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Naphthalene acetic acid as a potent elicitor of coordinated defense responses and terpenoid metabolism reprogramming in the medicinal succulent *Euphorbia trigona* Mill. (Euphorbiaceae)

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Original Research

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Abstract:

Plant growth regulators (PGRs) are effective tools for enhancing phytochemical production in medicinal plants. This study investigated the elicitation efficacy of gibberellin (GA_3) and auxins (IAA, IBA, NAA) at 250 mg/L on the medicinal succulent *Euphorbia trigona* Mill. (Euphorbiaceae). After seven months, NAA treatment significantly increased root biomass and uniquely triggered a coordinated defense response. This was evidenced by the significant upregulation of phenylalanine ammonia-lyase (PAL) and increased activities of the antioxidant enzymes peroxidase and superoxide dismutase. Consequently, NAA induced a substantial accumulation of defense-related metabolites, most strikingly a five-fold increase in terpenoids, along with elevated flavonoids and flavonols. GC-MS profiling suggested an NAA-induced reprogramming of the terpenoid profile. The results demonstrate that unlike GA_3 or other auxins, NAA is the most effective elicitor for enhancing bioactive compounds in *E. trigona* through a coordinated defense network.

Keywords: Coordinated response; Elicitation; Euphorbiaceae; *Euphorbia trigona* Mill.; Metabolic reprogramming; Naphthalene acetic acid (NAA); Phenylalanine ammonia-lyase; Secondary metabolism; Terpenoids

1. Introduction

Euphorbia trigona Mill., a densely branched, erect succulent shrub, holds significant medicinal and ornamental value and belongs to the prolific *Euphorbia* genus (Khan et al., 2020). Plants from this genus are renowned for producing a rich diversity of bioactive secondary metabolites, including terpenes, flavonoids, and sterols, which underpin their extensive pharmacological applications ranging from anticancer to antimicrobial activities (Kemboi et al., 2021; Amtaghri et al., 2022; Mustafa et al., 2022). For instance, latex extracts from various *Euphorbia* species have demonstrated efficacy in disrupting quorum-sensing and biofilm formation in pathogenic bacteria, highlighting their potential as novel antimicrobial agents (Nashikkar et al., 2011; Upadhyay et al., 2013). The biosynthesis of these valuable secondary metabolites is not constitutive but is highly inducible by both environmental stimuli and biotic/abiotic elicitors (Calvo et al., 2015; Pott et al., 2019). Among these elicitors, plant growth regulators (PGRs), traditionally known for their roles in growth and development, are increasingly recognized as potent modulators of plant secondary metabolism (Rui et al., 2020; Pérez-Llorca et al., 2023). The hormonal signaling networks involving auxins, gibberellins, jasmonates, and salicylates can significantly alter the flux through key biosynthetic pathways, thereby influencing the accumulation of defense-related compounds (Dermastia, 2019; Boncan et al., 2020). This crosstalk

between hormone signaling and secondary metabolism represents a strategic area for manipulating the phytochemical profiles of medicinal plants (Dai et al., 2006). A pivotal enzyme in this regulatory interplay is phenylalanine ammonia-lyase (PAL), which catalyzes the first committed step in the phenylpropanoid pathway toward the synthesis of phenolics, flavonoids, and lignins (MacDonald et al., 2007; Zhang et al., 2015). PAL activity is often a key determinant of a plant's defensive capacity, as its induction is closely linked to the production of antimicrobial and antioxidant compounds (Tao et al., 2011; Rasool et al., 2021). Recent evidence suggests that auxin signaling can significantly alter PAL activity, thereby influencing the accumulation of defense-related phenolics and flavonoids (Zhang et al., 2021).

Concurrently, the elicitation of defense responses is often accompanied by a controlled oxidative burst, which requires the activation of robust antioxidant systems. Enzymatic antioxidants such as peroxidase (POX) and superoxide dismutase (SOD) play a crucial role in maintaining cellular redox homeostasis and are integral components of the defense mechanisms in *Euphorbia* species (Medda et al., 2003; Pintus et al., 2008; Atzori et al., 2011). POX, in particular, has been noted for recognized dual role in cell wall fortification and ROS scavenging (Medda et al., 2011; Shukla et al., 2016).

Secondary metabolism can be strongly induced by environmental stimuli as well as by PGRs, which are increasingly recognized as effective modulators in medicinal plants (Khan et al., 2020). However, species-specific responses remain critically understudied, particularly in non-model succulent species with inherent metabolic richness such as *E. trigona*. PGRs are emerging as effective tools for eliciting defense responses and enhancing the production of valuable phytochemicals in medicinal plants (Pérez-Llorca et al., 2023). While the role of classic stress hormones like methyl jasmonate and salicylic acid in eliciting secondary metabolism is well-established (Sharma et al., 2020; Abdelsalam et al., 2023), the potential of more common PGRs —particularly auxins and gibberellin, gibberellins— to act as effective elicitors is less explored and often contradictory (Sedighi et al., 2014; Kulbat, 2016). Among auxins, naphthalene acetic acid (NAA) has been reported to significantly influence the flux through key biosynthetic pathways of secondary metabolites in various plant species (Li et al., 2022). Furthermore, a comparative study examining the effects of indole acetic acid (IAA), indole butyric acid (IBA), naphthalene acetic acid (NAA), and gibberellin (GA_3) on the coordinated defense response-encompassing enzyme activities and metabolic profiles—in a medicinal succulent like *E. trigona* is lacking. Most critically, there is a fundamental gap in understanding whether these hormones can trigger an integrated metabolon-like response-simultaneously upregulating key defense enzymes (PAL, POX, SOD) and channeling metabolic flux toward the biosynthesis of specific, valuable terpenoids and phenolics-rather than causing fragmented, isolated changes. This study aims to address this knowledge gap by identifying the most effective hormonal elicitor for *E. trigona* and elucidating the mechanistic interplay between hormone signaling, enzymatic activation, and subsequent metabolic reprogramming. This crosstalk between hormone signaling and secondary metabolism may lead to the formation of a 'defense metabolon'—a transient, multi-enzyme complex that channels substrates efficiently to enhance the production of protective compounds (Weng et al., 2019). The purpose of the present research was to investigate whether exogenous application of auxins and gibberellin could elicit such a coordinated response in *E. trigona*. The findings will provide a strategic framework for the targeted phytochemical manipulation of medicinal plants, moving beyond trial-and-error approaches to a more predictive elicitation strategy.

2. Experimental

2.1 Plant material and growth conditions

This study was conducted at the Farhikhtegan Greenhouse Town of Islamic Azad University, Gorgan Branch (Fig. 1), using a completely randomized block design with four replications. The plant specimen was identified by Marjaneh Aydani, a botanist at the Herbarium of the Medicinal Plants Research Center, Islamic Azad University, Gorgan Branch, following standard morphological identification keys (Eggli, 2002). A voucher specimen was deposited at the herbarium under voucher number MPRC-ET-1256. Key diagnostic characteristics included erect stems with a triangular cross-section, paired spines along the stem ridges, and small, lanceolate leaves. A stem incision test confirmed the exudation of white latex, a typical trait of the Euphorbiaceae family. Based on these characteristics, the plant was identified as *E. trigona* Mill. (Fig. 1).

Leaf-bearing shoot cuttings (13-15 cm in length, 7-10 mm in diameter) were collected from two-year-old *E. trigona* plants. The cuttings were shaded for two weeks to allow the cut ends to dry and stop latex bleeding before being planted in pots. The growth medium consisted of an 8:3 (*v/v*) mixture of cocopeat and perlite. The plants were maintained under controlled greenhouse conditions with day/night temperatures of 30 ± 5 °C/25 ± 5 °C and a relative humidity range of 60-75%. The first irrigation, which included a fungicide, was performed one week after planting. Irrigation was conducted once every two weeks at the beginning of spring and weekly during the summer.

2.2 Hormonal treatments

The hormones used in this study were purchased from Sigma Company and obtained through the authorized importer, ShimiGostarAran. Stock solutions of gibberellin (GA_3), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA) were prepared by initially dissolving each hormone in a minimal volume of 96% ethanol, followed by dilution with distilled water to a final working concentration of 250 mg/L. This concentration was selected based on preliminary dose-response assays and a review of relevant literature, confirming its efficacy in eliciting physiological responses in succulent plants without inducing phytotoxicity. Hormonal treatments, along with a fertilizer application (N20P20K20 fertilizer at 3 g/L), were initiated two months after rooting and applied as a

Figure 1. Left: An example of *Euphorbia trigona*. Right: Geographical location of Farhikhtegan Greenhouse Town in Iran (36.868152, 54.448608). (Taken from Google Maps link: https://maps.app.goo.gl/cofUiAbteicEP1Ew7).

foliar spray every 14 days. The control plants received the same irrigation, fungicide, and fertilizer regimen but were sprayed with distilled water only.

2.3 Growth parameter measurements

Seven months after the start of the experiment, the fresh weight (FW) of roots and shoots was recorded. Samples were then oven-dried at 90 °C for 24 hours, and the dry weight (DW) of roots and shoots was measured.

2.4 Extraction and quantification of secondary metabolites

2.4.1 Total terpenoid content

One gram of fresh plant sample was homogenized in 7 mL of 95% methanol using an ice-cooled mortar. After centrifugation at 4000 rpm for 15 minutes, 500 µL of the supernatant was combined with 3 mL of chloroform. The solution was vortexed and then left to stand for 3 minutes before 200 µL of H_2SO_4 was added. Following this, the solution was cooled in an ice bath for 15 minutes and subsequently kept in the dark at room temperature for 1.5 to 2 hours. The supernatant was slowly dried and then mixed with 3 mL of 95% methanol. Once the reddish-brown precipitate was completely dissolved, the optical density was measured at 538 nm. A 95% methanol solution was used as the blank, and linalool solutions in 95% methanol served as the standards. The total terpenoid content was calculated as milligrams of linalool per gram of fresh weight (Ghorai et al., 2012).

2.4.2 Total phenolic compounds

Total phenolic compounds were measured using Velioglu et al. (1998) method. Accrodingly, a 0.1 g sample was homogenized with 5 mL of a mixture containing 80% methanol and 1.0% hydrochloric acid. After incubation for 2 hours at room temperature, the mixture was centrifuged at 3000 g for 15 minutes. Then, 750 µL of Folin-Ciocalteu reagent was added to 100 µL of the supernatant, and the resulting mixture was allowed to stand at room temperature for 5 minutes. Subsequently, 750 µL of 6% sodium carbonate was added. After 90 minutes, the absorbance of each sample was measured at 725 nm. A standard curve was generated using gallic acid, and the total phenolic content was expressed as milligrams of gallic acid equivalents per gram of fresh weight.

2.4.3 Total flavonoid content

The total flavonoid content was determined using the aluminum chloride colorimetric method (Zhishen et al., 1999). A 0.1 g plant sample was ground in 10 mL of 80% methanol, and the resulting extract was centrifuged at 2000 g for 15 minutes. Then, 500 µL of the supernatant was mixed with 5 mL of distilled water and 300 µL of 5% sodium nitrite. After 5 minutes, 600 µL of 10% aluminum chloride was added to the solution. After an additional 6 minutes, 2 mL of 1 M sodium hydroxide and 2 mL of distilled water were added to the mixture. The absorbance of the solution was measured at 510 nm. Rutin solutions were used as standards, and the flavonoid content was expressed as milligrams of rutin per gram of dry weight.

2.4.4 Total flavonol content

One milliliter of 2.0% aluminum chloride and 3 mL of 5% sodium acetate were added to 1 mL of the plant extract (prepared as described for the total flavonoid measurement). After 150 minutes in darkness, the absorbance of the samples was measured at 440 nm. The total flavonol content was calculated using a quercetin standard curve and expressed as milligrams of quercetin per gram of dry weight (Akkol et al., 2008).

2.4.5 Total anthocyanin content

The anthocyanin content in the samples was measured according to the method described by Wagner (1979). A 0.2 g plant sample was homogenized in 3 mL of acidic methanol (prepared with a 99:1 *v/v* ratio of pure methanol to pure hydrochloric acid). The extract was kept in the dark for 24 hours at 25 °C. It was then centrifuged for 10 minutes at 6000 g, and the absorbance of the supernatant was measured at 550 nm. The anthocyanin concentration was calculated using a molar extinction coefficient of 33,000 $M^{-1} \cdot cm^{-1}$ and expressed as nanomoles per gram of fresh weight.

2.5 Enzyme activity assay

2.5.1 Phenylalanine ammonia-lyase (PAL) activity

A 300 mg fresh sample was homogenized in a chilled mortar with 6.5 mL of 50 mM Tris-HCl buffer (pH 8.8) containing 15 mM β-mercaptoethanol. The resulting extract was centrifuged at 50,000 g for 30 minutes at 4 °C using an Eppendorf 5804R centrifuge. The supernatant was collected as the enzyme extract. The reaction mixture, consisting of 1 mL of extraction buffer, 0.5 mL of 10 mM L-phenylalanine, 0.4 mL of deionized distilled water, and 1 mL of the enzyme extract, was incubated for one hour at 37 °C. The reaction was terminated by adding 0.5 mL of 6 M HCl. The resulting product was extracted with 15 mL of ethyl acetate. The ethyl acetate was evaporated, and the residual solid was dissolved in 3 mL of 0.05 M sodium hydroxide. The concentration of cinnamic acid produced from phenylalanine by PAL was calculated based on the absorbance at 290 nm and an extinction coefficient of 9,500 $M^{-1} \cdot cm^{-1}$. One unit of PAL activity was defined as the amount of enzyme required to produce one micromole of cinnamic acid per minute (Wang et al., 2006).

2.5.2 Peroxidase (POX) activity

One gram of fresh shoot sample was homogenized in 10 mL of 0.1 M phosphate buffer (pH 7.5). After filtration and centrifugation at 16,000 g for 25 minutes, the supernatant was used as the enzyme extract. The guaiacol reagent was prepared by adding 220 µL of guaiacol to 2 mL of 1.0% ethanol, and the final volume was adjusted to 100 mL with distilled water. The reaction mixture contained 3.8 mL of 0.2 M acetate buffer (pH 3.6), 0.2 mL of the enzyme extract, 0.1 mL of 20 mM guaiacol reagent, and 1 mL of 40 mM H_2O_2. Reactions were conducted at 35 °C. Peroxidase activity was determined by monitoring the change in absorbance at 470 nm for one minute (Nakano et al., 1981).

2.5.3 Superoxide dismutase (SOD) activity

Superoxide dismutase activity was measured using the photochemical method described by Beauchamp et al. (1971). The reaction mixture contained 1.3 µM riboflavin, 13 mM L-methionine, 63 µM nitroblue tetrazolium (NBT), and 0.05 M sodium carbonate. Then, 1.6 mL of this mixture was combined with 0.4 mL of the enzyme extract (the same extract used for the POX assay) and 1 mL of distilled water. The mixtures were illuminated in glass test tubes, while identical solutions kept in the dark served as blanks. The reaction was initiated and terminated by switching the light on and off, respectively. The reaction rate was determined by measuring the increase in absorbance at 560 nm over 8 minutes.

2.6 GC-MS metabolomic profiling and compound identification

Shoot samples from the control, NAA, and GA_3 treatments were dried away from sunlight and pulverized. For the GC-MS analysis, a single composite sample per treatment was created by thoroughly mixing an equal amount of dried shoot powder (1.0 g) from each of the five biological replicates, resulting in a representative 5.0 g sample for each treatment. All extraction steps were performed using glassware to prevent potential contamination with plasticizers. This composite sample was then subjected to Soxhlet extraction using 250 mL of 96% ethanol for 9 hours. The solvent was removed using a rotary evaporator. The dried extract was weighed to calculate the extraction yield, reconstituted in ethanol, and analyzed using an Agilent 7890B gas chromatograph coupled with an Agilent 5977B mass selective detector (Agilent Technologies, USA).

The GC-MS analysis was performed under the following conditions: An HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent J&W) was used. The carrier gas was ultra-high-purity helium at a constant flow rate of 1.0 mL/min. The injector temperature was set at 250 °C, and 1 µL of sample was injected in split mode with a split ratio of 1:50. The oven temperature program was as follows: initial temperature 60 °C (held for 5 min), increased to 280 °C at a rate of 5 °C/min, and finally held at 280 °C for 10 min. The transfer line temperature was set at 280 °C. The mass spectrometer was operated in electron impact mode at 70 eV. The ion source temperature was 230 °C, and the quadrupole temperature was 150 °C. The mass scan range was set from 40 to 600 *m/z*.

Identification of the volatile constituents was performed using the Agilent ChemStation data system. The mass spectra of the unknown compounds were acquired and automatically compared with reference spectra in two mass spectral libraries: The Wiley 9th Edition and NIST 14 (National Institute of Standards and Technology) databases. The search was configured to consider spectra with a minimum similarity match quality of 70%. A compound was considered confidently identified when the library similarity match factor was 80% or higher. The relative abundance of each compound was expressed as a percentage based on the peak area in the total ion chromatogram (TIC), calculated by the ChemStation integrator, without the use of correction

factors.

2.7 Statistical analysis

Values represent the mean ± [standard error/deviation] of four biological replications for growth parameters, total shoot secondary metabolites, and enzyme activities. The relative variability within each treatment group was assessed by calculating the coefficient of variation (CV) (Curran-Everett, 2018). Potential outliers were examined for each dataset using Dixon's Q test at a 95% confidence level (Dixon, 1950); no statistical outliers were identified, thus all replicates were retained for analysis. The significance of treatment effects was determined by one-way Analysis of Variance (ANOVA). The assumption of homogeneity of variances was verified using Levene's test. For datasets where this assumption was violated, the Games-Howell post hoc test, which does not assume equal variances, was employed. For datasets meeting the assumption, Tukey's Honestly Significant Difference (HSD) test was used. All mean comparisons were performed at a significance level of $p \leq 0.05$. All statistical analyses were conducted using IBM SPSS Statistics software (Version 23). For multivariate visualization, a heatmap with hierarchical clustering was generated using the web-based tool ClustVis (https://biit.cs.ut.ee/clustvis). The data matrix for the heatmap consisted of the mean values of all quantified parameters across the different treatments. Data were unit variance scaled (Z-score normalization) across parameters prior to analysis. Subsequently, a Pearson correlation matrix between all parameters was computed and visualized. Hierarchical clustering was performed on both rows (parameters) and columns (treatments) using the Euclidean distance measure and the complete linkage clustering method.

3. Results and discussion

3.1 Experimental findings

3.1.1 Growth parameters and terpenoid content

As summarized in Table 1, hormonal treatments did not significantly alter the fresh or dry weight of the aerial parts. In contrast, NAA application significantly enhanced root fresh weight compared to the control and other hormone treatments. Furthermore, NAA treatment resulted in a significant increase in root dry weight compared to the control group. The other hormones had no significant effect on root dry weight. Most notably, NAA treatment induced a dramatic and significant increase ($p \leq 0.05$) in the shoot terpenoid content compared to the control and all other hormones. In contrast, gibberellin, IAA, and IBA had no significant influence on terpenoid levels (Table 1). The magnitude of the increase in terpenoid production (five-fold) induced by NAA is comparable to effects reported for strong elicitors like methyl jasmonate, suggesting that synthetic auxins can be just as effective in manipulating valuable metabolites (Wasternack et al., 2017). The robust response of *E. trigona* to NAA highlights the inherent metabolic potential of *Euphorbia* species to produce a wide range of bioactive compounds, including terpenoids (Shi et al., 2008). The stronger response to NAA compared to endogenous IAA may be due to NAA's greater stability against degradation or its different affinity for specific auxin receptors that initiate signaling pathways leading to secondary metabolism (Simon et al., 2011).

3.1.2 Antioxidant enzyme activities

Plant hormones activate the antioxidative defense system, which includes SOD, to help plants cope with stress (Kapoor et al., 2014). Plant stress hormones such as abscisic acid (ABA), salicylic acid (SA), and brassinosteroids (BRs) significantly influence peroxidase activity in plants, particularly under stress conditions (Kellos et al., 2008; Kapoor et al., 2014; Nagar et al., 2021). Auxin application can enhance plant resistance to abiotic stresses (Zhang et al., 2022). Under stress conditions, hormones can enhance the activities of SOD and other antioxidant enzymes, thereby reducing oxidative stress damage (Kapoor et al., 2014; Zhang et al., 2015; Zhao et al., 2022). In this study, the activities of the antioxidant enzymes peroxidase (POX) and superoxide dismutase (SOD) in the shoots were significantly altered by the auxin treatments (Table 1). Both NAA and IAA treat-

Table 1. The effects of hormonal treatments on growth parameters, shoot terpenoid content, and antioxidant enzyme activities in *Euphorbia trigona*. Values are Mean ± SD (n = 4). Different letters within a row indicate significant differences according to Tukey's HSD test ($p \leq 0.05$).

Trait	Control	NAA (250 mg/L)	IBA (250 mg/L)	IAA (250 mg/L)	GA_3 (250 mg/L)
Shoot Fresh Weight (g)	60.57 ± 4.28a	81.14 ± 37.81a	70.82 ± 10.71a	65.08 ± 7.98a	71.53 ± 9.79a
Shoot Dry Weight (g)	3.01 ± 0.69a	4.59 ± 2.38a	3.52 ± 0.71a	3.60 ± 0.74a	3.2 ± 0.77a
Root Fresh Weight (g)	2.077 ± 0.43b	4.52 ± 1.04a	2.67 ± 0.48b	2.77 ± 0.77b	2.43 ± 0.47b
Root Dry Weight (g)	0.27 ± 0.04b	0.60 ± 0.18a	0.43 ± 0.14ab	0.49 ± 0.05ab	0.32 ± 0.07b
Shoot Total Terpenoid (mg/g Linalool eq)	7.65 ± 1.64b	38.76 ± 1.32a	9.35 ± 3.81b	12.76 ± 2.88b	9.83 ± 1.57b
Shoot POX Activity ($\times 10^{-3}$ ΔOD.g^{-1}FW·min^{-1})	9.00 ± 0.91b	14.75 ± 1.04a	6.75 ± 0.65c	13.38 ± 0.85a	8.75 ± 0.65b
Shoot SOD Activity (ΔOD·g^{-1}FW.8min^{-1})	0.21 ± 0.03b	0.29 ± 0.01a	0.32 ± 0.01a	0.32 ± 0.00a	0.23 ± 0.01b

ments resulted in a significant increase ($p \leq 0.05$) in POX and SOD activities. IBA treatment had a divergent effect: it significantly increased SOD activity but caused a significant decrease in POX activity. The increase in SOD and POX activities under NAA treatment indicates the induction of a controlled oxidative stress, which in turn activated these antioxidant enzymes. Gibberellin increases photosynthesis and the activities of antioxidant enzymes, which can improve overall plant health and yield (Guo et al., 2022). However, GA_3 did not affect the activity of antioxidant enzymes in the present work (Table 1).

3.1.3 Phenolic compound profiles

Among secondary metabolites, phenolic compounds play a significant role in plant defense mechanisms, including responses to biotic and abiotic stresses. Their synthesis is often triggered by these stress factors (Rasouli et al., 2016). Plant hormones significantly influence the production of phenolic compounds (Sedighi et al., 2014; Kulbat, 2016). In the present research, Gibberellin application caused a significant reduction in phenolic content compared to the control, and the effect of auxin treatments on total shoot phenolic compounds was not significant (Fig. 2). However, the content of shoot flavonoids and flavonols was significantly increased by NAA treatment (Fig. 3 and Fig. 4). The difference between NAA and IAA was not significant, nor were the differences between IBA, GA, and the control. In contrast, hormonal treatments had no significant effect on shoot anthocyanin content (Fig. 5). The stability of anthocyanin content across all hormonal treatments indicates the existence of independent and tightly regulated mechanisms for these pigments, likely due to their multiple essential roles in plant physiology (Landi et al., 2015). The observed increase in antioxidant enzyme activities and the accumulation of flavonoids and flavonols under NAA treatment suggest that the elicited oxidative stress contributed to the activation of both enzymatic and non-enzymatic defense pathways. IAA caused a significant increase in the shoot flavonol content, however, IBA did not cause a significant change in the content of the secondary metabolites measured in this study (Table 1, Fig. 2-Fig. 5). Sedighi et al. (2014) demonstrated that the type and concentration of PGRs, such as gibberellin, and auxin, affect phenolic substance production which is in agreement with the results of the present work.

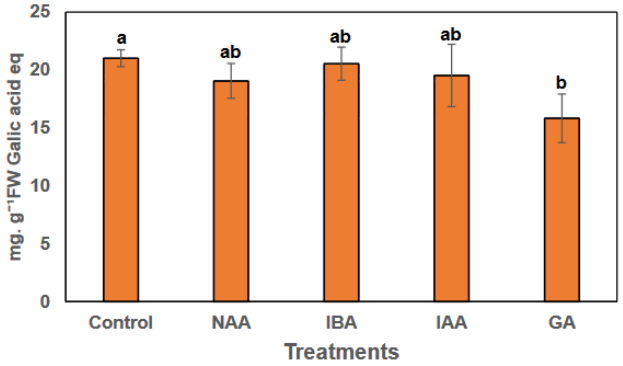

Figure 2. The effects of the hormones auxin and gibberellin on the total phenolics content of the shoots (Mean ± SE) in *Euphorbia trigona*. The same letters indicate no significant difference ($p \leq 0.05$).

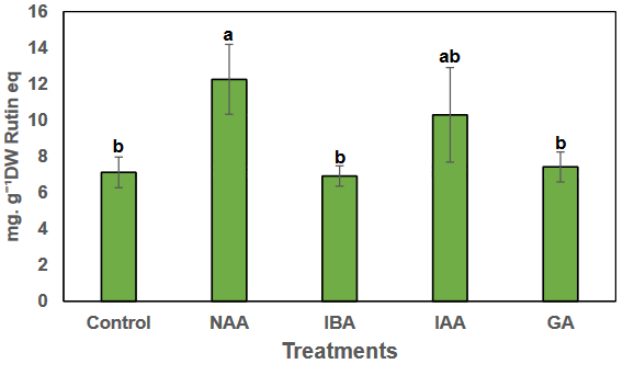

Figure 3. The effects of the hormones auxin and gibberellin on the total flavonoids content of the shoots (Mean ± SE) in *Euphorbia trigona*. The same letters indicate no significant difference ($p \leq 0.05$).

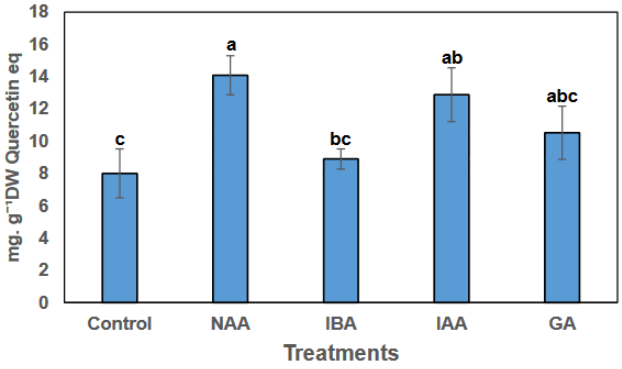

Figure 4. The effects of the hormones auxin and gibberellin on the total flavonols content of the shoots (Mean ± SE) in *Euphorbia trigona*. The same letters indicate no significant difference ($p \leq 0.05$).

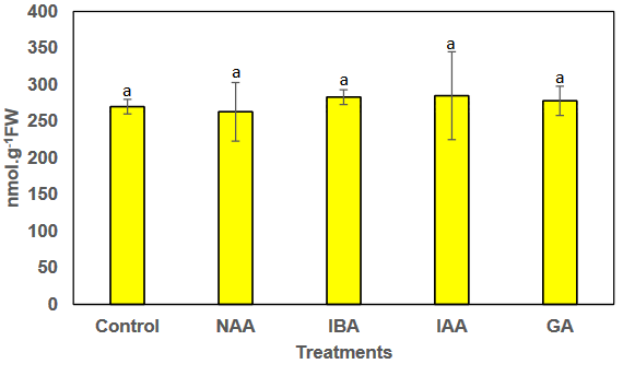

Figure 5. The effects of the hormones auxin and gibberellin on the total anthocyanins content of the shoots (Mean ± SE) in *Euphorbia trigona*. The same letters indicate no significant difference ($p \leq 0.05$).

3.1.4 Phenylalanine ammonia-lyase (PAL) activity

The activity of the key phenylpropanoid pathway enzyme, PAL, was significantly upregulated only in response to NAA treatment (Fig. 6). The activity in NAA-treated plants was significantly higher than in the control, IBA, and GA_3. All other hormonal treatments, had no significant impact on PAL activity. The suppression of total phenolic content by GA_3, without a concurrent decrease in the total contents of flavonoids, flavonols, anthocyanins, or PAL activity (Fig. 1, Fig. 2 to Fig. 5), suggests that gibberellin may selectively inhibit specific branches of the phenolic biosynthesis pathway. This selective inhibition may occur through post-

transcriptional mechanisms, a hypothesis that aligns with findings from other researchers (Akhtar et al., 2020).
The significant upregulation of PAL activity under NAA treatment is highly notable, as this enzyme is recognized as a key multifaceted regulatory node in the phenylpropanoid pathway and a common target for various elicitors (Zhang et al., 2015).

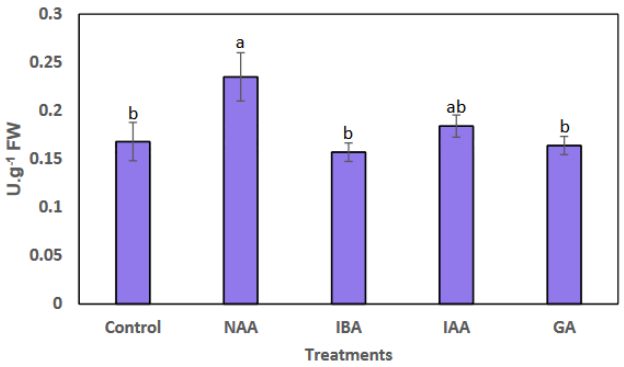

Figure 6. The effects of the hormones auxin and gibberellin on the phenylalanine ammonia lyase (PAL) activity of the shoots (Mean ± SE) in *Euphorbia trigona*. The same letters indicate no significant difference ($p \leq 0.05$).

3.2 Integrated multivariate analysis: Heatmap and hierarchical clustering

To integrate the multivariate physiological responses previously detailed in the statistical analyses (Table 1; Fig. 1, Fig. 2 to Fig. 5), a heatmap coupled with hierarchical clustering was constructed (Fig. 7). This visualization powerfully synthesizes the distinct response patterns elicited by the different hormonal treatments. The heatmap confirms the key findings from the univariate analyses, revealing a clearly segregated and tightly co-regulated defense module (right cluster in Fig. 7). This module, whose individual components were significantly upregulated by NAA in our earlier statistical tests, includes root biomass (Root FW, Root DW), key defense enzymes (PAL, POX), and protective secondary metabolites (Terpenoids, Flavonoids, Flavonols). The NAA treatment consistently shows the strongest positive Z-scores across this entire module, visually consolidating the statistically significant increases reported in Table 1 and Fig. 3, Fig. 4, and Fig. 6. For instance, the profound activation of this cluster under NAA is evidenced by high positive Z-scores for Terpenoids ($Z = +1.34$), PAL ($Z = +1.01$), POX ($Z = +1.35$), Flavonoids ($Z = +1.47$), and Root FW ($Z = +0.68$), aligning with the five-fold increase in terpenoids (Table 1), the significant upregulation of PAL activity (Fig. 6), and the elevated levels of flavonoids and flavonols (Fig. 3 and Fig. 4). In contrast, the pronounced negative Z-scores for these same traits under IBA and IAA treatments, such as Terpenoids ($Z = -1.67$ and -1.78, respectively) and PAL ($Z = -1.19$ and -2.02, respectively), reflect their general ineffectiveness or suppressive role, as previously established.
Conversely, a separate cluster of traits (left cluster in Fig. 7), including SOD activity, Anthocyanin, and total Phenolics, exhibited an independent regulatory pattern. This visualization aligns with the statistical results that showed these traits were either unresponsive (Anthocyanin, Fig. 5) or responded differently to NAA and other hormones. For example, the high Z-scores for Phenolics under GA_3 ($Z = +2.97$) and IAA ($Z = +2.36$) are consistent with the significant changes shown in Fig. 1, whereas NAA had a neutral effect (Phenolics, $Z = -0.50$). Similarly, SOD activity was highest under GA_3 ($Z = +2.61$) but showed a moderate response to NAA ($Z = -0.29$).
Therefore, the heatmap does not introduce new inferences but provides a consolidated visual proof of the coordinated defense response uniquely orchestrated by NAA, which was first established through rigorous statistical testing. The concurrent upregulation of the defense-related cluster supports the hypothesis that NAA triggers a controlled oxidative burst and coordinates a defense metabolon, as discussed previously (Mittler, 2017; Weng et al., 2019).

3.3 GC-MS metabolomic profiling

The GC-MS analysis of *E. trigona* has not been reported directly in other provided contexts. However, insights from related *Euphorbia* species can be inferred. *Euphorbia tirucalli* stem analysis identified 46 chemical constituents, with main components being lanosta-8,24-dien-3-ol (13.60%), (23S)-ethylcholest-5-en-(3β)-ol (7.02%), linoleic acid (2.96%), and viminalol (2.57%) (Yusoff et al., 2017). *Euphorbia hirta* methanol extract revealed 15 compounds, with glycol aldehyde dimer being the main constituent (41.22%) (Karki et al., 2020). *Euphorbia helioscopia* essential oil analysis identified thymol (48.36%), caryophyllene (23.57%), and carvacrol (6.70%) as major components (Beltagy, 2019). *Euphorbia heterophylla* essential oil analysis identified 35 compounds, with terpenoids being the main components (88.70%) (Elshamy et al., 2019). Generally, *Euphorbia* species contain a variety of terpenoids, fatty acids, and other bioactive compounds, which could be expected in *E. trigona* as well. GC-MS analysis was employed to profile the metabolic composition of the shoot extracts. This analysis identified that the main constituents in the shoot extracts of control plants were terpenoids, fatty acids, esters, and steroid derivatives (Table 2). The major compounds detected were bis(2-ethylhexyl) phthalate (9.56%), (23S)-ethylcholest-5-en-3β-ol (9.56%), epifriedelinol (8.37%), and 9,19-cyclolanost-25-en-3-ol, 24-methyl-, (3β,24S) (4.95%).
Hormone treatments affected the extract mass, diversity, and content of GC-MS detectable compounds. The mass of the dried extract obtained from 5.0 g of dry shoot powder was 0.2 g (4.0% yield), 0.34 g (6.8% yield), and 0.39 g (7.8% yield) for the control, NAA, and GA_3 treatments respectively, indicating that both hormonal treatments increased the total amount of extractable material. Among the detected compounds, six chemicals were considerable regarding their relatively high percentage in the extracts. As shown in the representative chromatogram (Fig. 8), a prominent peak was detected and identified as Bis(2-ethylhexyl) phthalate (DEHP), which was present in the extracts of the control and both hormone treatments at a retention time of approximately 26.8 minutes across all samples. NAA

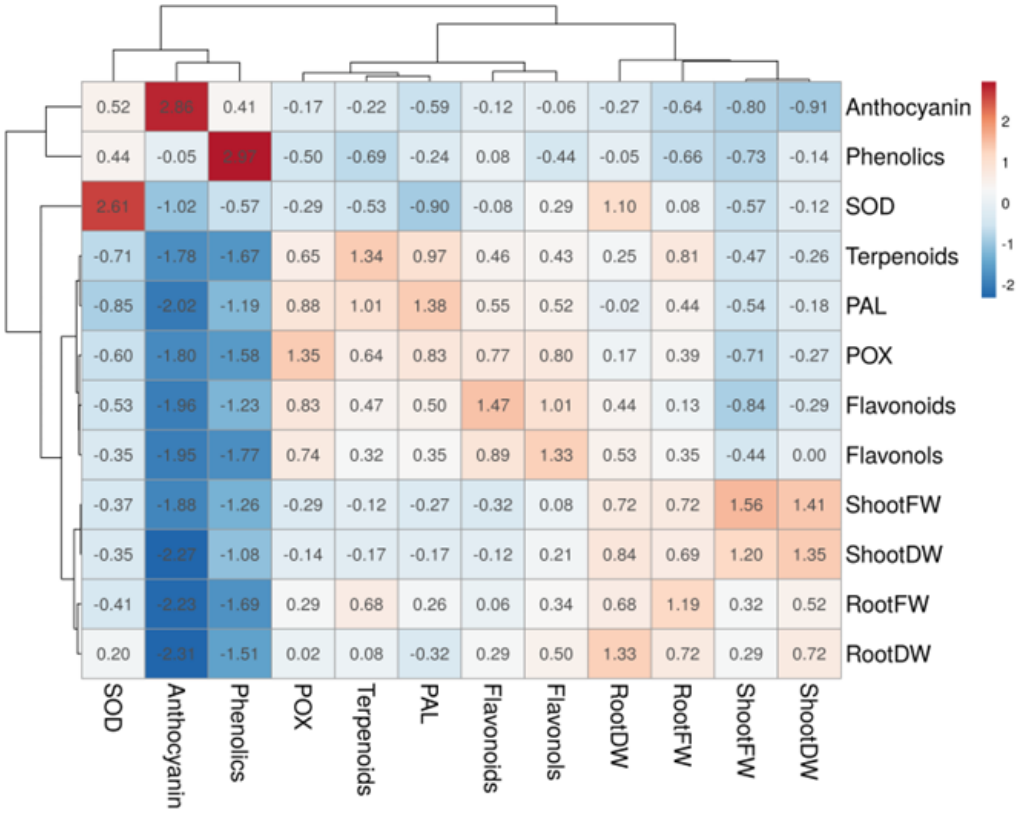

Figure 7. Heatmap with hierarchical clustering of morphological, biochemical, and physiological traits in *E. trigona* under different plant growth regulator treatments. Rows and columns are clustered based on Euclidean distance and complete linkage. The color scale represents Z-score normalized values, from blue (low) to red (high).

decreased the diversity of detectable ingredients, while it enhanced the relative percentage of the compound (49.31%) by about 4 to 5 times compared to control or gibberellin treatment. A profound increase in the content of DEHP under nitrogen or sulfur fertilizers was previously reported for succulent plants, *Agave americana* and *Euphorbia tirucalli* (Farnia et al., 2022; Jokar et al., 2023).

Bis(2-ethylhexyl) phthalate (DEHP) has been reported to exhibit antitumor and antileukemic properties (Javed et al., 2022). On the other hand, DEHP is classified as an endocrine disruptor due to its weak estrogenic and anti-androgenic properties (Lucaccioni et al., 2021; Tassinari et al., 2021).

Moreover, GC-MS preliminary screening in the present

Table 2. Relative abundance (%) of major bioactive compounds (> 2%) in GC-MS profiles of Euphorbia trigona shoot extracts from control and hormone-treated plants. Results are from a pooled sample analysis per treatment.

Number	Chemical Name	Treatments		
		Control	NAA	GA
1	Hexadecanoic acid	2.12	2.73	-
2	Bis(2-ethylhexyl) phthalate	9.56	49.31	11.27
3	Stigmasterol	-	-	4.51
4	(23S)-ethylcholest-5-en-3β-ol	9.56	-	8.14
5	Lup-20(29)-en-3-ol, (3.β)	2.12	-	-
6	Benzo[b]cyclopropa[1m]fluorenone	2.12	-	-
7	α-Amyrin	2.57	-	-
8	Lupeol	2.57	-	-
9	7-Methoxymethyl-1-(trimethylsilyl)-1-[1,1-bis(trimethylsilyl)ethyl]-2-oxa-1-sila	2.20	-	-
10	9,19-Cyclolanost-25-en-3-ol, 24-methyl-, (3β,24S)	4.95	-	-
11	Epifriedelinol	8.37	-	11.33
12	4-*epi*-Friedelin	2.47	-	3.20
13	6,10,14-Trimethyl-2-Pentadecanone,	-	3.16	-
14	Lanosta-8,24-dien-3-ol, (3β)	-	-	2.13
15	β-Amyrin	-	-	2.10
16	9,19-Cyclolanostan-3-ol,24-ethylene-, (3β)	-	-	3.51
17	14-Hydroxy-13-methoxy-8,11,13-podocarpatriene	-	-	9.06
18	D:A-Friedooleanan-3-one friedelin	-	-	3.20

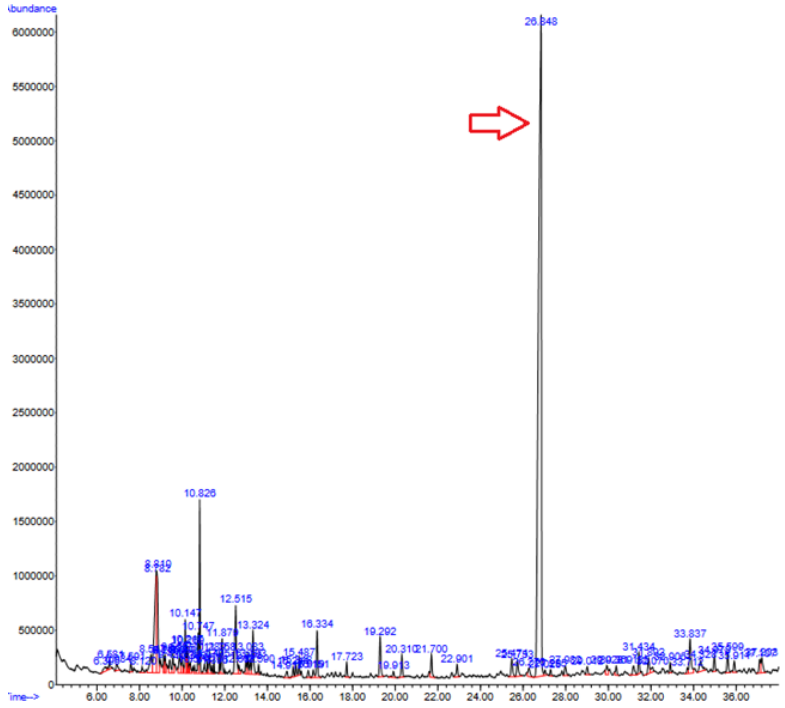

Figure 8. GC-MS analysis of *Euphorbia trigona* shoot extract under NAA treatment. The total ion chromatogram (TIC) reveals a complex metabolite profile. The compound eluting at 26.848 min (arrow) was putatively identified as Bis(2-ethylhexyl) phthalate (DEHP) by comparing its mass spectrum with those in the NIST14 and Wiley 9th Edition mass spectral libraries.

work suggests that NAA decreased the amount of Epifriedelinol and (23S)-ethylcholest-5-en-3β-ol in the extracts compared to control and gibberellin treatment.

Epifriedelinol, a triterpenoid compound, has several notable applications in various fields. It has demonstrated significant anticancer properties by inducing apoptosis in cancer cells, making it a potential candidate for cancer therapy. This compound exhibits strong antibacterial and antifungal activities, which can be leveraged in developing treatments for infections caused by pathogenic bacteria and fungi. Epifriedelinol has anti-inflammatory properties, which can be beneficial in treating inflammatory diseases and conditions. It has potential neuroprotective effects, which could be useful in the treatment of neurodegenerative diseases (Baldwin et al., 2022).

(23S)-Ethylcholest-5-en-3β-ol has several notable applications, particularly in the fields of pharmacology and medicine. This compound has shown potential in targeting various cancers, including breast, lung, and ovarian cancers. It is identified as one of the active compounds in ethanol extracts with potential anticancer properties (Sanora et al., 2019). It is part of the bioactive compounds in *Euphorbia tirucalli*, which is traditionally used for its antimicrobial properties (Yusoff et al., 2017). This compound is found in *Kalanchoe pinnata* leaves, which have demonstrated significant wound healing effects in experimental rats (Mohd-Jaafar et al., 2013).

In the present research, gibberellin showed a positive effect in enhancing the amount of stigmasterol (4.51%) and 14-hydroxy-13-methoxy-8,11,13-podocarpatriene (9.06%) in the extracts. Stigmasterol, a phytosterol found in various plants, has numerous applications across different fields due to its diverse biological activities. Stigmasterol exhibits significant anticancer properties by inducing apoptosis, cell cycle arrest, and autophagy in various cancer cell lines, including ovarian, skin, liver, and prostate cancers (Bae et al., 2020; Bakrim et al., 2022). It has potent anti-inflammatory effects, reducing the release of pro-inflammatory cytokines and inhibiting COX-2 and iNOS. Stigmasterol improves glucose metabolism by enhancing GLUT4 translocation and reducing insulin resistance. It shows potential in preventing Alzheimer's disease and other neurodegenerative conditions by regulating oxidative stress and inflammatory pathways (Bakrim et al., 2022). Regular consumption of stigmasterol can lower LDL cholesterol levels by 8-10%, reducing the risk of cardiovascular diseases (Bae et al., 2020).

Stigmasterol has demonstrated effectiveness against various Gram-positive and Gram-negative bacteria. It inhibits the growth of *Candida* species and certain parasites like *Trypanosoma congolense* and *Leishmania major* (Bakrim et al., 2022). In plant seeds, stigmasterol promotes cell proliferation, maturation, and germination, enhancing overall plant growth and productivity (Bae et al., 2020). Due to its high nutritional value and physiological activity, stigmasterol is used in dietary supplements to support overall health (Huang et al., 2024). Further research is recommended to explore its pharmacodynamics, pharmacokinetics, and toxicological profiles to fully harness its therapeutic potential (Bakrim et al., 2022).

14-Hydroxy-13-methoxy-8,11,13-podocarpatriene has been studied for its potential antifungal properties. The conversion of related compounds into derivatives with greater antifungal activity suggests that modifications of this compound could enhance its efficacy against fungal pathogens. Structural features similar to those found in 14-hydroxy-13-methoxy-8,11,13-podocarpatriene have been linked to

antibacterial activity. For instance, the presence of specific functional groups in related compounds has been shown to impart significant antibacterial properties, which could be explored in this compound as well. Compounds with similar structures have demonstrated phytotoxic effects, which could be relevant for agricultural applications, such as controlling plant diseases caused by pathogens (Masi, 2024).
Based on the results of the present work, NAA and gibberellin treatments decreased the amount of 9,19-cyclolanost-25-en-3-ol, 24-methyl-, (3β,24S) in the *E. trigona* extracts. This compound has several notable applications, particularly in the pharmaceutical and medicinal fields. It has demonstrated significant antibacterial properties, particularly against *Escherichia coli*, making it a potential candidate for developing antibacterial agents (Likittrakulwong et al., 2023). As a lanostane triterpenoid, it exhibits adaptogenic and anabolic activities, which help enhance the general performance of organisms under stress by normalizing physiological processes and various body functions. The compound's medicinal activities suggest its potential use in future drug discovery systems, particularly for developing treatments that require adaptogenic and anabolic properties (Qadir et al., 2018).

3.4 Evaluating the evidence for NAA as a potent elicitor of a coordinated defense metabolon

3.4.1 Integrated multivariate response to NAA

Clustering analysis (Fig. 7), alongside evaluation of hormone effects on various traits (Table 1, Fig. 2, Fig. 3, Fig. 4 to Fig. 6), confirmed that NAA elicited a response profile distinct from the control and other hormones, primarily by strongly upregulating a core network of correlated parameters. The uniform upregulation (red coloration, Fig. 7) of this cluster, particularly under the NAA treatment, indicates a strong positive correlation and co-regulation among these traits. Our finding that NAA (a synthetic auxin) was the most potent elicitor of the defense response aligns with new insights into the regulation of the phenylpropanoid pathway by auxin, which suggests auxin can unexpectedly modulate defense pathways (Zhang et al., 2021). However, this remarkable and distinct ability of NAA to elicit a coordinated defense response is an effect not shared by indole auxins (IAA, IBA) or gibberellin (GA_3). The distinctly different responses to the various auxins (IAA, IBA, NAA) observed in our study emphasize the signal-specific and species-specific nature of the auxin-mediated regulation of secondary metabolism, which has been noted in other studies (Li et al., 2022).The specific enhancement of root biomass by NAA suggests a strategic resource reallocation toward the root system, potentially to improve nutrient and water uptake to support the energetically demanding process of secondary metabolite biosynthesis, a phenomenon supported by theories on plant resource allocation under stress (Gómez et al., 2012; Erb et al., 2020; Pérez-Llorca et al., 2023).
The most compelling evidence for NAA's role as a potent abiotic elicitor is the synchronized upregulation of key defense components. This coordinated response included the induction of phenylalanine ammonia-lyase (PAL), the core enzyme of the phenylpropanoid pathway, the antioxidant enzymes peroxidase (POX) and superoxide dismutase (SOD), and the substantial accumulation of protective metabolites-most notably a five-fold increase in terpenoids, accompanied by elevated levels of flavonoids and flavonols. The strong coordination between the induction of PAL activity and the accumulation of terpenoids, despite a lack of common precursors, reinforces the hypothesis that NAA may stimulate the formation of a temporary "defense metabolon" to efficiently channel substrates toward the production of protective compounds (Weng et al., 2019). This suggests the activation of a convergent signaling network that co-regulates physical and biochemical defenses. The strong positive correlations within this network, vividly displayed by the hierarchical clustering analysis (Fig. 7), underscore a tightly coordinated response. We had previously reported that even short-term NAA pretreatments-involving immersion of the basal 2-3 cm of shoot cuttings in a 500 mg/L NAA solution for 3 minutes-resulted in a similar enhancement of total terpenoid content in subsequently grown *E. trigona* plants (Rezayi et al., 2022).
The central role of PAL is consistent with its established position as a regulatory node in plant defense, often targeted by various elicitors (Zhang et al., 2015; Rasool et al., 2021). It is also noteworthy to consider that *Euphorbia* peroxidase exhibits catalase-like activity, decomposing hydrogen peroxide into water and oxygen, and can also form superoxide anions through cyclic reactions (Mura et al., 2006). The concomitant rise in POX activity aligns with its documented function in *Euphorbia* species, where it contributes to both the defensive oxidative burst and the scavenging of resultant ROS (Medda et al., 2003; Pintus et al., 2008). Furthermore, our analysis reveals a more complex metabolic interplay. Specifically, based on the results of the hierarchical clustering analysis (Fig. 7), the strong and positive correlation between shoot terpenoid, flavonoid, and flavonol content and shoot phenylalanine ammonia-lyase activity cannot be attributed to a dependence on common precursors, as there is no correlation between the total content of terpenoids and total phenolic compounds. Therefore, it seems plausible that a considerable portion of the phenolic compounds dependent on phenylalanine ammonia-lyase activity are specialized phenolic compounds with branched or terpenoid side rings (such as terpenoid coumarins).

3.4.2 Contrasting responses to other hormones and independent pathways

In contrast, the responses to other hormones were fragmented. IAA elevated SOD and POX activities and flavonol content, indicating a mild oxidative stress response, but failed to trigger the full defensive metabolon. IBA's significant suppression of POX, despite increasing SOD, points to a potential disruption of the ROS scavenging system, highlighting the complex and sometimes antagonistic effects auxins can have on different components of the antioxidant apparatus (Kellos et al., 2008; Wang et al., 2013). The significant suppression of total phenolics by GA_3, without a corresponding decline in PAL-dependent flavonoids or PAL activity itself, is particularly insightful. It suggests that

GA_3 may selectively inhibit specific branches of phenolic biosynthesis independent of the PAL gateway enzyme, a finding that resonates with reports of variable hormonal effects on phenolic production (Sedighi et al., 2014; Kulbat, 2016). This fractionated regulation is further evidenced by the isolated clustering of total phenolics and anthocyanins from the core defense module elicited by NAA.

The segregation of total phenolics and anthocyanins into distinct clusters, separate from the core defense-growth network orchestrated by NAA, indicates that their biosynthesis in *E. trigona* is governed by regulatory mechanisms independent of this elicitation pathway. This physiological independence is further evidenced by the lack of significant response in anthocyanin content to any hormonal treatment, suggesting a critical evolutionary constraint to maintain the stability of these pigments due to their fundamental roles in plant physiology. The observed fractionated regulation within the phenolic spectrum is consistent with previous findings (*e.g.*, (Sedighi et al., 2014)), where gibberellin suppressed total phenolic content without affecting flavonoids, highlighting the complex and often compartmentalized nature of phenolic metabolism. Notably, the insensitivity of anthocyanin pathways to NAA, in stark contrast to the strong responsiveness of other flavonoids and flavonols, underscores the specificity of this elicitor and suggests that anthocyanin homeostasis is prioritized and tightly controlled under these experimental conditions.

The enzyme SOD also showed a more isolated pattern, suggesting a role potentially more related to general oxidative stress management than to the specific elicited defense pathway.

Beyond defense metabolism, GA also exhibited a divergent role in primary metabolism. We previously demonstrated that foliar application of GA_3 at 250 mg/L for *E. trigona* resulted in the most significant increase in chlorophyll a, b, total chlorophyll, and carotenoid content among all hormonal treatments (Rezaei et al., 2023). This finding highlights a divergent role of GA in primary metabolism (photosynthesis) versus secondary metabolism (defense-related terpenoid biosynthesis) in this species. The ability of GA to boost carotenoids (which are themselves terpenoid compounds) further illustrates the nuanced and compartmentalized nature of terpenoid metabolism regulation, where specific branches can be selectively activated by different elicitors.

3.4.3 Metabolic reprogramming and specificity of NAA

The GC-MS metabolomic profiling provides a deeper layer of evidence for NAA's profound impact, causing a marked shift in the shoot metabolome. The dramatic increase in total terpenoid content estimated by the colorimetric assay suggests a massive redirection of metabolic flux toward terpenoid biosynthesis pathways upon NAA treatment. However, GC-MS profiling revealed that this was not a uniform increase across all terpenoids. Instead, NAA caused a profound reprogramming of the terpenoid profile, characterized by a sharp increase in the relative abundance of Bis(2-ethylhexyl) phthalate and a decrease in specific triterpenoids like epifriedelinol. This indicates that NAA not only acts as a general booster of terpenoid production but also as a specific regulator that shifts the metabolic equilibrium toward the synthesis of particular compounds, potentially enhancing the plant's defense portfolio in a targeted manner. Similarly, Bagheri et al. (2025) demonstrated that fertilizer application could qualitatively reprogram the terpenoid profile in *Euphorbia tirucalli*, another succulent species within the same genus. Their GC-MS analysis revealed that combined iron chelate and NPK fertilizer treatment led to the production of 11 distinct bioactive terpenoids, compared to only 3 in control plants. This finding underscores that targeted biochemical interventions, whether hormonal or nutritional, can effectively shift metabolic flux in *Euphorbia* species toward a more specialized and therapeutically relevant chemical repertoire.

3.4.4 Proposed mechanism for NAA-induced coordinated response

Based on our integrated results, a mechanistic model for NAA's action as a potent elicitor in *E. trigona* can be proposed. Unlike the endogenous auxin IAA, the synthetic and more stable NAA is perceived by specific auxin receptors, potentially initiating a unique signaling cascade that mimics a mild abiotic stress (Simon et al., 2011; Zhang et al., 2022). This perceived stress triggers a controlled oxidative burst, evidenced by the significant upregulation of SOD and POX activities, which in turn acts as a secondary signaling node. A key event in this cascade is the specific and strong upregulation of PAL, the gateway enzyme to the phenylpropanoid pathway. We hypothesize that NAA signaling leads to the transcriptional activation of *PAL* genes, channeling metabolic flux toward the synthesis of flavonoids and flavonols (Zhang et al., 2015). Concurrently, NAA appears to profoundly reprogram terpenoid metabolism, likely through the transcriptional activation of key genes in the MEP (Methylerythritol Phosphate) or MVA (Mevalonate) pathways, such as those encoding terpene synthases (TPS) (Tholl, 2015). The remarkable five-fold increase in total terpenoids, coupled with the qualitative shifts observed in the GC-MS profile, strongly supports this notion of transcriptional reprogramming.

The co-regulation of PAL and terpenoid pathways, despite their distinct biochemical origins, suggests that NAA elicits a "defense metabolon" – a transient supramolecular complex that coordinates the flux of resources and energy toward the synchronized production of diverse defense compounds (Weng et al., 2019). This coordinated response is likely mediated by integrated hormone signaling networks, where NAA-triggered signals potentially cross-talk with other defense hormones like jasmonates (Pérez-Llorca et al., 2023). Furthermore, the significant investment in root biomass under NAA treatment indicates a strategic resource reallocation to support this energetically demanding biosynthetic effort (Erb et al., 2020). This model positions NAA not merely as a growth regulator, but as a master switch that activates a pre-programmed, coordinated defense network in *E. trigona*, enhancing the production of valuable bioactive compounds without incurring severe growth penalties.

3.5 Ecological and practical implications

The profound qualitative shift in the terpenoid profile revealed by GC-MS signifies not just a general increase in production, but a precise metabolic reprogramming that may be beneficial for shaping root microbiome composition or deterring specific pests (Jacoby et al., 2021). The increased peroxidase activity not only plays a role in scavenging ROS but also likely contributes to strengthening the cell wall through lignification and creating physical barriers against pathogens (Almagro et al., 2009). Farnia et al. (2022) reported that NPK fertilizer application significantly reduced root growth in *Euphorbia tirucalli*, and the addition of various sulfur fertilizers generally failed to mitigate these negative effects and even further suppressed shoot biomass. This parallels the growth-inhibitory or neutral effects we observed with gibberellin and IBA, underscoring that the response of succulent *Euphorbia* species to external biochemical stimuli is highly specific and can incur significant trade-offs with growth. Their findings reinforce the notion that the efficacy of an elicitor is not solely measured by its ability to enhance metabolite production but also by its minimal detrimental impact on plant vitality, a balance notably struck by NAA in our study. Our findings provide a strategic basis for using NAA as a cost-effective and powerful tool in tissue culture or greenhouse cultivation to enhance the production of valuable pharmaceutical metabolites in medicinal plants (Espinosa-Leal et al., 2018). It is important to note that bis(2-ethylhexyl) phthalate (DEHP) is also a common industrial plasticizer and environmental contaminant (Zanotelli et al., 2010; Kubwabo et al., 2013; Boran et al., 2019). However, its significant and treatment-specific increase ($\sim$ 5-fold under NAA), alongside its previously reported induction in other succulent plants under specific fertilizer treatments (Farnia et al., 2022; Jokar et al., 2023), strongly suggests a biological origin in this context rather than mere contamination. Fortunately, certain bacteria, such as *Mycobacterium* sp., can degrade DEHP into less harmful products, indicating potential for bioremediation (Nakamiya et al., 2005).

4. Concluding remarks

In conclusion, this study unequivocally positions naphthalene acetic acid (NAA) as a superior abiotic elicitor in *E. trigona*, distinct from other auxins or gibberellin. The novelty and principal finding of this work is the demonstration that NAA uniquely orchestrates a coordinated defense metabolon, integrating enhanced root biomass with the synergistic upregulation of key enzymes (PAL, POX, SOD) and the substantial accumulation of valuable bioactive compounds, most strikingly a five-fold increase in terpenoids. This coordinated response was not triggered by IAA, IBA, or GA_3, highlighting the specificity of NAA's action. Furthermore, the GC-MS analysis revealed a profound reprogramming of the terpenoid profile under NAA, moving beyond a simple quantitative increase to a qualitative shift in the plant's metabolic output. These findings establish a robust strategic framework for employing targeted NAA application as an effective and precise tool for the phytochemical enhancement of medicinal plants. Future research perspectives should focus on elucidating the underlying molecular mechanisms. This includes investigating the transcriptional regulation of key biosynthetic genes (*e.g.*, in the phenylpropanoid and MEP pathways) in response to NAA using techniques like RT-qPCR or RNA-seq. The intriguing hypothesis of a NAA-induced "defense metabolon" could be explored through protein-protein interaction studies. Furthermore, research should validate the efficacy of NAA-elicited plant extracts in vitro for enhanced pharmaceutical properties, such as antimicrobial or anticancer activities. Finally, exploring the synergistic effects of NAA with other established elicitors like methyl jasmonate could unlock even greater potential for optimizing the production of valuable metabolites in *E. trigona* and related species.

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Abbreviations

ANOVA: Analysis of Variance; **CV:** Coefficient of Variation; **DEHP:** Bis(2-ethylhexyl) Phthalate; **DW:** Dry Weight; **EI:** Electron Impact; **FW:** Fresh Weight; **GA_3:** Gibberellic Acid; **GC-MS:** Gas Chromatography-Mass Spectrometry; **IAA:** Indole-3-Acetic Acid; **IBA:** Indole-3-Butyric Acid; **NIST:** National Institute of Standards and Technology; **NAA:** 1-Naphthalene Acetic Acid; **PAL:** Phenylalanine Ammonia-Lyase; **PGRs:** Plant Growth Regulators; **POX:** Peroxidase; **ROS:** Reactive Oxygen Species; **SOD:** Superoxide Dismutase. **TIC:** Total Ion Chromatogram.

Authors contributions

Hakimeh Rezaei conducted the laboratory operations and parts of the statistical analysis, and wrote the initial draft of the manuscript. Aryan Sateei supervised the research, designed the experiments, contributed to parts of the statistical analysis, and performed editing, completion, and translation of the manuscript. Tahereh A. Aghajanzadeh supervised and provided guidance and consultation for certain measurements. Mehdi Ebadi provided guidance and consultation for certain measurements.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests

The authors declare that there is no conflict of interest.

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