INSTITUTION OF AGRICULTURAL TECHNOLOGISTS,

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EVALUATION OF RKVY PROJECTS

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UNIVERSITY OF AGRICULTURAL SCIENCES,

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"HIGHTHROUGHPUT FUNCTIONAL CHARACTERIZATION OF INSECTICIDAL/NEMATICIDAL MOLECULES TO CONTROL INSECTS AND NEMATODES"

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HIGHTHROUGHPUT FUNCTIONAL CHARACTERIZATION OF INSECTICIDAL/NEMATICIDAL MOLECULES TO CONTROL INSECTS AND NEMATODES

EXECUTIVE SUMMARY

Insects are vital to the overall order of many ecosystems and have lived collectively with the human population for centuries. While insects are viewed as beneficial in several facets of society, they also endanger the human population. As agricultural pests, insect species have been the cause of food source damage and depletion, resulting in substantial economic losses. Progress in crop protection by chemical has been extraordinary over the last decades, not only in the invention of new and selective active ingredients but also in the assessment of the behavior of these chemicals in the environment.

Promising control results with most insecticides, however, have been short-lived, with the development of insecticide resistance, the culmination of factors including pesticide misuse, lack of novel compounds in the pipeline, and a dearth of diversity in the mode of action. Insecticide resistance has been reported in areas worldwide, with the most commonly used compounds such as synthetic pyrethroids, organophosphates, and chlorinated hydrocarbons, being less effective in targeting and altering the insect nervous system (WHO 2014).

Insecticide resistance, therefore, has been a contributing factor to continued persistence of agricultural pests and the resurgence of vector-borne diseases, highlighting the importance of (i) identifying and developing insecticides with alternative modes of action and (ii) alternative control approaches.

Intensive agriculture, which is associated with heavy inputs of synthetic insecticides, has serious ecological impacts, leading to loss of vital ecosystem services including insect-mediated pest suppression. In recent years, efforts have been made towards obtaining safer options to chemical insecticides for sustainable pest management.

The use of plant extracts as botanical insecticides is also an important provisioning ecosystem service. Integrated pest management (IPM) is an example of redesigning intensive agricultural systems. Instead of relying principally on synthetic pesticides, IPM uses non-chemical or botanical insecticide measures to suppress pest population increase and a range of curative management tactics with synthetic pesticide use as last resort (Barzman et al. 2015). The declining availability of many pesticides due to resistance and deregistration, reflecting increasing awareness of their environmental and human health

consequences, has driven changes towards ecologically based practices (Barzman et al. 2015; Borel 2017; Chagnon et al. 2015; Li et al. 2017; Sumon et al. 2018).

Ecologically based pest management tactics such as conservation biological control have been shown to reduce the use of synthetic insecticides in a variety of cropping systems whilst maintaining or increasing crop yields and efforts are being made to up scale the practice globally (Pretty et al. 2018; Wyckhuys et al. 2013; Xu et al. 2017). Despite these advantages, however, uptake of conservation biological control on a wide scale is limited (Gurr et al. 2016). In cases where uptake has been strong, the vegetation used in habitat manipulation provides multiple ecosystem services rather than suppressing pests alone (Khan et al. 2006, 2012). To date, however, there is a major gap in knowledge about the possibility of habitat manipulation plants providing botanical insecticides. This is important because synthetic insecticides present significant risks to human health. Agricultural workers and consumers are at risk of being negatively affected by insecticide products, tank mixes, drift, residues and breakdown products, especially as a consequence of poor registration, storage and misuse (Eddleston et al. 2002). In agricultural areas where there are high illiteracy rates, and poor training and equipment, the impacts are especially high (Amoabeng et al. 2017; Williamson et al. 2008).

Many plants possess secondary metabolites such as alkaloids, phenols and terpenoids that can have insecticidal activity such as toxicity, repellency, feeding deterrence against insect pests (Koul 2004). Botanical insecticides, including extracts and essential oils of these plant species, have been used to protect crops against insect herbivory for many years (Belmain et al. 2012; Isman 2000, 2008). Synthetic insecticides often have lethal and sub-lethal effects on natural enemies (Desneux et al. 2007). Biopesticides are considered relatively benign to non-target species owing to their rapid breakdown, selectivity nature and reduced risk of insecticide resistance as plant extract, particularly crude extracts have multiple modes of action other than toxicity, such as repellency (Amoabeng et al. 2013; Dubey et al. 2011; Isman 2006; Koul et al. 2008; Tembo et al. 2018). Another important benefit of botanicals is that they tend to depend on "suites" of closely related active constituents rather than a single active ingredient; this diversity may delay or mitigate the development of resistance in pest populations to most botanicals (Koul 2004). Biopesticides have been used for centuries as means of managing pests until synthetic insecticides replaced plant extracts (Isman 1997). The interest in botanical insecticides is increasing but still accounts for less than 1% of crop protectants used globally (Isman 2008, 2017). In developing countries, plant extracts are often prepared from common weed species that grow around the field and obtained freely, with labour as the only cost, resulting in cheaper pest management option when compared with synthetic insecticides (Amoabeng et al. 2014; Isman 2017).

Highthroughput screening

Highthroughput screening is a method of scientific experimentation that comprises the screening of large compound libraries for activity against biological targets via the use of automation, miniaturized assays and large scale data analysis. High throughput is an adjective use before screening to become fastest - first and best. It represents high speed of screening throughout process and reflects what chemists can easily work upon. Use of *in-vitro* and *in vivo* assays against molecular targets for the evaluation of chemicals as lead structures in pesticide discovery (Duke J., and M. Bogenschutz, 2002). Traditional *in vivo* screening processes are time consuming, laborious, slow in process, requires larger space. *In vivo* high throughput screening permits rapidly screen large amounts of compounds for biological activity. The natural substance extracts are then automatically tested by ultra-high-throughput *in vivo* screening (UHTVS), which is performed for appropriate insecticidal, fungicidal and herbicidal efficacy. If the robots discover that a sample has the desired biological effect, it goes through further stages of isolation before being tested for its suitability as a possible pesticide (Shaon kumar Das, 2016).

Plant parasitic nematodes are harmful plant pathogens causing much more damage annually compared to insect pests, they cause projected yield loss of 12.3% ($157 billion dollars) worldwide. Out of which $40.3 million is reported from India (Singh et all., 2015). Farmers/growers identified insect pests, and other constraints as production problems but overlooked plant parasitic nematodes. Plant parasitic nematodes (PPNs) are causing serious yield loss in a wide range of plants and agriculture crops (Perry and Moens, 2011). Nematode diseases are difficult to control because of their hidden nature and hence, more often overlooked. Plant parasitic nematodes not only cause damage individually but form disease-complexes with other micro-organism and increased the crop loss.

Fungal diseases in developing countries demand special attention. The general impact of fungal pathogens on human health goes beyond the ability of fungi to infect humans, since they destroy a third of all food crops annually (Fisher et al., 2012), causing economical loss and impacting global poverty. Statistics from the 2009–2010 world harvest (www.fao.org or FAOSTAT1) suggest fungi-induced losses in five of the most important crops globally (rice, wheat, maize, potatoes, and soybean). If those losses were mitigated, these crops would have been enough to feed 8.5% of the seven billion populations in 2011 (Fisher et al., 2012). The most economically devastating fungi are *Magnaporthe oryzae*, affecting rice and wheat, followed by *Botrytis cinerea*, which has a broad host range and Puccinia spp., affecting wheat (Dean et al., 2012). Several high-value crops produced in the tropics, such as bananas, coffee, cacao, spices, mangos, and several nuts, are currently affected by fungal infections and these crops are not produced colder climates (Drenth and Guest, 2016).

Most of the crop plants are attacked by seed and soil borne diseases. Among those pathogenic fungi, Aspergillus flavus and Rhizoctonia bataticola are known to infect and causing heavy losses. The disease severity depends upon the temperature and moisture conditions. For the management of Aspergillus flavus and Rhizoctonia bataticola synthetic fungicides are known to be effective. However, the use of synthetic fungicides is limited by the emergence of resistant fungus strains and some fungicides possess considerable toxicity. Moreover, there is a growing public concern over the increased health and environmental hazards associated with synthetic molecules. While the scientific development of new insecticides has plateaued, great strides have been made in the field of insect genomics. Emphasis is given now to develop extracts from essential oils and plants for the management of Aspergillus flavus and Rhizoctonia bataticola.

Antifungal investigations have revealed that plant extracts like garlic extract, some essential oils from *Mentha spicata* L., *Foeniculum miller*, *Azadirachta indica*, *Conium maculatum* and *Artemisia dracunculus*, active ingredients of cinnamon like cinnamaldehyde and eugenol, different plant extracts like neem (*Azadirachta indica*) seed kernel extract (NSKE), Pongamia (*Pongamia pinnata*) oil and nimbicidin, aqueous extract of *Acacia nilotica, Achras zapota, Datura stramonium, Emblica officinalis, Eucalyptus globules, Lawsonia inermis, Mimusops elengi, Peltophorum pterocarpum, Polyalthia longifolia, Prosopisjuliflora, Punica granatum* and *Sygigium cumini* have very good antifungal activity against one or the other Aspergillus species tested.

Similarly, antifungal activity of aqueous extract and oil of neem (*Azadirachta indica*), leaves of *Allamanda cathartics* and *Artabotrys hexapetalus*, ajowain (*Trachispermum ammi*), lemon grass (*Cymbopogon citratus*), Tulsi (*Ocimum sp.*), mentha (*Mentha sp*.), *Rauwolfia sp*., mehandi (*Lawsonia inermins*), and samhalu (*Vertex trifolia*), 24 botanicals belonging to the family Compositae have been well researched and established.

Keeping the above in view, the project, “**HIGHTHROUGHPUT FUNCTIONAL CHARACTERIZATION OF INSECTICIDAL/NEMATICIDAL MOLECULES TO CONTROL INSECTS AND NEMATODES”** was taken up by Department of Molecular Biology and Agricultural Biotechnology, University of Agricultural Sciences, Raichur with Rashtriya Krishi Vikas Yojana funding. The project was implemented during 2016-17. The details of the project are as under:

1.	**Title of Project**	**:**	**"HIGHTHROUGHPUT FUNCTIONAL CHARACTERIZATION OF INSECTICIDAL/ NEMATICIDAL MOLECULES TO CONTROL INSECTS AND NEMATODES"**
2.	**Nodal officer and Principal Investigator**	**:**	**Dr. B. Kisan,** Asst. Professor and Head, Department of Molecular Biology and Agricultural Biotechnology, College of Agriculture, University of Agricultural Sciences, Raichur
3.	**Implementing Institution (S) and other collaborating Institution (s)**	**:**	Department of Molecular Biology and Agricultural Biotechnology, University of Agricultural Sciences, Raichur
4.	**Date of commencement of Project**	**:**	2016-17
5.	**Approved date of completion**	**:**	2016-17
6.	**Actual date of completion**	**:**	2016-17
7.	**Project cost**	**:**	Rs. 30 lakhs

The objectives of the project are as follows:

1. Obtaining chemical scaffolds/ molecules for screening.
2. Developing highthroughput screening platform.
3. Testing the molecules for their effectiveness.
4. Evaluation of efficacy on major insect/ pests.

The focus of Evaluation is:

i. To examine whether the screening procedure based on 96 well plate and utility of imaging and other methodologies adopted was able to expand the screening procedure faster, cheaper and in timely fashion.
ii. To evaluate plant extracts, essential oils and synthetic chemical compounds to screen for their activity against the nematodes and fungal pathogen inhibition.
iii. To evaluate the efficacy of molecules selected on control of diseases

The intention of the scheme is to develop a screening procedure using sophisticated systems and assays that can evaluate plant extracts, essential oils and synthetic chemicals for their activity against nematodes and fungal pathogens and evaluating the efficacy of the molecules for control of diseases in the field. The underlying logic is;

a. Screening of large compound libraries for activity against biological targets via the use of automation, miniaturized assays and large scale data analysis becomes fastest - first and best.
b. It represents high speed of screening throughput process and reflects what chemists can easily work upon.
c. Emphasis on use of plant extracts and essential oils in place of synthetic chemical compounds to screen for their activity against the nematodes and fungal pathogen inhibition will pave the way for development of pest management systems that minimize health and environmental hazards.

The research work was carried out during 2017-18 at the Department of Molecular Biology and Agricultural Biotechnology, College of Agriculture and Main Agricultural Research Station, University of Agricultural Sciences Raichur, Karnataka (India).

The identified pure culture of pathogenic fungi Aspergillus flavus and Rhizoctonia bataticola were collected from Department of Plant Pathology, College of Agriculture, UAS, Raichur for the study. The Aspergillus flavus and Rhizoctonia bataticola cultures were subcultured on PDA slants and kept at 28 ± 2 ºC for 7 days. Those slants were preserved in refrigerator at 4 ºC and maintained by sub-culturing once in a month in order to avoid a decline in strain viability. Such cultures were used throughout the study.

Twelve essential oils, 24 plants extracts and 64 synthetic chemicals were screened using microtiter plate method, food poison method, pyocynin assay and further confirmed by the Resazurin assay for activity against the pathogenic fungi Aspergillus flavus and Rhizoctonia bataticola.

FINDINGS AND DISCUSSION

Among the essential oils (EOs) tested, OL-2 and OL-12 combination of EOs for concentrations 0.25, 0.5, 1 and 2 per cent, cent percent mortality observed at time intervals 10 min onwards. The essential oils independently brought about 100% mortality within 12 hours of incubation.

Among the essential oil extracts screened with Food Poison Technique, inhibition of mycelial growth was found be significant with orange oil and cinnamon oil.

The results of screening a few essential oils showed that they inhibited the nematode In-vitro at 1% concentration on exposure within an hour of incubation and further the combination of different essential oils provided the effective control within 30 minutes of exposure and these tests were confirmed with model system *Caenorhabditis elegans* and further with M*eloidogyne incognetta*, the root knot causing nematode. The studies indicated that control of the nematode is possible by further formulating these essential oils and testing in pot and field conditions.

Further, studies are required to bring out the best combination of synthetic and organic based formulation to control the nematode population in the field as the seven chemical compounds, two essential oils showed the effectiveness in controlling nematodes at 10ppm concentrations.

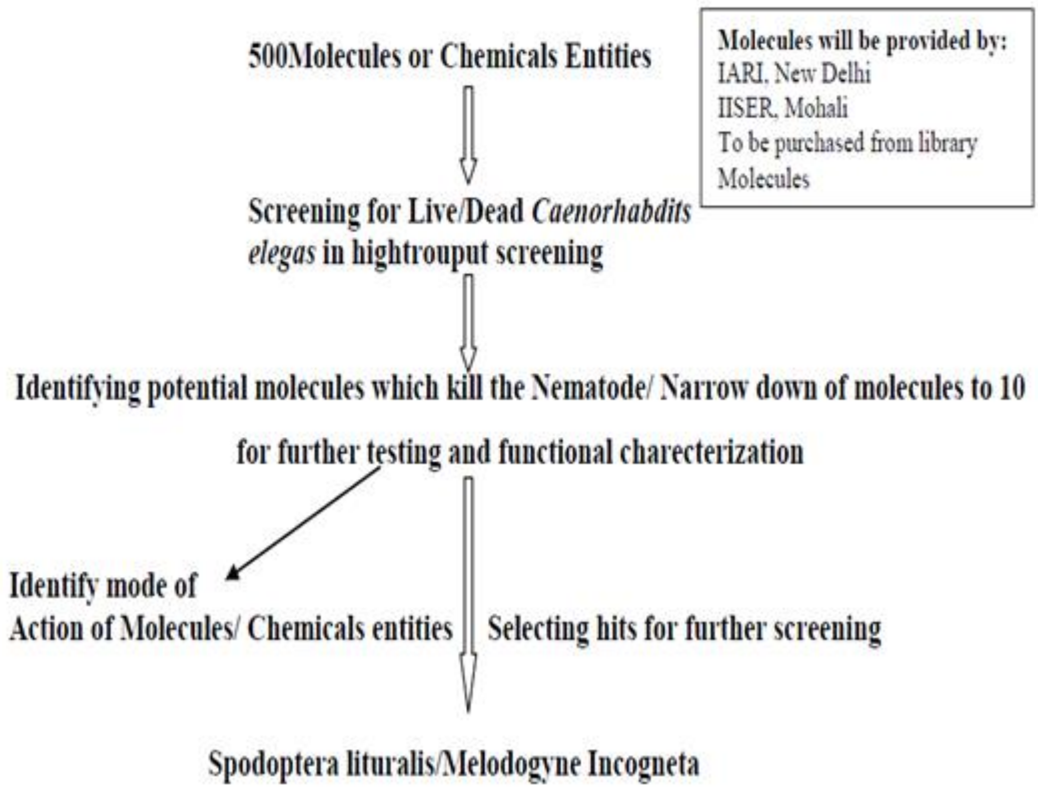
The studies will help the farming community by finding new solutions to control the disease causing nematodes and avoid loss of crop. It will also help in floriculture sector, horticultural crops and fruit crops by controlling the nematode infestation resulting in better yields and profit to farmers.

The screening for the fungicidal activity with essential oil was evaluated by microtiter method, food poison method, pyocynin assay and further confirmed by the Resazurin assay for controlling cell disruption there by controlling the fungal inoculum. Further testing in pot culture and field evaluation needs to be carried out.

Efficacy in field studies:

With good results in *in vitro* studies, experiments two formulations with the concentrations ranging from 0.5 to 10% were done by spraying in a small scale field trial to evaluate their performance in Chilli and Ber. During the study phytotoxicity was observed above 5% concentration of the spray. It was found that the formulations were able to control the powdery mildew of Ber and Chilli to the tune of 70%. These formulations are organic in nature. Hence, ease on environment, environment friendly, farmer friendly, do not cause any pollution and resistance development will not be possible as these essential oil and formulations will be having more than 30 compounds which will be acting simultaneously. Hence, longer utility and profitability to the farmers and farming community can be assured. This formulation is under field test in second year of evaluation on various vegetables and fruit crops. This formulation will boost the farmer's economy and saves the crop from loss on account of powdery mildew which is a major disease in chilli and other vegetables. This formulation also increases productivity by enhancing, retaining the chlorophyll for longer periods as the crop is protected from the powdery mildew and the fruit bearing capacity and size of the fruit is enhanced in these sprays.

During the implementation of the project, it has been possible to standardize the screening protocol with the help of Highthroughput screening using *Caenorhabdits elegans* as model nematode. Potential molecules which control the nematodes have been identified and narrowed down for further testing and functional characterization. Identifying the mode of action of molecules and selecting hits for further screening will have to be carried out.



ACTION POINTS

1. The project has been well conceived and systematically executed and has been successful in examining whether the screening procedure based on 96 well plate and utility of imaging and other methodologies adopted is able to make the screening procedure faster, cheaper and in timely fashion. This will help in screening molecules for action against insect pests and diseases.
2. As a future step arising out of the project, identifying the mode of action of molecules and selecting hits for further screening will have to be carried out.
3. Imaging of *Caenorhabdits elegans* using SPIM, Light Sheet Microscopy or Spinning Disk Confocal with Zyla 4.2 Plus and Sona sCMOS cameras has helped in high resolution imaging performance and Sona is the most sensitive sCMOS camera with a very large field of view, superior longevity and quantitative accuracy.
4. Molecular targets need to be established
5. Testing formulations to control fungal and nematode in pot cultures and field conditions needs to be carried forward for *Aspergillus flavus, Fusarium oxysporum*

uduum and *Rhizoctonia bataticola.* No clear description about major achievemets on anti parasitic molecule / extracts from highthroughput screening have emerged. The report highlighted that, very good antifungal activity against one or the other Aspergillus species tested. But to find utility of the above, more quantitative results are required. Otherwise, extending the same to practical application is difficult.

6. While several essential oils and plant extracts have been evaluated for activity against biological targets via the use of automation, miniaturized assays and large scale data analysis, there is need to identify the molecules responsible for effective control, mode of action and comparative costs to study the economics of disease management. The plant extracts obtained from Jain University is not even partially characterized. Further, the synthetic chemicals from Kuvempu university is not explained in detail about the chemistry and properties. The report is not providing more comprehensive quantitative result of potential individual essential oil/extract/synthetic chemicals with reference to any of the leading reference standards.
7. Specific role of the essential oils, plant extracts and synthetic chemicals are not well presented. Therefore, it is very difficult to draw any specific conclusion about the beneficial role of above chemicals, extracts, synthetic chemicals in comparison with a reference standard and their practical utility.
8. The infrastructural facilities created at the centre was used by three masters students for their research work. Two projects have been received from Department of Science and Technology, Government of India which will be taken up by using the facilities created by the project.
9. The facilities are also being used by students from various disciplines such as nematology, dental pathology, microbiology and biotechnology from different institutes. It is recommended that the facilities should be exclusively used for the development of agriculture by taking up more research in the field. If at all the facilities are spared, it should be on revenue generation method since the techniques involve expenditure.

RESEARCHABLE ISSUES

1. Relevance of secondary metabolites such as alkaloids, phenols and terpenoids which have both beneficial and harmful effects on insect control with reference to their control needs to be documented along with economics.
2. Development of standard protocols for screening compounds for biological targets is needed (especially for fungal diseases, nematodes and bacteria).
3. Need for efficiency and efficacy of plant metabolites in critical limits on pathogens under varied climatic conditions (i.e., temperature, rainfall and relative humidity).

4. Need for more basic and applied research work on hormones/ pheromones, i.e., phytochemical changes in plants due to climate change.