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RESEARCH ARTICLE

Assessing the allelopathic effect of *Chrysopogon zizanioides* (L.) Roberty root methanolic extract on *Brassica rapa* subsp. *chinensis* var. *parachinensis* using an untargeted metabolomic approach

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ABSTRACT

Chrysopogon zizanioides (L.) Roberty, or vetiver grass, is a deep-rooted perennial grass. An examination of allelopathy using a metabolomic approach offers valuable insights into vetiver extract's mechanism of action and phytotoxicity. This study utilised an untargeted metabolomics approach through the use of choisum (*Brassica rapa* L. subsp. *chinensis* (L.) Hanelt var. *parachinensis* (L.H. Bailey) Hanelt) as target plant because it is easier to cultivate and susceptible to the extract. Vetiver root methanolic extracts at various concentrations (0, 0.1, 1, 10, 50, and 100 mg mL^{-1}) were sprayed at 100 mL m^{-2} on choisum seedlings at the 2 to 3 leaves stage. After 21 d, the Soil Plant Analysis Development (SPAD) and chlorophyll content of exposed choisum were measured, and their metabolites were subjected to gas chromatography-mass spectrometer (GC-MS)-based metabolomics analysis. The result demonstrated that the 100 mg mL^{-1} methanolic extract significantly decreased SPAD reading by 57.29% and reduced chlorophyll content by 66.38% (chl *a*) and 73.49% (chl *b*) of choisum compared to the control. Furthermore, considerably reduced stomatal length of exposed choisum, up to 34.31%, was observed when exposed to maximum concentration (100 mg mL^{-1}). In total, nine metabolites with variable importance in projection (VIP) > 1 and $P < 0.05$ were found and identified as amino acids and carbohydrates. The highest concentration of extract enriched pathways of propanoate, Se-compound, cysteine, and methionine metabolism in choisum, suggesting the extract induced plant stress. The findings confirm the allelopathic potential of vetiver root and provide insight into the response of choisum to the allelopathic activity of vetiver grass root methanolic extract.

Key words: Allelopathy, *Brassica rapa*, *Chrysopogon zizanioides*, metabolomics, vetiver grass.

INTRODUCTION

Plants can synthesise diverse metabolites, which serve various purposes, such as promoting growth or providing defensive mechanisms. In plants, metabolites consist of primary and secondary metabolites, produced and modified by the metabolism of biological systems such as cells, tissues, or organisms (Oh et al., 2023), which change metabolic levels due to biotic and abiotic stress (Sharma et al., 2018). Examining the tolerance of plants to abiotic and biotic stress is essential to explore unknown regulatory networks that control plant growth and development in any critical biochemical process through the detection of metabolites (Razzaq et al., 2019). In contrast to microorganisms or animals, plants have been documented to possess over 200 000 metabolites, which play essential

roles in plant growth, development, and response to environments (Weng et al., 2021). Among these metabolites, allelochemicals are secondary compounds produced by plants and have detrimental effects on neighbouring flora and fauna. These allelochemicals can hinder germination, root or seedling growth, photosynthesis, enzyme activity, cell division, protein synthesis, and respiration, among other things (Lopes et al., 2022). In particular, medicinal plants are deemed a crucial reservoir of secondary metabolites and have been documented to exhibit robust allelopathic capabilities (Islam et al., 2018) and maybe a feasible alternative in bioherbicide development for sustainable weed management (Hasan et al., 2021). Herbicide overuse causes chemical expenses, groundwater leaching, and irrigation water recycling concerns (Poudyal and Cregg, 2019). New herbicides and biopesticides from natural sources may reveal new mechanisms of action and lead to new weed management strategies (Westwood et al., 2018).

Chrysopogon zizanioides (L.) Roberty, widely known as vetiver grass, is a perennial grass belonging to the Poaceae family. It has been widely introduced and is currently cultivated and naturalised in Asia, Africa, South America, Australia, and the Pacific (De Guzman and Oyen, 1999). Vetiver is a grass with a dense root system and aromatic properties that can grow up to 30 cm deep (Pareek and Kumar, 2013). The essential oil, sourced from the roots of vetiver, is extensively utilised in the perfumery, medicinal and cosmetic industries (Grover et al., 2021). The dense rooting system of vetiver grass makes it ideal for soil conservation and slope stabilisation. Concomitantly, the allelopathic potential of vetiver grass has been previously described. A study by Costa et al. (2020) indicated that vetiver grass exhibits allelopathic properties towards *Lactuca sativa*. Furthermore, an investigation conducted by Dlamini et al. (2022) observed a significant prevalence of uncovered terrain adjacent to the vetiver plant, demonstrating its inherent allelopathic potential. Meanwhile, the phytochemicals screening of root methanolic extract of vetiver grass by Muthukrishnan and Manogaran (2018) revealed positive results for saponins, flavonoids, steroids, glycosides and terpenoids.

Metabolomics is an emerging omics technology that employs analytical tools to analyse metabolites in biological samples (Pérez-Alonso et al., 2018; Sharanya et al., 2020). Gas chromatography-mass spectrometer (GC-MS)-based metabolomics is ideal for identifying and quantitating small molecular metabolites (< 650 Da). Metabolomics has been applied to allelopathy research to identify, quantify, and profile allelochemicals and determine their effects on plant interactions (Scavo and Mauromicale, 2021). Metabolomic analysis of allelopathy may yield crucial data for developing allelochemical-based herbicides and potentially offer novel alternatives to synthetic herbicides (Yu et al., 2023). There is a growing focus on the effect of allelopathy via metabolomics analysis. For example, Azizan et al. (2022) reported significant metabolite changes in the lettuce shoot when treated with *Wedelia trilobata* extracts. The study suggested changes in specific metabolic pathways as a response to enhance its tolerance against the treatment. Another study by Zhu et al. (2021) revealed that *Arabidopsis thaliana*, when exposed to *Eupatorium adenophorum* extract, indicated that the citrate cycle was suppressed, the metabolism of amino acids was hindered, and phosphate absorption was inhibited by the treatments applied.

In the present investigation, vetiver root methanolic extract was used to investigate the allelopathic mechanism. According to Azizan et al. (2015), the methanol extract exhibits higher activity and is recognised for its effective extraction capabilities across a range of samples. Furthermore, a study conducted by Chigayo et al. (2016) has revealed that methanol demonstrates the highest yield and concentration of phenolic and flavonoid compounds compared to alternative solvents. *Brassica rapa* L. subsp. *chinensis* (L.) Hanelt var. *parachinensis* (L.H. Bailey) Hanelt, locally known as caixin or choisum, is a widely cultivated vegetable in Asia (Li et al., 2023). Choisum was selected as the target plant based on its homogenous growth pattern, an abbreviated time period to reach maturity (30 d), and superior germination rate. Furthermore, the utilisation of *Brassica* spp. in allelopathy research has been substantial due to their susceptibility to allelopathic extracts, as demonstrated by Ali et al. (2017) and Yao et al. (2018).

The primary objectives of this study are to assess the allelopathic activity of *C. zizanioides* extract on *Brassica rapa* subsp. *chinensis* var. *parachinensis* (choisum) using a metabolomic method and to identify its impact on plant metabolism. Stomata and chlorophyll play vital roles in the process of photosynthesis. Consequently, any disruption to these components will inevitably have an impact on plant metabolism and growth. Thus, this study also aims to explore the allelopathic effect of the extract towards chlorophyll content and stomata of target plants.

MATERIALS AND METHODS

Plant material and methanolic extract preparation

Vetiver (*Chrysopogon zizanioides* (L.) Roberty) slips were obtained from the Department of Agriculture Malaysia and cultivated at the UniSZA herbal garden (05º45'10.0" N, 102º37'41.9" E). After 6 months, vetiver roots were harvested, washed and dried in an oven at 60 °C for 24 h. The dried vetiver roots were ground using a basic microfine grinder (MF 10, IKA, Staufen, Germany). The extraction of vetiver root was based on a modified approach as described by Aslani et al. (2014) and Ismail et al. (2016). In a beaker, 100 g powdered root were soaked in 1 L 80% methanol for 72 h at 4 °C. The extract was then filtered and evaporated to dryness in a rotary evaporator at 40 °C. Subsequently, 10 g crude extract was diluted in 100 mL distilled water to produce a stock solution (100 mg mL^{-1}). The stock solution was diluted with distilled water to obtain extract concentrations of 0.1, 1, 10, and 50 mg mL^{-1}. The choisum seeds for pot bioassay were bought from Leckat Corporation Sdn. Bhd., Kuala Lumpur, Malaysia.

Metabolomic experiment and metabolite extraction

Three choisum seeds were sown in a pot (15 cm height × 10 cm width) containing 800 g sandy loam soil (pH in H_2O 7.7, organic C: 2.47%, sand: 67.90%, clay: 17.30%, silt: 14.80%). The methanolic extract with different concentrations (0, 0.1, 1, 10, 50 and 100 mg mL^{-1}) was sprayed with a garden sprayer at 100 mL m^{-2} rate when choisum produced 2-3 true leaves. Two weeks after emergence, half-strength of Hoagland solution (20 mL) was applied to the pots. The experiment was carried out in randomised complete block design (RCBD) with five replicates under the greenhouse (temperature: 30.3 ± 3.3 °C, relative humidity: 66.82 ± 13.0%). Choisum sprayed with distilled water was used as a control (0 mg mL^{-1}). Metabolite extraction was performed based on Azizan et al. (2015). Briefly, the exposed choisum plants were harvested on the 21st day for metabolite extraction. The samples were ground in liquid nitrogen, and approximately 20 mg were weighed in an Eppendorf tube and extracted with methanol/water (80/20 v/v) and chloroform/water (50/50 v/v). Extracts were then sonicated for 15 min and centrifuged at 12000 rpm for 20 min. The derivatisation was done by mixing 50 µl BSTFA in pyridine. The samples were then incubated in a ThermoMixer C (Eppendorf, Hamburg, Germany) at 60 °C for 1 h before being injected into GC-MS. In all samples 20 µL ribitol were spiked as an internal standard.

GC-MS parameter

The samples were analysed in a randomised order. The GC-MS analysis of the samples was conducted using a GC/MS instrument (Agilent 7890B GC System, Santa Clara, California, USA). The analysis was performed on an Agilent HP-5MS column (30 m, 250 µm, 0.25 µm) with specific operating conditions as follows: Initial oven temperature was 80 °C held for 2 min, increased to 180 °C at the rate of 10 °C min^{-1} held for 7 min and the final oven temperature was 280 °C at the rate of 30 °C min^{-1}, held for 8 min. The helium flow rate was 1.0 mL min^{-1}. The splitless injection was used with the ionisation energy of 70 eV. The injector port and detector temperature were 250 and 280 °C, respectively.

Determination of chlorophyll contents

The SPAD value of choisum leaves was measured on the 21st day of the experiment by using a portable SPAD meter (502 plus, Konica Minolta, Tokyo, Japan). The chlorophyll content (chl *a* and *b*) was determined based on the method outlined by Lichtenthaler and Wellburn (1983). The 100 mg samples were extracted in 5 mL 80% acetone and the absorbance was read at 663 and 645 nm. The pigments were calculated based on the following equation:

$$\text{Chlorophyll } a \text{ (mg mL}^{-1}\text{)} = 12.7 \text{ (A663)} - 2.69 \text{ (A645)}$$
$$\text{Chlorophyll } b \text{ (mg mL}^{-1}\text{)} = 22.9 \text{ (A645)} - 4.68 \text{ (A663)}$$

Stomata length measurement

The abaxial of choisum leaves was observed under the scanning electron microscope (SEM) (JSm-636OLA, JEOL, Tokyo, Japan) to observe the effect of the vetiver root methanolic extract on the stomatal length of choisum. The sample fixation for SEM was based on Gowtham et al. (2016).

Data processing and statistical analysis

Data on chlorophyll contents and stomatal length underwent the Shapiro-Wilk test and ANOVA using Minitab 20.3 software (Minitab, State College, Pennsylvania, USA) and Tukey's test at a significance level of 0.05 to compare the means. The GC-MS data were processed using Agilent MassHunter Software, and compound identification was carried out by comparing the mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library (2014). The data matrix containing metabolite name and their corresponding peak area was then uploaded to MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) for integral normalisation, ANOVA with a significance level of 0.05 and pathway enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG). SIMCA-P + version 14.1 (Umetrics AB, Umea, Sweden) was used to perform multivariate analysis on the normalised and validated data matrix.

RESULTS AND DISCUSSIONS

Effect of vetiver root methanolic extract on chlorophyll content and stomata length

The findings illustrated in Figure 1A indicate that the treatments significantly impacted the SPAD reading of choisum plants. Specifically, the SPAD reading of choisum plants exposed to 100 mg mL^{-1} vetiver root methanolic extract exhibited a noteworthy reduction of 57.29% compared to the control group. The chlorophyll content (chl *a* and *b*) of choisum was significantly reduced when exposed to 100 mg mL^{-1} (Figure 1B). The choisum experienced a decrease compared to the control in chlorophyll levels, specifically 66.38% for chl *a* and 73.49% for chl *b*, upon exposure to 100 mg mL^{-1}. The reduction of chlorophyll eventually reduces the photosynthesis activity. The decrease in chlorophyll levels can be linked to reduced mineral absorption and disruptions in the regulatory mechanisms responsible for chlorophyll synthesis (Siyar et al., 2019). The reduction of chlorophyll content in target plants due to the allelopathy effect was similar to those reported by Ma et al. (2020), Li et al. (2022) and Singh et al. (2022). In addition, the experiment revealed that the length of stomata in the choisum compared to the control decreased by up to 34.21% when subjected to 100 mg mL^{-1} (Figures 2A-2B). According to the findings, the stomatal length reduced as vetiver root methanolic extract concentration increased. The cause of this phenomenon was attributed to the influence of allelopathy on the elongation of cells, as indicated by Šoln et al. (2022).

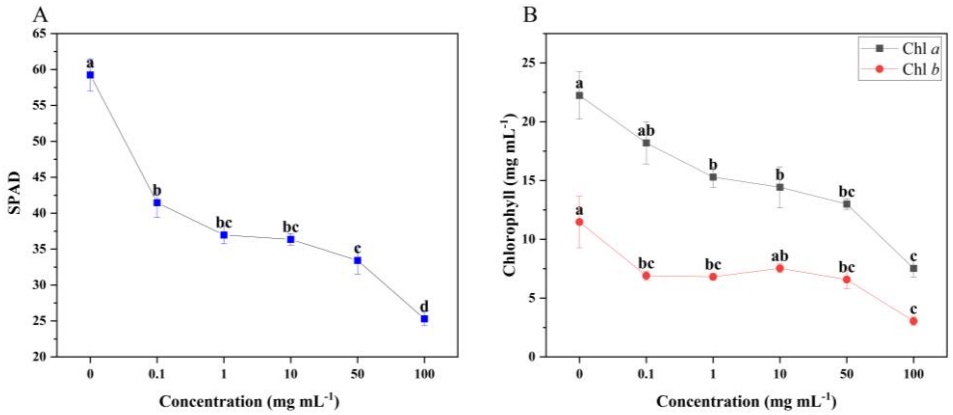

Figure 1. Effect of vetiver root methanolic extracts on the SPAD readings (A) and chlorophyll *a* and *b* (B) of choisum. Different letters on the points indicate significant differences according to the Tukey test ($P < 0.05$). Data presented as mean ± standard error from five replicates ($n = 5$). Bars indicate standard errors.

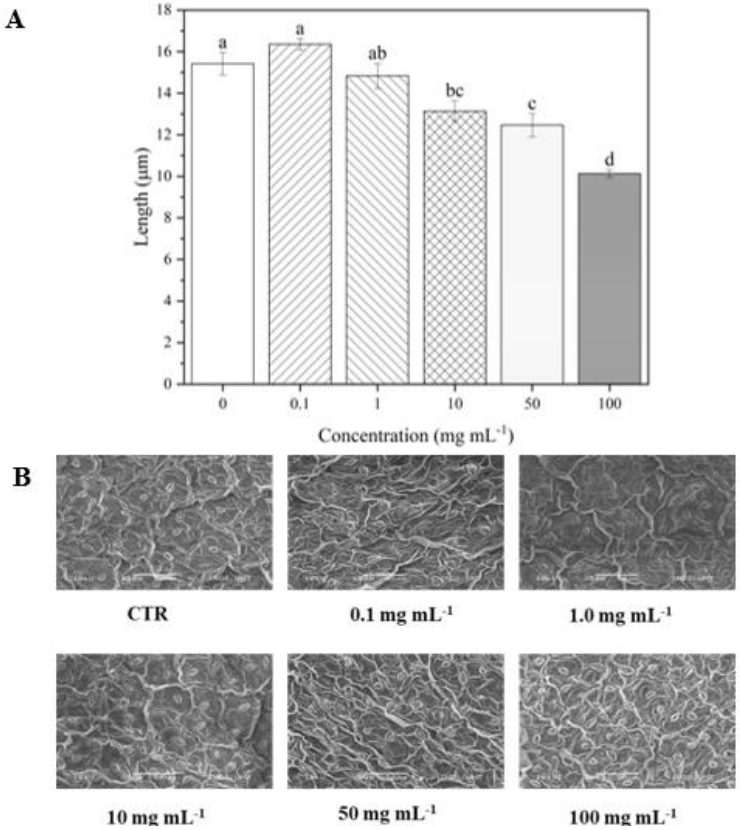

Figure 2. A) The impact of varying concentrations of the vetiver root methanolic extract on the length of choisum stomata. Different letter on the bar indicates significant differences according to the Tukey test ($P < 0.05$). Data presented as mean ± error ($n = 9$), bars indicate standard error. B) The stomata of choisum under scanning electron microscopy (SEM) at 500X magnification. CTR: Control.

Metabolites analysis in choisum

The GC-MS was used to analyse the metabolite changes and metabolic pathways in exposed choisum. Based on the one-way ANOVA, there were 26 significant metabolites identified in the target plant (Table 1). Unsupervised principal component analysis (PCA) revealed that choisum exposed to 100 mg mL^{-1} was distinctly differentiated from the control group (Figure 3A). The first two PCA gave R^2X (cum) of 0.571 and Q^2 (cum) of 0.414. The PCA was deemed to attain optimal fitting performance when the total variance values (R^2) surpassed 50% and were larger than the Q^2 value (Mamat et al., 2020). The PCA score plot clearly indicated the effect of different vetiver root methanolic extract concentrations on the metabolite composition in choisum. The contribution of metabolites to the separation between different concentrations of vetiver root methanolic extract was illustrated in the biplot (Figure 3B).

Table 1. One-way ANOVA showing choisum (*Brassica rapa* subsp. *chinensis* var. *parachinensis*) significant metabolites at $P < 0.05$ according to the Tukey test. TMS: Trimethylsilyl.

Nr	Metabolites	F-value	P-value
1	L-Rhamnose, 4TMS derivative	1369.3	0.00000
2	Butanedioic acid, 2TMS derivative	674.41	0.00000
3	L-Alanine, 2TMS derivative	642.92	0.00000
4	Serine, 3TMS derivative	634.38	0.00000
5	Heneicosane	39.081	0.00000
6	2,3,4-Trihydroxybutyric acid tetrakis(trimethylsilyl) derivative, ((R*,R*)-)	24.959	0.00000
7	L-Sorbopyranose, (1S,2R,3S)-, 5TMS derivative	21.212	0.00000
8	Citric acid, 4TMS derivative	20.504	0.00000
9	12-Hydroxyoctadecanoic acid, 2TMS derivative	12.047	0.00001
10	Glyceric acid, 3TMS derivative	9.5405	0.00004
11	Palmitic acid, TMS derivative	9.3978	0.00005
12	D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 1)	9.2019	0.00005
13	Eicosane	8.7942	0.00008
14	Myo-inositol, 6TMS derivative	8.2660	0.00012
15	Stearic acid, TMS derivative	8.1164	0.00013
16	D-Glucose, 5TMS derivative	7.7698	0.00018
17	D-Gluconic acid, 6TMS derivative	7.6305	0.00021
18	Sucrose, 8TMS derivative	7.3556	0.00026
19	Malic acid, 3TMS derivative	6.8959	0.00041
20	L-Threonine, 3TMS derivative	6.2234	0.00078
21	2-*O*-Glycerol-alpha-d-galactopyranoside, hexa-TMS	5.8870	0.00110
22	1-Monopalmitin, 2TMS derivative	5.7540	0.00126
23	D-Fructose, 5TMS derivative	5.2165	0.00222
24	Maltose, 8TMS derivative	5.1211	0.00246
25	D-Glucitol, 6TMS derivative	3.2921	0.02101
26	Dodecanoic acid, TMS derivative	3.2749	0.02147

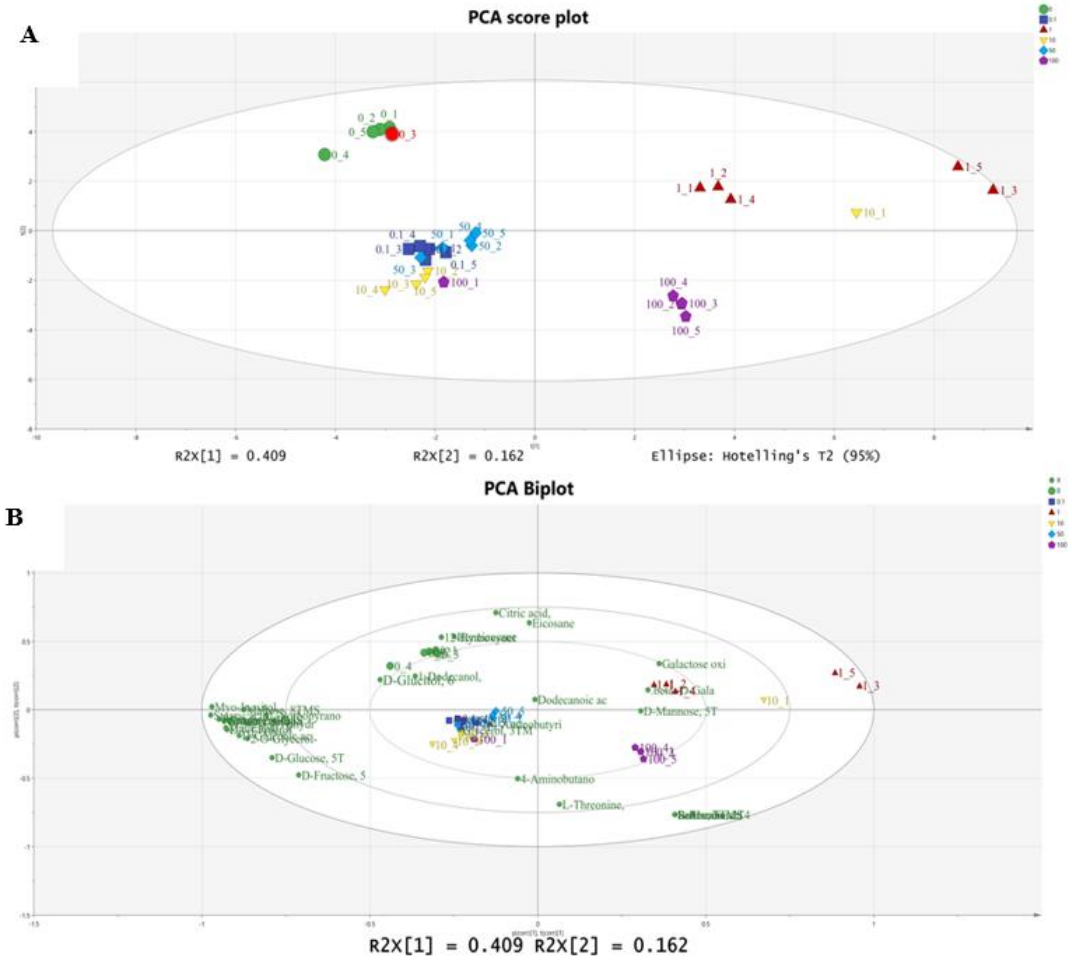

Figure 3. A) PCA score plot and biplot derived from the metabolites of choisum exposed to the vetiver root methanolic extract at different concentrations. B) Biplot of samples and metabolites of choisum upon application of vetiver root methanolic extract.

Supervised partial least squares discriminant analysis (PLS-DA) was performed to enhance the separation and identify the metabolites responsible for the segregation (Figure 4A). The score plot for PLS-DA exhibited a favourable level of agreement with an R^2 value exceeding 50% and larger than Q^2 values. The PLS-DA is a method of supervised analysis that computes variable importance in projection (VIP) values through a targeted emphasis on identifying and differentiating chemical markers or biomarkers. Based on the VIP score greater than 1 (VIP > 1) and $P < 0.05$, nine metabolites were found to contribute to the separation among different concentrations of vetiver root methanolic extract. The pathway enrichment analysis was then performed to identify pathways enriched in the metabolites list with VIP > 1 and $P < 0.05$. It should be noted that 4-aminobutanoic acid was excluded from the study because its P-value was greater than 0.05. The results indicated that choisum treated with 100 mg mL^{-1} vetiver root methanolic extract experienced a rise in the levels of free amino acids such as alanine, L-serine, and L-threonine (Figure 4B). The findings corroborated those of a previous study by Batista-Silva et al. (2019), which posited that plants exhibit a marked increase in the concentration of free amino acids in response to diverse abiotic stress conditions. Thus, this finding suggested that the vetiver root methanolic extract induced a stress response in the target plant. Carbohydrates such as D-fructose were also low in choisum exposed to 100 mg mL^{-1} (Figure 4B). The reduction of stomata length and chlorophyll content can hinder photosynthesis, thus reducing

carbohydrate production. The results align with a study by Huang et al. (2020) that reported sugar reduction in target plants due to the *Cinnamomum septentrionale* allelopathic effect. A decrease in the level of citric acid in choisum when exposed to 100 mg mL^{-1} could indicate alterations in the tricarboxylic acid cycle (TCA) pathway. The TCA pathway is vital for plant energy production, C metabolism, and macromolecule biosynthesis. The alteration of the TCA pathway may hinder the photosynthesis process (Zhang and Fernie, 2018). The TCA cycle is associated with the Calvin cycle, which converts CO_2 into glucose during photosynthesis (Sweetlove et al., 2010). Therefore, any disturbance in the TCA cycle can lead to a decrease in glucose production.

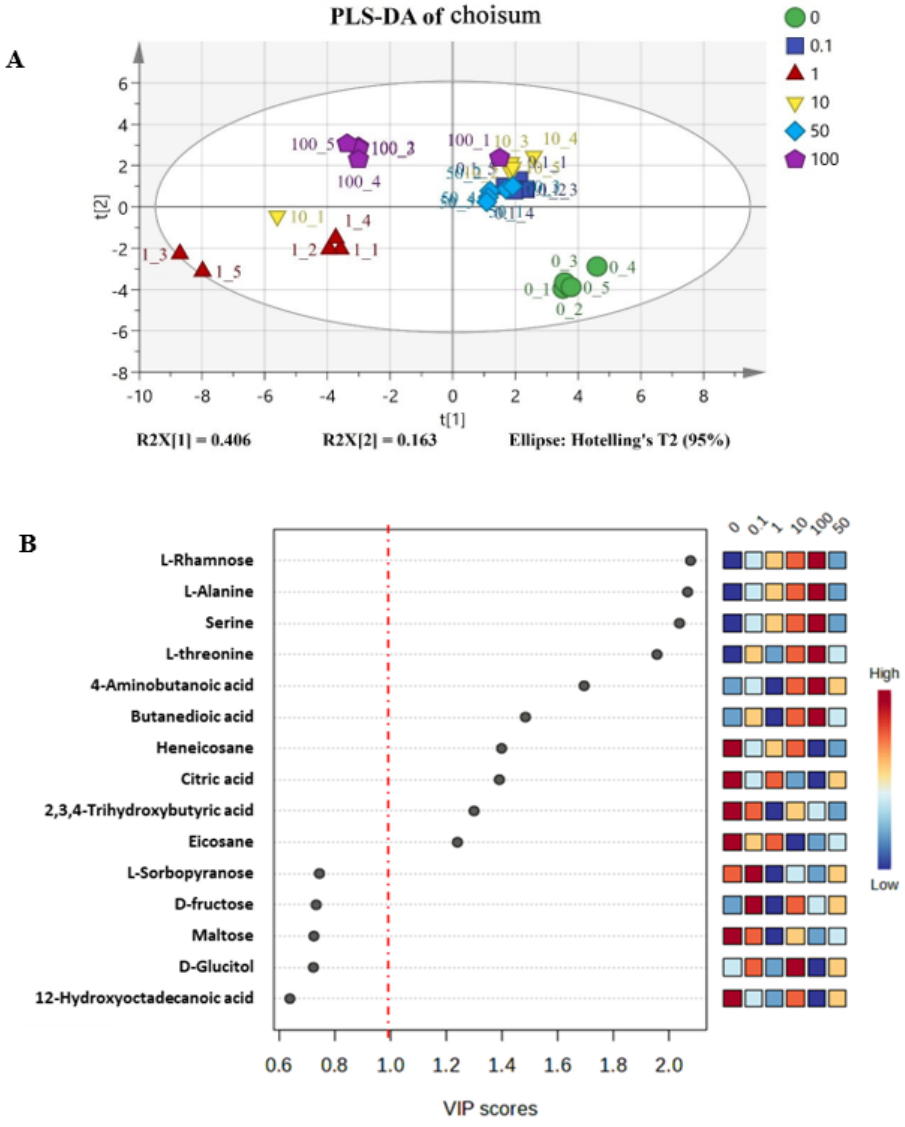

Figure 4. A) Partial least squares discriminant analysis (PLS-DA) score plot of choisum exposed to the vetiver root methanolic extract at different concentrations. B) Variable importance in projection (VIP) scores from PLS-DA of metabolites profile choisum treated with vetiver root methanolic extract.

Based on pathway enrichment analysis, the exposure of 100 mg mL^{-1} on choisum strongly enriched three pathways: Propanoate metabolism, Se-compound metabolism, and cysteine and methionine metabolism (Figure 5). The increment of succinic acid (butanedioic acid) in choisum treated with 100 mg mL^{-1} suggested that choisum was in stressed condition and thus enriched the propanoate metabolism. In plants, succinic acid acts as an osmotic regulator, promoting increased adaptability to abiotic stress conditions (Zhang et al., 2021). Meanwhile, amino acids comprise a vast array of substances that participate in the primary metabolism of plants and vital functions in plant physiological processes. For example, amino acids can function as osmotic regulators, govern stomatal opening, and serve as precursors for synthesising defence-related metabolites and signalling metabolites (Florencio-Ortiz et al., 2018). Cysteine and methionine are two essential amino acids in plants that play a vital role in metabolism, especially in plant S metabolism. Plant S metabolism plays a crucial role in numerous biological responses within plants, specifically in regulating abiotic and biotic stress through 3'-phosphoadenosine 5'-phosphate (PAP) and controlling redox through glutathione, as highlighted by Kopriva et al. (2019). The elevation of cysteine and methionine levels may be attributed to plant stress responses due to the effect of vetiver root methanolic extract on choisum. The upregulation of Se-compound metabolism in choisum is related to the coping mechanism of plants towards reactive oxygen species (ROS). The Se-metabolites can function as a plant stress modulator by preventing the accumulation of reactive oxygen species (ROS) during stress (Chauhan et al., 2019).

Figure 5. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway enrichment analysis of choisum when treated with vetiver root methanolic extract.

CONCLUSIONS

Based on the findings, the methanolic extract of vetiver root exhibited allelopathic effects towards choisum. The application of the extract led to a decrease in SPAD readings and chlorophyll content of the target plant. In addition, the allelochemicals present in the extract have substantially reduced the stomata length by 65%. Thus, it can be suggested that the methanolic extract of vetiver root may potentially impede cell elongation. Meanwhile, the extract derived from vetiver root has been observed to enhance the levels of free amino acids, namely alanine, L-serine, and L-threonine in choisum. Additionally, the pathway enrichment analysis showed enriched pathways were involved in regulating plant stress, such as propanoate, Se-compound, and cysteine and methionine metabolism, suggesting a plant defence mechanism to alleviate the allelopathic effect of vetiver root.

Author contribution

Conceptualisation: M.A.S.S., N.Y. Methodology: M.A.S.S., N.Y. Software: M.A.S.S., K.A.A. Validation: M.A.S.S. Formal analysis: M.A.S.S., N.A.M. Writing-original draft: M.A.S.S., N.A.M. Writing-review & editing: N.Y., K.A.A. Visualization: M.A.S.S. Supervision: N.Y., K.A.A. All co-authors reviewed the final version and approved the manuscript before submission.

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