiles of the green leaves which possessed the compounds such as nonanal, decanal, hexanal, and (Z)-3-hexenylacetate rather than the dry leaves, forming the vital constituent for the minimal attraction of the insect (Qiao *et al*., 2012; Syazwan *et al*., 2019). Finally, as the *Aquilaria* tree matures, it closes its exterior entry and exit hole leaving a distinct lesion (Figure 4b).

The microorganisms present in the gut of the *Z. conferta* might also have an indispensable role in infecting the *Aquilaria* trees as they are assigned with various significant roles in their host metabolism, physiology, growth, reproduction **(**Breznak, 1982; Chen and Purcell, 1992; Lemke *et al*., 2003). The larvae might act as a vector and release potential microbes to induce the infection through the excretion process during the tunnelling pursuit. Study on the diversity of the microorganisms associated with the gut of the larvae and comparative analysis with the microbes associated with *Aquilaria* trees and in resinous agarwood might help us to understand and co-relate the mechanism of insect-plant and microorganism's interactions. Analysis on the potential role of the endophytic microbes associated with the *Aquilaria* trees in producing odorous compounds will help us in a deeper understanding of its capability of alluring the *Z. conferta* towards *Aquilaria* trees. Furthermore, the study of the difference between the insect-infested and non-insect infested *Aquilaria* trees will also contribute to a broader way of understanding of the mechanism of insect infestation, as *Z. conferta* is not found to invade all the *Aquilaria* trees in the same environment.

PRESENT AND FUTURE PERSPECTIVES

1. *Zeuzera conferta – a pest?*

The association of the *Z. conferta* with the *Aquilaria* trees can be referred to as "necessary evil". The infestation by the borer in the young *Aquilaria* plants causes a major a threat to its survival as the activity of tunnelling by the borer damages the tissues of the plants. However, its desideratum cannot be ruled out, as agarwood of good grades is obtained only when the trees are infested by it (Hoque *et al*., 2019). The damage caused by the *Z. conferta* in *Aquilaria* trees is still considered as moderate level apart from the other 19 pests that are found to associate with the trees (Syazwan *et al*., 2019). The people familiar with the *Aquilaria* habitats and plantations are very much cognizant of the necessity of its association with the *Aquilaria* trees. Even the ill-educated in the rural areas are familiar with its essentiality. Though the scientific knowledge of different mechanisms of formation of agarwood is unknown to them.

Despite having a prominent role in assisting fine agarwood formation, it remains as a major drawback for most of the *Aquilaria* cultivators owing to the damage it causes by infesting in the young *Aquilaria* plants leading to the stunted growth and sometimes death of the plants. The term pest is reckoned for an insect or a microbe in the agricultural practices if it interrupts the progression or the development of the plant (Syazwan *et al*., 2019). The commencement of the infestation of the pest *Z. conferta* is observed in the plants attaining 5 years, and maximum in the trees of age group 8-16 years and moderately above 16 years (Kalita *et al*., 2015). The cultivators have therefore taken quite a few measures to protect the *Aquilaria* plants from infestation by the *Z. conferta*. Some of the measures are (i) Spraying of the insecticides on the surface of the tree (ii) spraying of the insecticides directly on the holes made by the borer on trees (iii) Killing of the insect directly when found (iv) Killing the female moth before oviposition. The measures taken are performed seasonally based on the availability of the insects. The event of killing is intermittent as it is performed only when the insects are found. However, the spraying of the insecticides is performed when the rate of an infestation is found to be higher based on the observation. Few other management practices that have hitherto been executed are, trimming and removal of the infected branches, shutting off the hole made by the insect with plasticine by the application of the liquid-based pesticides, and application of the granule-systemic based pesticides (Syazwan *et al*., 2019). Pheromones, mass trapping, mating disruption, entomopathogenic fungi, and nematodes are other control measures that have been successful in controlling the lepidopteran pest (Ibrahim *et al*., 2019, Ong *et al*., 2010). The practise of spraying insecticides is carried out when the trees are young and are heavily infested by *Z. conferta*. However, when the trees attain its maturity, the incidence of the *Z. conferta* is not much of a concern and the practice of spraying insecticides lessens as the tree becomes less vulnerable to breakage and damage. The insecticides used and their effectiveness in controlling the incidence of *Z. conferta* is needed to be analysed in detail. However, the application of the pesticides in controlling the incidence of the pest has become increasingly knotty due to concern about human health hazards, environment and pest resistance (Atreya *et al*., 2012). Furthermore, the obscure habitat of the larvae inside the trees and prolonged ovipositional period makes the chemical treatment less successful (Shamseldean *et al*., 2009).

2. *Frass*

The frass of the *Aquilaria* trees acts as an indicator of the incidence of the *Z. conferta* (Figure 5). Frass are the excrement or the excreted pellets which are released by the larvae after feeding on their food source. In wood borers the frass from the tunneling activity is expelled out from the entry and exit hole on the ground (Ong *et al*., 2010). The expelled frass helps the seeker, seeking the activity of *Z. conferta* in the *Aquilaria* trees for confirmation about the infestation just by scrutinizing the presence or absence of the frass on the ground surrounding the *Aquilaria* trees. The frass varies in shape and color with some spherical or oval in shape and white in color and some wet sticky powdered with brownish appearance. In assessing between the old and the new infestations by the *Z. conferta*, frass plays a prominent role through its morphological appearance. Dry frass and dimmed colored (Figure 5a) indicates old infestation, whereas wet and bright colored indicates the new ones (Figure 5b). According to the cultivators, the trees which expels brownish coloured frass after the insect infestation produces a better grade of agarwood (Figure 5c). Ants and spiders are later reported to occupy the abandoned stem to take refuge (Ong *et al*., 2010). According to Reynold and Hunter, (2004) frass also provides important source of nutrients in the soil system which increases the diversity of the soil invertebrates. The deposition of the frass in the forest floor increased the nitrogen content excessively. Collembola, fungal-feeding nematodes, bacterial-feeding nematodes, and prostigmatid mites are some of the soil invertebrates that have significantly increased in the Southern Appalachians due to the depositions of the frass in the forest floor (Reynolds *et al*., 2003). Careful investigations on frass can lead to future diagnostic mechanisms on staging insect infestations leading to agarwood resin formation. Biochemical, microbiological and image analysis of frass appear to be suitable initial analytical candidates in this regard.

3. Artificial Rearing

The incidence of *Z. conferta* is not observed in all the *Aquilaria* trees that is cultivated. The incidence or the prevalence of the pest attack in the *Aquilaria* trees is mostly observed in monocultures (Ong *et al*., 2014). The practise of growing as monocultures by the farmers into small or a large-scale came into existence due to the overexploitation outside of their natural habitat (Irianto *et al*., 2011). However, there are some regions where the incidence of the insect is not observed despite being grown as monocultures or mix cultures. Owing to that innumerable artificial techniques are applied for production of agarwood, as naturally the formation of agarwood very much relies on the insect and microorganism’s interactions (Syazwan *et al*., 2019). Despite its success in production of agarwood through artificial means, quality has always been a matter of concern for the cultivators as the superior grades are only harvested from the insect infected ones (Kalita *et al*., 2015; Hoque *et al*., 2019). The technique of artificially rearing the insect might be a key alternative to overcome this hurdle. The artificially reared *Z. conferta* larvae can be introduced in the *Aquilaria* trees where the incidence is not generally observed. Consequently, the larvae might select suitable site in the tree introduced and initiate its tunnelling activity and execute its role in insect-plant and microorganism’s interaction leading to the agarwood formation. The latest study carried out by Borthakur *et al*., (2021) on the life cycle of the *Z. conferta* bestows hope of artificially rearing the insect and making it available is in close proximity. This technique can be an alternative source of livelihood and also help in catalysing the production of quality agarwood, generating more advantage to the cultivators. Moore and Navon, (1966) were the first researchers to develop artificial medium for the wood borers, specifically for the leopard moth *Zeuzera pyrina* L. The artificial media of their preparation comprised of a basal medium of three variants, composed of full fat soya meal (30.0g); Sucrose (48.0g); Brewer's yeast (24.0g); Agar-agar flakes (24.0g); Nipagin (1.5g); Acetic acid 20% v/v (30.0ml); Sodium ascorbate 10% w/v (30.0ml); Pear bark homogenate (10g/70ml H_2O); and Distilled water. The media was successful in raising the successive generation of the leopard moth considerably within a short duration of 3-4 months than in nature which took a year. However, the artificially bred larvae were found glabrous and they differed in color as compared to those developed in woods. Moreover, apart from the laboratory conditions the rearing can also be tried in its natural state by providing the cambium portion of the *Aquilaria* stem as the larvae depends on it as food source (Borthakur *et al*., 2021). Therefore, the introduction of the larval stage of the *Z. conferta* at the right age of the *Aquilaria* plant might be a solution to the problem of lack of insect incidence and also might be less susceptible to breakage and death as matured trees are less vulnerable to pest (Ong *et al*., 2014).

Conclusion

Agarwood resin formation which is a unique phenomenon is still not clearly understood by science. The study of the insect should secure equal eminence with other areas of research associated with *Aquilaria* trees. The superior quality of agarwood garnered after the insect infestation makes the need to study the insect *Z. conferta* highly significant. Since the accumulation of the oleoresin is observed along the tunnels generated by the insect, there is also a possibility that the microbes existing in the gut of the larvae might as well initiate the microbial infection through the excrement of the larvae. The possibility of the involvement of chemical signalling in the ecology of the insect and its interaction with the plants and microorganisms further strengthens the argument that *Z. conferta* plays a pivotal role in the famous agarwood aroma development. Future studies are likely to unravel the intricacies of this involvement. The phrase "Necessary evil" parallels in describing the *Z. conferta*, as the infestation is a critical necessity to fine agarwood formation but also remains a major threat for the plantation when infestation occurs at early age. The selectiveness in infesting a particular *Aquilaria* tree, environmental conditions, and soil quality of the regions where the insect infestation is observed could be interesting themes of future research. Possibly the insights from the future research on *Z. conferta* and the plant-insect-microbe continuum can help to understand the aroma of agarwood better.

Abbreviations

VOCs: Volatile Organic Compounds.

HIPVs: Herbivore Induced Plant Volatiles.

MVOCs: Microbial Volatile Organic Compounds.

COI: Cytochrome Oxidase subunit I gene.

DAMPs: Damage-Associated Molecular Patterns.

HAMPs: Herbivore- Associated Molecular Patterns.

FACs: Fatty acid-Amino acid Conjugates.

LOX: Lipoxygenase.

CNS: Central Nervous System.

Acknowledgment

We are grateful to Mr. Abul Hussain, local trader, Namti, Sivsagar district Assam for rendering help during our field surveys and also to Ms. Rene Barbie Brown, Research Scholar, Assam Don Bosco University for assisting with the figures.

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Figure 1: Larva of *Zeuzera conferta* Walker: The process of extracting larvae from the *Aquilaria* trees firstly involves selecting the trees with the help of frass. The texture of the frass are checked to differentiate between the old and the new infestations. Dimmed coloured and dry frass indicates the old infestations whereas brightly coloured and wet with moisture intact indicates the new ones. The newly infected trees are selected and cut off and are brought into the agarwood processing centres. The stems are first cut into sections horizontally with the help of the chainsaw, few centimetres below and above the infestation point. The later are split into many symmetrical parts from top to the bottom edge vertically, thoroughly until the larvae comes out.

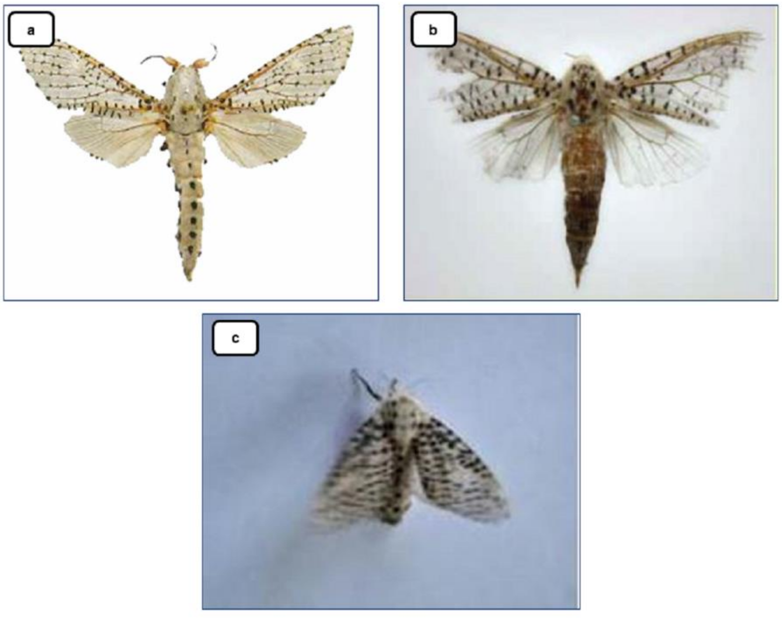

Figure 2: Morphologies of Adult *Zeuzera conferta* Walker. (a) Male (♂) *Z. conferta* Walker specified by Yakovlev (2011) from Sylhet region of Bangladesh, host plant unspecified. (b) Female (♀) *Z. conferta* Walker specified by Ong et al. (2010) from *Rhizophora apiculata* plant in Malaysia. (c) *Z. conferta* Walker specified by Borthakur et al. (2021) from *Aquilaria malaccensis* plant in India, gender (♂/♀) Unspecified. [Photographs reproduced with permission from respective publishers]

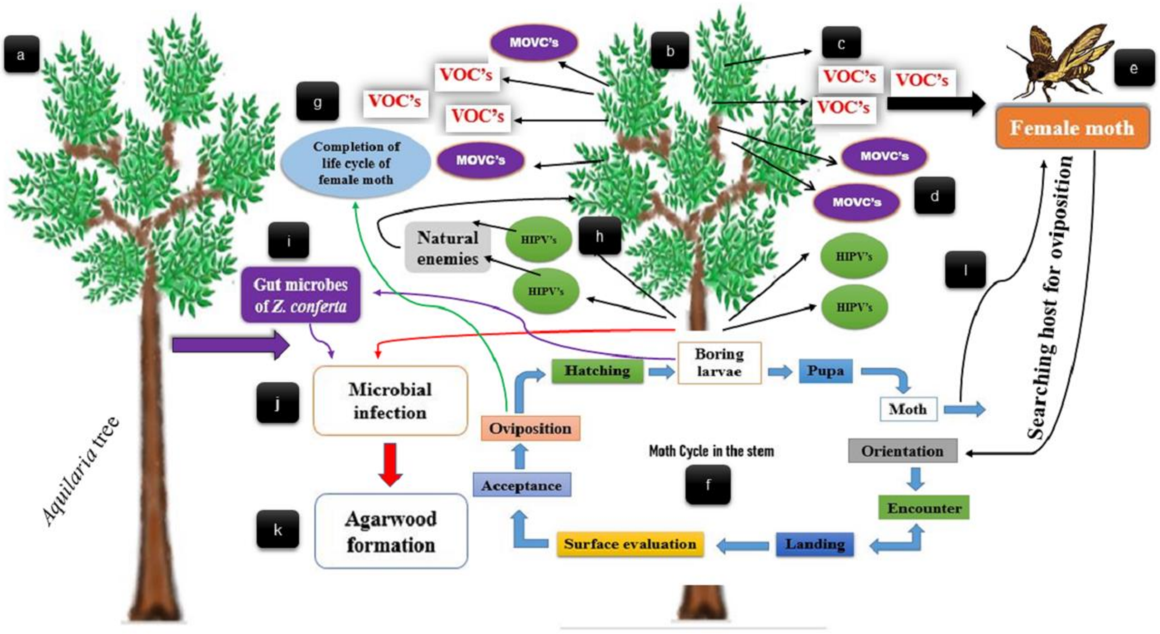

Figure 3: Interactions of *Zeuzera conferta* Walker linked in agarwood formation: (a) *Aquilaria* tree (b) Insect mediated agarwood formation in the *Aquilaria* tree through series of interactions between insect (*Z. conferta*)-plant (*Aquilaria*) and microorganisms. (c) The *Aquilaria* tree releases VOCs (Volatile Organic Compounds) (d) Microorganisms from the host tree also releases MOVCs (Microbial Organic Volatiles Compounds). (e) The female moth perceives the volatile compounds through their olfactory system from the surroundings. (f-g) Using volatile compounds the moth seeks for the host location and undergoes different series of events preceding the oviposition, checking the suitability for oviposition at the favourable site in the stem. On finding the suitable site the female moth lay eggs and completes its life cycle. The hatched larvae then commence the activity of the infestation by forming tunnels in the stem. (h) The tree releases HIPVs (Herbivore Induced Plant Volatiles) after the infestation to attract the natural enemies (e.g., carnivorous insects) of the larvae and establish a mutualistic relationship with the tree. (i-j) Microbial infection occurs along the tunnels created by the larvae, where the accumulation of resin occurs. The gut microbes of the *Z. conferta* also act as a source of microbial infection. (k) The accumulated resins across the tunnels are later called as agarwood. (l) The larvae emerge out as an adult moth from the exit hole after completing its life cycle.

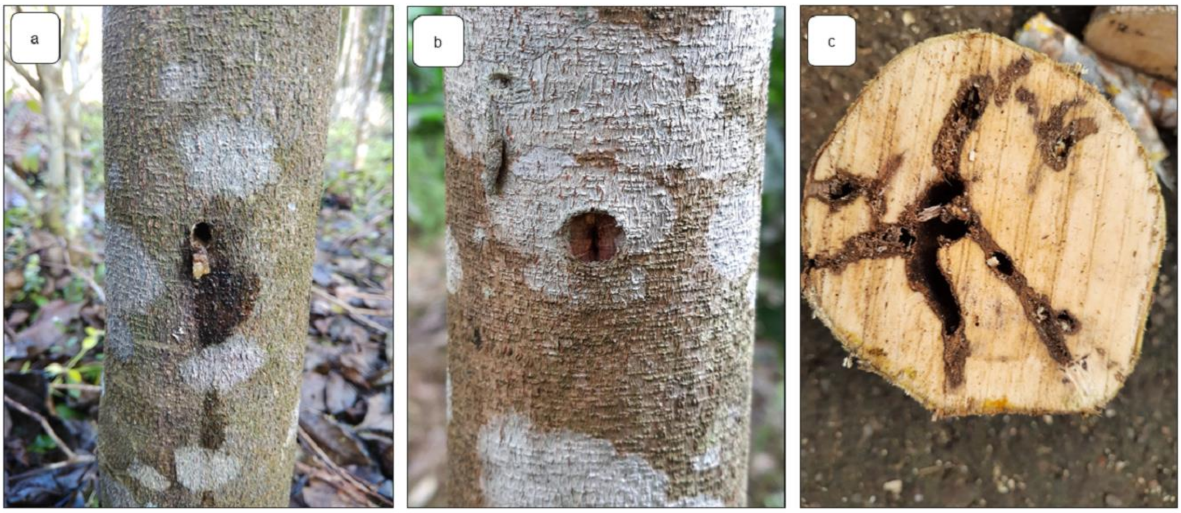

Figure 4: Resin formation at site of infestation: (a) Spherical wound created by *Zeuzera conferta* infestation. (b) Closure of the wound as the tree attains maturity. (c) Formation of agarwood along the tunnels created by *Zeuzera conferta* inside the stem of the *Aquilaria* tree.

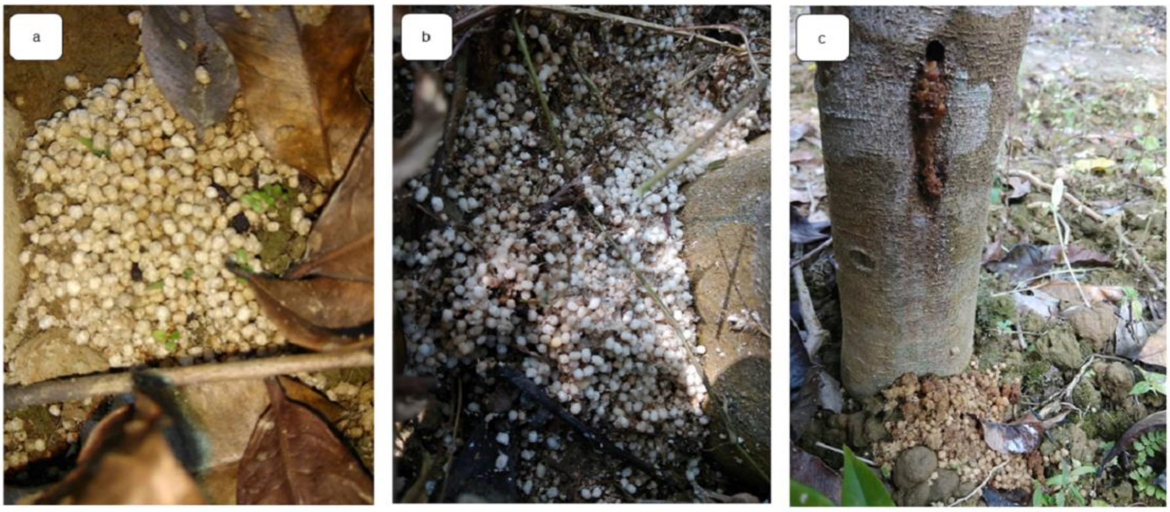

Figure 5: Insect frass as indicator: Frass or faecal pellets emancipated after the *Zeuzera conferta* infestation in the *Aquilaria* trees. (a) Dry, dimmed coloured frass (b) Whitish, freshly released frass (c) Sticky, brownish coloured frass.