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ODORANT BINDING PROTEIN IN PHEROMONE DETECTION AND DEVELOPMENT OF BIOSENSORS FOR INTEGRATED PEST MANAGEMENT

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ABSTRACT

Pests of plants pose a serious threat to the crop yield all over the globe, reducing the output by a large percentage every year. Hence it is important to develop novel and bio-friendly methods for monitoring and elimination of pests, while reducing the use of chemical pesticides. One such approach is the development of a biosensor which is based on odorant binding proteins immobilized on glass chips combined with electrodes for detection of insect semiochemicals, which are mainly volatile pheromones**.** Hence to develop such gadgets we must have a clear idea about pest insect pheromones, its characteristics and molecular, biochemical and physiological aspects of the entire perception procedures of these compounds. Considering the fact that insects use these chemicals for various functions in their life cycle, the knowledge about the chemistry and techniques for isolation and detection of these chemicals is of great significance. Since there is a lack of such tools that could actually be used instantaneously on agricultural field till in India for integrated pest management program. The high-throughput analytical technology is the basic tool required for selective and efficient detection and identification of pheromones for development of such efficient biosensors.

Keywords: Odorant-binding protein, pheromones, biosensor, Quartz crystal microbalance, pest management.

I. INTRODUCTION

The mode of communication in insects have been an important research area in the past few decades, particularly the family of semiochemicals that includes pheromones which are known to influence a large variety of behavioral aspects in insects. These aspects include sexual reproduction, social organization and most importantly the survival of various insect species[1]. Thus, pheromones can be used in a technology based approach or rather, as tools for controlling and managing pests of agricultural crops, and various species-specific strategies can be developed to eradicate numerous invasive species. Disruption of mating, mass trapping and attract-and-kill, are some of the first-hand pest control devices that depend on the use of pheromones[2][3]

A paper published in 1959 by Karlson and Luscher described “pheromones” as substances secreted by an individual of one species, received by another individual of that species, which is used as chemical cues for intraspecific signaling for communication and decision making [4]. These signals are received via selective detectors of such chemicals, that are found in insects and are known to posses highly adaptive olfactory system. These systems are characterized by their ability to process information by complex interaction between proteins and ligands which are then converted into appropriate behavioral signals[5].

Hence, it is important to deduce the chemistry of such pheromone molecules and employ various analytical methods to extract, identify and quantify these molecules to further develop sensors (both biological and chemical) for detection of such volatiles so that it can be used in agricultural fields to detect, control and monitor pest infestations. The fact that pheromones are very small compounds and generated in minuscule quantities (in parts per billion) and are highly specific (since it does not have any effect on non-target organisms), allows for rapid detection of contagions in agricultural fields aiding to risk-management analysis[6].

The buildout of various detection systems based on electrochemical sensors and specifically biosensors by mimicking the biological olfaction have been used for detection of key ligands in composite environment, leading to disclosure of novel techniques that employ chemical cues as way to detect pests in fields**.** Besides these novel technologies, pheromone traps have already been employed successfully across the world to captivate and monitor pest infestations. For instance, pheromone traps have been employed to confine pests of

lepidopteron and coleopteran species which include bollworm (*Helicoverpa armigera*) and leafworm *(Spodoptera littoralis),* beetles *(Harmonia axyridis), w*eevils *(Cosmopolites sordidus*) etc [3], [7]–[10]

In this review we will discuss in depth about the basis of insect olfactory system and odorant binding proteins, the various detection techniques of pheromones and development of biosensors for use in integrated pest management programme (IPM).

INSECT OLFACTION AND PHEROMONE PERCEPTION BY ODORANT BINDING PROTEINs :

The process of pheromone and other odorant perception occurs via a complex series of events. The olfactory system in insects is mostly located in the antennae, which is generally covered with minute sensilla, and the morphological features of these structures varies with species but the organizational and functional features are conserved [11], [12]. The signal chemicals access the sensillum through small cuticular pores and are transported by odorant-binding-proteins (OBPs), that are located in fluid in the sensillum lymph, to the odorant receptors (ORs)[13]. The odorant is encapsulated by OBPs and translocated to the dendritic membrane (location of ORs) where delivery of odorant occurs via a conformational change and release which arise due to interaction with negatively charged regions in the receptor. On binding to the receptor an ion channel unlocks and relays signals to OR neuron. The odorant then goes through an enzyme specific degradation [14], [15],

In insects, these ORs are G-protein-coupled receptor with seven transmembrane domains which is different from animal GPCRs [16] and pose an ideal target for insect specific signal inhibition that can be deployed for pest control management. But our focus here is the application of OBPs as biosensing elements and develop biosensors that can be exploited to detect odorants and pheromones in natural environment due to some of its remarkable properties and characteristics[17].

OBPs show incredible stability at high temperatures (up to 70 degree C), therefore can be utilized in challenging conditions. These proteins can easily be expressed and purified in bacterial and eukaryotic systems using standard protocols, with very high yields. Various denaturing agents can be used to alter the conformation of OBPs and it easily refolds back to its original conformation on removal of the impairing agent. So, it can be said that these proteins are perfect candidates to sense and trap environmental chemical odors due to low dissociation constant at a micromolar range and its natural affinity towards odorant and pheromones [18].

TYPES AND CHEMISTRY OF PHEROMONE MOLECULES :

Secretion of pheromones causes two types of effects in insect, either a definitive behavioral change with immediate consequence on the receiver or a change in developmental physiology of the recipient, the former known as releaser pheromone and the later known as primer pheromone. A number of studies in recent years have classified pheromones according to its specific roles and chemistry [19].

One of the most interesting types are the aggregation pheromones found in gregarious insects like beetles, locusts, termites and bees, where these pheromones are responsible for mate selection, foraging and collective gathering. A study of this type of pheromones in termites *Reticulitermes speratus* was conducted and results suggested that these chemicals consisted of six compounds - an aromatic group (2PhC11-2-phenylundecane), two linear-chain alkanes (C25 and C27), saturated and unsaturated carboxylic acids (palmitic acid and tVA respectively), and cholesterol[20] [21].

Alarm pheromones employed by both eusocial and solitary species are produced in response to traumatic interactions and results in either the dense or dispersion of producing individuals. These signals can be monoterpenes, sesquiterpenes or short-chain aliphatic hydrocarbon [22]. In major pest, the *Ostrina nubilanis* (European corn borer) egg laying behaviour is determined by oviposition-deterrent pheromones, which leads to an increased spatial distribution of egg-laying across the fields. These pheromones were detected to consists of methyl palmitoleate, palmitate, oleate and stearate when tested in microgram amounts in egg masses [23].

When it comes to sex pheromones, there is a huge structural diversity that varies among orders. In case of lepidopterans, sex pheromones are generally exuded by females and can be distinguished into two broad structural groups. The first group or type includes sex pheromones with a linear carbon structure (C10 to C18) with almost three double bonds and a terminal group (acetate, alcohol or acetaldehyde) and are generally derived from fatty acids such as palmitic or stearic acids. On the other hand the second type of sex pheromones

consists of a longer, saturated or unsaturated hydrocarbon chain, generally with odd number of carbons(C17 to C23) with its oxides [24].

Trail pheromones found in most social insects are used for recruitment and pathway marking towards resources and are often released along with alarm pheromones. In termites, analysis of these trail pheromones revealed it to be a multicomponent blend of various semiochemicals including decadienol, C18 aldehyde, dodecanal, neocembrine + dodecatrienol and other two or three component mixtures [25].

When it comes to reproductive division of labor in eusocial insects, the queen or royal pheromone plays a significant role. For ants, wasps and bumblebees these queen pheromones are commonly cuticular hydrocarbons such as C25 CHC pentacosane or methyl-hentriacontane. Queen bees (*Apis mellifera*) have special queen mandibular glands which secrete queen pheromones, where coniferyl alcohol is the key component, and other components include methyl oleate (methyl (Z)-octadec-9-enoate), linoleic acid and hexadecan-1-ol [24], [26]. Since insect pheromones are mostly volatile compounds and are released in microscale quantities, its extraction and study is a laborious and demanding process and takes some unique techniques for rapid detection and identification. Development of such techniques is therefore of extreme fascination, some of which are discussed here.

II. METHODOLOGY

TECHNIQUES USED TO EXTRACT, QUANTIFY AND IDENTIFY PHEROMONE MOLECULES:

The inspection and exploration of volatile semiochemicals bank on highly diversified technologies for improvement of knowledge and awareness of interaction biology among insect species and have gained great importance in agronomic ecology and pest management in biological research

The general approach to inspect volatiles from field samples follows few basic steps. First, chemical and behavioural evidences of semiochemicals interaction is collected from the field. Then, the collected extracts are sampled followed by bioassay on insect behaviour and evaluation when subjected to the said extracts. Finally, the volatiles are identified by analytical techniques and the active components are recognised. The disclosure of the chemical nature of the volatiles assists in chemical synthesis in increased quantity for further evaluation [27].

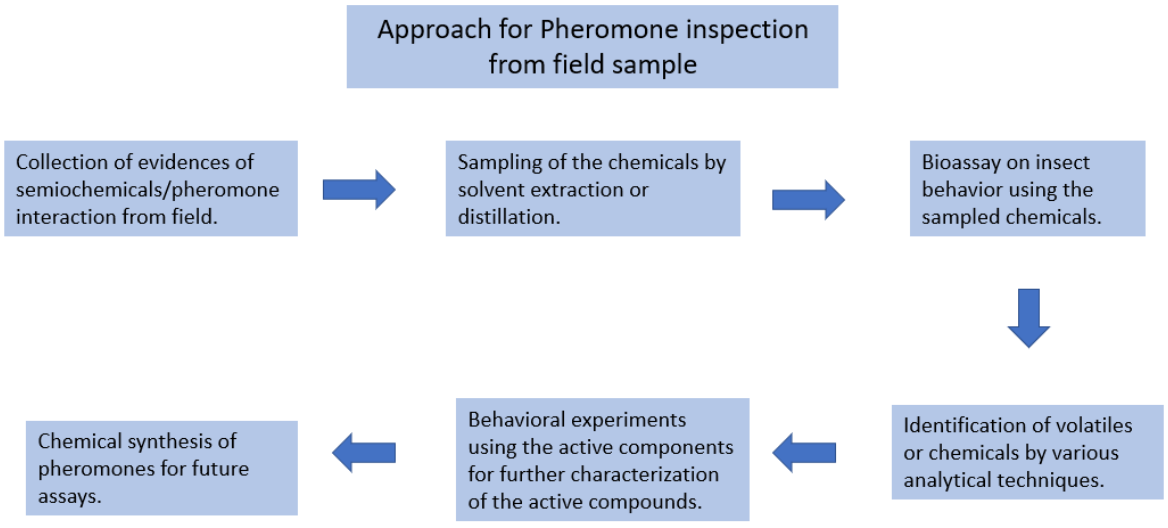

Figure 1: Workflow showing the conventional approach for pheromone extraction and identification.

Extraction and sampling methods:

Sampling of semiochemicals and pheromones is based on its extraction and collection, but a prior knowledge about the evidence of semiochemical production, behaviour and morphology of insect and various other sources is required. The most frequently used extraction techniques for retrieving of semiochemicals are solvent extraction and distillation [28].

The separation of semiochemicals obtained from biological sources is most extensively done by solvent extraction method. This technique is based on solvent-contact with the biological sample (can be the entire insect, glands, specific organs or tissues) for which extraction is performed. The solvent that is used, infiltrates the matrix containing the sample and elutes out with dissolved volatiles, depending on the solubility characteristics of the volatile in question. Thus, unrefined extract containing the solvent enriched with chemical metabolite is obtained after separation from the original matrix. The solid-liquid version is the preferably used in most cases, but various other versions like liquid-liquid extraction can also be used, and based on the effectiveness, this technique varies according to sample and correct choice of solvent [27]. The efficiency of solvent extraction can be increased by manipulating the physical or chemical properties of solvent via approaches like the solvent extraction assisted with microwaves, in which heat radiation is used for calefaction of the sample and limiting the duration of the entire operation [29].

The classic procedure of distillation is also carried out for the volatile extraction. The various methods include hydro-distillation, steam distillation and distillation coupled with solvent extraction. In case of hydro-distillation, the working sample is submerged in water and heated to paroxysm, where the steam generated would break cellular structure and the pheromones or volatiles are released. In case of steam distillation, heated vapour is passed through sample to pull out the volatiles [30]. The solvent distillation extraction is based on the principle where the sample is placed in a water containing receiver and another receiver contain the solvent, both are heated, until the vapor phases of water and solvent combine and thereby the volatiles are captured, condensed and separated [27].

The collection or sampling and trapping of the extracted volatile pheromones are mediated via various volatile chemical entrapment techniques. These include Static headspace sampling, dynamic headspace sampling (coupled with gas chromatography) and purge method. Headspace sampling is used separate the analyte from the non-analyte before gas chromatography is done. The entire sample matrix is held in gas tight vials while the most volatile component is transferred to chromatographic inlets or columns. This transfer is either via static collection where the components to be analysed in its vapor phase are accumulated and captured by sorption on suitable materials, or by dynamic sampling, which employs an inert or carrier gas flow to assist the extraction and accumulation of the volatiles scraped by the gas and then retained in adsorptive traps. There are various other methods for the collection process and once the volatile sampling is carried out, the next step is to analyse the volatiles by means of suitable techniques according to desired intentions [31].

Analytical techniques for identification :

To assess the volatiles obtained with its functional activities or response generated in insects, two elemental features are incorporated, first, is the chemical profiling of the collected compound and second is the complete identification of its active components.

Gas chromatography is the first technique of choice in such studies. It is an instrumental technique used to separate and analyse target volatile components in sample mixtures. The separation or partitioning of components is achieved when the sampled mixture is subjected at the opening of a chromatographic column, where the passage of mixture is aided by inactive-gas flow. While passing, the components associate differentially with the material in the inner column at a molecular level in accordance to the affinity of compound to the column, which is definite for each of the components at fixed conditions, leading to their separation. Thus, the desired components are separately eluted out of the column for rapid detection and analysis of signals specific to compound on a chromatogram[32].

For complete analysis, this technique requires specific detectors for the recognition, identification and quantification of the separated components. The most fitting detector choice in experiments like these depends on the physical and chemical properties of the obtained compound, concentration, etc. In the examination of pheromones/volatiles with biological activity on insects, the detectors used (or coupled with gas chromatography), with more frequency are the mass spectrometry (MS) detector for identification of compounds via molecular weight determination, flame ionization detector or FID for detection and quantification of virtually any organic compounds containing carbon atoms[33], and the electroantennography (EAG) detector for a more congruent analysis of active pheromone molecules obtained by gas chromatography by detection of volatiles perceived by antennal olfactory apparatus of insects [34].

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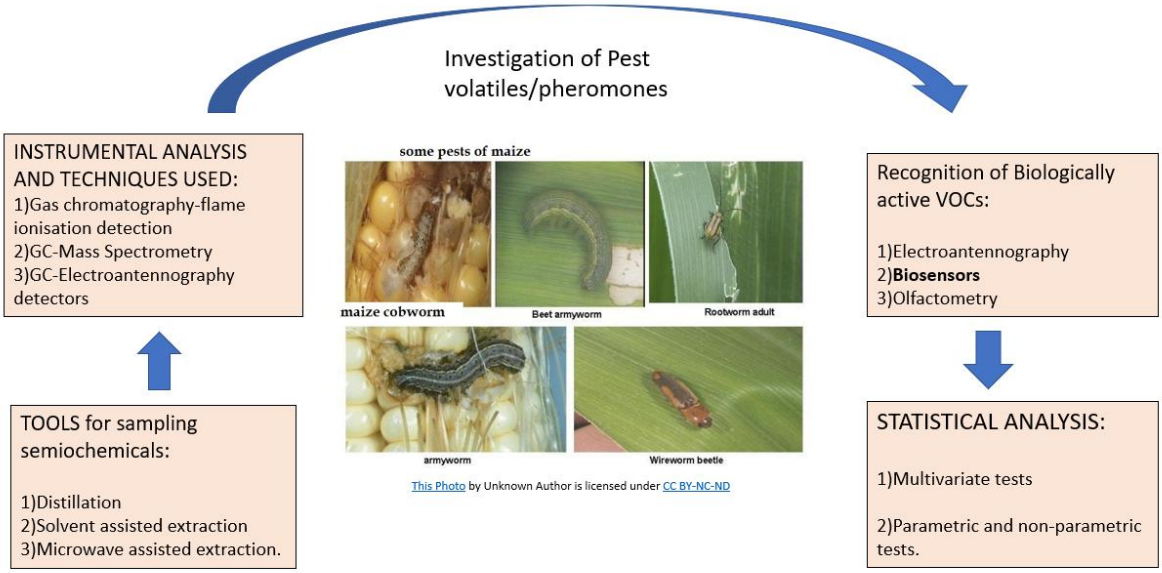

Figure 2: Overview of tools for sampling and analysis of semiochemicals

Now that we have a clear idea about the perception procedure, chemistry and the basic identification techniques of pheromones, let us discuss about the alternative technologies that have been developed already or are emerging at the moment for in-field detection of pheromones to track pest population and various other fields where these semiochemicals or pheromones are involved.

III. APPLICATION

DEVELOPMENT OF SENSORS BASED ON ODORANT BINDING-PROTEIN :

Over the years various research investigations has been carried out to demonstrate the conversion of reception signals by OBPs, of different species immobilised on various signal transducers to construct sensors able to sense volatile organic chemicals. In a research conducted in 2015, scientists were able to graft and immobilise mammalian OBPs on a polycrystalline diamond (PCD) to develop an array of sensors for artificial olfaction [35]. Another recent study in 2019, which was based on two classes of OBPs, pheromone binding protein and general OBP of *Bombyx mori,* immobilised on a quartz crystal microbalance (QCM), was pheromones to differentiate pheromones at parts per billion concentrations[36]. Thus, development of sensors based on this principle can be used as an extremely effective tool for detection of minute quantities of molecules in the environment, thereby giving leads in application to detect unwanted pests in agricultural fields.

As discussed earlier, it is known that OBPs are present in abundant amount in the antennae of insects and is critical for odour detection. The basic characteristics of this class of protein have been determined by structural studies, which established OBPs to be low molecular weight proteins (<35 kDa), having six cysteine residues that form three disulphide bonds (a criteria adequate to categorise a protein molecule as OBP)[37].

The entire process of developing such sensors is both very elaborate and can be summed up in the following steps:

1) Since, in this review we are dealing with pests so the first step would be the identification of the insects and expression of OBP genes by sequencing of RNA extracted from insect antennae. The genes identified here either by RNA-sequencing or using bioinformatics tool are characterised and quantified using qPCR. The proteins are usually expressed and purified by cloning, where recombinant plasmid vectors with the gene construct is transformed into bacterial cell where the protein is expressed and purified using set protocols[38].

2) The purified OBPs are used to screen its affinity in binding to various semiochemicals (that are available from commercial or in-house libraries), by protein-ligand binding kinetic assays. Here in-silico methods such as molecular docking is used to determine the active sites and conserved amino acid sequence of the protein, the suitable ligands(semiochemicals) for that particular OBP is determined so that the sensor developed would be used to detect pests releasing those particular volatiles[39][40]

Another approach to obtain the required OBP is the use of protein engineering technique such as site-specific protein mutation, which can be used in tailoring of the protein at the ligand binding pockets. This approach is based on the studies which suggested that the binding sites or pockets for various volatiles in the OBPs of different organisms are somewhat similar, hence a mutated protein would be the best choice to develop sensor arrays that can discriminate a wide range of volatiles from different pests.[41]

3) Now in-vitro studies like fluorescent based protein-ligand interaction assays are conducted with selected chemicals to confirm if the interaction dynamics of the OBP and its ligand is feasible enough to be further used in the development of the sensing device[40].

4) Next step in this process is the immobilisation of the OBP and subsequent construction of the biosensor. The self-assembled monolayer (SAM) technique was used to immobilise OBP on the gold-plated surface of 20MHz Quartz crystal microbalance, the process is known as grafting. These SAMs are generally organic molecules that consists of head group (usually thiols or phosphonates) and tail group. The head group bonds with surface of the substrate (QCM) by chemisorption and the tail-ends are generally carboxyl group which is activated by coupling using carbodiimide solution, and on activation it forms covalent bonds with free amino groups of the protein[36][40].

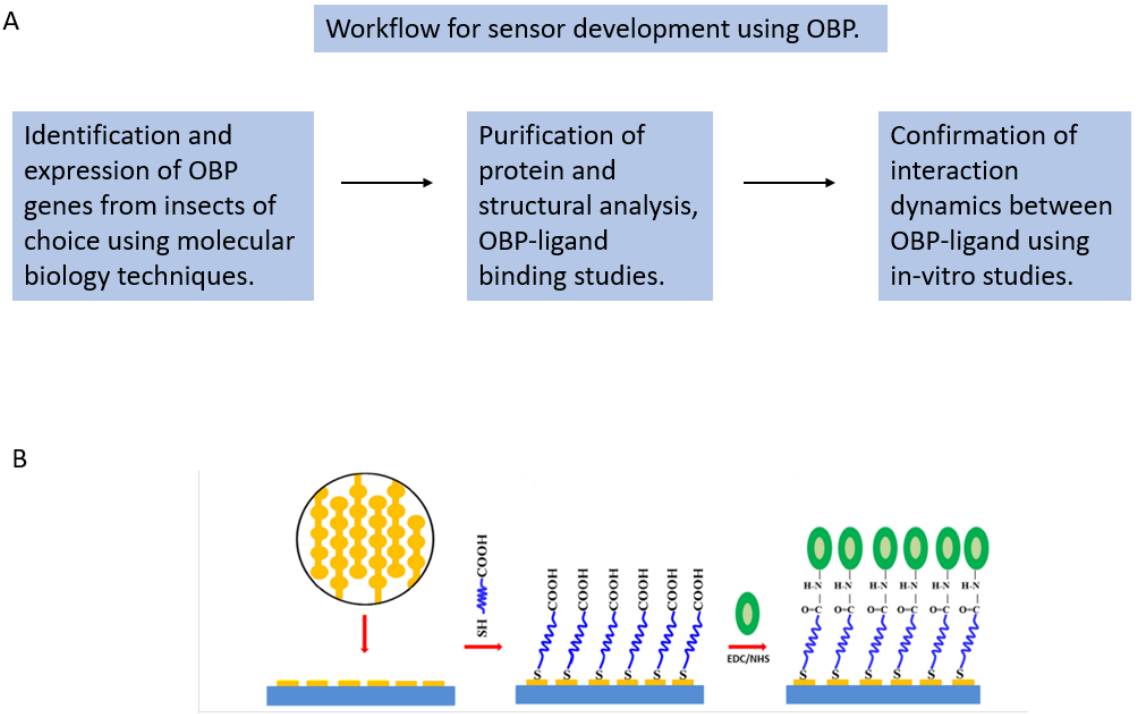

Figure 3: A) Schematic for reverse chemical ecology to express OBP and study OBP and its interaction with ligands(pheromones). B) Immobilisation of OBP by self-assembly with polyethylene glycol on interdigitated electrodes of a glass substrate layered with gold (QCM). *(Brezolin et al 11 June 2018 Springer-Verlag GmbH Germany, part of Springer Nature 2018)*

Thus, the biosensor developed is tested for its response to varying concentration of different semiochemicals. So how does the QCM-OBP-biosensor work? The QCM is basically a piezoelectric sensor, where the crystal is placed between electrodes, one of which is coated with active gold connected to the quartz disc. When this disc to connected to alternating electric field, any deposition of ligand molecule on the sensor would decrease the frequency of oscillation over time. This is how it detects any change in mass when it is subjected to any volatile component binding to the OBP active site immobilised on it[42]. The response generated by the biosensor is then compared using statistical tools, with the results of fluorescent based ligand interaction assay. If the results are satisfactory, this highly sensitive biosensor (detection range in ppb) is then ready for application for

monitoring pest population and estimating the range of infestation so as to take suitable measures to eliminate the pests[36].

Drawbacks : These sensors, although effective to a certain level are not as efficient and complex as natural olfactory systems. The fact that in natural system there are enzymes that degrade OBPs after it delivers the ligand to ORs, cannot be replicated in case of biosensors, hence might detect the same molecule multiple times, hence it remains a huge drawback. There is also a chance of damage of the biological components of these sensors when exposed to external environment.

IV. CONCLUSION AND FUTURE PROSPECTS

With the increase in demand for production of agricultural crops two has led to an explosion of huge number of pest species. A single rice field can be infested by two hundred different types of pest, which is the case for other important crops as well. So, increase in pests led to proportional increase in the use of pesticides which poses serious threat to the environment due to the bioaccumulation of these harmful pesticides in the food chain. On the other hand, the use of pesticides also leads to elimination of insects beneficial for farming industry. Thus, newer approaches like the use of pheromone traps and other detectors to detect specific insects considered as harmful pests has been an important area of research in developing countries. As discussed, the mode of communication of insects is extremely sophisticated, and the use of diverse range of semiochemicals to alter behaviour and physiology are the reason that more and more research is being done in this field. The use of quintessential techniques to investigate and improve the apprehension of the olfactory systems of insects, the mechanism of perception, the variety of proteins and substrates involved, has already led to the development of routine methodologies to be applied for agrarian interests in detection of pests prior to actual infestations.

The development of OBP based QCM biosensor is one promising approach owing to its greater sensitivity towards pheromones in parts per billion range, than GC/MS instruments. Thus, further development of such sensors may prove to be a feasible approach in the discussed agricultural problems, where monitoring of both beneficial and harmful pests would be possible.

There is a huge research gap in the field of an alternative and eco-friendly way of management of agricultural pests. The detection, close observation and elimination of pests by the use of pheromone detectors and conjugating it with other control strategies (like pest specific pesticides) is far from being practically implicated. Many sensor systems for detection of volatiles are under development and some have shown potential to detect on-site pheromones. Since, the traditional systems of detection (GC or GC-MS) require large and complex setups, biomimetic detector systems which are supposedly more stable and reliable are being developed. These systems avail various physicochemical properties of volatile detectors (such as ion mobility spectrometer or conductive sensors) to transduce into perceptible signals and real-time detection of pheromones[43].

A research conducted in 2016 by a group of Indian scientists, successfully developed a bio-engineered silicon dioxide based microelectromechanical system for detection of sex pheromone of female *Helicoverpa armigera* (pest of tomato and several other crops). This system consisted of specific covalently functionalized microcantilever sensors for detection of pest specific pheromones. This device gave a robust response data on optical detection via the measurement of resonant frequency from the developed device (MEMS) via a Laser Doppler Vibrometry (LDV). This device could measure femtograms of pheromones[44]

Thus, development of unique systems for sensitive and cost-efficient and real time detection of pheromones should be one of the priorities in the scientific community.

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