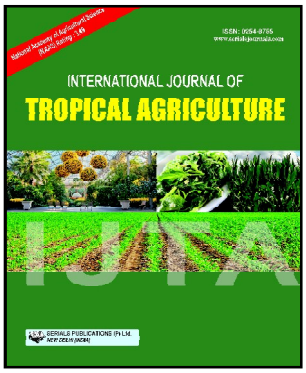

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Sulfoquinovosyl Diacylglycerol A Larvicidal Molecule in *Aadinkombu* An Aa *Musa* Cultivar Induces Allelopathy in *Odoiporus Longicollis* [Olivier]

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Abstract: *Aadinkombu* an indigenous *Musa* cultivar (AA), common in Pathanamthitta District of Kerala, an agro ecosystem which is ecotone with tropical evergreen forest (Konni forest Division, Kerala) is resistant to infestation by *Odoiporus longicollis* Olivier. Sulfoquinovosyl diacylglycerol (SQDG) isolated and identified from the pseudostem of this cultivar is highly toxic to the endophytic larvae at a low concentration of 1ppm. Hemolymph of the affected larvae showed hyperproteinaemia, inability to maintain free amino acid pool and increased catabolism of amino acids. Toxicity by SQDG has resulted haemocytopaenia with selective enhancement on the number of granulocytes, together with sharp depletion on the number of plasmatocytes. Cytopathological changes such as lack of plasma membrane integrity, nuclear fragmentation and abnormal staining patterns of hemocytes were observed. Cessation of feeding, caused by SQDG may be due to synthesis of 20-hydroxyecdysone at extremely high concentration through elevated activity of 20-hydroxyecdysone monooxygenase. Inhibition of pupation and slow death of 4th instar *O.longicollis* larvae, intoxicated with 1ppm of SQDG may be due to imbalance in the hormone profile and free amino acid pool.

Keywords: *Odoiporus longicollis*, *Musa* cultivar, *Aadinkombu*, Sulfoquinovosyl diacylglycerol, Hemocytopaenia, Hyperproteinemia, 20-hydroxyecdusone.

INTRODUCTION

Banana and plantains are one of the earliest group plants to be domesticated and play a pivotal role in human welfare, since time immemorial. *Musa* cultivars are the major fruit crops, globally cultivated and consumed in more than 128 countries,

throughout tropics and subtropics (Denham *et al.,* 2003).India is the largest producer of banana in the world and they are cheapest, plentiful and most nourishing of all fruits. Plantains and banana promise to meet the demand for vital needs such as fruits, fibre and fuel for growing population all over the world. Owing to the multifaceted uses high economic returns and high socio economic significance banana is often referred to as 'Kalpatharu' or plant of virtues (Sundararaju, 1998).

Globally bananas are the fourth largest agriculture commodity in the world trade and totally 98 million tons of fruits are produced annually. Among 87% of all banana produced in the world are cultivated by small scale farmers (Frison *et al.,* 1997) and the above statement is absolutely correct in the South Indian state, Kerala (Kavitha *et al.,* 2015; Kavitha *et al.,* 2017)).Major banana growing states in India are Maharashtra, Tamil Nadu, Gujarat, Assam, Karnataka, Kerala, Bihar, West Bengal, Andhra Pradesh and Orissa. Production is highest in Maharashtra (65.70tones/hectare) against a national average of 35.50 tones/hectare (Resmi *et al.,* 2016).India is believed to be one of the biodiversity centers of origin of *Musa* cultivars, possessing immense diversity (Ashalatha *et al.,* 2005; Mukundakumar *et al.,* 2013).

Infestation of *Odoiporus longicollis*, Olivier (Class: Insecta, Order: Coleoptera, Family: Curculionidae) is a major problem faced by Indian farmers doing commercial cultivation of *Musa*. Larval stages of this pest are purely endophytic and the whole life cycle of this pest is completed within the pseudostem of *Musa* cultivars (Padmanabhan and Sunararaju, 2001). During the course of field study in various agro ecosystem of Kerala, it was found that mother weevil are highly judicious in selecting host plants for oviposition and they showed extreme preference to some *Musa* cultivars and extreme non preference or avoidance to some other cultivars for oviposition (Kavitha *et al.,* 2015a; Kavitha *et al.,* 2015b). Detailed analysis of the different *Musa* cultivars revealed that they possessed high variation on the distribution of secondary metabolites such as total phenols(TP), total flavonoids (TF) and activities of related enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (POX) and the mother weevil showed extreme preference to those cultivars which possessed very low contents of TP, TF and low activity of PAL, PPO and POX. They also showed non preference or avoidance to those *Musa* cultivars which possessed very high content of TP, TF and elevated activity of related enzymes such as PAL, PPO, POX (Ajitha *et al.,* 2018).

Rearing of endophytic larvae of *O.longicollis* in some *Musa* cultivars which possessed high content of TP, TF and elevated activity of PAL, PPO and POX has resulted 100 % mortality of the larvae in one week and symptoms such as metabolic disorders, changes in hemolymph protein profile and cytopathological changes was observed on the fourth day (Kavitha *et al.,* 2016). *Aadinkombu* is commercially non-viable *Musa* cultivar with genome constitution AA possess small fruit bunch with erect pointed tips like that of horns of goat (photo plate 1). Rearing of *O.longicollis* larvae in this cultivar has resulted allelopathy (Kavitha *et al*., 2015a). Isolation, identification and mode of action of the active principle from this cultivar forms the subject matter of this paper.

MATERIALS AND METHODS

Experimental Organism: Endophytic larvae of *Odoiporus longicollis* were dissected out from the infested pseudostem of susceptible cultivars were used. Fourth instar larvae are preferably large and are voracious feeders and hence that larvae were used for the study.

Rearing of larvae in the live pseudostem: Four month old *Musa* cultivar with a trunk circumference of 25-30 cm were used for the study. The crown of the plant was cut in such a way that the live stump

of 1 meter height remains in viable state. Larvae of *O. longicollis* (Six number) were carefully put on the free end and allowed them to bore into the pseudostem. The free end was covered with a piece of mosquito net and kept undisturbed for eight days. On the eighth day the pseudostem was cut 15 cm below the first cut and the larvae were carefully taken out and observed and compared with larvae in susceptible cultivars. If all the larvae were dead on the eighth day the cultivar was considered as resistant to *O. longicollis* infestation (Kavitha *et al.,* 2015a).

Phytochemical isolation of Compounds

Pseudostem of *Musa* cultivar which are highly susceptible or resistant to infestation was cut vertically into small chips and dried under shade (while ambient temperature was 28-33^{0}C) for three weeks after which it was ground to a fine powder in electric pulveriser and sieved and 400 gm powder was kept overnight in organic solvents, first extracted by petroleum ether, followed by acetone and finally with methanol. The extracts were filtered and concentrated in rotary vacuum evaporator and larvicidal activity was done. As the larvicidal activity was located only in acetone extract it was subjected to column chromatography using suitable solvent system. Elution was carried out by gradient polarity system starting from 100% Petroleum ether to 100 % Methanol. Each fraction was monitored by TLC and were combined, based on their TLC profiles, which resulted 9 fractions. Larvicidal activity of each fraction was carried out. As the 8th fraction gave larvicidal activity that fraction was again subjected to column chromatography which resulted isolation of the compound in pure form.

Spectral Analysis of Active molecule

NMR spectrum (^{1}H, ^{13}C, HMBC & HSQC) and LC-Q-T of-MS of active compound were done in IIRBS and School of Environmental Science, Mahatma Gandhi University, Kottayam, Kerala, The fatty acid moiety of the compound was isolated and were subjected to GC-MS analysis was done in Care Keralam, KINFRA park, Koratty, Trissur.

Testing of Larvicidal activity

Freshly cut pseudostem of 100gm pieces of circular rods were used for the study. Active compound or extract was dissolved in water containing 0.5% Tween. The dissolved extract (2 ml) from susceptible or resistant pseudostem or active molecule in known concentration were injected into the 100 gm piece of pseudostem evenly at 10 sites using insulin syringe. Each larvae was allowed to bore into the pseudostem.

Every day each piece containing larva inside was kept very close to the ear for hearing the carving sound, which is the indication of health of the larva inside. Each piece containing larva was kept in a plastic container covered with a cheese cloth and kept in dark, cool place. Fresh piece of pseudostem, administered with the test sample was provided once in two days. On 8 th day the pseudostem piece were carefully dissected to observe the larvae. Minimum quantity of active compound required for mortality within 7 days was studied. As 100% mortality of the larvae in live pseudostem of pest resistant *Aadinkombu Musa* cultivar occurred between 7th and 8th day, 7days was taken as limit of activity for toxicity (Kavitha *et al.,* 2015a).

Assay of larval enzymes

Cell free hemolymph of fourth instar larvae was used for the assay. Bioassays of Protein (Lowry *et al.,* 1951), Total free Amino acids (Spice, 1957), Transaminases (Aspartate amino transferase [E.C. 2.6.1.1] and Alanine amino transferase [E.C. 2.6.1.2](Reitman and Frankel, 1957), Leucine Amino peptidase [E.C. 3.4.11.1](Amador *et al.,* 1963) and Cathepsin D [E.C. 3.4.23.5](Mycek, 1970).Enzyme immunoassay of 20-Hydroxyecdysone as per enzyme kit(Porcheron *et al.,* 1989) purchased from Bertin Pharma, (France) was carried out using standard protocols. Uric acid

content was estimated using a standard assay kit purchased from Span diagnostics.

Electrophoresis of cell free hemolymph of larvae was carried out by the method devised by Laemmeli (1970).12% resolving gel and 4% stacking gel were used for SDS PAGE.

Haematological studies

The larvae on the 4th day of toxicity (before death) were used for study. The larvae were kept on a glass plate kept above ice cubes and small cut was given on the ventral side with much care, not to puncture the gut, to collect hemolymph. Total haemocyte count was done by Neubauer haemocytometer. The number of hemocytes per cubic millimetre (mm^3) was calculated using the formula suggested by Jones (1963). A thin film of smear was prepared using hemolymph to study the differential hemocyte count. The smear was air dried and stained using Giemsa stain for 20 minutes. The slide was then washed in running water to remove excess stain. The slides were then air dried and viewed under high power of a compound microscope (LABOMED) for the identification of the cells. The number of different types of hemocytes of treated and control samples were counted. The haemocytes were identified by their distinguishing characters as described by Wigglesworth (1972), Patton (1963). The percentage of different types of haemocytes in both the treated and control samples were calculated.

STATISTICAL ANALYSIS

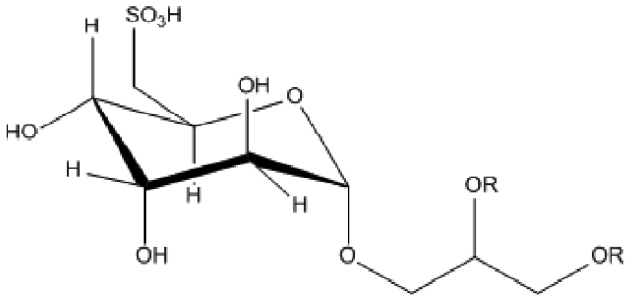
The data obtained are represented as Mean ± Standard Deviation. To test the significance of the data obtained, statistical analysis were carried out using ANOVA ($p \leq 0.05$) using SPSS 21 software (Daniel, 2006).

RESULTS

Musa cultivars which is resistant to infestation by *Odoiporus longicoliis* is an indigenous cultivar, locally known among folk-lore as *Aadinkombui*, with genome constitution AA is indigenous to Pathanamthitta district of Kerala. Acetone extract of the dry powder of the pseudostem showed larvicidal activity against the endophytic larvae. Column chromatographic separation of the extract using different proportion of chloroform-methanol mixture has resulted nine different fractions. Among the different fractions 8th fraction (2:98 methanol: chloroform mixture) showed larvicidal activity, which on sub fractionation has resulted isolation of the active molecule in pure form.

Structure of the active molecule, elucidated through analysing the spectral characters is given as Fig. 1. The molecule is Sulfoquinovosyl diacylglycerol (SQDG) attached with two unsaturated fatty acids. Six different types of fatty acids were identified through GC-MS analysis which are also given along with the structure. The active molecule is present in the pseudostem at an extremely low concentration and the yield was 0.002%.

Sulfoquinovosyl diacylglycerol has induced hyperprotenemia of the hemolymph (Fig. 2) with qualitative changes in the protein, evidenced by changes in the protein profile on the electropherogram (Fig. 3). In accordance with



Fatty acid composition (R)
Palmitic acid
Stearic acid
Lauric acid
Myristic acid
Pentadecanoic acid
9-Hexadecenoic acid

Figure 1: Structure of Sulfoquinovosyl diacylglycerol

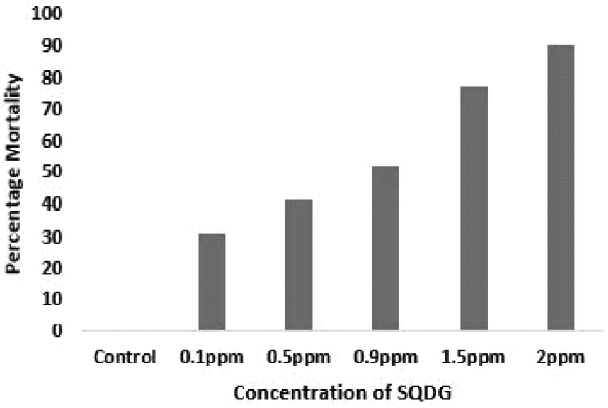


Figure 2: Showing the percentage mortality of Sulfoquinovosyl diacylglycerol on *O.longicollis*

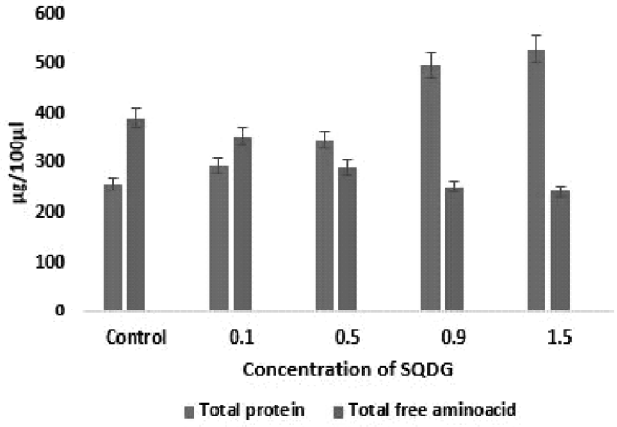
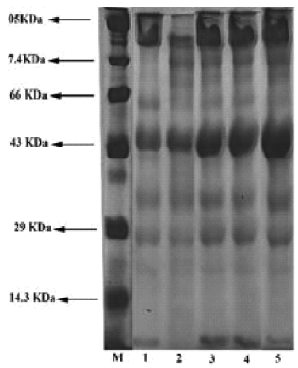


Figure 3: Effect of Sulfoquinovosyl diacylglycerol on Total protein and Total free amino acid



M-Marker, 1-Control, 2-0.1ppm, 3-0.5ppm, 4-0.9ppm, 5-1.5ppm

Figure 4: Electropherogram showing the effect of Sulfoquinovosyl diacylglycerol on the hemolymph protein

elevation of protein contents, a sharp decline on the total free amino acid content was observed (Fig. 2). Activity of transaminases, both aspartate aminotransferase (AsAT) and alanine amino transferase (AlAT) showed dose dependent elevation during the course of toxicity (Fig. 4). Activity of cathepsin D showed a dose dependent elevation during toxicity but at a same time activity of leucine amino peptidase (LAP) showed a significant inhibition of activity (Fig. 5 & 6). The content of hemolymph uric acid showed a sharp elevation (Fig. 7).

The larvae intoxicated by different concentration of the active compound has resulted sharp hemocytopenia on the fourth day (Fig. 8). Differential hemocyte count of healthy larvae revealed six different types of hemocytes. Larvae

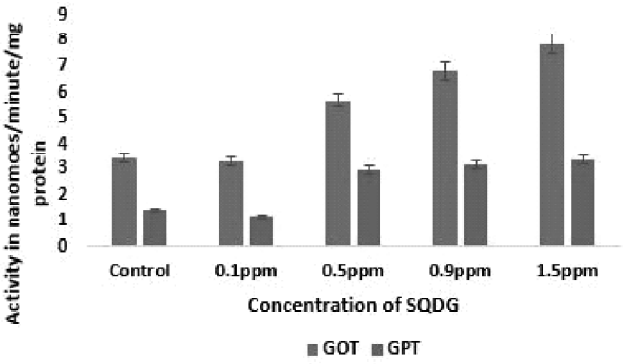


Figure 5: Effect of Sulfoquinovosyl diacylglycerol on the activity of Transaminases

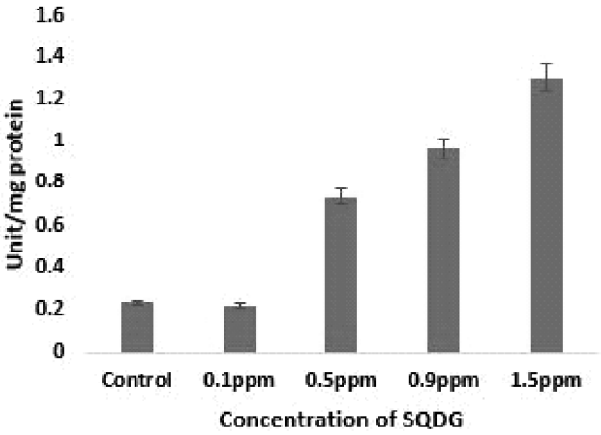


Figure 6: Effect of Sulfoquinovosyl diacylglycerol on the activity of Cathepsin D

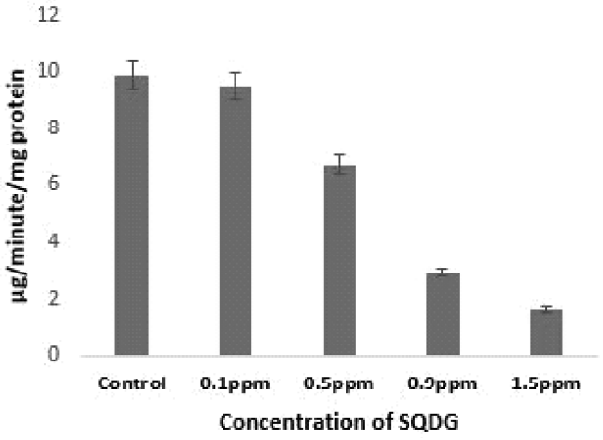


Figure 7: Effect of Sulfoquinovosyl diacylglycerol on the activity of Leucine aminopeptidase

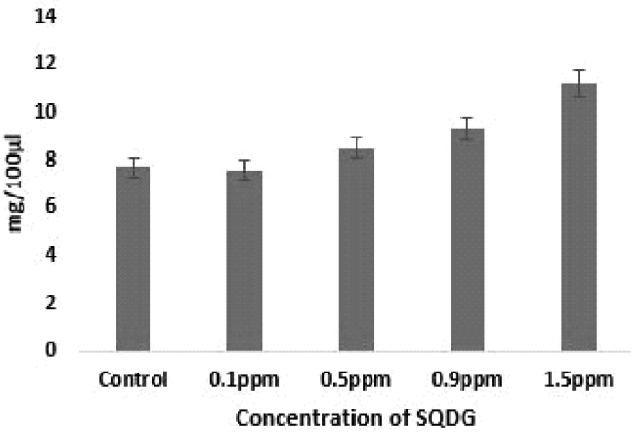


Figure 8: Effect of Sulfoquinovosyl diacylglycerol on Uric acid

under toxicity showed a sharp elevation in the number of granulocyte together with steep decline on the number of plasmatocytes. (Fig. 9).

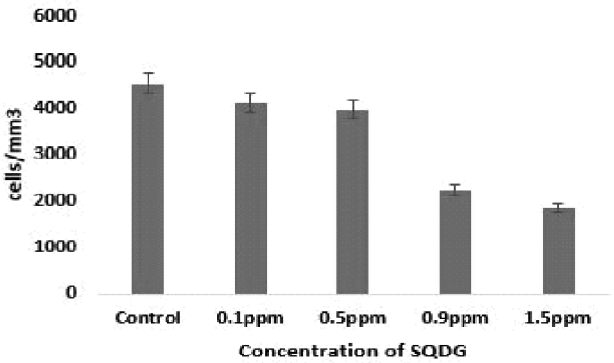


Figure 9: Effect of Sulfoquinovosyl diacylglycerol on total hemocyte count

Cytopathological studies revealed wide spread changes in the plasma membrane, loss of nucleus and abnormal staining pattern (Photoplate 2a&2b).

The active compound has induced a dose dependent elevation on the activity 20-hydroxyecdysone monooxygenase, resulting as outburst on the release of the active 20-hydroxyecdysone in the hemolymph (Fig. 10).

DISCUSSION

Diversity of *Musa* cultivars is so high in southern state like Kerala and three year field study in various agro ecosystem of Kerala has resulted in the

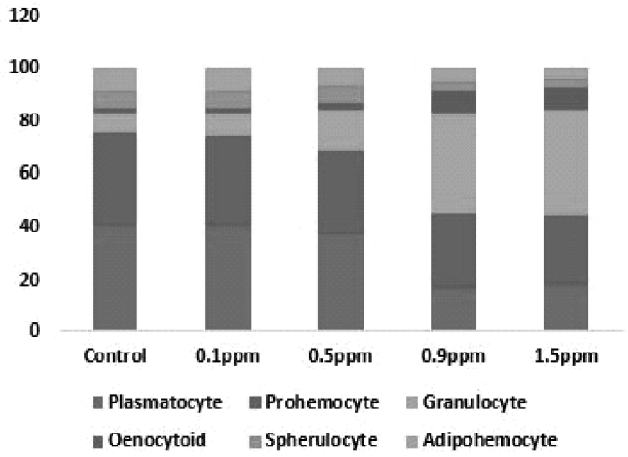


Figure 10: Effect of Sulfoquinovosyl diacylglycerol on total hemocyte count

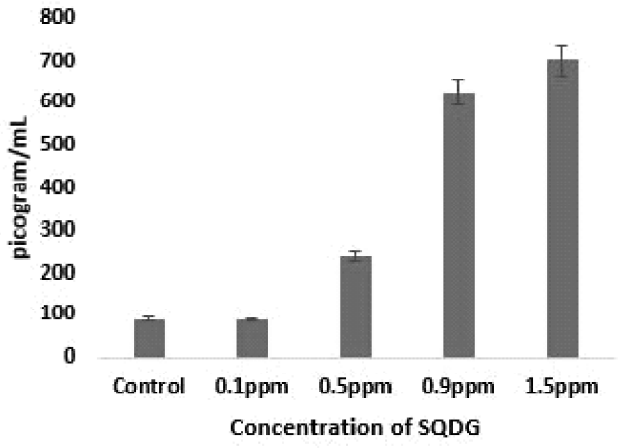


Figure 11: Effect of Sulfoquinovosyl diacylglycerol on 20-hydroxyecdysone

Photo plate 1: Aadinkombu

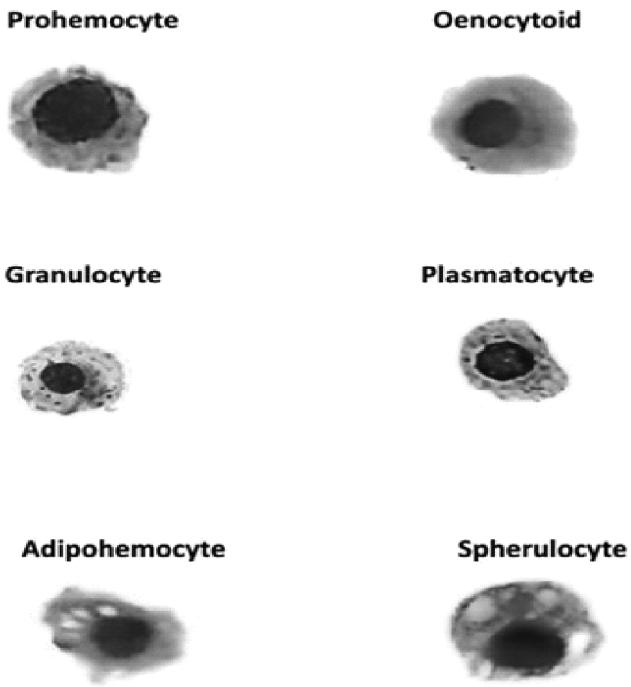
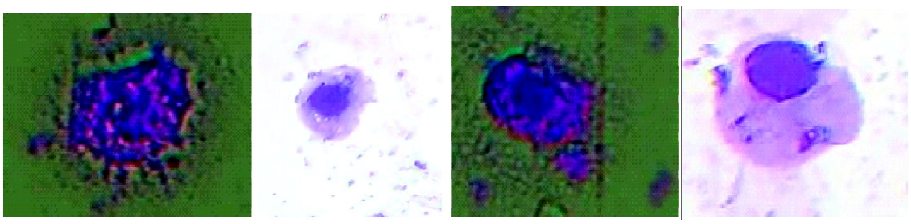
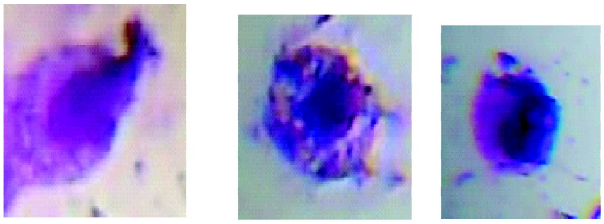


Photo plate 2a: Different Types of Hemocytes in *O.longicollis* larvae



Nuclear fragmentation and Denucleation in the Hemocyte



Cytoplasmic Vacoulation Cell Membrane Rupturing

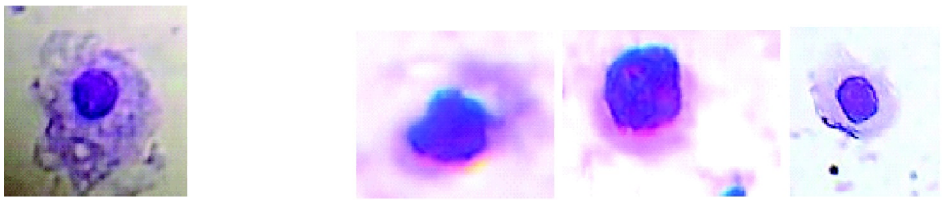


Photo plate 2b: Cytopathological Changes induced by Sulfoquinovosyl diacylglycerol

identification of 85 *Musa* cultivars and their unique features such as genome constitution, commercial viability and pest status have been described(Kavitha *et al.,* 2017). Interaction with old traditional farmers revealed that wide spread conversion of many agro ecosystem in long duration crops like rubber plantations has resulted a sharp reduction on the diversity of *Musa* cultivars during the recent past of two decades (Kavitha *et al.,* 2017). Now most of the farmers during commercial cultivation of *Musa* are very particular and they are interested in cultivating commercially viable cultivars (CVC) only. The features of CVC are large fruit bunch, short duration to harvest, palatable ripe fruits, familiarity among public and high market value(Kavitha *et al.,* 2015a).Unfortunately all the CVC are highly susceptible to infestation by *O.longicollis* because of low contents of TP, TF and very low activity of PAL, PPO and POX (Kavitha *et al.,* 2015b; Ajitha *et al.,* 2018) and hence farmers aggressively apply systemic insecticides and often injection of aqueous suspension of monocrotophos in the pseudostem to keep away this endophytic pest and to get a good harvest.

Aadinkombu (AA) is a *Musa* cultivar which possess small fruit bunch, long duration to set flower, long fruit ripen period, long duration of the replanted suckers to sprout and low market value, all of these characters which makes it as commercially non-viable cultivars (Kavitha *et al.,* 2015a). Strong allelopathy shown by this cultivar on the endophytic larvae of *O. longicollis* was identified as because of the presence of SQDG and it was confirmed through due to its spectral characteristics.

Hyperprotenemia showed by *O.longicollis* larvae under toxicity by the allelopthic molecule may be as extreme step of stress response to a lethal toxin. Along with elevation of protein, a sharp decline on the content of total free amino acids were observed. Similar observations were reported in other insect larvae such as *Oryctes rhinoceros* exposed to cold shock (Adhira *et al.,* 2010) and during infestation by *Bacillus thuringiensis* (Adhira and Evans 2012), exposure of *Spodoptera littura* to various toxins (El-Shershaby *et al.,* 2008) resulted sharp elevation of protein.

Transaminases are responsible for converting amino acids to ketoacids and subsequent entry into Kreb's cycle for energy release. Elevation of AsAT and AlAT under toxicity of Sulfoquinovosyl diacylglycerol may be for meeting the increased energy demand, because the larvae during toxicity stops feeding. Increased catabolism during toxicity was evidenced through elevation of uric acid in the hemolymph. Elevated transaminase activity was reported in *Spodoptera litura* on infection by *Bacillus thuringiensis* (El-Shershaby *et al.,* 2008).

The activity of LAP was inhibited by the active compound, sulfoquinovosyl diacylglycerol. Another proteolytic enzyme, which is more related to internal reorganisation of the body such as moulting and pupation is an aspartate protease, cathepsin D, which became elevated sharply during the toxicity. The antagonistic activity of two parallelly working enzymes under healthy state of larvae have shifted their activity during the course of toxicity and this phenomenon may have some adaptic significance in the body of intoxicated larvae. This type of antagonistic response of these two enzymes was observed in *Oryctes rhinoceros* under various stress conditions (Adhira and Evans 2012), and also in other insects *Musca domestica* intoxicated with cyfluthrin (Saleem *et al.,* 2004) *Bombyx mori*, exposed to H2O2 (Kim *et al.,* 2010).

Hemocytopaenia and various cytopathological changes were induced by Sulfoquinovosyl diacylglycerol. This type of hemocytopaenia was observed in *Dysercus sirgentatus* and *Papilo demoleus* on intoxication by azadirectin (Pandey *et al.,* 2011). Cytopathological studies revealed that most of the cell affected during toxicity was plasmotocytes and

this may be the reason for the low number of plasmatocytes observed during differential count. The debris of the lysed plasmatocytes in the hemolymph may be the reason for elevation in the number of granulocytes. This type of observation were also observed in *Rhyncophores ferrugineus*, experimentally infected by *B.thuringiensis* (Manachini *et al.,* 2011).

Toxicity has resulted sharp elevation of enzyme 20-hydroxyecdysone monooxygenase which resulted sharp accumulation of 20-hydroxyecdysone in the hemolymph. Gradual increase of 20-hydroxyecdysone just before pupation in *Bomyx mori* (Muramatsu *et al.,* 2008) and receptors of the epidermis became insensitive to juvenile hormone which lead to commitment peak of 20-hydroxyecdysone and successful pupation in *Drosophila melanogaster* are common process in holometabolous insects (Wang *et al.,* 2010). Sharp increase of 20-hydroxyecdysone in the fourth instar larvae during toxicity by SQDG might have caused hormonal imbalance in *O.longicollis* larvae. 20-hydroxyecdysone in excess has an ability to inhibits food consumption, which is a common physiological and behavioural change shown by pre pupae of almost all holometabolous insects (Warren *et al.,* 2006). The observed inhibition of feeding activity by active compound may be due to excess production of 20-hydroxyecdysone. Hormonal imbalance and inhibition of feeding may facilitated death of the larvae in presence of other reasons such as increased catabolism of amino acids and upset of protein turn over in the body.

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