https://doi.org/10.33307/entomon.v48i1.842
ENTOMON 48(1): 39-50 (2023)
Article No. ent. 48105

Antixenosis of Thodan (AAB), a resistant *Musa* cultivar to *Odoiporus longicollis* (Olivier) and characterization of larvicide molecules

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ABSTRACT: Banana pseudostem borer (BPB) *Odoiporus longicollis* (Olivier) (Coleoptera, Curculionidae) is a serious pest of *Musa* cultivars. Experimental maintenance of larvae in the live pseudostem of cultivar Thodan, a resistant AAB Musa cultivar has resulted antixenosis followed by death of larvae within a week. Antixenosis was characterized by significant decrease of total hemocytes and sharp changes on the proportionate distribution of different types of hemocytes in larvae. As the number of plasmatocytes, prohemocytes, splenocytes and adipohemocytes decreased the number of granulocytes and oenocytes increased. Antixenosis also caused accumulation of 20-hydroxyecdysone (20E) and significant inhibition on the activities of trypsin like serine protease (TlSP) and phenoloxidase (PO). Phytochemical analysis of Thodan resulted characterization of three larvicides such as Betulinic acid (BA), Stigmasterol-3-O-glucoside (SOG) and Sulfoquinovosyl diacyl glycerol (SQDG), and the content in the pseudostem ranged 0.0027 to 0.007 per cent. All the three larvicides were highly toxic to the larvae with LD_{50} of 0.38 ppm for SOG, 0.41 ppm for SQDG and 0.83 ppm for BA. Simultaneous action of three larvicides in the live pseudostem resulted resistance in Thodan against infestation by BPB. Susceptible Nendran showed negligibly low content of SOG (0.0011%) and SQDG (0.0013%) but no detectable quantity of BA. Intoxication by all the three larvicides caused significant changes on the proportionate distribution of hemocytes, accumulation of 20-hydroxy ecdysone (20E) and inhibition of TlSP and PO, the enzymes involved in larval metamorphosis and cuticle sclerotisation. This study demonstrated that resistance of Thodan against *O. longicollis* is due to adverse effect of these larvicides on endocrine system, cuticle development and cytotoxicity of hemocytes. As these larvicide molecules are stable compounds, there is scope for them to be used as substitutes in place of deleterious insecticides for the management BPB.
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KEY WORDS: Betulinic acid, Stigmasterol–3-O-glucoside, Sulfoquinvosyl diacylglycerol, endocrine system, cytotoxicity, metamorphosis

INTRODUCTION

Banana and plantains are important fruit crops of tropical and subtropical regions of the world and India is one of the major Centres of diversity of these plants (Bhat and Jarret, 1995; Reshma *et al.*,

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2016). *Odoiporus longicollis* (Olivier) (Coleoptera, Curculionidae), the banana pseudostem borer (BPB) is a major problem in the commercial cultivation of banana and plantains (Padmanaban *et al*., 2001; Kavitha *et al*., 2015a, b; Ajitha *et al*., 2017). Larvae are the destructive stages in the lifecycle and they are feeding on the pseudostem. If infestation is in the early growth phase, the plant will not set fruit and if it is in the late vegetative phase the plant topples down before the maturity. In the absence of proper control measures yield loss will be 80 per cent or more (Visalakshi *et al*., 1978; Padmanaban *et al*., 2001). Since the pest is an internal feeder, topical application of insecticides is futile and farmers are adopting pseudostem injection of insecticides (Aishwarya, 2019; Kavitha, 2019). Field study conducted in various agroecosystems of Kerala, India proved *O. longicollis* is extremely host specific (Kavitha *et al*., 2015a, b; Ajitha *et al*., 2017). India has rich diversity of more than 512 *Musa* cultivars and Kerala, a southern state of India has 217 *Musa* cultivars (Uma, 2010; Reshma *et al.,* 2016; Ajitha *et al*., 2018a). Among these diverse cultivars only few are commercially viable (CV) and are abundant in agroecosystem (Kavitha *et al*., 2015a, b). All the CV *Musa* cultivars are highly susceptible to BPB. Activity of supporting enzymes such as phenyl alanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (PO) are extremely low in CV cultivars (Ajitha *et al*., 2018a, b; Kavitha *et al*., 2020a). Most of the commercially nonviable (CNV) *Musa* cultivars are seen in villages and crushed juice extracted from the fresh pseudostem of some of these *Musa* cultivars are folk lore remedy for urolithiasis, hyperacidity and diabetes. CNV *Musa* cultivars are rare in agroecosystems and interestingly they face sporadic or no infestation by BPB (Kavitha *et al*., 2015a, b; Ajitha *et al*., 2018a, b). Experimental maintenance of *O. longicollis* larvae in the live pseudostem of some of the CNV *Musa* cultivars resulted 100 per cent mortality of larvae within a week (Kavitha *et al.,* 2020a, b, 2021). Those *Musa* cultivars which caused death of the larvae within a week of experimental maintenance are termed as resistant to BPB and larvae taken out on the fourth day of were weak, flaccid and hemocytes of the larvae showed cytopathological changes (Kavitha *et al.,* 2016). Kavitha *et al.* (2020b, 2021) and Ajitha *et al.* (2023) reported antixenosis on the pest, is characterized by sharp decrease of total hemocyte count, quantitative and qualitative changes on protein, sharp decrease of free amino acids and imbalance on the activities of enzymes such as transaminases, leucine aminopeptidase and cathepsin, and disruption of carbohydrate metabolism.

All the BPB resistant *Musa* cultivars possessed extremely high content of phenols, flavonoids and elevated activities of PAL, PPO and PO, compared to that of susceptible *Musa* cultivars. Phytochemical analysis of the pseudostem of resistant *Musa* cultivar Thenkaali (an indigenous triploid AAB cultivar) resulted in the identification of Stigmasterol-3-O-glucoside (SOG) and Pisanglilin (an exotic diploid AA cultivar) resulted characterization SOG and Sulfoquinovosyl diacylglycerol (SQDG) as larvicidal agents. Experimental rearing of *O. longicollis* larvae for three days in the excised pseudostem pieces of susceptible *Musa* cultivars, which were impregnated with either SOG or SQDG also resulted antixenosis and death (Kavitha *et al.,* 2020b, 2021). Thodan, a giant CNV plantain (an indigenous triploid AAB cultivar) was not infested by BPB under field condition and experimental maintenance indicating strong antixenosis (Ajitha *et al.,* 2018a, 2023). Bioassay guided isolation, identification and mode of action of larvicides responsible for the antixenosis on *O. longicollis* larvae were identified and reported in this paper.

MATERIALS AND METHODS

Actively feeding larvae of *O. longicollis* (fourth instar) collected from susceptible *Musa* cultivar banana (Nendran) were used for bioassay. Younger instars are small and highly sensitive to handle and fifth instar larvae are not in active feeding state and hence they were not used for bioassay (Kavitha *et al*., 2020b). Thodan, a BPB resistant *Musa* cultivar which was used for bioassay guided extraction and isolation of active larvicidal molecules. The cultivar, indigenous to Vettikavala Grama Panchayath of Kottarakara Taluke in

Kollam district of Kerala, India was identified as resistant to *O. longicollis* through field observation and subsequent laboratory observations (Ajitha *et al*., 2017, 2018a). Nendran *Musa* cultivar, which is highly susceptible to BPB was used as control.

Experimental maintenance of larvae in the live pseudostem: Four month old Thodan and Nendran cultivars, maintained in the College campus were used for the study. Crown of the cultivars with trunk circumference of 25 to 30 cm was cut down in such a way that the live stump of 1.0 m remained in viable state. Fourth instar larvae of *O. longicollis* (six numbers) were carefully placed on the free cut end of the pseudostem and allowed them to bore themselves into it. Cut end was covered with a piece of mosquito net and kept undisturbed for four or seven days. Either on the fourth day or on the seventh day, pseudostem bearing larvae was cut 25 cm below the free cut end and observed. Active wriggling movements of exposed larvae, in search of pseudostem to bore into it and creamy white colour indicate health of the larvae but flaccid body with bluish white colour indicates death of the larvae. As antixenosis by the resistant Thodan caused 100 per cent mortality of them within seven days of experimental period (Ajitha *et al.,* 2018a) larvae were removed on the fourth day of experimental period from the live pseudostem of Thodan and used for collecting hemolymph for further analysis.

Bioassay guided extraction and isolation of larvicide molecules: Fresh pseudostem of healthy Thodan cultivar weighing 50 kg was chopped into small pieces and kept for drying under shade for one week at room temperature of 26 to 31^{0}C. Dry pieces were powdered in electric motor with mesh size of 0.5 mm. Pseudostem powder (1kg) was sequentially extracted with hexane, chloroform and methanol in soxhlet and 2L of solvent was used for each extraction under controlled condition of 60^{0}C. Extracts were evaporated to dryness in rotary vacuum evaporator, limiting the temperature to 50^{0}C.

Fractionation of active extract: Chloroform extract was fractionated by column chromatography (silica gel 60-120 mesh) with solvent mixture at different proportion *viz.,* 1. hexane-chloroform (50:50), 2. hexane-chloroform (25:75), 3. hexane-chloroform (0:100), 4. Chloroform-methanol (90:10), 5. chloroform-methanol (80:20) and 6. chloroform-methanol (0:100). Volume of each elution was limited to one litre.

Sub fractionation of active fraction: Fraction 5 (active larvicidal fraction) was sub fractionated with following solvent mixture with silica gel (60-120 mesh) as stationary phase. 1. hexane-ethyl acetate (45:55), 2. hexane-ethyl acetate (35:65), 3. hexane-ethyl acetate (25:75), 4. hexane-ethyl acetate (0:100), 5. ethyl acetate-methanol (99.5:0.5), 6. ethyl acetate-methanol (85:15) and 7. ethyl acetate-methanol (80:20). Volume of each solvent mixture was limited to 500 ml.

Isolation of active compounds from 5th sub fraction: Sub Fraction 5 (active larvicidal sub fraction) was again fractionated with ethyl acetate-methanol (99.5:0.5), which resulted isolation of three compounds. Phytochemical isolation was done in the Phytochemistry and Phytopharmacology Division of Jawaharlal Nehru Tropical Botanic Garden Research Institute, Palode, Thiruvananthapuram, Kerala.

Spectral analysis and structural elucidation: Compounds were subjected to ^{1}H NMR, ^{13}C NMR and HRMS. One of the three compounds possessed attached fatty acids, which were identified by GC-MS. All spectral analysis was performed in the sophisticated Instrumentation Centre, University of Kerala, Kariyavattom, Thiruvananthapuram, Kerala. The structural elucidation of compounds was done by using the *chemdraw* software with the expertise of Phytochemistry and Phytopharmacology Division of Jawaharlal Nehru Tropical Botanic Garden Research Institute, Palode, Thiruvananthapuram.

Experimental maintenance of larvae in pieces of excised pseudostem: Each extract/ fraction/ active compound was dissolved in 0.5 per cent Tween80 in such a way to get 1 to 20 mg extract/ mL water. Aqueous suspension of extracts (1.0 mL) was injected evenly at 10 sites into 100g fresh pseudostem pieces of susceptible Nendran cultivar.

A small hole was made on the pseudostem piece and a single larva was allowed to bore into the pseudostem. Pseudostem injected with 0.5 per cent Tween (1.0 mL) was used as control. Every day each piece of pseudostem containing a single larva inside was kept very close to the ear of the investigator to listen the feeding sound of the larva, which is the indication of the health of the larva inside. Each observation lasted for five minutes and each piece with larva was observed six times a day. Fresh pseudostem impregnated with test material was provided every two days by gently taking the larvae out of the old pseudostem and allowing them to bore into the new one. Ability of the larvae to bore themselves into the pseudostem is also an index on the health of the larvae (Kavitha *et al.*, 2020b, 2021). On the seventh day of experiment, control and treated larvae were taken out with much care and percentage of mortality was tested as described in detail (Kavitha *et al.*, 2020b). All the larvae of the treatment group were either became immobile or dead within one week.

Determination of LD_{50}: Toxicity study and percentage mortality were calculated as described below (Kavitha *et al.*, 2020b, 2021) and LD_{50} values determined by Probit analysis.

$$\frac{\text{Percentage of test mortality} - \text{Percentage of control mortality}}{100 - \text{Percentage of control mortality}} \times 100$$

Mode of action studies on larvicide molecules: Larvicide molecules at the dose of their LD_{20} concentration were used for mode of action study on the fourth day of toxicity. Betulinic acid (0.4 ppm), Stigmasterol-3-O-glucoside and Sulfoquinovosyl diacylglycerol (0.2 ppm) are the doses fixed. Larvae were carefully taken out from the pieces of pseudostem, washed in distilled water, blotted in tissue paper and hemolymph was collected. A small slit was made on the ventral portion of neck, without puncturing the gut and hemolymph was collected in chilled Eppendorf tubes and stored in deep freezer at -20^{0}C. The samples were centrifuged in refrigerated centrifuge at 1000 g and used for biochemical estimations. Hemolymph samples without centrifugation were used for the study of haemocytes.

Estimation of 20-hydroxyecdysone (20E) : Cell free hemolymph 50µl was diluted to 0.5 ml using methanol, mixed well and centrifuged at 1000 g for ten minutes. Supernatant was collected and allowed to evaporate gradually in dust free condition at room temperature. The dry sample was dissolved in Enzyme Immunoassay Buffer provided in the assay kit. 20E of hemolymph was estimated by Enzyme immunoassay (Porcheron *et al.*, 1989) using an assay kit (A05120, Cayman Chemicals, France), provided with 96 well micro plate and absorbance measured at 405 nm by Micro plate reader.

Assay of Trypsin like Serine Protease: Activity of this enzyme was done as described in standard protocol (Erlanger *et al.*, 1961). Cell free hemolymph 100µl was diluted to 1.0 mL with ice cold, Tris HCl buffer (0.05 M, pH 8.2) and used as enzyme for the assay. The enzyme 100µl was made up to 1.0 ml with buffer and was incubated at 37^{0}C for 90 minutes. 1.0 ml of BApNA (40 mg of N-benzoyl-dl- arginine-paranitroanilide dissolved in 0.5 ml dimethyl sulfoxide and then made up to 100 ml with Tris HCl buffer) was added to the above solution and incubated at 37^{0}C for 60 minutes. The reaction was stopped by the addition of 30 per cent acetic acid. The solution was centrifuged at 12,000 g for 4 minutes and supernatant was read at 410 nm. Trypsin standards were run alongside.

Assay of Phenol oxidase: Phenoloxidase (tyrosinase) was measured colorimetrically using dopa as substrate (Lerch, 1987). Cell free hemolymph 100µl was diluted to 1.0 ml of phosphate buffer (0.1 M, pH 6) under ice cold condition and kept in the refrigerator until use. Diluted hemolymph 100 µl was used enzyme, which is applied into 2.9 ml of substrate solution (L-3,4 dihydroxyphenylalanine, 0.8 mg ml^{-1} in buffer), followed by rapid mixing. Enzyme reaction took place at 30^{0}C and read at 475 nm. One unit of enzyme activity corresponds to absorption change of 0.6/minute. A small aliquot of enzyme was subjected to protein estimation. Activity of enzyme is expressed as Units/minute/mg protein.

Estimation of Protein: The quantification of protein in the cell free hemolymph was estimated using Folin-Ciocalteu reagent (Lowry *et al.*, 1951).

Cell free hemolymph 100 µl was made up to 1.0 mL with distilled water and 1.0 ml of 10 per cent aqueous trichloro acetic acid was applied. The mixture was kept in ice bath for complete precipitation of protein, followed by centrifugation at 1000 g for ten minutes. Supernatant was decanted and precipitate was dissolved in 1.0 ml of 0.1 N NaOH and used for estimation. The dissolved sample of protein was treated with 3.5 ml of alkaline $CuSO_4$ solution, followed by 0.5 ml of Folin's reagent. The colour developed was estimated spectrophotmetrically at 620 nm.

Hemocytes count: Total hemocytes count of the collected hemolymph was carried out by adopting the protocol of Jones (1963) using Neubauer Hemocytometer, after diluting five times the hemolymph, with phosphate buffered saline (PBS, pH 7.2) possessing 0.1 per cent Giemsa stain.

Differential hemocytes count: A small drop of hemolymph was placed on a clean dry glass slide and a thin film of it was prepared with the help of another glass slide by drawing the second slide across the first one at an angle of 45^0. The smear was air dried for five minutes and stained using Giemsa stain for 15 minutes. Excess stain was washed in running water and slides were observed under microscope at 45x. Different types of hemocytes were identified by their distinguishing features (Wigglesworth, 1972).

Statistical analysis: SPSS 24.0 software (IBM, USA) was used. Data obtained was expressed as mean ± standard error of six numbers of observations. One way Analysis of variance (ANOVA) was applied on the data to assess the effect of individual larvicides and antixenosis exhibited by Thodan with respect to susceptible Nendran cultivar (control).

RESULTS

Antixenosis of resistant Thodan on *O. longicollis*

Thodan (AAB) is a tall, giant *Musa* cultivar which is resistant to infestation by *O. longicollis*. The name Thodan (meaning of *thodu* in local language is covering) was originated because of the exceedingly thick rind of the fruits. Ripe fruits are not as palatable as fruits of CV cultivars and hence they are used as vegetable. Nendran *(*AAB) the most common CV cultivar of Kerala (India) is highly susceptible to infestation by BPB. Experimental maintenance of *O. longicollis* larvae in the live pseudostem of resistant *Musa* cultivar Thodan with three days existence caused strong antixenosis characterized by weak, flaccid and unable to feed the tender pseudostem and their hemolymph exhibited significantly high content of 20-hydroxy ecdysone (20E) compared to that of the larvae in the susceptible, control pseudostem. Elevation of 20E was more than two-fold with that of control. Activity of trypsin like serine protease (TlSP) and phenol oxidase/tyrosinase (PO) showed significant inhibition in larvae maintained in cv Thodan, compared to susceptible cv Nendran (Table 1). Total hemocytes count of larvae maintained in the resistant pseudostem showed significant decrease (70%) in number than control. Proportionate distribution of different types of hemocytes also showed significant difference between the test and control larvae. In healthy larvae, plasmatocytes occupy major proportion and is almost 40 per cent of total hemocytes. After three days of existence larvae in resistant Thodan, plasmatocytes decreased to 18.75 per cent. The second major population of hemocytes is prohaemocytes which accounts to 35 per cent of total cells, decreased to 26 per cent. In healthy normal larvae, proportion of spherulocytes and adipohaemocytes occupy 6 and 8 per cent respectively was decreased to 3.5 per cent. Interestingly two types of cells such as granulocytes and oenocytes showed significant increase during antixenosis. In normal healthy state, proportion of granulocytes is only 8 per cent, which increased to 38 per cent (Table 2). Among the hemocytes, the oenocytes accounts to 2 per cent in normal healthy larvae, which exhibited fourfold increase in larvae under antixenosis.

Bioassay guided phytochemical analysis of pseudostem

Extraction of pseudostem powder of Thodan with organic solvents resulted localization of larvicidal activity in chloroform extract. Fractionation of chloroform extract by column chromatography

Table 1. Antixenosis by a resistant *Musa* cultivar and its larvicide molecules on 20-hydroxyecdysone and activities of enzymes related to cuticle sclerotisation in *Odoiporus longicollis*

Host Plant/ Larvicide molecules	20-hydroxy ecdysone	Activity of Trypsin like Serine Protease	Activity of Phenol Oxidase
Susceptible cv.Nendran (Control)	96.38 ± 06.42	0.96 ± 0.05	48.52 ± 3.91
Resistant cv Thodan	202.19 ± 14.38*	0.42 ± 0.03*	33.41 ± 3.01*
Betulinic acid	142.16 ± 10.54*	0.61 ± 0.04*	36.53 ± 3.23*
Stigmasrerol-3-O-glucoside	168.33 ± 12.18*	0.53 ± 0.04*	37.80 ± 3.02*
Sulfoquinovosyl diacyl glycerol	136.47 ± 11.70*	0.51 ± 0.03*	34.71 ± 2.96*

*Values are mean ± SE; *Values are significantly different from control, $p \leq 0.05$, n = 6; Quantity of 20-hydroxyecdysone is expressed as picogram / mL Hemolymph; Activity of Trypsin like serine protease is expressed as Units/ mg of protein; Activity of Phenol oxidase is expressed as Units / mg of protein; (BA, SOG and SQDG were administered into pieces of pseudostem at their LD_{20} concentration)*

Table 2. Effect of antixenosis by resistant host plant and its individual larvicide molecules on the total and differential hemocytes count in *Odoiporus longicollis*

Host Plant/ Larvicide molecules	Total hemocyte count	Differential hemocyte count (%)					
		PLA	PRO	GRA	OEN	SPL	ADH
Nendran (Susceptible)	4540.57 ± 33.45	40.82 ±3.80	34.69 ±3.05	8.15 ±0.63	2.04 ±0.21	6.12 ±0.56	8.17 ±0.71
Thodan (Resistant)	1877.34 ± 16.67*	18.75 ±1.60*	26.57 ±2.34*	38.12 ±3.11*	9.33 ±0.83*	3.42 ±0.27*	3.63 ±0.21*
Betulinic acid	2011.18 ± 18.12*	20.19 ±1.86*	27.83 ±2.41*	36.42 ±3.23*	8.37 ±0.67*	8.37 ±0.65*	3.43 ±0.25*
Stigmasteol-3-O-glucoside	1938.32 ± 17.34*	18.68 ±1.61*	25.24 ±2.30*	39.21 ±3.82*	9.23 ±0.78*	9.23 ±0.79*	4.61 ±0.40*
Sulfoquinovqsyl diacyl glycerol	1630.23 ± 14.56*	14.59 ±1.04*	26.38 ±2.14*	40.16 ±3.90*	12.14 ±0.98*	10.15 ±0.97*	4.33 ±0.38*

*PLA –Plasmatocyte, PRO – Prohaemocyte, GRA – Granulocyte, OEN – Oenocyte. SPL – Splenocyte, ADH – Adipohaemocyte; Values are mean ± SE; n=6, *Values significantly different from control, $p \leq 0.05$; (BA, SOG and SQDG were administere into pieces of pseudostem at their LD20 concentration)*

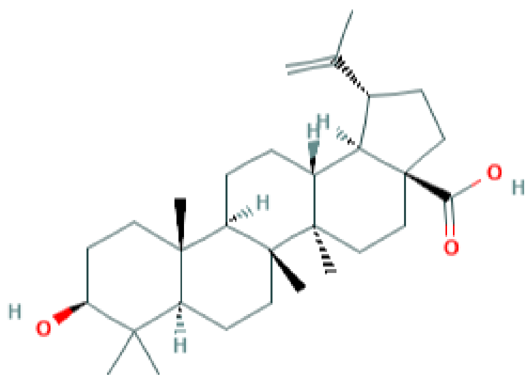

Fig. 1 Structure of Betulinic acid

resulted six fractions and among them 5th fraction showed larvicidal activity. Sub fractionation of 5th fraction gave seven sub fractions, of which 5th sub fraction exhibited larvicidal activity (Table 3). Further chromatographic separation of the 5th sub fraction has resulted isolation of three larvicides in pure form. Spectral analysis of the isolated compounds revealed that they are Betulinic acid (BA), Stigmasterol-3-O-glucoside (SOG) and Sulfoquinovosyl diacylglycerol (SQDG).

Intoxicated larvae with isolated compounds were weak, flaccid and unable to feed depending on the concentration of the molecules. At LD_{20} concentration (0.2 ppm for SOG and SQDG and 0.4 ppm for BA), larvae stopped feeding on the fifth day and were unable enter into the pseudostem provided on sixth day. Control group of larvae developed into pre pupa. Larvae allowed to feed pseudostem impregnated with different concentration of individual larvicide molecules did not develop into pre pupa and died within two weeks.

SOG is the most toxic of all the three larvicide molecules followed by SQDG and BA. SQDG is the most abundant larvicide (0.007%) followed by SOG (0.0036%) and BA (0.0027%). Pseudostem powder of susceptible cv Nendran was also subjected to extraction and fractionation by column chromatography. The cultivar showed the presence of SOG (0.0011%) and SQDG (0.0013%). No detectable quantity of BA was yielded from this cultivar. Fresh pseudostem weighing 50 kg yielded only 1.0 kg of dry powder and hence the presence of larvicides molecules in the live pseudostem ranged between 0.000054 and 0.00014 per cent (Table 3).

Betulinic acid (BA) is chemically 3-b-hydroxyl-lup-20(29)-en-28-oicacid. It is a pentacylic lupine type triterpene (Fig. 1). The compound is present at a very low concentration of 0.0027 per cent on dry weight basis with LD_{50} of 0.832 ppm (Table 3). The spectral characteristics as per ^{13}C NMR, ^{1}HNMR and HRMS of BA is explained below.

_δC(125 MHz): C-1(40.0), C-2 (29.3), C-3 (79.7), C-4 (39.9), C-5 (56.8), C-6 (19.5), C-7 (34.4), C-8(40.8), C-9 (49.2), C-10 (38.1), C-11 (24.4), C-12 (126.7), C-13 (139.8), C-14 (43.3), C-15(27.9), C-16 (25.4), C-17 (47.1), C-18, (54.5), C-19 (40.4), C-20 (40.5), C-21 (31.9), C-22 (38.2), C-23 (28.8), C-24 (16.4), C-25 (16.1), C-26 (17.9), C-27 (21.7), C-28 (182.3), C-29 (17.7), C-30 (24.1), C-1' (145.5), C-2' (123.4), C-3' (150.1), C-4' (127.0), C-5' (128.7), C-6' (143.8), C-7' (170.4).

^{1}H NMR ($CDCl_3$): 0.65, 0.77, 0.98, 1.14 and 1.34 (5s, 15H, all tertiary $–CH_3$), 1.37 (m, 2H, H-21), 1.38 (m, 2H, H-16), 1.45 (m, 2H, H-20), 1.51 (m, 4H, H-18, H-19 and H-15), 2.09 (m, 3H, H-1 and H-9), 2.13 (m, 2H, H-14), 3.17 (t, 2H, *J* = 7 Hz, H-2), 3.38 (s, 2H, H-7), 4.56 (s, 2H, H-11), 4.59 (s, 2H, H-12).

Table 3. Larvicidal fractions and isolated active compounds from chloroform extract of Thodan against *O. longicollis*

Fractions/Active compound	Fraction / compound (mg)	Yield (%)	LD_{50} (ppm)
Fraction 05	2100	0.21	2.6
Sub Fraction 05	180	0.018	1.21
Betulinic acid	27.3	0.0027	0.832
Stigmasterol-3-O-glucoside	35.8	0.0035	0.380
Sulfoquinovosyl diacylglycerol	72.7	0.0073	0.411

Larvae intoxicated by BA at LD_{50} concentration of 0.8 ppm stopped feeding on the third day of toxicity. They were unable to bore themselves into the pseudostem after 72 hours of toxicity at 0.4

ppm BA. Larvae allowed living for 48 hours in pseudostem with 0.4 ppm BA and later life in control pseudostem could not recover to normal life and die within one week, without undergoing development. During the course of study control larvae successfully moulted into pre pupa.

Stigmasterol-3-O-glucoside is a glycosylated sterol (Structure: Kavitha *et. al*., 2020b) in which glucose is linked with third carbon of the sterol ring with the hydroxyl group of the first carbon of glucose. It is a stable compound with 29 carbons, forming three hexacarbon rings and other carbon atoms are attached with it as a pentameric ring and as free carbon skeleton. The compound is present at a very low concentration (0.0035% on dry weight basis) and possessed LD_{50} of 0.38 ppm and (Table 3) and LD_{20} of 0.2 ppm. The spectral characteristics as per ^{13}C NMR, ^{1}HNMR and HRMS are described below.

_δC(125 MHz): C-1(37.20), C-2 (31.83), C-3 (78.84), C-4 (42.10), C-5 (140.34), C-6 (121.82), C-7 (31.72), C-8(31.83), C-9 (50.19), C-10 (36.60), C-11 (18.96), C-12 (38.50), C-13 (42.10), C-14 (56.72), C-15(24.11), C-16 (28.82), C-17 (55.91), C-18, (11.79), C-19 (18.57), C-20 (39.61), C-21 (20.93), C-22 (138.33), C-23 (129.19), C-24 (51.28), C-25 (31.83), C-26 (19.35), C-27 (18.96), C-28 (24.11), C-29 (11.79), C-1' (101.09), C-2' (73.58), C-3' (76.12), C-4' (70.22), C-5' (77.19), C-6' (61.61)) .

_δH(500 MHz): 1.04(1H, m) H-2, 1.51 (1H, m) H-3, 5.19 (s, 1H) H-6, 0.70 (3H, s) H-18, 1.02 (3H, s) H-19, 1.6 (3H, d, J=4Hz) H-21, 1.18 (2H, m) H-28, 0.84 (3H, t, J=8Hz) H-29, 4.39 (1H, d, J=8Hz) H-1', 3.30 (1H, m) H-2', 3.30 (1H, m) H-3', 3.31 (1H, m) H-4', 3.39 (1H, m) H-5', 2.31 (1H, dd, J=4Hz, 3.6Hz) H-6'. From all the above spectral data, structure of the compound is identified as stigmasterol-3-O- glucoside, which is perfectly matching with the previous reports (Kojima *et al*., 1990) and that of a single larvicide identified in another pest resistant *Musa* cultivar (Kavitha *et al*., 2020b)

Sulfoquinovosyl diacyl glycerol is a sulfur containing compound and is characterized as (2S) -1-2-DL-O palmitoyl-3. O-(6-sulfo-a-D-quinovodiacsyl) glycerol (Structure: Kavitha *et.al*., 2021) It has six fatty acids in which two of them are attached with this compound at a time and they are Lauric acid, Myristic acid, Palmitic acid, Stearic acid, Penta decenoic acid and 9-hexadecnoic acid. The compound is present at 0.007 per cent in pseudostem on dry weight basis and showed LD_{50} of 0.41 ppm (Table 2). The spectral characteristics as per ^{13}C NMR, ^{1}HNMR and HRMS are described below.

It is amorphous white powder; 197-198^{0}C; positive ESI-MS 795 $(M+1)^{+}$; IR (KBr) *v* (cm^{-1}) 3426, 2919, 2851, 1736, 1634, 1465, 1221, 1170, 1110, 1060, 1038, 764, 720.

^{1}H NMR (Methanol-d_4, 400 MHz): ^ (ppm) 5.76 - 5.72 (m, 1H), 5.48 (dd, J = 15.3, 7.3 Hz, 1H), 5.26 (t, J = 4.5 Hz, 1H), 4.66 (s, 1H), 4.15 (d, J = 7.8 Hz, 1H), 4.00 - 4.04 (m, 2H), 3.86 - 3.89 (m, 2H), 3.73 (d, J = 11.5 Hz, 1H), 3.35 - 3.40 (m, 2H), 3.24 - 3.33 (m, 1H), 2.10 - 2.11 (m, 5H), 2.01 - 2.03 (m, 1H), 1.27 - 1.41 (m, 2H), 0.90 (s, 39H), 0.87 (t, J = 6.5 Hz, 6H).

^{13}C NMR (METHANOL-d_4, 100 MHz): ^ (ppm) 175.8, 133.5, 130.8, 130.36, 103.0, 77.8, 77.6, 76.32, 73.4, 71.9, 71.7, 68.2, 61.3, 53.2, 34.4, 32.4, 32.3, 32.0, 31.7, 29.6, 29.5, 29.4, 29.0, 27.1, 26.5, 25.9, 25.0, 22.5, 13.6. Also, the ^{13}C NMR showed two distinct set of peak regions corresponding to fatty acids (14.5 - 35.9) and sugar (62.7 - 78.0). Attachment of sulfur to sugar was observed via a peak at 54.6 in ^{13}C NMR spectrum. The two methyl group protons at 0.84 in the ^{1}H NMR spectrum and 14.5 in the ^{13}C NMR spectrum revealed the molecule must be diacylated. The values at 62.7, 69.8 and 71.6 are characteristics of glycerol unit in SQDG (Sulfoquinovosyl diacylglycerol). Based on the above molecular features the compound is identified as Sulfoquinovosyl diacyl glycerol. The spectral characters are identical with that of the larvicide molecule identified in a diploid AA *Musa* cultivar Pisanglilin (Kavitha *et al*., 2021).

Toxicity by individual larvicides

Larvae allowed to exist in the live pseudostem of Thodan for three days were weak and flaccid.

Identical changes were observed in larvae which were maintained in 100 g pieces of pseudostem with individual identified larvicide molecules at LD_{20} (BA at a dose of 0.4 ppm or SOG or SQDG 0.2 ppm). Hemolymph of weak and flaccid larvae, which were either taken out from the live and resistant pseudostem or intoxication by larvicides showed sharp significant decrease on total hemocytes count. Differential hemocytes count of control and intoxicated larvae showed sharp difference on the percentage distribution of hemocytes (Table 2). In the affected larvae granulocytes increased from 8 to 38 per cent. Proportion of oenocytes was also increased from 2 to 8 per cent. Significant decrease on the number of plasmatocytes (50 to 60% decrease) and prohemocytes (20 to 25% decrease) were also observed.

Toxicity by individual larvicides molecule resulted exponential increase on the content of 20E in the hemolymph. Accumulation of 20E was significantly high in larvae existed for three days in live pseudostem than toxicity by individual larvicide molecule. Content of 20E in non- feeding pre pupae was estimated as 136.07±10.32 ng ml^{-1} of hemolymph, which is almost equal to 20E of fourth instar larvae under intoxication by larvicides molecules. Significant inhibition on the activities of trypsin like serine protease (TlSP) and phenoloxidase (PO) were also observed in the hemolymph of the affected larvae. TlSP exhibited 60 per cent inhibition while the larvae in the live and resistant pseudostem for three days (Table 2). Individual larvicide molecules extracted from the resistant plantain at the dose LD_{20} caused similar effect but inhibition of TlSP activity was only 40 per cent. Under identical experimental condition activity of PO exhibited 25 to 30 per cent inhibition.

DISCUSSION

Ajitha *et al*. (2018b) and Kavitha *et al*. (2020b, 2021) reported *Musa* cultivars Yangambi, Thenkaali and Pisanglilin showed resistance to the BPB, as they showed 100 per cent mortality on the reared larvae. Hemolymph of the larvae which spent three days in the live pseudostem of Thenkaali and Pisanglilin showed rupture of hemocytes, qualitative and quantitative changes in the protein profile of hemolymph and imbalance in the activities of enzymes regulating free amino acid pool (Kavitha *et al*., 2020b, 2021). In the present study, larvae spent three days in the live pseudostem of Thodan exhibited severe hemocytopenia with sharp increase on certain hemocytes such as granulocytes and oenocytes. Granulocytes are cells which are actively involved in cellular immune response such as nodulation, encapsulation, phagocytosis of pathogen and removal of necrotic cells (Youngwoo and Sayoull, 2019). Numerical increase of granulocytes in the hemolymph of *O. longicollis* larvae in Thodan may be for the removal of dead cells formed as a result of antixenosis by the resistant host plant.

Recent studies on the mechanism of pest resistance of *Musa* cultivars revealed the presence of a single larvicide molecule (SOG) in the pseudostem of Thenkaali (AAB) (Kavitha *et al*., 2020b) and two larvicide molecules such as SOG and SQDG in a diploid AA *Musa* cultivar Pisanglilin (Kavitha *et al*., 2021). Present study in Thodan revealed that in addition to SOG and SQDG it possessed BA as third larvicide molecule. Besides the larvicide molecules Thodan possessed very high quantities of other secondary metabolites such as phenols and flavonoids in the pseudostem, compared to susceptible *Musa* cultivars. Activities of supporting enzymes such as PAL, PPO and PO were also high in Thodan, compared to susceptible cultivars (Ajitha *et al*., 2018a). In the present study the susceptible Nendran also possessed low content of SOG (40%) to that of its content in resistant Thodan.

Bioassay guided isolation and characterization of phytocompounds responsible for resistance exhibited by *Musa* cultivar Thodan revealed the presence of three larvicides in the pseudostem. Among them, two larvicides isolated from Thodan such as SOG and SQDG are highly toxic to *O. longicollis* and are previously reported from two indigenous *Musa* cultivars of Kerala (Kavitha *et al*., 2020b; Kavitha *et al*., 2021). SOG was isolated first time from the bark of *Prunella vulgaris* as a glycosylated sterol (Kojima *et al*., 1990) and in the present study the spectral

characters obtained in respect of SOG was identical with that of the previous study. SOG has no mammalian toxicity and exhibited antihyperglycaemic (Panda *et al*., 2009), hypocholesterolaemic (Chandler *et al*., 1979) and hepatoprotective activity (Kaur *et al.,* 2011) in humans and animal models.

SQDG exhibited cytotoxicity on the haemocytes and resulted lysis of the midgut epithelium of the intoxicated larvae of *O. longicollis* (Kavitha *et al*., 2021). SQDG was isolated first time as a sulfur containing lipid (Benson, 1959) of the thylacoid membrane (Kaichiro *et al*., 2016). Lysis of midgut cells and hemocytes in *O. longicollus* larvae during toxicity by SQDG may be due to inhibition of DNA polymerase action. Among the three larvicides, SQDG is the most abundant and its toxicity was almost equal to that of SOG (Kavitha *et al.,* 2021). BA the third larvicide molecule identified from Thodan, is a known insect growth regulator seen in many plants which did not exhibit any attack by insects, but the presence of this compound in resistant *Musa* cultivar is new to science. Larvae of *Callasobruchus chinensis,* treated with BA resulted wide spread abnormalities and death (Madhavi *et al*., 2019). Treatment with BA on the larvae of *Spodoptera littoralis* resulted cytotoxicity of hemocytes (Coloma *et al*., 2011) and in larvae of *Papilio demoleus,* BA caused strong anti-feedant action (Srinivasa Rao *et al*., 2014). In the present study, larvae of *O. longicollis* in pseudostem administered with 0.4 or 0.8 ppm BA stopped feeding on the fifth and third day of toxicity respectively. Exposure of larvae to BA at 0.8 ppm for 24 hours in control pseudostem was sufficient for inhibiting their development to 5th instar (pre pupa). Larvae did not die within seven days due to toxicity by BA at a dose of 0.4 ppm (LD_{20} concentration) but they were unable to feed or develop into pre pupa within 14 days.

Intoxication by all the three larvicides or three days existence in resistant host plant resulted accumulation of 20E in the hemolymph of fourth instar larvae of *O. longicollis*, which is almost equal to the quantity of 20E in the non-feeding pre pupa (Kavitha, 2019). Cessation of feeding, observed in larvae during the initial phase of toxicity may be due to accumulation of 20E. In *Helicoverpa armigera*, it was observed that 20E binds to dopamine receptor, a G protein to stop larval feeding and promote pupation (Xin *et al.,* 2019, 2021). Another effect observed in the hemolymph of intoxicated larvae is the inhibition of TlSP and PO. These two enzymes play important role in cuticle sclerotisation and melanisation and also important in defense against parasites (Amado *et al*., 2004). In *Drosophila ananassae* and *Culex quinquefasciatus* TlSP and PO are parallely acting enzymes and are exhibiting very high activity in larvae and negligibly low in five day old mosquitoes and seven day old flies. Elevation of temperature in *C. quinquefasciatus* larvae (Ayana Gayathri and Evans, 2018) and transgeneration effect of BA in *D. ananassae* (Anuji, 2021) caused increase of pigmentation in adults and elevation of both enzyme activities. In Monarch butterfly *Danus plexipuss,* infection by a protozoan caused abnormal melanisation and elevation of PO activity (Cecilia *et al*., 2021). All these reports prove the active role of these enzymes in cuticle development and inhibition of TlSP and PO activities in the inhibition of cuticle development.

ACKNOWLEDGEMENTS

Authors thank Kerala State Council for Science, Technology and Environment for funding a research scheme entitled Molecular mechanism of pest resistance of certain *Musa* cultivars against infestation by *Odoiporus longicollis*. Authors are indebted to Dr. Sabulal, Division Head, Phytochemistry and Phytopharmacology Division, Jawaharlal Nehru Tropical Botanic Garden Research Institute, Palode, Thiruvananthapuram, for his guidance in the phytochemical studies.

REFERENCES

Adamo S.A. (2004) Estimating disease resistance in insects: phenoloxidase and lysozyme like activity and disease resistance in the cricket *Gryllus texenisis*. Journal of Insect Physiology 50: 209–216. doi.org/10.1016/j.insphys.2003.11.011.

Aiswarya D. (2019) Studies on sub lethal toxicity of carbosulfan on adults of banana pseudostem

weevil *Odoiporus longicollis* (Oliver), Ph.D thesis, University of Calicut. Kerala.

Ajitha T., Kavitha K.J., Shabithraj K., Murugan K. and Evans D.A. (2017) Pest status of certain *Musa* cultivars on Kerala with special reference to infestation by *Odoiporus longicollis*(Oliver) and genome constitution of the cultivars. Journal of Entomological Research 41: 399–404. doi:10.5958/0974-4576.2017.00064.0.

Ajitha T., Kavitha K.J., Shabithraj K. and Evans D.A. (2018a) Distribution of flavonoids, phenols and related enzymes in *Musa* cultivars and their role in resisting infestation and causing mortality in *Odoiporous longicollis* Oliver, International Journal of Tropical Agriculture 36: 37–44 .

Ajitha T., Kavitha K.J., Shabithraj K., Bharya L.R., Vishnu Dhas E.H. and Evans D.A (2018b) Secondary metabolites of *Musa* cultivars confer resistance against infestation by stem weevil *Odoiporus longicollis* (Oliver). ENTOMON 42: 301–310.

Ajitha T., Ayana Gayathri R.V. and Evans D.A. (2023) Antixenosis of a resistant Musa cultivar on *Odoiporus longicollis* Olivier and expression of alpha amylase by the pest. Journal of Biosciences 48: doi: 10.1007/12038-023-00331-9.

Anuji T.K. (2021) Transgenerational effect of Bisphenol A in *Drosophila ananessae* (Doleschall) and its influence on protein turn over and antioxidant enzymes. Ph.D. Thesis, University of Kerala, Kerala, India.

Ayana Gayathri R.V. and Evans D.A. (2018) *Culex quinquefasciatus* Say larva adapts to temperature shock through changes in protein turn over and amino acid catabolism. Journal of Thermal Biology 74: 149–159. doi:10.1016/j.therbio.2018.03.016.

Benson A.A., Daniel H. and Wilser R.A. (1959) Sulfolipid in plants. Proceedings of the National Academy of Science USA 45: 1987–1592.

Bhat K.V. and Jarret R.L. (1995) Random amplified polymorphic DNA and genetic diversity of Indian *Musa* germplasm. Genetic Resources and Crop Evolution 42: 107–118.

Cecilia A.S. and Sonia A. (2021) Thermal tolerance and environmental persistence of a protozoan parasite in monarch butterflies. Journal of Invertebrate Pathology 183: 107544. doi: 10.1016/j.ip.2021.107544.

Chandler R.F., Hoper S.N. and Ismail H.A. (1979) Antihypercholesterolemic studies with sterols and stigmasterol. Journal of Pharmaseutical Science 68: 245–247. doi.org/10.1002/jps.2600680235.

Coloma A.G., Balboa C.L., Santana O., Reina M. and Fraga B. (2011) Triterpene based plant defenses. Phytochemistry Reviews 10: 245–260. doi:10.1007/s 11101-010-9187-8.

Erlanger B.F., Kokowsky N. and Cohen E. (1961) The preparation and properties of two new chromogenic substrates of trypsin. Journal of Archives in Biochemistry and Biophysics 95: 271–278. doi.org/10.1016/0003-9861(61) 90145X.

Jones J.C. (1963) Hemocytes of *Rhodnius prolixus* Stal. American Zoologist 4: 282–294.

Kaichiro E., Koichi K. and Hajimi W. (2016) Sulfoquinovosyl diacylglycerol has essential role in *Thermosynechococcus edongates* under phosphate deficient medium. Plant Cell Physiology *57*: 2461–2471.doi.org/10.1093/pcp/pcw 159.

Kavitha K.J. (2019) Allelopathic interactions of certain *Musa* cultivars against *Odoiporus longicollis* (Olivier), and molecular mechanism of pest resistance, Ph.D. thesis, University of Kerala, Kerala.

Kavitha K.J., Murugan K.and Evans D.A. (2015a) Screening of Wild Cultivars of banana in Kerala, India using score card method and its resistance against *Odoiporus longicollius* Olivier. Phytomorphology 65: 121–126.

Kavitha K.J., Shabithraj K. and Evans D.A. (2015b) Allelopathic interactions of certain *Musa* cultivars certain against *Odoiporus longicollis* Oliver. ENTOMON 40: 209–220.

Kavitha K.J., Murugan K. and Evans D.A. (2016) Cytopathological and haematological changes in *Odoiporus longicollis* (Olivier) grub by Aadinkombu and Thenkaali, the two pest resistant *Musa* cultivars identified in Kerala. Journal of Entomological Research 40: 27–33. doi:10.5958/0974-4576.2016.00005.0.

Kavitha K.J., Shabithraj K. and Evans D.A. (2020a) Allelopathic effects of high contents of phenols and flavonoids in *Musa paradisiaca* L. cultivars on banana borer *Odoiporus longicollis* (Olivier). Allelopathy Journal 49: 99–113. doi.org/10.26651/allele.j/2020-49-1-1257.

Kavitha K.J., Anil John J. and Evans D.A. (2020b) Stigmasterol-3-O-glucoside, an allelopathic molecule responsible for pest resistance Thenkaali AAB, a *Musa* cultivar against *Odoiporus longicollis* (Olivier). Current Science

118: 946–953. doi: 10.18520/cs/v118/i6.

Kavitha K.J., Sibina L. and Evans D.A. (2021) Allomones confer resistance to *Musa paradisiaca* L. cultivar Pisanglilin against infestation by *Odoiporus longicollis* Olivier and characterization of allomones. Allelopathy Journal 53: 35–52. doi.org/10.26651/allele.j/2021-53-1-1-1326.

Kaur N., Jasmine C., Akash J. and Lalith Kishore T. (2011) Stigmasterol a comprehensive review. International Journal of Pharmaceutical Science Research 2: 2259–2265.

Kojima H., Sato N. and Ogura H (1990) Sterol glucosides from *Prunella vulgaris.* Phytochemistry 29: 2351–2355.

Lerch K. (1987) Monophenol monoxygenase from *Neurospora crassa.* Methods in Enzymology 142: 165–168.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951) Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265–275. doi.org/10.1016/0303-3894(92)8711-4.

Madhavi M., Babu S., Rao G. and Srinivas B. (2019) Morphological abnormalities of Betulinic acid from *Zizphus jujube* against *Callasobruchus chinensis*. Bioscience and Biotechnology Research Asia 16: 36–43. doi:10.13005/bbra/2756.

Padmanaban B., Sundararaj B. and Sathiamoorthy S. (2001) Incidence of banana pseudostem borer *Odoiporus longicollis* Olivier (Coleoptera, Curculionidae), in banana peduncle. Indian Journal of Entomology 63: 203–205.

Panda S., Jafri M., Kaur A. and Mehitha B.K. (2009) Thyroid inhibitory, antiperoxidative and hypoglycaemic effects of Stigmasterol isolated from *Butea monosperma.* Fitoterapia 80: 123-126. doi.org/10.1016/fitote.2008.12.002.

Porcheron P., Moriniere M., Grassi J. and Pradelles P. (1989) Development of an enzyme immunoassay for ecdysteroids using acetylcholine esterase as label. Insect Biochemistry 19: 117–122. doi.org/10.1016/0020-1790(89)90081-4.

Reshma L., Nair A.R. and Nair A.S. (2016) Population genetic structure and diversity analysis of South Indian Banana cultivars. Journal of Plant Breeding and Crop Sciences 8:1–12. doi: 10.5897/JPBCS2015.0519.

Srinivas Rao V., Nageswara Rao A. and Sabitha R.S. (2014) Effect of Betulinic acid on feeding deterance of *Papilio demoleus* L. (Lepidoptera, Papilionidae) larva. Indian Journal of Fundamental and Applied life Sciences 4: 43–48.

Uma S. (2010) Annual Report of NRCB - 2010, National Research Centre on Banana - ICAR, Thiruchirapalli, Tamil Nadu.

Visalakshi A., Nair G.M., Beevi S.N. and Kamalamma A.N. (1978) *Odoiporus longicollis* (Coleoptera, Curculionidae), a pest of *Musa* cultivars in Kerala. ENTOMON 14: 366–369.

Wigglesworth V.B. (1972) Principles of Insect Physiology, 7th Edition, Chapman and Hills, London. pp 433–440.

Xin L.K., Jun-Ying Zhang, Di Wang, Yu Meng Zhao, Xian-Lin Han, Jin-Xing Wang and Xiao-Fan Zhao (2019) The stored hormone 20-hydroxyecdysone binds to dopamine receptor to repress lepidopteran insect feeding and promote pupation. PLoS Genetics 14: 15(8): e 1008331. doi: 10.1371/journal.p gen.1008331 .

Xin L.K., Yan Xue Li and Yan Li Li (2021) Holotetramerization of GPCR transmits 20-hydroxyecdysone and increases its entry into cells for insect metamorphosis. Development 148, dev.196667. doi: 10.1242/dev.196667.

Youngwoo C. and Sayoull C. (2019) Hemocyte – hemocyte adhesion by granulocytes is associated with cellular immunity in cricket *Gryllus bimaculatus.* Scientific Reports 9: 18066. doi: 10.1038/s 41598-019-54484-5.

(Received October 19, 2022; revised ms accepted January 10, 2023; published March 31, 2023)