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The role of copper sulfate in modulation of mechanical stress responses and ethyl acetate emission in date palm tissues

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Abstract
In this study, copper sulfate ($CuSO_4$) was evaluated as affected factor on date palm plantlet in tissues culture on emission of ethyl acetate which considers the important volatile organic compounds (VOCs) of kairomone for red palm weevil, some chemical compounds and some activity of antioxidant enzymes. Plantlets were treated with different concentration with $CuSO_4$ (0, 5, 10, 20mg/L tissue culture media). The treated plantlets with each concentration of $CuSO_4$ were divided to healthy plantlets and mechanically injured and incubated at growth room. GC-MS analysis was used to estimation of ethyl acetate emission in plantlets samples and the high level of emesis ethyl acetate at injured plantlets with free of $CuSO_4$. Biochemical compounds were estimated and the results referred to high level of phenol was at the treated plantlets with $CuSO_4$ (5mg/l) with injured while high level of total indol and GA_3 were at treated plantlets with $CuSO_4$ (5mg/l) with uninjured. High level of amino acid and flavonoids were recorded at untreated plantlets with $CuSO_4$ in uninjured plantlets in amino acid and injured in flavonoids. High level of activity antioxidants enzymes (SOD, CAT, and ADH) and the proline levels were recorded in the injured plantlets with 20mg/l of $CuSO_4$. Chlorophyll a and b levels were the highest at treated plantlets with 20mg/L $CuSO_4$ with uninjured samples

Keywords: *Phoenix dactylifera* L., ethyl acetate, phytohormones, GC-MS Analysis, volatile compounds, antioxidant enzymes

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the earliest cultivated plants that is a valuable agricultural crop in arid and semi-arid regions (Johnson, 2011) [24]. It is a vital part of rural economies, the environment, and ensuring food security in North Africa and the Middle East (Mendel *et al.* , 2024) [28]. Date palm cultivation has increased lately because of its unique ability to tolerate hostile growing conditions and its high nutritional and economic value. Traditional date palm propagation, which involves using offshoots (suckers) to propagate palms vegetatively, is still a common method in commercial date palms since it preserves the genetic identity and performance of elite cultivars (Johnson, 2011) [24]. Offshoots are axillary buds originating from the lower portion of the mother palm and act as the primary planting material in a typical commercial orchard (Chouibani *et al*., 2022) [10]. Sustainable offshoots are therefore essential for effective production, crop selection, and reducing pest spread (Johnson, 2011) [24]. One of the most significant challenges to date palm farming among biotic restrictions is the red palm weevil (RPW; *Rhynchophorus ferrugineus* Olivier), the most devastating insect pest in the world (Faleiro, 2006) [16]. The pest has expanded quickly since its introduction in the Middle East and central Mediterranean basin (Al-Dosary *et al.*, 2016) [4], severely harming many date palm varieties economically. The destructive pests are mostly caused by the introverted feeding habits of larvae within the palm tissues, causing delayed detection of the infection (Wakil *et al*., 2024) [39]. Females prefer to lay eggs in wounds, pruning sites, and soft palm tissues like near the base of the offshoots in suitable feeding sites. After hatching, the larvae silently mine inside the upper tissues, disintegrate the conducting tissues, and weaken the palm character precipitating its collapse often before control measures can be applied (Faleiro, 2006) [16].

The host-location response of the red palm weevil is predominantly mediated by kairomones, volatile organic compounds (VOCs) emanating from the healthy, wounded or fermenting date palm tissues (Hoddle *et al.* , 2024) [22]. Volatiles like ethanol, ethyl acetate as well as other fermentation products inhibit the host location and trap attractiveness response synergistically to the aggregation pheromone ferrugineol, promote enhanced host location and oviposition response (Faleiro, 2006 and Favaro *et al.* , 2024) [16, 17]. Mechanical injuries like pruning or offshoot removal also found to trigger microbial invasion and fermentation of host palms tissues promoted by the mechanical injury, this way resulting in facilitated production of attractive kairomone volatiles. As a result, protecting the wounds of the palms has been viewed as an important element of any strategies targeting generic integrated pest management (IPM) control of the weevil populations. Copper sulfate ($CuSO_4$), or other copper compounds, being a widely employed fungicidal and bactericidal agent, may be efficient in reducing the microbial invasion as well as fermentation rate in palm tissues, this way, much declining the production

of kairomone related volatiles in the host palms tissue without interfering with the integrity of the insect olfaction capable receptors (Hoddle *et al*., 2024) [22].

The present study is for addressing the relation between copper supplementation, date-palm physiology and emission of an RPW-associated chemical cue. The role played by Cu in the regulation of ethyl acetate emission has been successfully incorporated into RPW kairomone trapping systems, these two research areas have largely been explored separately.

Material and methods

1. Experimental Design, Plant Material, and Copper Sulfate Treatments

Phoenix dactylifera L. plantlets (35cm in length) were continuously subcultured on MS basal medium with 3% (w/v) sucrose and solidified with 0.7% (w/v) agar. The pH value of medium was adjusted to 5.8 before autoclave (121ºC, 20 min). For *in vitro* cultures, the temperature was 25±2º C with a 16/8h photoperiod.

For this study, copper supplement was added to MS medium to test the impacts on volatile emission levels and physiological and biochemical parameters. $CuSO_4$ was added to a MS base at 0.025 mg/L 1of Cu, which is the normal Cu level in MS medium, creating four treatments: C0, which is the normal MS without added copper (control); C1, MS with an addition of 5mg/L 1$CuSO_4$; C2, MS with an addition of 10 mg/L$CuSO_4$; and C3, MS with an addition of 20mg/L$CuSO_4$.

Three culture jars represented three biological replicates per treatment, with five plantlets in each culture jar. After the $CuSO_4$ treatments, plantlets were either mechanically wounded or not wounded for volatile collection.

Mechanical injury, as encountered in routine micropropagation work, was mimicked by preparing standardized wounds on leaves of mature *in vitro* date palm plantlets. Each leaf was wounded with a sterile scalpel by making three horizontal incisions about 5 mm long. Wounded and unwounded plantlets were then transferred to growthroom.

For volatile collection, individual plantlets were enclosed in 500-ml sealed glass jars fitted with inlet and outlet tubes. Volatiles within the jar headspace were collected immediately after mechanical injury to analyze wound induced volatiles. The volatile samples were then analyzed using GC-MS.

2. GC-MS Analysis of Ethyl acetate

The ethyl acetate as a volatile organic compound released from date palm plantlets were analyzed using a gas chromatography mass spectrometry (GC-MS) system with a capillary column (HP-5MS, 30m, 0.25 mm 25 m film thickness, or equivalent). Helium was employed as the carrier gas, which passed at a steady flow rate (1 ml/min).The injector temperature was 250ºC.The oven temperature was 40ºC for 2 min and then increased to 200ºC at a rate of 5 C/min. The oven temperature was then increased at a rate of 15ºC min to 250 ºC. Spectra (mass to charge ratio, m/z) were recorded in the electron ionization (EI) mode at 70 eV. For qualitative analysis, the retention time and mass spectral data were compared with authentic compounds and the mass spectrum to NIST library of mass spectral data was used to confirm the identity of EAD. Quantification of acetate was achieved using an external standard calibration curve constructed with authentic acetate. The amount of acetate was normalized by fresh tissue weight and sampling period (e. g00 $^{-1}$ FW $^{h-1}$).

Date palm plantlets were evaluated for total chlorophyll level with a portable chlorophyll meter (CCM-200 Plus Chlorophyll Content Meter; Opti-Sciences Inc.). The indicator values on the combined samples corresponding on the biological replicates were used. Results were shown as g/ fresh weight (FW).

3. Determination of chemical compounds

Sample preparation:

Fresh material of date palms (1 gm) was grounded in a chilled mortar and pestle with 10 ml of 80% ethanol. Samples were incubated overnight in darkness at 4ºC. Samples were then centrifuged at 13,000Xg for 5 minutes and the supernatants collected. Supernatant was used for detection of total phenol, total indoles, total free amino acids, total flavonoids and GA3.

a. Proline Content:

The free proline content was determined following the method described by Bates *et al* (1973) [5]. Fresh plant tissue was extracted and reacted with the corresponding ninhydrin based reagent. The chromophore was extracted and its absorbance was measured spectro-photometrically at 520 nm. The proline content was calculated and presented in g FW.

b. Phenolic compound analyses:

Total phenolics were measured as described by Ainsworth and Gillespie (2007) [2]. One ml of the ethanolic extract was pipetted into a tube and mixed with 5 ml of distilled water and 500L of Folin Ciocalteu reagent and shaken for 5 min. A further addition of 1.5 ml of sodium carbonate (20%) was added and the total volume was made up to 10 ml with distilled water. The reacting mixture was allowed to stand in the dark at room temperature for 2h and absorbances were taken at 765 nm with spectrophotometer.

c. Total free amino acids

Total free amino acids was measured using McGrath (1972). One ml of ethanolic extract was diluted in 4 ml of deionized water, 1 ml of ninhydrin reagent was added, and the samples were then placed in a boiling water bath at 100ºC for 15 min before cooling to room temperature.

After, 1 ml of 50% ethanol was added to the samples before measuring the absorbance at 570 nm.

d. Photosynthetic pigments

According to Wettstein (1957) [40], the plant pigments such as total carotenoids, chlorophyll a, and chlorophyll b were

measured. Fresh leaf tissue (1g) was homogenized in 85% acetone with calcium carbonate ($CaCO_3$) and sterile sand. After filtering the homogenate. An Orion AquaMate 8000 spectrophotometer was used to detect absorbance at 440, 640, and 660 nm after the filtrate volume was adjusted to 15 mL with 85% acetone. The amounts of carotenoid, chlorophyll a, and chlorophyll b were then determined using the formulas provided by Wettstein (1957) [40].

4. Determination of total Indoles

The total indole assay was carried out following the method described by Selim *et al.* (1978) [31]. One ml of ethanolic extract was combined with 4 ml of para-dimethylaminobenzaldehyde (PDAB) reagent and incubated in 37ºC for 1 h. The absorbance was measured at 530 nm using spectrophotometer.

5. Extraction and quantification of gibberellic acid (GA 3)

GA 3 was isolated as previously described for GA 3 by Sayed *et al* (2019) [30]. 1 N Hcl was used to lower the extract pH to 2.8, and then the extract was washed three times with ethyl acetate. the lower aqueous phase was discarded and the ethyl acetate fractions mixed and used for the determination of GA3. The concentration of GA3 was evaluated as described by Udagawa and Kinoshita (1961) [35]. The ethyl acetate extract (300L) was mixed with 1 ml of 10N HCl and following 1 ml of 25% a 10 N HCl solution was added.

The mixture was vortexed thoroughly and left in a boiling water bath for 5 min. It was then brought down to room temperature before absorbance was read at 750 nm with spectrophotometer.

All biochemical and enzymatic analyses were carried out on three biological replicates for each treatment and expressed on a fresh weight basis where relevant.

6. Activity of antioxidant enzymes SOD, CAT and ADH

Superoxide dismutase (SOD) activity was estimated by nitro blue tetrazolium (NBT) method of photochemical reduction of NBT (Beauchamp and Fridovich, 1971 [6]) using the enzyme extract, 0.10M potassium phosphate buffer, methionine (1.5m M), Triton X-100, NBT and riboflavin. The reaction mixtures were photosensitized by keeping them in light, absorbance at 560 nm was taken with the help of spectrophotometer. SOD activity was reported as units (Ug^{-1} FW).

Catalase (CAT) Activity was determined using a method modified from Putter (1974) by measuring the breakdown of H_2O_2 into water and oxygen.The reduction in absorbance was measured spectrophotometrically at 240nm. CAT activity was expressed as Ug^{-1} FW.

Alcohol dehydrogenase (ADH) activity was measured based on procedure outlined by Chung and Ferl (1999) [11]. Spectrophotometrically enzyme activity was determined by rate of NAD ^+ /NADH-dependent reaction at 340nm. ADH activity was calculated as: Ug^{-1} FW.

7. Statistical analysis

The experimental design was a completely randomized experiment with three replications. The SPSS program was used to evaluate the data. Data were subject to analysis of variance (ANOVA) to compare the effects of the copper sulfate levels and mechanical damage. The differences among the means were compared using Least significance difference (LSD) multiple range tests.

Results

1. GC–MS analysis of ethyl acetate emission

GC–MS analysis revealed a markedly response pattern for ethyl acetate emission using different copper treatments under normal and wounding stress condition. Under non-stressed conditions, ethyl acetate concentrations were 330, 0.22234, 0.31202, and 0.23410 ppb in C0, C1, C2, and C3, respectively. Mechanical stress increased ethyl acetate emission in all corresponding treatments, reaching 0.45227, 0.94135, 0.48750, and 0.40050 ppb in C0*, C1*, C2*, and C3*, respectively.

The strongest response was therefore observed in the low-$CuSO_4$ stressed treatment (C1*), in which ethyl acetate emission increased approximately 4.2-fold relative to the corresponding non-stressed C1 treatment and approximately 2.9-fold relative to the untreated control. This result identifies C1* as the most responsive treatment for ethyl acetate production despite its comparatively moderate physiological stress profile.

The increasing $CuSO_4$ concentration under mechanical stress causes progressive reduction of ethyl acetate emission although several stress-associated physiological responses have enhanced concomitantly. The C1* treatment with the lowest $CuSO_4$ concentration, the maximum level of ethyl acetate is 0.941 ppb, while the inermediate and highest $CuSO_4$ the emissions reduce to 0.487 and 0.400 ppb respectively, representing a decrease of approximately 48.2% and 57.5% in relative terms to C1*.It is important that this reduction happened even though $CuSO_4$ at the highest concentration triggered intense activation of stress-responsive metabolism. C3* was the only sample that exhibited highest proline concentration (12.4 g g^{-1} FW), SOD activity (154 U g^{-1} FW), Cat activity (145 U g^{-1} FW), ADH activity (51.7 U g^{-1} FW), and because of this revealed the tissues were still metabolically responsive and highly expressed the antioxidant and stress-adjustment systems. As such, the reduction of ethyl acetate emission in connection to higher $CuSO_4$ concentrations cannot be tied to a loss of plant physiological activity. These results suggested that increasing $CuSO_4$ leads to a decrease in the metabolic processes related ethyl acetate formation and release while the broader stress-defense pathways are being activated. The ethyl acetate emission suppression at a $CuSO_4$ treatment is an mainly significant finding of the current study since it reflects the capability of a $CuSO_4$ treatment to modulate a volatile chemical compound possibly involved in date palm, red palm weevil chemical communication.

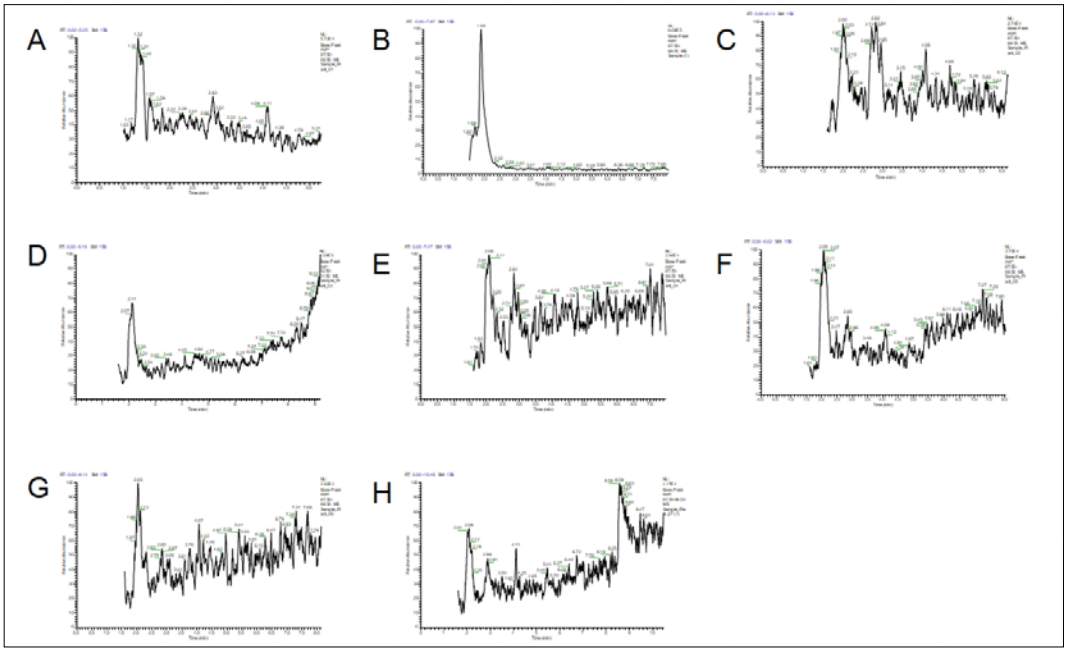

Fig 1: Effect of $CuSO_4$ treatment and mechanical stress on ethyl acetate emission from date palm tissues detected by GC–MS. Ethyl acetate emission was analyzed in date palm tissues exposed to different concentrations of $CuSO_4$ (0, 5, 10, and 20 mg L^{-1}), either in the absence or presence of wounding stress. Control represents untreated, non-stressed tissues, whereas Control*, 5 mg*, 10 mg*, and 20 mg* represent the corresponding $CuSO_4$ treatments subjected to mechanical wounding. Ethyl acetate was identified by GC–MS based on its characteristic mass spectral profile and chromatographic retention time, and its relative abundance was determined from the detected chromatographic signal.

The physiological and biochemical responses of date palm tissues to $CuSO_4$ concentration and mechanical wounding was examined separately and in general agreed with a cascade of responses assuming distinct patterns, increasing $CuSO_4$ concentration raised many stress associated response parameters such as Proline and SOD, CAT and ADH activities and total phenol, while chlorophyll cell content remain unaffected in the non-stressed tissues compared to stressed tissues, though the significant response was found to differ widely across the various parameters. Mechanical wounding appeared to have altered the parameters as well, usually leading to elevation or production of selected defence associated metabolites and sometimes suppression of selected growth and metabolism associated parameters.

Proline accumulation was also more distinct than for chlorophyll content in the non-wounded treatments per $CuSO_4$ concentration, reflecting that in this case, proline metabolism in date palm tissues is highly sensitive to Cu stress. In the non-wounded treatments, proline concentration was progressively higher in the increasing from 1.3 at C0 up to 2.0, 6.0, 6.9 at C 1, C2 and C 3 respectively. Mechanical injury caused a pronounced increase in proline level at all $CuSO_4$ concentrations. Wounded treatments C0*, C1*, C2* and C3* of 2.5, 4.5, 10.6 and 12.4 were greater by assumed 2, 2.3, 1.8 and 1.8 fold respectively than corresponding non-wounded treatments.

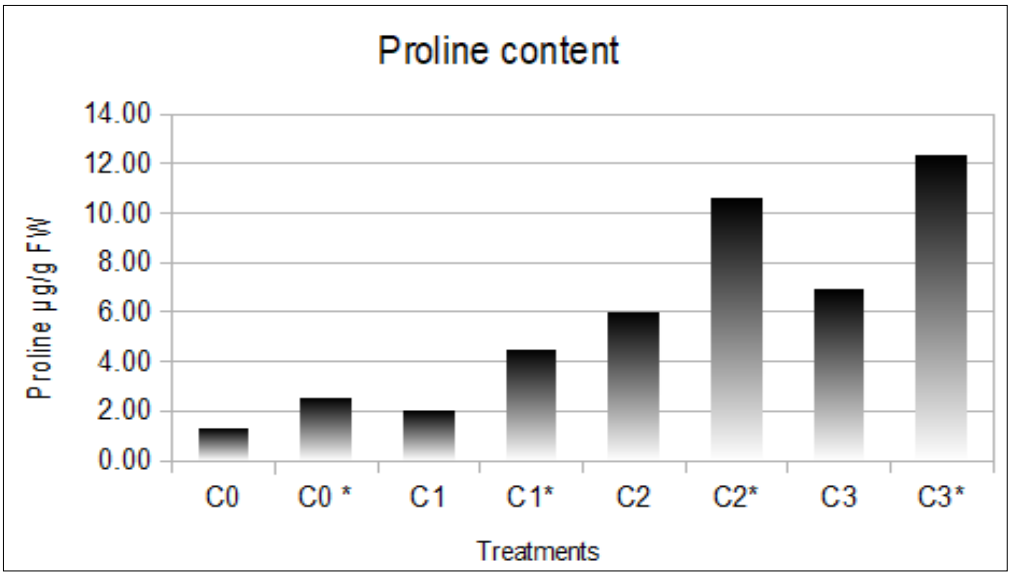

Fig 2: Effect of copper sulfate treatment and tissue injury on proline content in date palm tissues. The proline content were determined in date palm tissues subjected to different concentrations of $CuSO_4$ C0, C1, C2 and C3(0, 5, 10, and 20 mg L^{-1}). Control represents untreated tissues, whereas Control*C0, C1*, C2* and C3*(0 , 5 mg, 10 mg, and 20 mg) represent the corresponding $CuSO_4$ treatments following tissue wounding. Values are expressed as mg g^{-1} fresh weight (FW).

SOD activity was increased significantly in response manner with $CuSO_4$ ranging from 60U g $^{-1}$FW in C0 in to 75, 85 and 110U g $^{-1}$ FW in C1, C2 and C3 respectively. Mechanical stress resulted in significant up regulation 75, 90, 100 and 154U g $^{-1}$ FW in C0*, C1 *, C2* and C3* respectively, with the maximum in C3* (2.8 times C0). CAT activity was also quite a bit induced at C1, C2 and C3 70, 120 and 145U g $^{-1}$ FW respectively from 70U g $^{-1}$ FW in C0, a factor of 2.0 and 2.1 respectively, and C3* respectively, with a maximal activity of 145U g $^{-1}$ FW (2.1 times control).

ADH activity was also markedly induced at C1, C2 and C3 22.0, 35.0 and 51.7 U g $^{-1}$ FW respectively (2.1 times control). These values were also significant (p<0. 001) increased over addition of $CuSO_4$ alone (Figure 3). As $CuSO_4$ concentration increased SOD activity progressively increased from 60 Ug $^{-1}$FW in C0 to 75, 85 and 110 U g $^{-1}$FW in C1, C2 and C3, respectively.

Mechanical damage further increased SOD activity to 75 90 100, and 154 U g ^{1} FW respectively in C0*, C1*, C2*, C3*, the highest activity being in C3* (2.6 times the control). Analogously the activity of catalase (CAT) increased from 70 U g ^{1}FW in C0 to 120 U g 1 FW in C3 and reached a value of 145 U g ^{1} FW in C3* approximately twice that of control (Figure 3). An increase in alcohol dehydrogenase (ADH) activity was also noted from 22. 0 U g ^{1} FW in C0 to 35.0 U g ^{1} FW in C3 and 51.7 U g ^{1} FW in C3*, this was 2.4 times of the control.

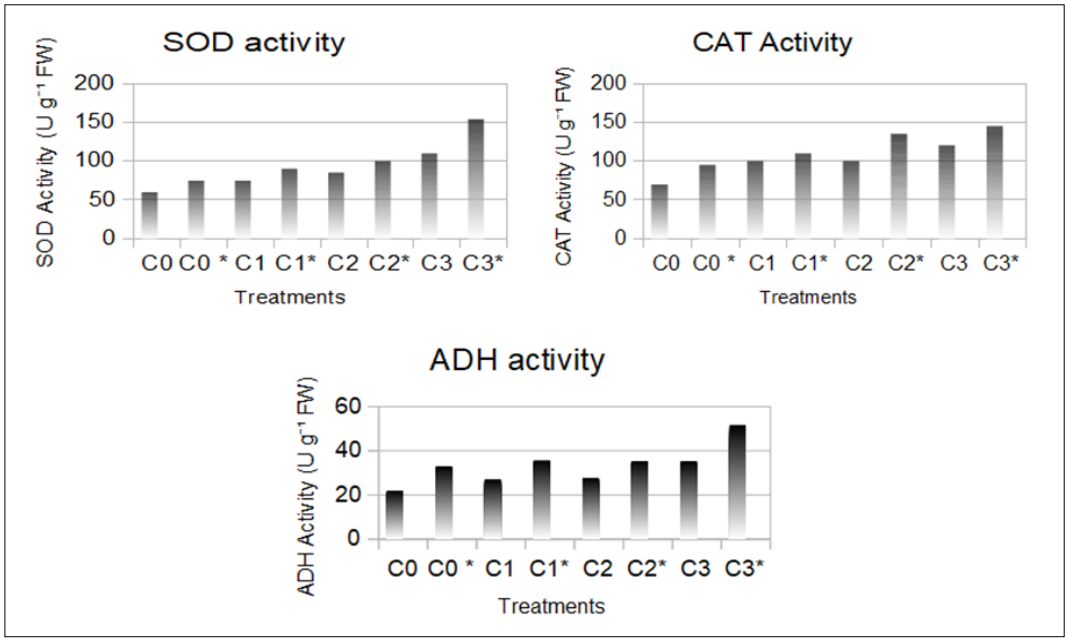

Fig 3: Effect of copper sulfate treatment and tissue injury on ROS activity (SOD, CAT and ADH activity) in date palm tissues. The SOD, CAT and ADH activity were determined in date palm tissues subjected to different concentrations of $CuSO_4$ C0, C1, C2 and C3(0, 5, 10, and 20 mg L^{-1}). Control represents untreated tissues, whereas Control*C0, C1*, C2* and C3*(0, 5 mg, 10 mg, and 20 mg) represent the corresponding $CuSO_4$ treatments following tissue wounding. Values are expressed as mg g^{-1} fresh weight (FW).

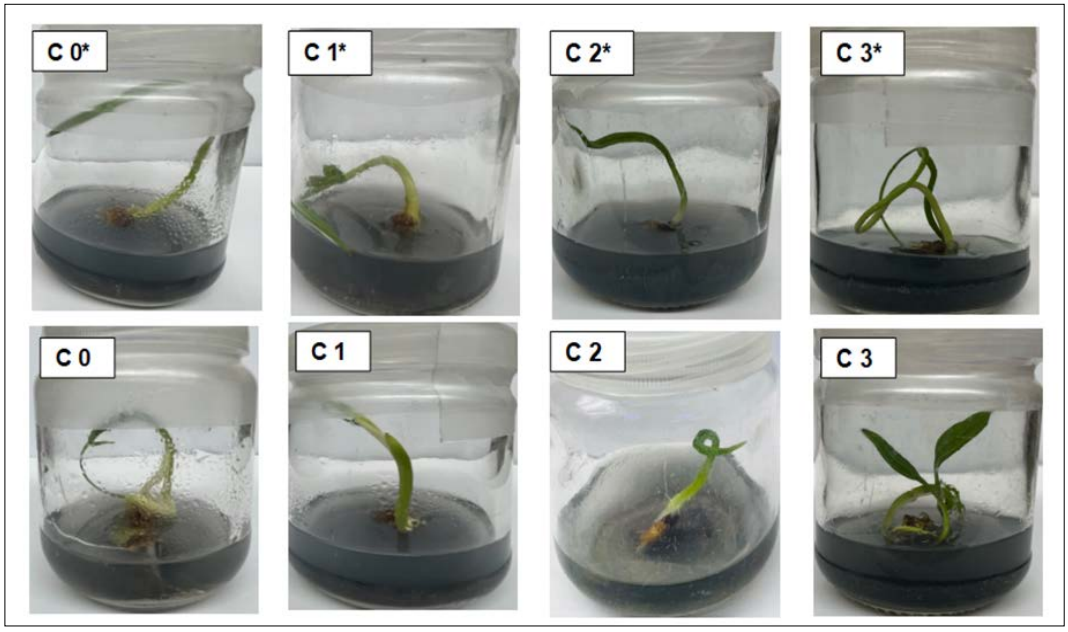

Fig 4: Effect of copper sulfate treatment and tissue injury o n date palm tissues. The date palm tissues subjected to different concentrations of $CuSO_4$ C0, C1, C2 and C3(0, 5, 10, and 20 mg L^{-1}). Control represents untreated tissues, whereas Control*C0, C1*, C2* and C3*(0 , 5 mg, 10 mg, and 20 mg) represent the corresponding $CuSO_4$ treatments following tissue wounding.

The biochemical metabolites that affect the growth and defense the overall biochemical profile also exhibited strong treatment-dependent $CuSO_4$ and mechanical stress responses. The highest antioxidant activity value of the non-wounded treatments was generated under the 5 mg L $^{-1}$ $CuSO_4$ was 91.2% concentration while the 5 mg L $^{-1}$ $CuSO_4$ concentration exhibited the highest indole concentration (0.08 mg g $^{-1}$) for all treatments and was statistically similar to the untreated control (0.08 mg g $^{-1}$). Yet the 20 mg L $^{-1}$ $CuSO_4$ concentration treatment generated the highest concentration of GA 3 (56.7 mg g $^{-1}$), total amino acid content (8.2 mg g $^{-1}$), chlorophyll a (7.2 mg g ^{1}), and chlorophyll b (8.9 mg g $^{-1}$). Mechanical wounding caused a significant change in this biochemical profile. The wounded C1* treatment (5 mg L $^{-1}$) had the greatest quantity of total phenolics (7.4 mg g $^{-1}$), compared to 2.8 mg g $^{-1}$ found in the corresponding non-wounded treatment, representing a 2.6-fold increase in phenolics following wounding. This is in agreement with the generally accepted role of wound signaling in the activation of phenylpropanoid metabolism and phenolic biosynthesis. Mechanical wounding is known to activate ROS-, ethylene- and jasmonate-mediated signaling pathways that can then in turn activate phenylpropanoid related metabolism and causes the formation of phenolic compounds that provide protection and defense at the wound site. Though, the biochemical antioxidant activity responded distinctly. At 5 mg L $^{-1}$ $CuSO_4$, the antioxidant activity decreased from 91.2% in the non-wounded treatment to 62.6% after wounding. In the same way, at 10 mg L $^{-1}$ and 20 mg L $^{-1}$ $CuSO_4$, the antioxidant activity also decreased from 82.8% to 57.8% and from 73.5% to 53%, respectively.

GA_3 concentrations also varied with $CuSO_4$ treatment. The non-wounded 20 mgL $^{-1}$ treatment had the highest value of GA3 (56.7 mg.g $^{-1}$). The mechanical wounding for this concentration was found to have decreased to 29.9 mg.g $^{-1}$). Similar results found for the control were seen at the 5 and 10 mg.g $^{-1}$ concentrations with levels dropping from 42.9 to 14.1 mg.g $^{-1}$ and from 32.4 to 22.1 mg.g $^{-1}$ respectively. This indicated that the mechanical wounding affected the flux balancing growth associated and defense associated metabolism.

Total amino acids followed a similar pattern also, the non-wounded 20 mg.g $^{-1}$ treatment had the highest value of all treatments (8.2 mg.g $^{-1}$), and by wounding this value was decreased to be 3.5 mg.g 1. The C2 treatment (10 mgL $^{-1}$) increased after wounding from 1.27 mg.g $^{-1}$ to 2.1 mg.g $^{-1}$. The C1 treatment increased after wounding from 0.28 mg.g $^{-1}$ to 0.65 mg.g $^{-1}$. Wounding found to be did not have an entirely inhibitory effect on amino acid metabolism and its effect was concentration-dependent.

Flavonoids also responded to treatments. The control treatment (C0) had 3.7 mg.g $^{-1}$, and the wounded control had the highest value of 4. 2 mg.g $^{-1}$. The C3 treatment also also found to be increased from 1.2 mg.g $^{-1}$ to 3.4 mg.g $^{-1}$ for the wounded plants. These results, with the phenolic response, confirms that mechanical injury had a specific, rather than generalized, effect on secondary metabolism.

Table 1: Effect of copper sulfate treatment and tissue injury on biochemical properties; phenol, flavonoides, total amino acid, indol and GA3 content

compouds	Phenol mg/g	Indol mg/g	GA3 mg/g	Amino acid mg/g	Flavonoids mg/g
Control	4.78 ^{d}	0.08 ^{a}	38.79 ^{b}	8.09 ^{a}	3.67 ^{b}
Control*	5.49 ^{c}	0.04 ^{b}	34.73 ^{c}	2.93 ^{d}	4.22 ^{a}
C 1(5 mg)	2.84 ^{g}	0.08 ^{a}	42.86 ^{a}	0.197 ^{h}	2.49 ^{e}
C1*(5 mg)	7.35 ^{a}	0.04 ^{b}	14.11 ^{h}	0.649 ^{g}	3.17 ^{c}
C2 (10 mg)	3.65 ^{f}	0.04 ^{b}	32.37 ^{d}	1.265 ^{f}	1.23 ^{g}
C2 *(10 mg)	5.7 ^{b}	0.02 ^{c}	22.1 ^{f}	2.1 ^{e}	2.2 ^{f}
C3 (20 mg)	5.83 ^{b}	0.013 ^{c}	56.67 ^{a}	8.191 ^{a}	3.41 ^{c}
C3* (20 mg)	4.08 ^{e}	0.013 ^{c}	29.92 ^{e}	3.492 ^{c}	1.20 ^{g}
LSD	0.36	0.0158	0.52	0.38	0.36

The data were determined in date palm tissues subjected to different concentrations of $CuSO_4$ C0, C1, C2 and C3 (0, 5, 10, and 20 mg L^{-1}). Control represents untreated tissues, whereas Control*C0, C1*, C2* and C3*(0, 5 mg, 10 mg, and 20 mg) represent the corresponding $CuSO_4$ treatments following tissue wounding. Values are expressed as mg g^{-1} fresh weight (FW).

Photosynthetic pigments and carotenoids the biochemical pigment analysis showed a concentration-specific relationship that supported the physiological measurements of chlorophyll. The 20 mgL $^{-1}$ copper sulphate ($CuSO_4$) non-wounded treatment had the highest concent of the Chlorophyll a (7.2 mg.g $^{-1}$) and chlorophyll b (8.9 mg.g $^{-1}$).

These values were halved (3.9 mg.g $^{-1}$, 6.3 mg.g $^{-1}$ for chlorophyll a and b) post physical wounding. Conversely, concentrations of chlorophyll a and b of the C1 treatment were comparatively low (1.6 and 3.3 mg.g $^{-1}$, respectively) and concentration of carotenoids was at its highest value (1.1mg g $^{-1}$). Mechanical stress resulted in a decreasing the carotenoid concentrations in the C1 treatment to be 0.3 mg g $^{-1}$.

According to the previous results, the pigment responses may have been regulated by the level of $CuSO_4$ rather than the dose, since non-wounded plants grown on 20 mgL $^{-1}$ $CuSO_4$ produced the most chlorophyll while the lower $CuSO_4$ dose generated the greatest amount of carotenoid pigmentation. This may be indicative of the ability of date leaf tissues to sustain activities related to photosynthetic pigment formation with partaking in stress-meabition pathways upregulated at the tested treatments. Copper is a micronutrient involved in photosynthetic electron transport and that of pigmentation metabolism, though a high tissue Cu concentration is proven to inhibit the photosynthesis in Cu tolerant plants once a tissue dose exceeds the air plant's physiological threshold.

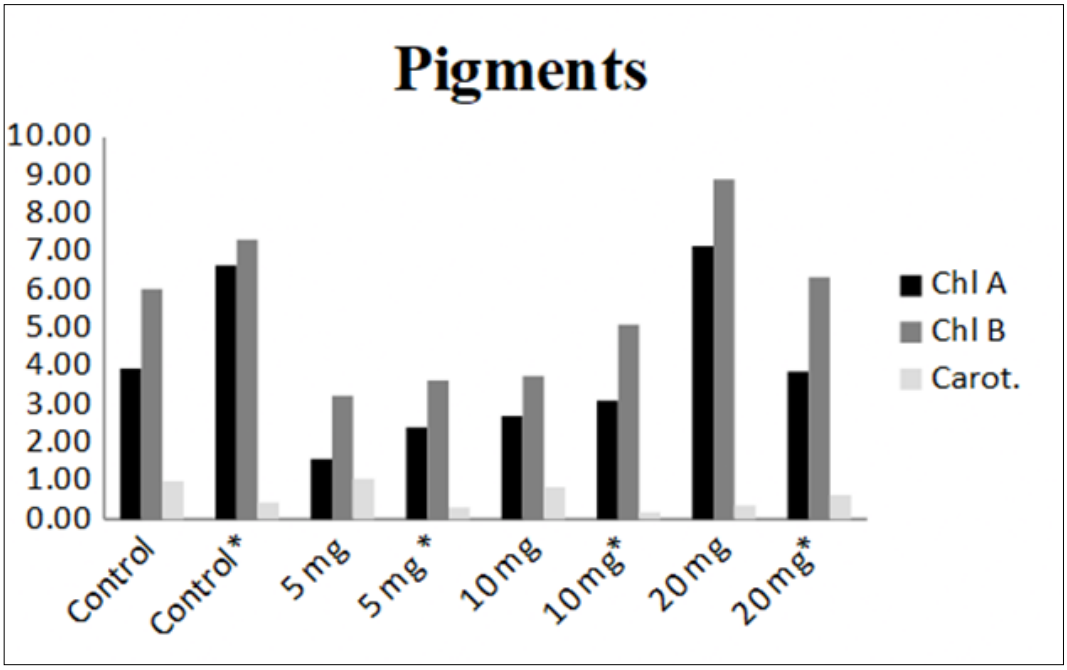

Fig 5: Effect of copper sulfate treatment and tissue injury on Photosynthetic pigments and carotenoids in date palm tissues. The chlorophyll a and b content (chl a and chl b) and carotenoids (carot.) were determined in date palm tissues subjected to different concentrations of $CuSO_4$ C0, C1, C2 and C3(0, 5, 10, and 20 mg L^{-1}). Control represents untreated tissues, whereas Control*C0, C1*, C2* and C3*(0 , 5 mg, 10 mg, and 20 mg) represent the corresponding $CuSO_4$ treatments following tissue wounding. Values are expressed as mg g^{-1} fresh weight (FW).

Discussion

The data of this study showed that the interaction between $CuSO_4$ concentration and mechanical wounding resulted in different physiological, biochemical and volatile phenotypes in tissues of date palm.

The GC-MS analysis revealed that mechanical wounding much affected the emission of ethyl acetate, and the response was highly affected by the $CuSO_4$ concentrations. The non-wounded tissues emitted between 0. 222 and 330 ppb of ethyl acetate. Wounding resulted in increased emission of ethyl acetate and post-wounding concentrations of 0.452, 0.941, 0.487, and 0.400 ppb of ethyl acetate emitted in C0*, C1*, C2*, and C3*, respectively. The enhancement of emissions following wounding revealed that injure to tissues stimulated the production and/or release of certain volatile compounds. Wounding elicits rapid changes in secondary metabolism and volatile emissions, which are part of the plant's response to injury (Isah, 2019) [23].

The maximum ethyl acetate emission was for sample C1* (0.941 ppb) which was approximately 4. 2 times greater than the corresponding non-wounded treatment C1. Increasing the concentration of $CuSO_4$ under wounded conditions resulted in continual decrease in the amount of ethyl acetate emitted. The emission was decreased from 0.94135 ppb in C1* to 0.487 and 0.400 ppb respectively in treatments C2* and C3*, representing decreases in emissions of approximately 48.2% and 57.5% respectively.

The decline in ethyl acetate emission at high $CuSO_4$ concentrations occurred even though the physiological stress reactions still appeared strong in C3* in particular, with the highest proline level and highest SOD, CAT and ADH activities. Because of this the activity of ethyl acetate emission was not simply a matter of no physiological activity occurring; rather it may represent a reallocation of metabolism at high levels of Cu. Copper is one of the most important micronutrients, with a multifaceted role in plant physiology, but excess Cu may upset cellular redox status and initiate anti-oxidative and detoxification activity (Wang *et al.*, 2024) [38]. More recently Cu availability is proven to also control plant secondary metabolism and other defense-related metabolism activity (Akhtar *et al*., 2026) [3].

Ethyl acetate is one of the palm originated volatile esters and was identified from the tissues of the date palm. Ethyl acetate was shown to evoke antennal responses in the weevil Rhynchophorus ferrugineus. The behavioral activity of ethyl acetate turned out concentration-dependent with high level of interactions with other volatiles. Vacas *et al.* (2014) [36] have identified ethyl acetate as one of the palms' complex mixture of esters in association with olfactory responses in ferrugineus. While, Vacas *et al.* (2017) [37] have demonstrated that the combination of ethanol with ethyl acetate acts as a synergist in improving the effectivity of ferrugineol-based traps. The previous finding and data illustrate that physical damage elicits ethyl acetate emission while increasing levels of $CuSO_4$ gradually reduces this emission. The greatest inhibition of ethyl acetate release was observed at the highest $CuSO_4$ level, where the weevil response may be reduced despite the presence of stress cues. These results indicated that $CuSO_4$ can alter the volatile metabolism of date palm and that means potentially alter the signaling mechanisms involved in red palm weevil plant communication.

A gradual increases of promoting effects of $CuSO_4$ concentration on proline accumulation and the activities of SOD, CAT and ADH were observed with mechanical stress, implying that the value of which may be optimal for stimulation of antioxidative systems and metabolic adjustment following mechanical injury.

The increasing in proline concentration in high $CuSO_4$ treatments suggests that an increased exposure to $CuSO_4$ elicited a progressive response of the tissue's biochemical stress response. The accumulation of proline within plant tissues in response to abiotic stress is a common occurrence

throughout plants and within many species which offers numerous benefits including osmotic adjustment, cellular homeostasis, ROS scavenging, membrane and protein protection and enzyme redox regulation (Ghosh *et al*., 2022) [19].

Also the marked response of proline accumulation in tissues subjected to injury indicated that this parameter could have been stimulated by such additional stress signal, and that it would have developed an even more intense biochemical response to $CuSO_4$. Highest proline content was observed for C3* (12.4), and it clearly revealed that $CuSO_4$ at the highest level combined with the physical damage caused the most severe metabolic disturbance among all treatments. Such a pattern aligns with other lines of evidence in other plants as well (Spormann *et al*., 2023) [33].

The simultaneous rise of SOD and CAT values would represent a stimulation of enzymatic antioxidant machinery as a response to the increasing $CuSO_4$ levels. Excess Cu has the capacity to interfere with cellular redox homeostasis and to induce ROS formation, this way leading to a stimulation of antioxidants; the cascade of enzymatic stimulation from SOD, which catalizes the transformation of superoxide radicals into H_2O_2 to CAT, which detoxifies the latter, would contribute to limit the ROS formation at higher oxidative stress levels (El-Beltagi *et al*., 2020; Chen *et al*., 2022) [9, 14]. The more noticed effect mainly in C3*, would points out a denser stimulation of antioxidant machinery after wound application in the presence of $CuSO_4$. The boost of ADH activity, mainly in C3*, would also indicate a more generalized modulation of stress-induced function, as this enzyme plays a role in cell redox regulation and primary metabolism, and is proven to be inducible following several kind of stresses (Tola *et al*., 2021) [34].

The total phenolic content was also found to be significantly influenced by both copper sulfate application and mechanical wounding. The wounded plants treated with 5 mg/l $CuSO_4$ considered the highest value of phenolic contents, when the non-wounded plants showed the lowest phenolic content. These results referred to the wounded activated phenylpropanoid pathway which play role to biosynthesis of phenolic contents that consider an important as part of the plant defense system by activating phenylalanine ammonia-lyase (PAL) enzyme that regulating phenolic metabolism and increasing the production of phenolic compounds involved in cell wall reinforcement, reactive oxygen species (ROS) (Ding and Ding 2020 and Sharma *et al.,* 2019 [12, 32]).

In contrast, El Hadrami and Al-Khayri (2012) [15] and Favaro *et al.* (2024) [17] reported that mechanical wounding promotes phenolic accumulation in date palm and other plant species as an adaptive response to oxidative stress and tissue repair. Copper is an essential micronutrient that plays a significant role in enhancing antioxidant metabolism under optimal conditions while amplifying defense responses under stress, as agreed with (Yruela, 2013 [41] and Hasanuzzaman *et al.,* 2018) [20]. Consequently, the high level of phenols in injured plantlets and treated with $CuSO_4$ may improve and cure the wound

Indole-3-acetic acid (IAA) play an important role for elongation, cell division, and tissue regeneration. The concentrations of 5 mg/l of $CuSO_4$ increased IAA to the high value and get the high value in antioxidant activity too. Indicating that low concentration of $CuSO_4$ promoted cellular homeostasis without disrupting endogenous growth regulator metabolism these results agreement with (Casanova-Sáez *et al.,* 2021 [8]; Li *et al.,* 2017). In antioxidants activity, Liu *et al.,* (2026) [26] reported that copper availability improved the antioxidant activity.

Gibberellic acid (GA_3) and total amino acid contents were increased in sample treated with 20 mg/l of copper sulfate. These results referred that high concentration of copper sulfate enhance some of plant physiology and metabolism. Gibberellins regulate cell division, elongation, enzyme activation, and biomass accumulation, while copper nutrition improves photosynthetic efficiency, thereby promoting the biosynthesis of endogenous growth regulators as mentioned by Yruela, (2013) [41] and Broadley *et al.,* (2012 [7]). According to Hasanuzzaman *et al.* (2018) [20] and Hildebrandt *et al.* (2015) [21], the high value of amino acid was at concentration 20 mg/l $CuSO_4$ that indicated to enhance nitrogen absorption and metabolic activity under optimal copper availability.

Reactive oxygen species (ROS) scavenging, cellular membrane protection, and stress tolerance are all important functions of flavonoids, and their production is further stimulated by wound-induced activation of the phenyl propanoid pathway. In this study the wounded control plants get the high value of flavonoids which meaning the copper sulfate decrease the stress of flavonoids production. These results agreement with Agati *et al*., (2012) [1]; Sharma *et al.,* (2019), and Dong and Lin (2021 [13, 32]).

In pigments, Concentration of $CuSO_4$ (20 mg/l) get high value of chlorophyll a and chlorophyll b. Copper sulfate play role in improving photosynthetic performance, electron transport, chloroplast development, and chlorophyll biosynthesis. These results were agreement with (Yruela, 2013 [41]; Broadley *et al.,* 2012 [7]). While mechanically wounded plants generally exhibited lower chlorophyll contents than the corresponding non-wounded treatments, reflecting the negative effects of wound-induced oxidative stress on chloroplast integrity (Hasanuzzaman *et al.,* 2018 [20]). In case of carotenoids, the high level of copper sulfate reducing carotenoids accumulation. Their reduction following wounding may reflect increased utilization during oxidative stress (Nisar *et al*., 2015; Foyer and Noctor, 2016 [18, 29]).

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