

Dissipation of emamectin benzoate in okra and its residual impact on okra metabolome

G.T. Pradeepkumar^{1*}, K.P. Subhash Chandran² and V. Vijayasree^{3*}

¹Research and development center, Bharathiar University, Coimbatore, India.

²Kerala University of Fisheries and Ocean Studies (KUFOS), Kochi, India.

³College of Agriculture, Kerala Agricultural University, Vellayani, India.

Email: pradeepgt@iisertvm.ac.in; kpsubhash@rediffmail.com; vijayasree.v@kau.in

ABSTRACT: Pesticide absorption is a complex process in which pesticides undergo transformation and affect plant metabolic processes and hence investigated the metabolic fate of emamectin benzoate, a macrocyclic lactone insecticide. Residues of emamectin benzoate followed first order kinetics in field with pre-harvest intervals of 1.96 and 2.54 days respectively, at single and double dose of applications. Both in leaves and fruits of okra, the emamectin benzoate metabolites, emamectin B1a1 and emamectin B1b1 accumulated up to third day and the metabolite 4''-deoxy-4''-epi-N-methylaminoavermectin-b1-benzoate was detected only in fruits and was absent in okra leaves. A comprehensive evaluation of metabolic variations in okra induced by emamectin benzoate compared to the unsprayed plant samples was conducted by UHPLC-ESI-Q-Orbitrap high-resolution mass spectrometer (HRMS) and data analysed by compound discoverer 3.3. The metabolomic results suggested that emamectin benzoate could affect the plants defence mechanism, and a total of 818 metabolites including organic acids, amino acids, phenolics, flavonoids and fatty acids, were clearly altered. The content of defence-related L-proline and other proline related compounds were significantly increased by emamectin benzoate. Many phenolics and flavonoids, such as hyperoside and quercetin, were significantly accumulated, which could benefit the plant to alleviate the stress conditions due to the application of insecticide. © 2025 Association for Advancement of Entomology

KEY WORDS: Macrocyclic lactone insecticide, metabolites, plant defence mechanism, phenolics, flavonoids

INTRODUCTION

Biotic stress especially damage caused by insect pests displays a crucial role in crop loss worldwide. Chemical pesticides are important in the ever-evolving scenario of modern agriculture, to control pest and ensure the productivity of the agricultural crops. Frequent and excess application of these pesticides might result in harmful residue accumulations in the commodities at harvest and is

having a detrimental impact on human health, environment and related ecosystem services. This also causes deviations in the normal metabolic pathways and results in the increase in concentration of some metabolites while the others may decrease. Due to the application of different neonicotinoids an increase in the content of soluble protein in okra was observed (Preetha and Stanley, 2012). Not only the pesticide concentration but also other abiotic factors are involved in pesticide uptake

* Author for correspondence

and its metabolism in the plant system (Steffens and Wieneke, 1975; Farha *et al.*, 2016).

Emamectin benzoate (EB) with new mechanism of action is an insecticide with high selectivity having strong activity against Lepidoptera (Fanigliulo and Sacchetti, 2008). As it is one of the most promising avermectin insecticide finds its place in the arsenal against agricultural pests, it becomes imperative to scrutinize its journey within the plant, especially in crops destined for human consumption. Among these crops, okra (*A. esculentus*) holds a unique position due to its cultural significance, nutritional value, and widespread consumption especially the red/purple variety (Tyagita *et al.*, 2021). EB is a synthetic derivative of abamectin, created by the fermentation of the soil actinomycete *Streptomyces avermitilis*, which consists of about 90 per cent avermectin B1a and 10 per cent avermectin B1b (Dong and Zhang, 2022). Jyot *et al.* (2014) reported that EB residues dissipated below the limit of quantification (LOQ) of 0.05 mg kg⁻¹ after 5 days at 68.1 and 136.2 g a.i. ha⁻¹ in okra. The quick degradation, absorption and translocation of EB may lessen the amount of insecticide deposited on the plant surface hence reducing the contact of natural enemies contacting the insecticides and suitably higher insecticide inside the leaf tissues effectively controlling the pest (Sun *et al.*, 2022). The residues of EB present on the plant parts are absorbed and the plants reduce the toxicity of these xenobiotic by metabolic activity. Yoshii *et al.* (2001) developed an HPLC method to analyse EB and its metabolites such as 8,9-Z-isomer, N-demethylated, N-formylated, and N-methyl formylated emamectin in different vegetables. The chemical characteristics of xenobiotic substances such as pesticides, environmental factors, metabolic variables, or the regulatory expression of different biochemical processes, all have an impact on the diversity of metabolic pathways in plants (Zhang *et al.*, 2021; Barchanska *et al.*, 2023). Environmental and physiological factors also influence plant detoxification systems (Huynh *et al.*, 2021). The level of secondary metabolites like flavonoids, hydroxycinnamic acids, catechins, anthocyanins, etc. may influence the crop plant physiology and metabolism. Zhang and Yang (2021)

found that salicylic acid, jasmonic acid, and brassinosteroids were involved in the metabolism and decontamination of pesticides. Understanding pesticide metabolism in plants is crucial step for the modification, safe and effective use of these substances, and bioremediation of polluted soil and water (Hoagland *et al.*, 2001; Van Eerd *et al.*, 2003). The metabolites variation due to the effect of pesticides on plant is mainly depend on the plant physiological properties, variety, field and soil conditions (Barchanska *et al.*, 2023).

Lei *et al.* (2020) studied the EB induced metabolic alterations in leaves of maize seedlings and found positive correlation of peroxidase, total soluble sugar, total soluble protein and proline with increase in concentration of EB. This proved the presence of oxidative metabolism to reduce the effect of exposure to EB in maize. Zhang *et al.* (2022) found that fenvalerate exposure decreased the flavonoid content by 25 per cent in lettuce leaves. The LC-HRMS study showed imidacloprid exposure increased metabolism of aminoacids (arginine, cysteine, homoserine and 4-hydroxyisoleucine) while decreased metabolism of carbohydrates (Zhang *et al.*, 2022). Huynh *et al.* (2021) studied metabolism of cyantraniliprole in tomato, where 21 metabolites were tentatively identified which varied as a function of tissue types and their developmental stages. The major transformation pathways were characterized by ring closure, followed by N-demethylation, and glycosylation.

The studies on changes in distribution patterns of the EB remains and metabolites in different crops is meagre. The knowledge of absorption, translocation and metabolism of EB in okra under natural conditions, and how the degradation products influence the formation of secondary metabolites involved in various pathways, is decisive in reducing the pesticide levels and hence, the investigation was undertaken.

MATERIALS AND METHODS

Chemicals and reagents: EB (purity 99.4%) is ISO 17034 certified reference material from M/S Sigma-Aldrich, while Proclaim 5 SG formulation was acquired from Syngenta India Ltd. LC-MS

grade methanol, acetonitrile, and anhydrous MgSO_4 , ammonium acetate (AA) were procured from Merck in Darmstadt, Germany. Reagent grade formic acid (FA) was purchased from JT Baker, New Jersey, United States. Primary Secondary Amine (PSA) was procured from Agilent Technologies, California, USA. Type 1 water was produced in the lab using Milli-Q, Direct 8 from Merk Millipore. PVDF filter (13 mm 0.22 micron) was purchased from Rankem, Haryana, India.

Field experiment: The red hybrid variety of okra (*Abelmoschus esculentus*), Aruna developed by Kerala Agricultural University was used. The field was prepared in laterite soil, in randomised block design with 25 to 30 plants in each plot. Plants were grown as recommended by Kerala Agricultural University in accordance with package of practice (Estelitta *et al.*, 2016). The field experiment was conducted during November to December 2023. The average temperature is 26 with min 24 and maximum 30°C. Average rain fall was 80mm with humidity 95 percentage at 8.46/77.07 latitude/longitude. Insecticide, EB was sprayed at the rate of 11 and 22g a.i.ha⁻¹ for three times as per the recommendation. Three weeks after germination first spray was given, second spray at fruit setting time and then third spray was given ten days after second spray. Third spray was administered after confirming the presence of sufficient fruits for harvest on days 0, 1, 3, and 5 after spraying. All harvested fruits were tender, edible and 5–6 days of maturity. The control plot where water instead of EB was sprayed. Samples were collected one hour after third spray, 1, 3, 5, 10, 15, 20, 25 and 30 days after third application. Fruits (approx. 500g) and leaf samples (fifth leaf from the apex, 10 leaves from different plants) were collected in triplicate per sample. The whole sample was ground well to get a homogenous uniform paste and 50g was stored in a deep freezer below -20°C until extraction.

Validating the method for the analysis of EB:

To evaluate the effectiveness of extraction and clean-up as well as to standardize the process for residue quantification of EB, method validation was carried out. To prepare the reference solutions needed for a calibration curve, a stock solution of

EB (1000 $\mu\text{g mL}^{-1}$) was made in HPLC grade methanol (Vijayasree *et al.*, 2014) and serially diluted (0.50, 0.25, 0.10, 0.075, 0.05, 0.025 and 0.01 $\mu\text{g mL}^{-1}$). EB was spiked into okra to test the method's repeatability and accuracy at concentrations of 0.01, 0.02, 0.05 and 0.1mg kg⁻¹. Method efficiency was verified as per SANTE 11312/2021 guideline.

Sample preparation and analysis for the assessment of pesticide residue: QuEChERS

(Pradeepkumar *et al.*, 2023; Sharma *et al.*, 2024) method of extraction was followed to extract EB (metabolites B1a1 and B1b1) in okra samples. Ten gram of the ground sample was extracted using 20ml of HPLC grade acetonitrile, cleaned up by dispersive solid phase extraction (DSPE) using PSA and MgSO_4 . The residue was then reconstituted in 2 mL of methanol and filtered through a 0.2-micron filter for UPLC-MS/MS analysis. The details of the method described elsewhere (Vijayasree *et al.*, 2014, 2015; Pradeepkumar *et al.*, 2023). Waters Acquity UPLC system equipped with a reverse phase Atlantis C-18 (2.1 mm $\times$ 100 mm, 5-micron particle size) column used for chromatographic separation. Mobile phase A is 10 per cent methanol in water/ 0.1 per cent formic acid/5m M ammonium acetate and B is 10 per cent water in methanol + 0.1 per cent formic acid + 5m M ammonium acetate. The gradient elution was given with total run time of 8 minutes. The flow rate was 0.8mL min⁻¹ and injection volume was 10 μL . The column temperature was kept at 40°C. The effluent from the LC system was introduced into AB Sciex API 3200 MS/MS system equipped with an electrospray ionization interface (ESI), operating in the positive ion mode. The source parameters were temperature 600°C; ion source gas (GS1) 50 psi, ion source gas (GS2) 60 psi, ion spray voltage 5,500 V, curtain gas 13 psi. Qualitative and quantitative ion pairs for B1a1 is 886.6:158.2, 886.6:126.2 and B1b1 is 872.5:158.2, 872.5:126.2. The retention time is 4.4 min and 4.5 min for B1a1 and B1b1, respectively. Using Microsoft Excel 2016, the quantitative findings were tabulated and the dissipation rate, half-life, and waiting period were analysed using linear regression analysis in accordance with the protocol described by Hoskins (1961).

Sample preparation for metabolite analysis and instrument settings: Sampling and sample preparation are as same as in residue analysis. After thawing the frozen sample, 5g of fruit and 4g of leaf sample were weighed into a 50mL centrifuge tube. This was extracted with 15mL methanol by vortex (Spinix vortex, Tarsons) shaking for 3 minutes and sonicated (Sonorex RK31, Bandlin) for 30 minutes. Centrifuged the sample at 7000 rpm for 5 minutes. Filtered through 0.22-micron filter and collected in 1.5mL glass LC vials with pre slit silicon septa for metabolite analysis.

For the investigation of metabolism, a high resolution mass spectrometer UPLC-ESI-Q-Orbitrap (Ultimate 3000 UPLC with Q-Exactive, Thermo Fisher Scientific) was utilized (Pan *et al.*, 2022). Using a flow rate of 0.2mL min⁻¹, the metabolites were separated using a Thermo Fisher Hypercil Gold C-18 column (150 mm x 2.1 mm, 1.9µm particle size). The column temperature was maintained at 40°C. Multistep gradient flow was used to elute the metabolites with mobile phase A, 0.1 per cent HCOOH in water and mobile phase B is methanol with 0.1 per cent HCOOH. The gradient flow was given as follows, 0 min to 2 min 5 per cent B, 10 min 70 per cent B, 20 min to 28min 98 per cent B, 29 min to 30 min 5 per cent B. HRMS data collected in both positive and negative ionisations separately. The heated ESI operated with capillary temperature of 320°C and auxiliary gas heater temperature of 250°C (Li *et al.*, 2020). The sweep cone was set to zero flow, the sheet gas flow rate was forty, and the auxiliary gas flow rate was five (All gas flows are in arbitrary units). The spray voltage was 4 KV. The full MS-ddMS2 spectra were collected at a resolution of 70000 in the mass range of 80 -1000 m/Z. dd-MS2 spectra were acquired at resolution of 17500 in centroid mode with collision energy 20 and loop count 5. EB and its known metabolites were included in the inclusion list and an exclusion list was prepared after analysing reagent blank in the same method. The mass calibration in positive and negative mode was performed prior to injection of samples.

Statistical analysis for metabolite profiling: Thermo Q Exactive tune and Xcaliber software package

was used to acquire and initial process of the LC-HRMS data. The ddMS2 chromatograms were processed to find the metabolites using compound discoverer 3.3 with work flow, untargeted metabolomics workflow (Fig. S-1). The differences between samples were identified, and performed retention time alignment (RT tolerance <0.2 min) with ChromAlign algorithm, unknown compound detection (mass tolerance <5 ppm), and compound grouping across all samples. Then elemental compositions for all compounds were predicted, filled gaps across all samples, and subtract chemical background (using Blank samples) (Deore *et al.*, 2024). The compounds were identified with respect to mzCloud (ddMS2) database and isotopic pattern matched with ChemSpider database (formula or exact mass). Using the mzCloud database, a similarity search was carried out with ddMS2 spectra for every compound for confirmation (Souza and Patti, 2021). Then differential analysis was performed (one-way ANOVA model with Tukey as post-hoc test), for determining p-values, p-values adjusted using Benjamini-Hochberg correction for the false discovery rate. Additionally, heat map-based hierarchical cluster analysis (HCA) to understand the variation in concentration of metabolites between samples and principal component analysis (PCA) to group similar samples, were carried out. Pathway analysis was performed using KEGG database through the Metabolika pathway finder, mzCloud and ChemSpider databases to identify compounds.

RESULTS AND DISCUSSION

Method validation of EB in okra: EB was estimated as sum of its metabolites (emamectin B1a + emamectin B1b). Matrix matched calibration was performed to reduce the matrix error. The matrix effect was determined by comparing the signal of EB prepared in pure solvent to okra matrix and found to be -9.5 to 14 per cent. Negative values show the reduction in signal observed with matrix. The analysis of the data on the recovery of EB fortified at four different levels indicated a mean recovery of 102 per cent (Table 1). The recovery percentage ranged from 80.0 to 118 with a standard deviation ranged from 8 to 17 per cent for EB for

the adopted method. LOQ determined as 0.01mg kg⁻¹. Given the reasonable recovery percentage (70–110) and RSD values (below 20), the approach can be deemed suitable for estimating the amount of EB residues in okra (SANTE, 2021).

Dissipation kinetics of EB in okra fruit: The initial deposit of emamectin B1a in fruits of okra for single and double dosages was 0.15 and 0.22mg kg⁻¹ and of emamectin B1b was 0.02 and 0.03mg kg⁻¹ which reached below quantitation limit after third day of spraying for both the metabolites and dosages (Table 2). The initial deposit of EB in cowpea pods, which was 0.073 and 0.153mg kg⁻¹ on the fifth and seventh day, dissipated below the quantitation level at 11.0 and 22.0g a.i.ha⁻¹, respectively (Vijayasree *et al.*, 2014). Half-life of 1.97 and 1.91 days and waiting period of 1.96 and 2.54 days, were recorded respectively, for single and double dosages where the FSSAI-MRL for EB in okra is 0.05mg kg⁻¹. EB degraded fast in rice, remaining residue become less than 0.1mg kg⁻¹ in 7 days and its half-life was 0.8–2.8 days (Deng *et al.*, 2020). From these result it is concluded that EB is dissipating or metabolising very fast in plants and can be considered as a safe insecticide.

Profiling of metabolites in okra fruit and leaf after EB treatment (Targeted compounds): The EB and its metabolites considered as target compounds are listed in Table S-1. Emamectin B1a and emamectin B1b accumulated predominantly in one hour after third spray both in leaves and fruits of okra, however was negligible from third day on okra fruit. The metabolite 4'-deoxy-4'-epi-N-

methylaminoavermectin-b1-benzoate (MFB1a, C₅₀H₇₅NO₁₄, RT 20.869 min, m/z 913.51) was detected only in fruits as sodium adduct on second and third day and was lacking in leaves of okra. This metabolite formed by substitution of methyl and aldehyde groups in amino group of emamectin B1b. According to Hatamleh *et al.* (2022) pesticides negatively impact the biochemistry, physiological apparatus, and cellular metabolism of plants. A very close position of the EB metabolites in the PCA loadings plot (Figs. 1A, D) and low value of log₂ fold change after 3 days (Fig. 4) proved the positive correlation and complete degradation of pesticide after the 3rd day. The results were highly agreeable to the dissipation study of EB.

Metabolic profiling of non-targeted metabolites after EB treatment: Through the analysis of changes in metabolites of okra, metabolic profiling was utilized to observe the distinct behaviours of above-ground plant tissues under insecticide-induced stress as well as in unsprayed plant samples at various intervals. A total of 81 metabolites, including amino acids, nucleosides, and fatty acids were observed in addition to flavonoids. Flavonoids were highlighted in this manuscript because their presence is indicative of stress-associated metabolites produced by plants as part of their recovery and defence responses. Lin *et al.* (2025) reported the impact of pesticides on secondary metabolism of plants, especially azadirachtin increased the content of flavonoids while Tolclofos-methyl, flutolanil, carboxin, and thiram caused a significant decrease. When the non-targeted metabolites on okra fruit and leaf

Table 1 Method validation (Recovery and precision in estimation of emamectin benzoate in okra fruit)

Spiked level	0.01 mgkg ⁻¹	0.02 mgkg ⁻¹	0.05 mgkg ⁻¹	0.1 mgkg ⁻¹
Average recovery % + RSD	95.5 + 16.8	105.1 + 11.6	102.3 + 7.7	101.2 + 12.7
Matrix effect%	-14	-12	-11	-9.5
Linearity, R ²				0.995
*LOD				0.005 mgkg ⁻¹
Deviation in retention time				0.014 minutes
**LOQ				0.01 mgkg ⁻¹
Deviation in ion ratio				0.24%

*LOD – Limit of detection **LOQ – Limit of quantitation

Table 2 Persistence of residues of emamectin benzoate in/on okra and its half life

Day	Total mean residue + SD (mg kg ⁻¹)	
	Single dose	Double dose
Day 0	0.171 + 0.035	0.25 + 0.040
Day 1	0.077 + 0.039	0.142 + 0.026
Day 3	0.05 + 0.023	0.065 + 0.020
Day 5	<LOQ	<LOQ
MRL (mg kg ⁻¹)	0.05	0.05
Half-life (Days)	1.97	1.91
Waiting period (Days)	1.96	2.54
Regression equation	y = -0.307 X+1.303	y = -0.315 X+1.502

*Single dose (11.0g a.i. ha⁻¹); Double dose (22.0g a.i. ha⁻¹)

were subjected to PCA, PC1 and PC2 together explained more than 61.1 per cent (PC1–46.5 and PC2–14.6%) and 58.4 per cent (PC1–30.2 and PC2–28.2%) of the total variance, respectively. In PCA of fruit (Fig. 1A), the signals of day 0 to day 5 shows a similar variance for PC1 but significant variance in other direction. Signals of day 15 to 30 were similar but it significantly different from day 0 to day 5. This shows the significant effect of EB in the metabolism of okra. In the case of okra leaf (Fig. 1C) signal variation observed after 10 days along PC1. PCA loadings plots showed that the flavonoid compounds are susceptible to EB treatment in okra. The flavonoid content in the initial days after spray is positively correlated with EB, (Fig. 1B) but after tenth day okra showed similar content of flavonoids as control (Fig.3). Zeng *et al.* (2021) reviewed similar results of flavonoid content in different vegetables and plants as a result of intoxication of different type of insecticides. Amiri *et al.* (2022) also reported increase in flavonoid content after pesticide treatment by chemical analysis in potato plants. EB can activate the phenolic biosynthetic pathways in okra and which help the plant to reduce the stress conditions arise due to EB and get more protection from insects. Sharma *et al.* (2019) reported that plants produce increased levels of polyphenols to reduce the abiotic stress by radical scavenging effect. Qiao *et al.* (2024) reported the storage of okra at 4°C activates

flavonoid synthesis pathway. While analysing PCA of leaves (Fig. 1C), signals up to 10 days grouped together, this showed EB persisted in leaves for more days compared to fruits and there after it varies along PC2. Pan *et al.* (2022) reported 61.8 per cent of variance was explained by PCA analysis in his study on metabolism changes by pymetrozine and tebuconazole on cucumber.

In the HCA of non-targeted compounds from okra fruit and leaf, we checked 28 compounds each having more than 80 per cent match in m/z cloud results with minimum two positive annotations (Fig. 2A). While clustering along Y axis, compounds with similar metabolism patterns were clustered together forming dendrogram. The red colour indicates higher concentration and the green colour lower content. The HCA of okra fruit have 6 branches, emamectin branch include scopoletin and epigallocatechin as sub branch. This shows the similarity in concentration of flavonoid with EB. L-norleucine, L-phenylalanine, Anthranilic acid, 9-oxo-ODE, trans-3-indoleacrylic acid, indole-3-acetic acid were clustered together and showing similar pattern in concentration changes. The first two are amino acids and other compounds related to plant growth. On third day after spraying they are increased and their concentration is similar to control thereafter (Fig. 2A). 8-hydroxyquinoline, morin, hyperoside, quercetin, quercetin-3-O-rhamnoside-7-O-Glucoside were clustered and showing higher concentration on third day after EB treatment. Most of the polyphenols (catechins, quercetins etc.) showed similar pattern of higher concentrations from the day of spraying and observed a reduction in concentration after 10th day (Figs. 2, 3). Amino acids and its derivatives clustered together and showing similarity in pattern with choline in leaf and grouped in the same child branch of the dendrogram. Choline serves as a synthetic precursor for other essential cell components and signalling molecules so it was clear that amino acid synthesis was similarly regulated and overcome the stress from EB. According to Pan *et al.* adding fertilizer and insecticides together to cucumbers increased their chlorophyll content and antioxidant content (Pan *et al.*, 2022).

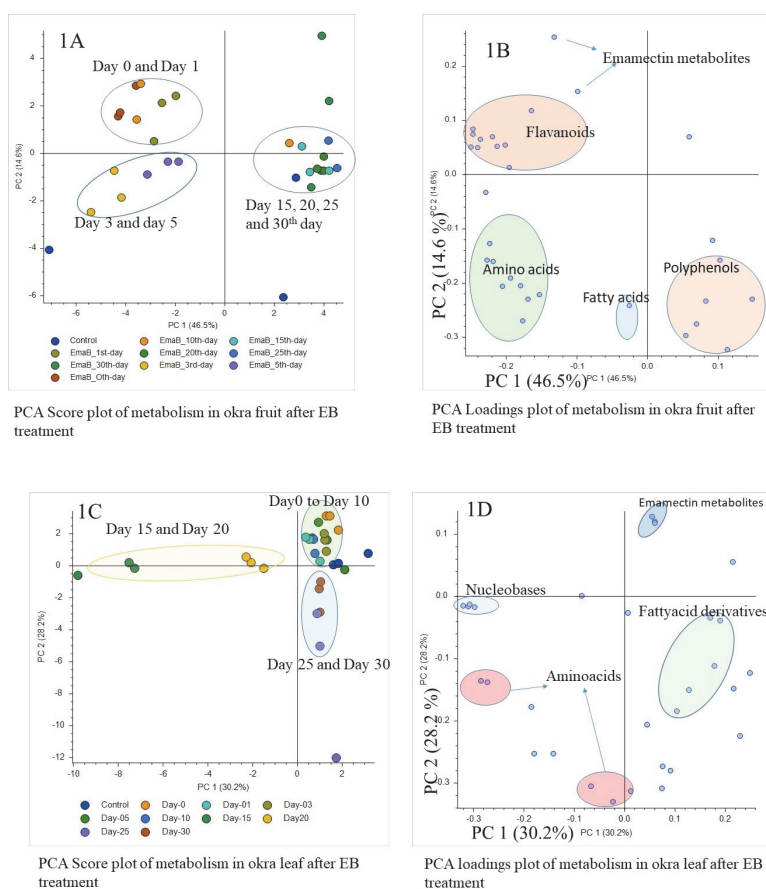


Fig. 1 PCA score plots and PCA loadings plot of metabolism in okra fruit and leaf after emamectin treatment. 1A and 1C are PCA score plots of fruit and leaves respectively. The dots with same colour represent replicates. The data variation through PC1 and PC2 were grouped. 1B and 1D are PCA loadings plots of fruit and leaves respectively. This represents the correlation between the compounds.

Compounds nearer are positively correlated and in opposite quadrants are negatively correlated. Flavonoids are positively correlated to EB while amino acids are negatively correlated to EB. The control samples were collected each day and a pooled control sample prepared and used in the study.

Differential analysis: The differential analysis results of metabolites in okra fruit after EB treatment of different days vs control. Three compounds up regulated and one down regulated in 0th day vs control, both metabolites of EB upregulated ($\log_2$ fold x $-\log_{10}$ P is 9×13.4 and 10×10.4) along with epigallocatechin (Fig. 4A). That is on the day of EB application its concentration is very high and significant. Three compounds L-phenylalanine, L-norleucine and indole-3-acetic acid have $-\log_{10}$ P-value less than 0.05, they are less significant. 11 other compounds show a $\log_2$ fold change greater than or equal to 1, in that

quercetin, scopoletin, morin, hyperoside and quercetin-3-O-rhamnoside-7-O-glucoside have $\log_2$ fold value more than 2, that is these compounds are very significant in okra metabolism under EB stress. The only compound down regulating is (+)-ar-turmerone. On third day (Fig. 4B) fold change values of EB decreased significantly but still up regulating and number of compounds have $\log_2$ fold value greater than 1 become 13. Similar results observed on fifth day but on 10th day EB have no significance in okra metabolism or it dissipated completely and epigallocatechin shows a higher $\log_2$ fold value of 7.8.

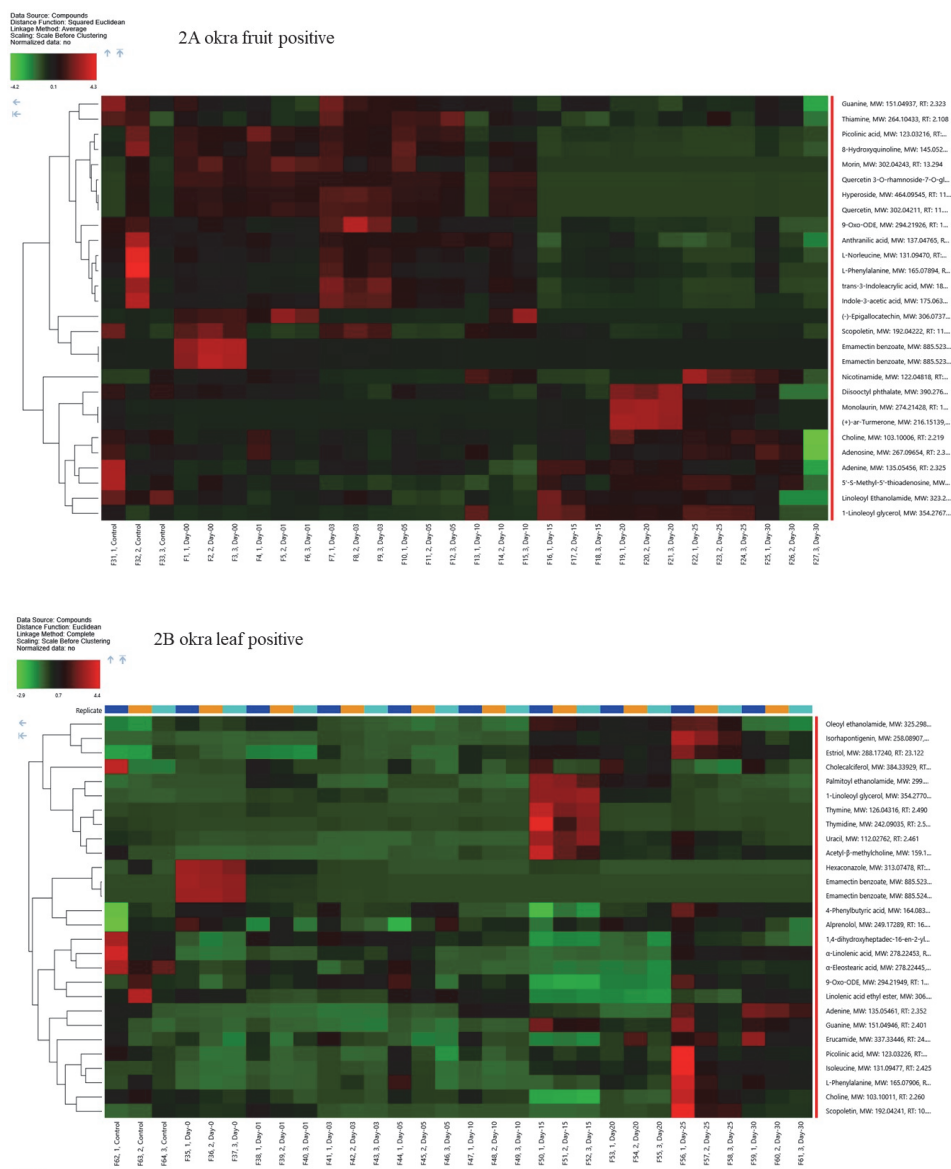


Fig. 2 heat map of metabolites in okra fruit and leaf clustered related to its area concentration in different days after emamectin benzoate treatment. Green colour represents less concentration and red colour high concentration of metabolite. In X axis labelled with day after treatment. In Y axis compounds represented in rows, and they clustered based on their similarity in concentration when days go on.

In metabolite identification, we followed the requirements for putative identification by accurate mass measurement, MS/MS spectrum comparison with database for fragment matching and retention time comparison (Fig. 3) (Zhang *et al.*, 2018; Jan *et al.*, 2021). Indole-3-acetic acid (IAA) (RT 12.34 min, $[M+H]^+$ m/z 176.0705) identified in okra fruit with other adducts $[M+Na]^+$ m/z 198.0525, $[M+H-H_2O]^+$ m/z 158.0810, and MS² fragments at m/z 130.0651 and m/z 176.0706. IAA has a complicated

stress managing mechanism and has significant role in plant growth and abiotic stress relief (Zhang *et al.*, 2022). (-)-Epigallocatechin (RT 8.54 min, $[M+H]^+$ m/z 307.0817) an important flavonoid identified in okra fruit, other adducts are $[M+Na]^+$ m/z 329.0631, $[M+K]^+$ m/z 345.0370, $[2M+H]^+$ m/z 613.1566 and MS² fragments identified are m/z 169.0496, 163.039 and 139.0391 (Fig. 5). Catechins synthesis is enhanced due to the abiotic stress factors like heat, draught, heavy metal

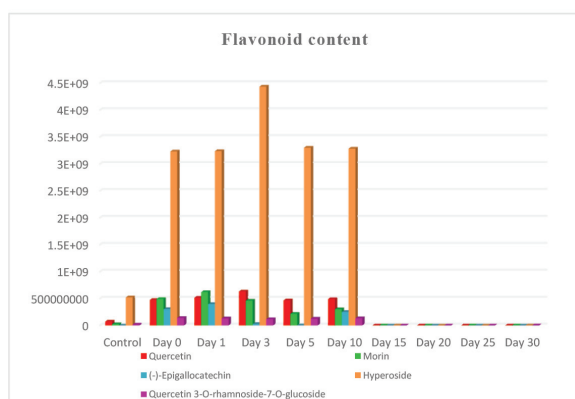


Fig. 3 Flavonoid content (Relative) in okra fruit after emamectin benzoate treatment. X- axis days after treatment of EB, Y- axis area under each peak. All the flavonoids present in okra fruit showing same trend of increasing concentration up to 10th day. There after it reduces and become similar to control.

contamination or insect attack in plants especially in tea (Ahamed *et al.*, 2023). Methyl á-eleostearate (RT 15.25 min, $[M+H]^+$ m/z 293.2473), a conjugated polyunsaturated fatty acid in higher amount on thirtieth day (73% relative variation with control) due to the response against insecticide stress. There was not much variation in its quantity on other days when compared with the control. Citral, an acyclic monoterpene aldehyde (RT 29.96 min, $[M+H]^+$ m/z 153.1273) with good stress amelioration efficacy was detected with slightly higher quantity on fifteenth and thirtieth day (26 to 28%). Melatonin and its precursor tryptophan have a significant role in enhancing plant stress tolerance to various environmental conditions, such as water scarcity (Sadak and Ramadan 2021). Quoc *et al.* (2024) reported that D-glucuronic acid, D-

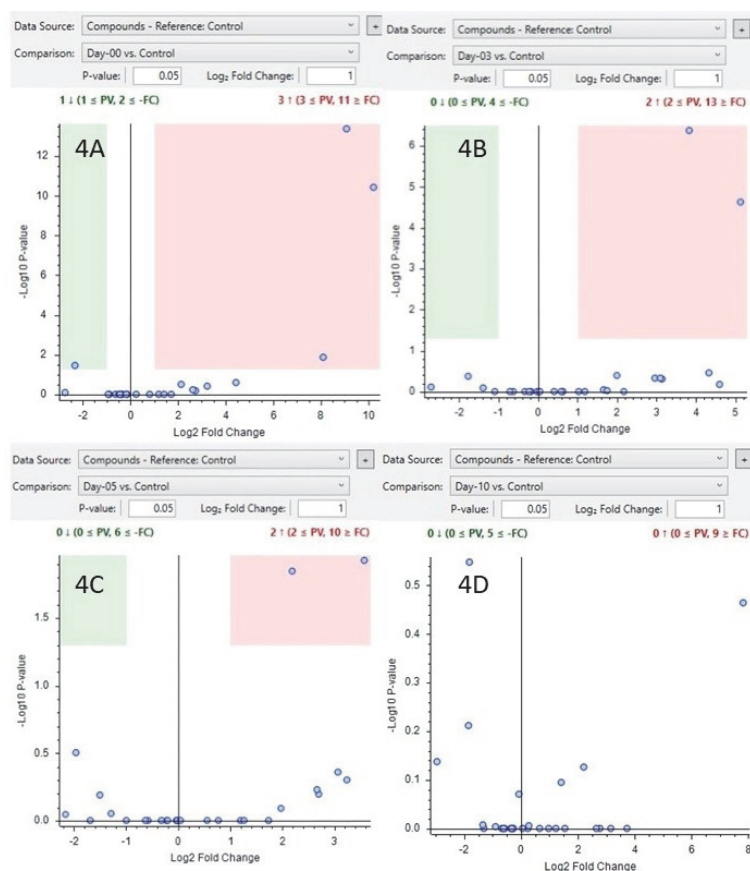


Fig. 4 Differential Analysis result of okra fruit after EB application, different day vs control. X axis of each graph is the log₂ fold change in concentration and Y axis -log₁₀ P-value. Compounds in the red box are upregulated and in green box are down regulated.

FC is the number of significant compounds have log₂ fold value greater than 1. PV is the number of significant compounds but -log₁₀ P value less than or equal to 0.05. Blue dot represents each compound

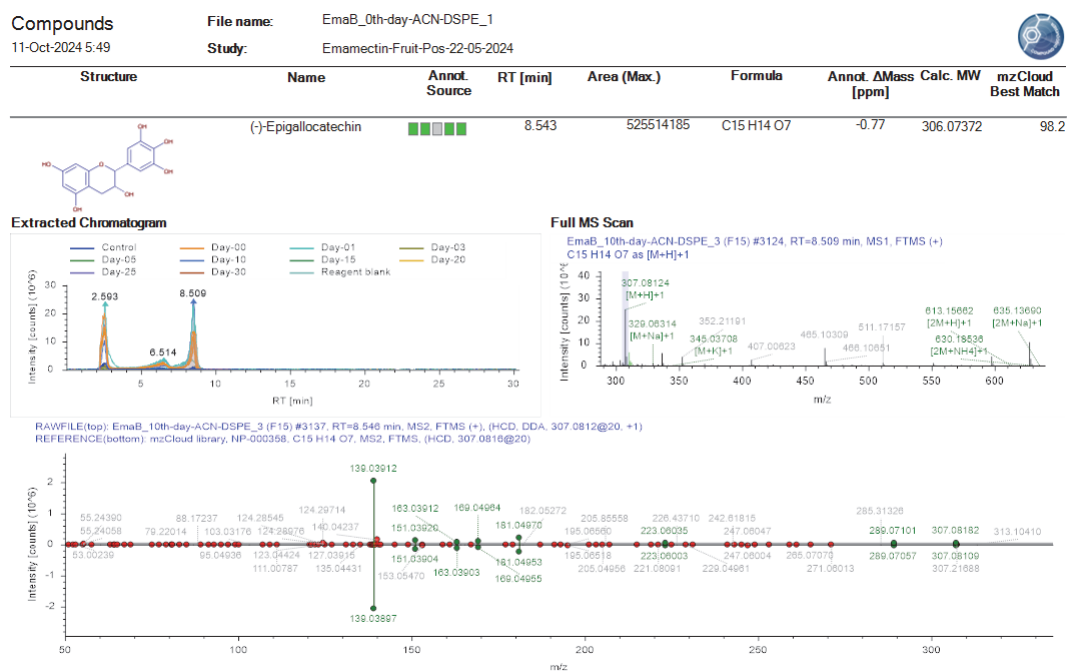


Fig. 5 The identification and confirmation of flavonoid (Epigallocatechin) from high resolution mass data ($M+H$)⁺, similarity of MS/MS fragmentation pattern with mzCloud database (98.2%) and retention time is 8.5 min

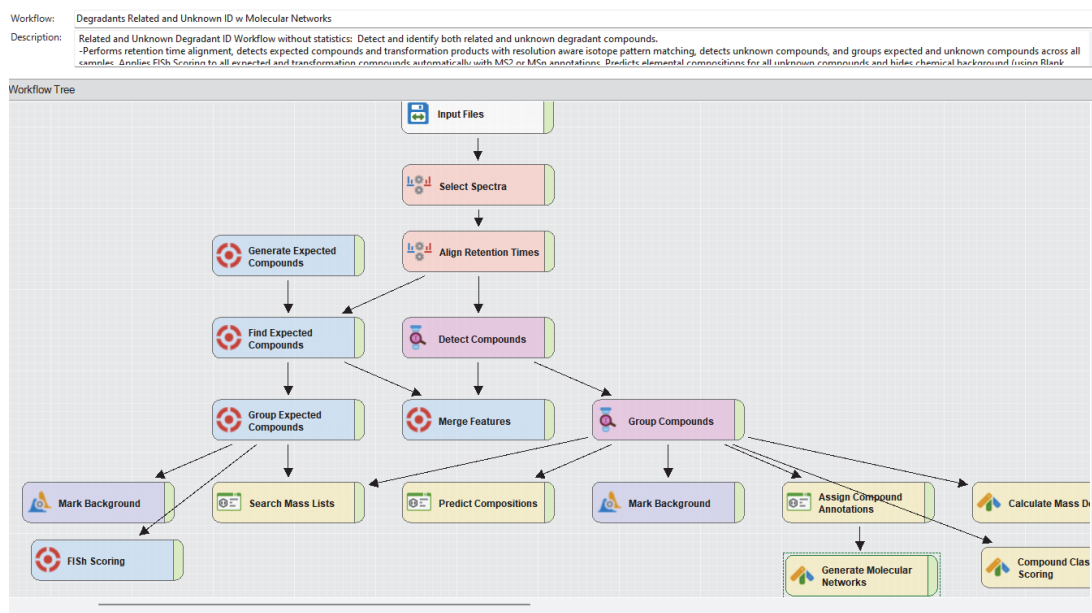


Fig. S-1 Workflow diagram given in Compound Discoverer 3.3 for target and non-target metabolomics

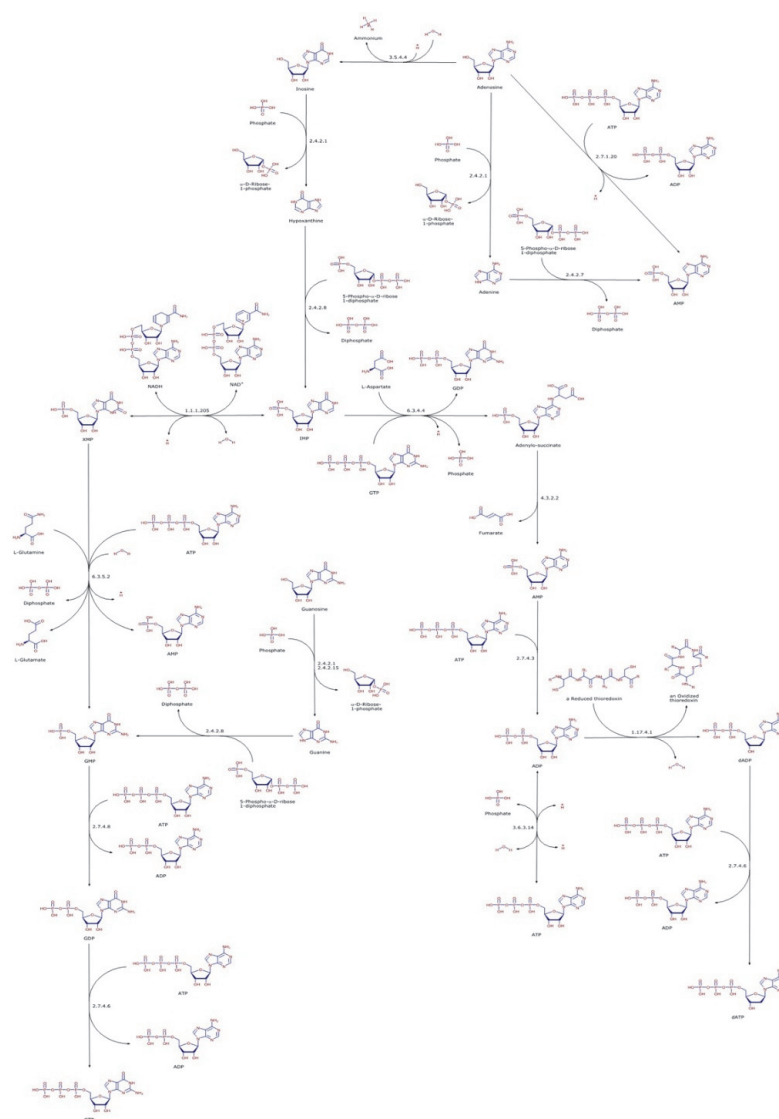


Fig. S-2 Super pathway of purine nucleotide salvage

Under insecticide-induced stress elevated levels of amino acids show a fundamental role in supporting plant metabolism. Accumulation of α -amino acid, D-Lactose monohydrate (RT 2.24 min, $[M+H]^+ m/z$ 360.1499) in EB sprayed fruits of okra was found instantly after spray and on the tenth day (30 to 34 %). L-Tyrosine (RT 2.4 min, $[M+H]^+ m/z$ 182.0811) content was found lower after fifth day of spraying when compared to the control. Higher levels of other

Due to insecticide toxicity, plants defence mechanism gets activated, which involves production of more signal molecules in diverse physiological processes. L-Proline (RT 2.47 min,

Table S-1. The list of metabolites analysed for profiling of metabolic changes of emamectin benzoate after application on okra fruit and leaves

No	Metabolite	Molecular formula	Molecular weight & monoisotopic mass (g mol ⁻¹)	Identified monoisotopic mass (g mol ⁻¹)	Structural change from B1b
1	Emamectin B1a	C ₄₉ H ₇₅ NO ₁₃	886.12 & 885.5238	885.5230	Methyl group on C-26
2	Emamectin B1b	C ₄₈ H ₇₃ NO ₁₃	872.09 & 871.5081	871.5078	
3	AB1a	C ₄₉ H ₇₅ NO ₁₃	886.12 & 885.5238	Not found	Methyl addition at amino group of EB1b
4	MFB1a	C ₅₀ H ₇₅ NO ₁₄	914.13 & 913.5187	913.5181	Methyl and aldehyde addition at amino group of EB1b
5	FAB1a	C ₄₉ H ₇₅ NO ₁₄	900.10 & 899.5031	Not found	Aldehyde addition at amino group of EB1b

Table S-2. Metabolic pathways identified in metabolomics study of okra fruit during EB metabolism

No.	Metabolic pathway identified	No. of compounds	Compounds matched	Identified compounds
1	Superpathway of flavones and derivatives biosynthesis	147	22	Rutin, Quercetin, Quercetin-7-O-glucoside, Quercetin-3-glucoside, Kaempferol 3,7-o-diglucoside
2	Superpathway of aromatic compound degradation via 2-oxopent-4-enoate	102	7	Anthranilate, Trans-o-hydroxybenzylidene pyruvate
3	Anaerobic aromatic compound degradation (Thauera aromatica)	74	7	Anthranilate, L-phenylalanine
4	Superpathway of purine nucleotide salvage	54	5	Adenosine, Adenine, Guanine
5	Superpathway of indole-3-acetate conjugate biosynthesis	48	6	L-Leucine, Phenylalanine, Indole-3-acetate
6	Superpathway of lipoxygenase	33	18	9,10-epoxy-10, 12Z octadecadienoate, Colneleate

[M+H]⁺ m/z 116.0709, identified in chemspider database) is such a compound which is greatly associated with stressful environment as defence molecule against biotic as well as abiotic stresses. On comparing with control, proline content was highly noticed in the emamectin sprayed fruits immediately and on the next day of spraying. Due to heavy metal-induced toxicity >20-fold increase in the free proline content was found in the leaves of metal non-tolerant *Silene vulgaris* (Sharma and Dietz 2006). Accumulation of other proline related compounds i.e., (4R)-4-(1-Aminoethyl)-L-proline (RT 2.95 min, [M+H]⁺ m/z 159.1126), prolintane (RT 13.943 min, [M+H]⁺ m/z 218.1901), (4R)-4-Fluoro-L-proline (RT 2.032 min, [M+H]⁺ m/z

305.0713) were also seen under stress conditions by emamectin spray.

The ability of amino acids, phenolics and flavonoids to scavenge free radicals makes them useful antioxidants that help plants to withstand a variety of abiotic challenges, such as metal stress (Drzewiecka *et al.*, 2018). Hyperoside (RT 11.85 min, [M+H]⁺ m/z 465.1027) is an unique flavonoid in okra whose content was higher up to the tenth day (10 to 27%) and was untraceable after it. Quercetin (RT 11.81 min, [M+H]⁺ m/z 303.0494) is another bioactive natural compound with antioxidative property which will be modifying plant physiology (Singh *et al.*, 2024). Plants exposed to high levels of pesticides showed a notable increase

in antioxidant levels in order to withstand the oxidative stress caused by the pesticides (Shakir *et al.*, 2018). It has been found that quercetin alleviates the toxicity of EB. In the present study, a significant amount of Quercetin 3-O-rhamnoside-7-O-glucoside (RT 11.87 min, $[M+H]^+$ (m/z 611.1604), was quantified up to the tenth day (15 to 22%) and undetectable thereafter in okra.

In this study it was verified the metabolites of EB formed during its dissipation in okra and variation in metabolomics due to EB stress. The only metabolite of EB, found in fruit is 4''-deoxy-4''-epi-N-methylaminoavermectin-b1-benzoate other than the sprayed components, EB1a and EB1b. 81 metabolites identified in total from okra fruit and leaf out of those 35 metabolites observed in both fruit and leaf of okra. Flavonoid content was increased as a result of stress from EB and scopoletin and epigallocatechin can be used as marker to EB stress. Fatty acids are comparatively less susceptible to EB stress. For the assessment of EB induced metabolite variations through metabolomics-based strategy, which included untargeted chemical profiling, and multivariate statistical analysis, the current study methodically reconnoitred the changes in metabolic pathways (list of metabolic pathways identified including the list of compounds putatively identified in each pathway is given in Table S2 and Super pathway of purine nucleotide salvage represented in Fig. S2) and distribution patterns of EB residues and metabolites in okra. Correlation of compounds in the pathways is analysed using R programme, epigallocatechin, quercetin, quercetin-3-O-Rhamnoside-7-o-glucoside have positive correlation with EB while Choline monolaurin are negatively correlated with EB stress. More information and studies may be required to find common markers for other insecticides and on different varieties of okra or other vegetables.

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