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LC-MS/MS Chemical Profiling of *Chrysopogon Zizanioides In Vitro* Shoots Propagated on Different Elicitors

Mahmoud A. M. Arafat[a,*] , Mohammed N. A. Khalil[b], Engy A. Mahrous[b,c], Essam M. Abd El-kader[d] and Seham El-Hawary[b]

[a] *Pharmacognosy Department, Faculty of Pharmacy, Heliopolis University, 11361, Egypt*

[b] *Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, 11562, Egypt*

[c] *Pharmacognosy Department, Faculty of Pharmacy, Ahram Canadian University, Giza, 12451, Egypt*

[d] *Timber Tree Research department, Horticulture Research Institute, Agriculture Research Center, Cairo, 12619, Egypt*

Abstract

Chrysopogon zizanioides (L.) Nash is widely used in *Ayruveda* for treatment of various ailments. Despite numerous *in vitro* protocols, no report had studied the chemical composition of the *in vitro* shoots culture. Hereto, shoots were induced from crown explants on MS (Murashige & Skoog) medium and propagated on MS (0.5 mg IAA/L) medium. Various additives levels of phenylalanine (Phe) and proline (Pro), alone or in different combinations with salicylic acid (SA), polyethylene glycol (PEG) were tested. Highest shoot multiplication was attained on MS (100 mg/l Phe + 500 mg/l PEG). Highest yield of total flavonoids (32.82 ± 0.08 catechin equivalent) and phenolics (286.76 ± 1.55 gallic acid equivalent) were achieved on MS (100 mg/l Phe + 100 mg/l SA) and MS (50 mg Pro/l), respectively. Shoots from these two media were chemically profiled in comparison to the control media using LC-MS/MS. Nine flavone glycosides, three lignans and flavolignans, four phenolics, three carboxylic acids and three fatty acids were the most abundant in the three extracts. Treatments and elicitors didn't affect the qualitative composition of shoot extracts but did influence the polyphenolics yield. This study paves the way for more research on *in vitro* biosynthesis and production of bioactive phytochemicals in vetiver shoots.

Keywords: Vetiver, *Chrysopogon zizanioides*, *in vitro*, shoots culture, total flavonoids and phenolics, LC–MS/MS.

1. Introduction

Vetiver grass, *Chrysopogon zizanioides* L. (CZ), is a perennial grass that is native to the South East Asia region [1]. It can help prevent sheet erosion [2]. Vetiver roots contain an essential oil whose composition could amount to more than 200 compounds [3]. It is used as a component in industry, perfumery, aromatherapy and cosmetics as well as in traditional medicine. Vetiver grass can reach heights of up to 2 meters whose chemical composition was previously studied [4], [5], proving that aerial parts contained carbohydrates, flavone glycosides, lignans, flavolignans, carboxylic acids and fatty acids. Several *in vitro* studies focused on callus formation and shoots initiation of vetiver.

Callus was induced using different explants and different medium composition [6]–[9]. Callus was cultured and used to initiate plantlets formation using Murashige and Skoog (MS) free medium [10] and medium supplemented with naphthalene acetic acid (NAA), kinetin [11] or benzyl adenine (BA) [7], [11]. Additionally, shoots formation was initiated from

*Corresponding author e-mail: mahmoud.anter@hu.edu.eg.;(Mahmoud A. M. Arafat).

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crown explant cultured on MS media supplemented with BA [12]. Alternatively, shoots formation and number were increased using MS media supplemented with colchicine or benzyl amino purine (BAP) [13] when using shoots as initial explants. The inhibitory effect of gamma irradiation on the *in vitro* shoots growth and multiplication was studied [14]. *In vitro* techniques facilitate the accelerated propagation of a significant volume of uniform and highly efficient biomass. No study had explored the chemical composition of the *in vitro* systems although the cultivation methodology employed and the composition of the growth medium emerge as pivotal factors dictating the synthesis of secondary metabolites within *in vitro* cultivated biomass [15].

Elicitors refer to compounds added to the culture medium to stimulate the production of secondary metabolites and they can be classified as either biotic or abiotic substances [16]. Elicitation induced the modulation and augmentation of plant secondary metabolites through the activation of genes responsible for synthesizing enzymes engaged in the biosynthesis of flavones, phenolics, and other compounds associated with defense mechanisms [17]. The successful elicitation of secondary metabolites hinges on selecting an appropriate elicitor, determining its concentration, and identifying the optimal timing for treatment. Salicylic acid (SA) served as an endogenous growth regulator capable of influencing the physiological and biochemical functions of plants, thereby modulating plants *in vitro* growth [18]. SA functions as a signaling molecule pivotal in regulating the expression of stress-related genes in plant cells which served to stimulate the biosynthesis of secondary metabolites under stress conditions induced by both abiotic and biotic factors [19]. SA had been employed to enhance the production of flavonoids and polyphenols in cell suspensions, calli, and tissue cultures spanning various plant families [20].

Drought stress triggers osmotic stress which disrupted cellular homeostasis and ion distribution in plants [21]. The introduction of elicitors induced stress conditions, prompting plants to naturally elevate the production of secondary metabolites as a defense mechanism [22]. Polyethylene glycol (PEG) was utilized as a suitable agent to induce drought stress by reducing the osmotic potential in the growth medium [23]. The inclusion of PEG decreased the water potential of the culture medium, leading to inhibited explant growth and increased accumulation of secondary metabolites [24].

Another significant strategy involves incorporating precursors into nutrient media to augment the synthesis of secondary metabolites. The aromatic amino acid phenylalanine (Phe) was employed as a precursor for phenolic compounds within the plant's phenylpropanoid biosynthesis pathway. Flavonoid synthesis initiates from Phe within the shikimate pathway, wherein Phe is converted into 4-coumaroyl-CoA which subsequently enters the flavonoid biosynthesis pathway through a chalcone intermediate [25]. Proline (Pro) is another amino acid involved in primary metabolism as a proteinogenic component [26]. A scheme involving the continuous cycling of Pro was proposed and suggested a connection between free Pro (either stress-induced or exogenously added) and enhanced phenolic metabolism occured through the stimulation of carbon flux through the oxidative pentose phosphate pathway[27].

In the current study, we developed an effective *in vitro* multiple shoot induction and user-friendly plant regeneration system for CZ using crown explants. Then the levels of phenolics and flavonoids in the *in vitro* plantlets were estimated under different

concentration of Phe and Pro alone or in combination with SA and PEG. Chemical profiling using LC-ESI-MS/MS was performed for the best treatments against control culture. The current work is the first report about the flavonoids and phenolic contents of the *in vitro* regenerated shoots of CZ.

2. Materials and methods

2.1. Plant materials and explant sterilization

Aerial parts were harvested from CZ grown at the medicinal and aromatic plants farm, Gzerat El-Sheer in El-Qanater El-Khayreya (voucher specimen no. 1.12.2022, deposited in the Herbarium of Pharmacognosy Department, faulty of Pharmacy, Cairo University). Samples were washed for an hour under running tap water after being soaked and shaken in soapy water with septol soap for 20 minutes. The explants were disinfected by soaking in 70% ethyl alcohol for 30 seconds under aseptic circumstances in a laminar air-flow cabinet. This was followed by sterilization with Clorox (10% v/v) (NaOCl, 5.25%) for 10 minutes, and finally with mercuric chloride (0.1% w/v) combined with a few drops of Tween-20 for 10 minutes. The explants were rinsed three times with sterile distilled water following their disinfection procedures before culturing on hormone-free MS solid medium containing 3% (w/v) sucrose, pH 5.6. Cultures were incubated at 26 ± 2ºC under 16 h light/day using fluorescent lighting lamps (2000-2500) lux for 4-6 weeks. Plantlets were used as a material for shoot induction.

2.2. Shoot induction and multiplication

CZ crown explants from *in vitro* plantlets were cultured on MS solid medium with 0.5 mg IAA/L for shoot multiplication and incubated at the same growth conditions.

2.3. Elicitor's treatment

Different elicitors were added for shoot multiplication and enhancement of total flavonoid and phenolic contents **Table (2)**, **(Fig. 3)**. MS solid medium was supplemented with 0.5 mg IAA/L and treated with different concentrations of elicitors namely; Pro (25, 50, 100 mg/L), Phe (25, 50, 100 mg/L) alone and in combination with SA (100 mg/L) and PEG 400 (100 mg/L). MS medium supplemented with IAA at 0.5 mg L^{-1} was used as a control.

2.4. Preparation of plant extracts

After 8 weeks of culture on the medium, shoot samples of CZ were collected from *in vitro* regenerated plants, homogenized using pestle and mortar then soaked in methanol for 24 h in dark. The extracted material was dissolved in methanol following evaporation. The mixture was centrifuged at room temperature for 15 minutes at 8,000 rpm. Then, filtered through a 0.22 µm membrane filter, the filtrate was subjected to phenolics, flavonoids and LC-ESI-MS/MS, analysis.

2.5. Determination of total flavonoid

Using an aluminum chloride ($AlCl_3$) colorimetric assay, the total flavonoid content was determined in accordance with [28]. In summary, 100 µL of extract was combined with 300 µL of 5% sodium nitrite ($NaNO_2$). After 6 minutes, 300 µL of a 10% $AlCl_3$ solution was added and distilled water was used to increase the volume to 2.5 mL. Samples were centrifuged at 5000 g for 10 minutes after adding 1.5 mL of 1 M NaOH after 7 minutes. At 510 nm, the supernatant's absorbance was measured in relation to the solvent blank. Using a calibration curve made using catechin as a standard; the total flavonoid content was calculated and represented as milligrams of catechin equivalent (mg CE) per 100 gram of sample.

2.6. Determination of total phenolics

The Folin-Ciocalteu method was used to estimate the total phenolic content [28]. In short, 250 µL of Folin-Ciocalteu reagent was added to the test tube containing the extract (100 µL), and the volume was adjusted to 3.5 mL using distilled water. After five minutes, 1.25 mL of a 20% aqueous sodium carbonate (Na_2CO_3) solution was added to the mixture to neutralize it. The absorbance was measured at 725 nm in relation to the solvent blank after 40 minutes incubation in dark. Using a calibration curve made using gallic acid; the total phenolic content was calculated and represented as milligrams of gallic acid equivalent (mg GE) per 100 gram of sample.

2.7. Liquid chromatography–electrospray ionization–tandem mass spectrometry

The sample was analyzed using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) with an

ExionLC AC system for separation and SCIEX Triple Quad 5500+ MS/MS system equipped with electrospray ionization (ESI) for detection. Ascentis® Express 90 Å C18 Column (2.1×150 mm, 2.7 µm) was used for separation. The mobile phase consisted of two eluents, A (5 mM ammonium formate pH 8) and B (acetonitrile, LC grade). The mobile phase gradient was programmed as follows: 5% B at 0-1 min, 5-100% B at 1-20 min, 100% B at 20-25 min, returning to 5% B at 26 min and held until 30 min. The flow rate was 0.3 ml/min and the injection volume was 5 µl. For MS/MS analysis, negative ionization mode a scan (EMS-IDA-EPI) was conducted from 100 to 1000 Da for MS1 with the following parameters: curtain gas: 25 psi; Ion Spray voltage: -4500 kv; source temperature: 500°C; ion source gas 1 & 2 were 45 psi. MS2 was conducted from 50 to 1000 Da with a declustering potential of -80 and collision energy of -35.

2.8. Statistical analysis

Each experiment was performed as triplicate per treatment. The data on number of shoots, weight, total flavonoids and total phenolics were analyzed using one way analysis of variance (ANOVA) and means were compared using Tukey's multiple comparisons test at 5% level of significance (p=0.05).

3. Results

3.1. Effect of elicitation on shoot induction and multiplication

Shoots induction was traced after (1, 4 and 8 weeks) from crown explants of CZ cultured on MS solid medium (0.5 mg IAA/L) supplemented with different concentrations of Pro and Phe alone or in combination with SA (100 mg/L) or PEG (100 mg/L) **Figure (1).** As shown in **Table (1)** and **(Fig. 2)**, precursors (Pro or Phe) at different concentrations with/without elicitors (SA or PEG) significantly affected shoot proliferation rate (in the form of shoot number per explant) and shoot growth (in the form of shoot fresh weight). Statistical analysis indicated that there was no significant difference in shoot number and weight among the treatments compared to the control, with the exception of the medium containing 25 mg/l Pro and 100 mg/l SA, which exhibited the highest fresh weight (3.87±0.37g). The highest significant shoot number/explant (11.1±1) was observed for explants grown on medium contained 100 mg/l Phe + 500 mg/l PEG. While the lowest one (2±1.53 shoot / explant) was recorded for those cultured on medium with 50 mg/l Phe + 500 mg/l PEG.

It could be noticed that adding SA or PEG to medium contained the same concentration of Pro or Phe lead to various growth responses of CZ. As for shoot fresh weight, explants growing on medium enrichment with 25 mg/l Pro + 100 mg/l SA recorded the highest fresh weight (3.87±0.37g). On the other hand, the lowest shoot fresh weight (0.42±0.12 g) was measured for shoots growing on medium contained 50 mg/l Pro. From tabulated data, it could be observed that increasing amino acids concentration in growth medium contained SA inversely proportional with shoot fresh weight.

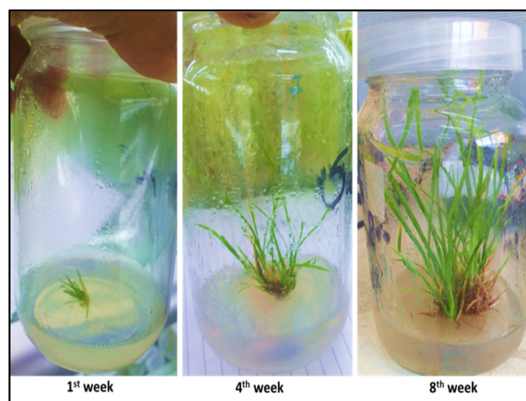

Figure 1: *In vitro* shoots induction (after 1, 4 and 8 weeks) from crown explants of CZ cultured on control medium.

Table 1: Number and weight of the propagated shoots of *C. zizanioides* on MS medium containing different elicitors

	Shoots number (Mean±SD)	Shoots weight (gm) (Mean±SD)
Control	**8±2.12**	**1.62±0.65**
25 mg/l Pro	**6±1.15**	**0.99±0.23**
50 mg/l Pro	**3±1.41**	**0.42±0.12**
100 mg/l Pro	**9±1.53**	**2.80±0.63**
25 mg/l Phe	**4±0.71**	**0.34±0.03**
50 mg/l Phe	**7±1.41**	**1.48±0.33**
100 mg/l Phe	**5±1.41**	**0.87±0.29**
25 mg/l Pro + 100 mg/l SA	**9±2.12**	**3.87±0.37**
50 mg/l Pro + 100 mg/l SA	**9±2.08**	**2.47±0.56**
100 mg/l Pro + 100 mg/l SA	**3±1.41**	**0.50±0.01**
25 mg/l Phe + 100 mg/l SA	**8±2.12**	**2.23±0.71**
50 mg/l Phe + 100 mg/l SA	**6±0.71**	**1.93±0.05**
100 mg/l Phe + 100 mg/l SA	**4±2.12**	**0.24±0.16**
25 mg/l Pro + 500 mg/l PEG	**3±0.71**	**0.48±0.06**
50 mg/l Pro + 500 mg/l PEG	**6±1.41**	**0.73±0.20**
100 mg/l Pro + 500 mg/l PEG	**4±1.50**	**0.61±0.14**
25 mg/l Phe + 500 mg/l PEG	**4±1.41**	**0.55±0.11**
50 mg/l Phe + 500 mg/l PEG	**2±1.53**	**0.70±0.57**
100 mg/l Phe + 500 mg/l PEG	**11±1**	**2.25±0.29**

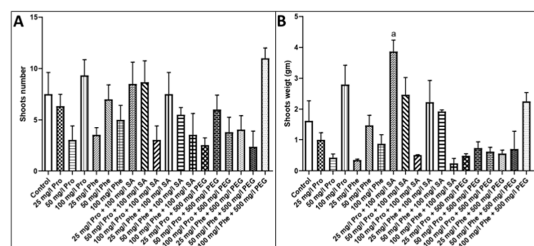

Figure 2: Effect of different concentration of Pro and Phe alone and in combination with SA and PEG on the number (A) and fresh weight (B) of *in vitro* shoots of CZ. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. [a] Significantly different from control at $p < 0.05$, (n=4).

Total flavonoid and phenolic contents

Total flavonoid and phenolic contents of CZ extract of the *in vitro* propagated shoots elicited with different concentrations of elicitors are presented in **Table (2), (Fig. 3)**. The results showed that the production of flavonoids and phenolics were significantly ($p < 0.05$) affected by the type and concentration of elicitors used. The highest significant increase in a flavonoid content (32.82±0.08 mg CE/100 g) was observed in shoots treated with combination of Phe (100 mg/L) and SA (100 mg/L) while the lowest one (0.93±0.02 mg CE/100 g) was recorded with combination of Pro (25 mg/L) and PEG (500 mg/L). The highest significant increase in a phenolic content (286.76±1.55 mg GE/100 g) was observed with shoots treated with Pro only (50 mg/L) while the lowest one (1.86±0.09 mg GE/100 g) was observed with Pro (100 mg/L).

Table 2: Total flavonoids and phenolics of the propagated shoots of *C. zizanioides* under different elicitors' treatments

	Total flavonoids Catechin equivalent (mg/100 gm)	Total phenolics Gallic acid equivalent (mg/100 gm)
Control	**4.98±0.05**	**30.04±0.27**
25 mg/l Pro	**1.78±0.06**	**21.93±0.07**
50 mg/l Pro	**28.70±0.50**	**286.76±1.55**
100 mg/l Pro	**1.20±0.02**	***1.86±0.09***
25 mg/l Phe	***1.07±0.03***	**40.17±0.45**
50 mg/l Phe	**6.30±0.22**	**31.21±0.57**
100 mg/l Phe	**10.96±0.01**	**35.65±0.66**
25 mg/l Pro + 100 mg/l SA	**2.85±0.01**	**43.02±0.21**
50 mg/l Pro + 100 mg/l SA	**1.51±0.03**	**39.36±0.16**
100 mg/l Pro + 100 mg/l SA	**11.42±0.02**	**155.46±1.34**
25 mg/l Phe + 100 mg/l SA	**5.93±0.08**	**82.83±0.46**
50 mg/l Phe + 100 mg/l SA	**4.69±0.09**	**50.97±0.42**
100 mg/l Phe + 100 mg/l SA	**32.82±0.08**	**218.78±2.77**
25 mg/l Pro + 500 mg/l PEG	**0.93±0.02**	**25.54±0.37**
50 mg/l Pro + 500 mg/l PEG	**6.27±0.08**	**14.49±0.39**
100 mg/l Pro + 500 mg/l PEG	**3.98±0.12**	**22.87±0.42**
25 mg/l Phe + 500 mg/l PEG	**6.00±0.04**	**31.49±0.63**
50 mg/l Phe + 500 mg/l PEG	***1.07±0***	**16.34±0.12**
100 mg/l Phe + 500 mg/l PEG	**1.94±0**	**17.76±0.15**

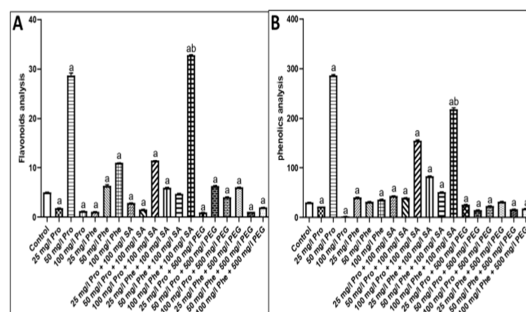

Figure 3: Effect of different concentration of Pro and Phe alone and in combination with SA and PEG on total flavonoids (A) and total phenolics (B) of CZ in vitro shoots. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test. [a] Significantly different from control at $p < 0.05$. [b] Significantly different from Pro (50 mg/L) at $p < 0.05$.

3.2. Chemical profiling using LC-ESI-MS/MS analysis

To provide insights into the chemical composition of *in vitro* regenerated shoots extracts from crown explants pretreated with Pro (50 mg/L) (ProE) or Phe (100 mg/L) combined with SA (100 mg/L) (PheSAE) compared to control (CE), LC-ESI-MS/MS technique was used in negative ionization mode, (**Fig. S1.1, S1.2, S1.3**). The components of ProE, PheSAE, and CE were identified by comparing their molecular formula with the metabolites listed in the dictionary of natural products (https://dnp.chemnetbase.com/faces/chemical/ChemicalSearch.xhtml) and analyzing their fragmentation patterns in relation to previously documented literature. As shown in **Table (S1)**, 22 distinct metabolites were identified in the extracts which belong to different chemical classes including nine flavone glycosides, three lignans and flavolignans, four phenolics, three carboxylic acids and three fatty acids, **Figure (4)**.

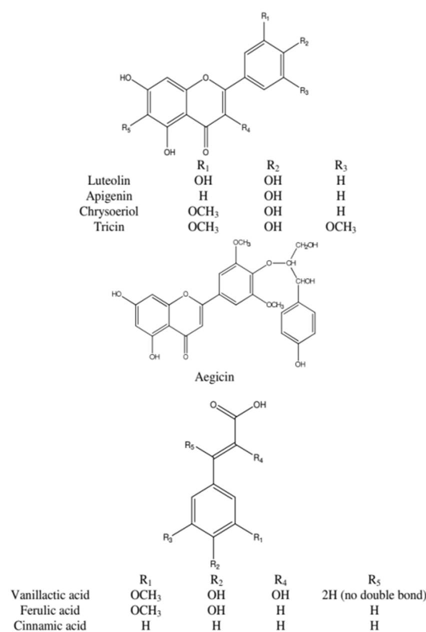

Figure 4: Selected structures of phenolic metabolites identified in *C. zizanioides in vitro* shoots different extracts.

Chemical profiling showed that the three compared extracts had very similar composition except for minor differences. It can be concluded that the studied treatments affected the yield of phenolics and flavonoids rather than the produced metabolites. Comparing LC-ESI-MS/MS results of three extracts, as shown in **Table (S1)**, nine flavone glycosides, three lignans and flavolignans, four phenolics, three carboxylic acids and three fatty acids were most abundant in three extracts, **Fig. 4**. All identified chemical compounds were detected in three extracts except chrysoeriol-*C*-arabinoside-*C*-glucopyranoside, luteolin 6-*C*-arabinoside 8-*C*-glucoside and aegicin that couldn't detected in PheSAE. Additionally, tricin-*O*-rutinoside and tricin 4'-*O*-(guaiacylglyceryl) ether couldn't be identified in PE. 2,7'-Cyclo-3',4,4',5,5',6,7-heptahydroxy-8,8'-lignan-9',9-olide-4,5-Methylene, 3',4',5',6-tetra-Me ether and feruloylquinic acid were detected only in PheSAE. In the coming sections, the details of metabolites identification are listed.

3.2.1. Identification of flavonoid glycosides

Nine flavonoids were identified in the negative ionization mode. The majority of these flavonoids were *C*-glycosides of apigenin, luteolin, and chrysoeriol, whereas the *O*-glycosides were primarily derivatives of tricin, **Table (S1)**. This aligns with earlier findings which have recognized flavones as the primary flavonoid category within the Poaceae family [29]. Peak 1 was tentatively identified as luteolin-6,8-di-*C*-pentoside, with a molecular ion $[M-H]^-$ at *m/z* 549.0 $(C_{25}H_{25}O_{14})^-$, based on the presence of fragment ions *m/z* 399 and *m/z* 369 representing $[Ag+113]^-$ and $[Ag+83]^-$, respectively. Additionally, the fragments at *m/z* 489 $[M-H-60]^-$ and 459 $[M-H-90]^-$ suggested cross-ring cleavages [O-C1 & C2-C3] and [O-C1 & C3-C4] in the sugar ring, which are characteristic of *C*-pentoside. Signals at *m/z* 399 and 369 were attributed to the second *C*-pentoside cleavage, namely, [459-60] and [459-90] (**Fig. S1.4**) confirming the *C*-glycosylation of luteolin with two pentoses, as previously identified in vetiver aerial parts [4]. Peak 2 showed molecular ion at *m/z* 562.9 $(C_{26}H_{27}O_{14})^-$ identified as apigenin-*C*-hexoside-*C*-

pentoside. This identification was based on the relative high abundance of signal *m/z* 443 $[M-H-120]^-$, which is indicative of *C*-hexoside (**Fig. S1.5**) based on their fragmentation patterns [30]. Apigenin-*C*-hexoside-*C*-pentoside was previously isolated from family Poaceae [31]. Peak 3 was identified as chrysoeriol-*C*-hexoside-*C*-pentoside, supported by the presence of $[Ag+113]^-$ and $[Ag+83]^-$ ions at *m/z* 413 and 383, respectively, which are indicative of chrysoeriol-di-*C*-glycosides (**Fig. S1.6**) which was previously detected in vetiver aerial parts [4]. Peak 4 exhibited fragment ions at *m/z* 383 $[M-H-90-60]^-$, 353$[M-H-90-90]^-$ which are characteristic of apigenin-*C,C*-di-pentoside, suggesting a predicted molecular formula of $[C_{25}H_{25}O_{13}^-]$. Consequently, peak 4 was designated as apigenin-*C*,*C*-di-pentoside (**Fig. S1.7**) as previously identified in vetiver aerial parts [4]. Peak 5 was determined to be luteolin 6-*C*-arabinoside-8-*C*-hexoside (also known as isocarlinoside) with a molecular ion at *m/z* 579.0 $(C_{26}H_{27}O_{15})^-$ which was previously isolated from aerial parts of vetiver [5]. The fragment ions observed at *m/z* 399 and 369 suggested $[Ag+113]^-$ and $[Ag+83]^-$, respectively, with "Ag" representing the aglycone, which are typical for luteolin-di-*C*-hexoside [30]. The *C*-arabinosyl group was assigned to position C-6 due to the comparatively lower abundance of the fragment at *m/z* 459 [M-H-120], coupled with the higher abundance of the fragment at *m/z* 489 [M-H-90] (**Fig. S1.8**).

Furthermore, three tricin hexosides were distinguished, each exhibiting a distinct glycosylation pattern. The tricin aglycone (peak 9) was detected at *m/z* 329, accompanied by two fragments indicating the sequential loss of two methyl groups at *m/z* 314 $(329-15)^-$ and 299 $(314-15)^-$ (**Fig. S1.12**). similarly, the identical pattern was discerned in the mass spectrum of peak 7 following a mass loss of 162 amu, corresponding to the loss of a hexose unit (491-hexose). Consequently, this peak was recognized as tricin hexoside, with a molecular ion at of *m/z* 491.0 $(C_{23}H_{23}O_{12})^-$ (**Fig. S1.10**). In the same vein, peak 8 was determined to be tricin-*O*-rutinoside, as evidenced by a mass loss of 308 amu, aligning with the rutinose moiety. The base peak of the aglycone at *m/z* 329 indicated a monosubstituted tricin-*O*-hexoside (**Fig. S1.11**). It's notable that both tricin hexoside and tricin rutinoside have been previously isolated and identified in rice leaves, belonging to the Poaceae family [32].

3.2.2. Identification of flavolignans and lignans

Lignans and flavolignans are frequently present in various Poaceae plants, such as rice and rye [32], [33]. Mass fragmentation patterns of lignans typically involve the loss of certain functional groups, including water (-18 amu), methyl moiety (-15 amu), carboxyl group (-44 amu), formaldehyde (-30 amu), and CH_2CHOH (-44 amu) [33]. Peak 10 was identified as aegicin, specifically hydroxyphenylglyceryl tricin, exhibiting its base peak at *m/z* 329 [M-H-166] due to the loss of the hydroxyphenylglyceryl moiety. Meanwhile, daughter ion at m/z 135 resulted from loss of CH_2OH from the monohydroxyphenylglycerol (**Fig. S1.13**). Aegicin was isolated from *Aegilops ovate* as the major tricin-lignan [34], [35]. Guaiacylglycerol tricin was determined in peak 11 whose mass fragmentation pattern revealed a base peak corresponding to the tricin aglycone at *m/z* 329 resulted from the characteristic loss of 196 amu attributed to the guaiacylglycerol moiety (**Fig. S1.14**). Guaiacylglycerol tricin was previously isolated form Poaceae [36]. Peak 12 was determined to be 2,7'-Cyclo-3',4,4',5,5',6,7-heptahydroxy-8,8'-lignan-9',9-olide-4,5-Methylene,3',4',5',6-tetra-methyl ether [*m/z* 443.0 $(C_{23}H_{23}O_9)^-$] (**Fig. S1.15**) which was previously detected in aerial parts of vetiver [4].

3.2.3. Other phenolic compounds

Ferulic acid derivatives were detected within three peaks, notably encompassing feruloylquninic acid (Fig. S1.16), vanillactic acid (peak 14, *m/z* 211.0) which undergoes dehydration to yield the ferulate moiety at *m/z* 193, (**Fig. S1.17**). This compound was detected previously in vetiver aerial parts [4]. Moreover, feruloyl quinic acid was discerned within peak 13, where the molecular ion at *m/z* 367.0 ($C_{17}H_{19}O_9^-$) underwent the loss of the quinyl moiety (-174 amu), resulting in the ferulate ion at *m/z* 193, as illustrated in **Fig. S1.16**. This compound has been previously isolated from the Poaceae family [37]. Peak 16 was characterized as feruloylhexose, determined by the molecular ion at *m/z* 355.1 ($C_{16}H_{19}O_9^-$) and the subsequent observation of the ferulate moiety fragment at *m/z* 193 following the elimination of hexose (-162) [38], **Fig. S1.18**. Peak 15 was determined to be 4-hydroxycinnamic acid [*m/z* 163.0 $(C_9H_7O_3)^-$], which was previously isolated from the Poaceae family [39], **Fig. S1.19**.

3.2.4. Identification of carboxylic acids

Peak 17 was determined to be quinic acid [*m/z* 191.0 $(C_7H_{11}O_6)^-$], with a fragment ion observed at *m/z* 111 resulting from the loss of CO_2 (-44 amu) after dehydration [40], **Fig. S1.20**. This compound was identified previously in vetiver roots and aerial parts [4]. Peaks 18 and 19 were recognized as gluconic acid [*m/z* 195.0 $(C_6H_{11}O_7)^-$] **Fig. S1.21** and shikimic acid [*m/z* 173.0 $(C_6H_{11}O_7)^-$] **Fig. S1.22**, respectively, both were previously isolated from the Poaceae family [41], [42].

3.2.5. Identification of fatty acids

Three fatty acids were distinguished through mass fragmentation analysis and comparison with databases, with unsaturated fatty acids predominating. Among these, trihydroxyoctadecenoic acid (20), hydroxyoctadecatrienoic acid (21), and hydroxyoctadecadienoic acid (22) were identified, as indicated by their respective masses at *m/z* 327.1, 293.1, and 295.1. Their predicted molecular formulae were $(C_{18}H_{31}O_5)^-$, $(C_{18}H_{29}O_3)^-$, and $(C_{18}H_{31}O_3)^-$, respectively.

4. Discussion

Amino acids, which are organic nitrogenous compounds, play a crucial role in stimulating plant cell growth. The exogenous application of amino acid solutions enhances plant growth by improving photosynthesis rates, chlorophyll biosynthesis, stomatal function, and gene expression within the plant [43]. SA is one of the plant hormones that modern research began to address its significant role in regulating many physiological processes, e.g., ions absorption, plant growth and development. Some studies have indicated that the external application of SA stimulated growth in maize [44]. SA significantly enhanced *in vitro* shoot multiplication of *Berberis asiatica* [45]. On the other hand, applying PEG significantly reduced growth and development of banana shoots *in vitro* [46]. They also declared that these negative effects depended on PEG concentration. Results of shoot growth, as illustrated in **Table (1)** and **Fig. (2),** revealed that CZ exhibited various responses according to the type and concentration of the applied amino acids, which may have a synergistic or an antagonistic relation with PEG or SA.

Table (2), **Fig. (3)** Demonstrated the effect of the precursors (Pro or Phe) at different concentrations with/without elicitors (SA or PEG) on flavonoids and phenolics production compared to the control. The experimental results revealed significant variations in the production of flavonoids and phenolics. This indicated that these precursors and elicitors have a

notable impact on the biosynthesis of these secondary metabolites. The control group represented the baseline levels of flavonoids and phenolics in the sample without any treatment. Treatments with Pro and Phe at various concentrations showed significant alterations in both flavonoid and phenolic content compared to the control.

Pro is frequently employed as an elicitor in the production of phenolic compounds. It has been shown to activate the pentose phosphate pathway, which subsequently feeds into the shikimate and phenylpropanoid pathways, leading to an increased accumulation of phenolic compounds [27], [47]. The results depicted in **Fig. (3)** and **Table (2)** demonstrate a concentration-dependent effect of Pro on the biosynthesis of secondary metabolites, particularly flavonoids and phenolics. At lower concentrations, Pro exerted a suppressive influence, resulting in reduced levels of these metabolites. In contrast, higher concentrations of Pro significantly stimulated their biosynthesis, leading to a marked increase in flavonoid and phenolic content relative to the control. Interestingly, when Pro was applied at an even higher concentration (100 mg/l), an opposite trend was observed, with a reduction in the biosynthesis of both phenolics and flavonoids. This paradoxical effect of Pro on the biosynthesis of secondary metabolites, particularly at varying concentrations, is consistent with the findings from previous studies. While exogenous proline at low concentrations has been reported to enhance stress tolerance [48], [49], possibly by modulating reactive oxygen species (ROS) scavenging and maintaining ROS homeostasis, higher concentrations of proline have been associated with toxic effects. These findings suggest that the role of proline in plant stress responses and secondary metabolite production is complex and highly dependent on its concentration [50].

Phe, an essential amino acid, also demonstrated concentration-dependent effects on the biosynthesis of flavonoids and phenolics. Consistent with the trends observed for Pro, lower concentrations of Phe led to a reduction in flavonoid levels relative to the control, suggesting an inhibitory influence at suboptimal concentrations. In contrast, as the concentration of Phe was increased, there was a marked enhancement in the synthesis of both flavonoids and phenolics. This suggests that at higher concentrations, Phe may act as a potent inducer of secondary metabolite biosynthesis, potentially by serving as a precursor or by modulating key enzymes involved in the phenylpropanoid pathway. Several studies have previously examined the effect of Phe on flavonoids and phenolics. [51] demonstrated that Phe increased the production of flavonoids and phenolics in callus of *Sequoia sempervirens*. Phe also enhanced flavonoid production in microshoots of *Scutellaria lateriflora* [52]. Moreover, Phe promoted the production of luteolin and rutin in root callus of *Rumex hastatus* [53]. Total phenolics were increased by Phe in adventitious roots of *Rubia tinctorum* [54]. Also, combination of dark with Phe enhanced the production of both flavonoids and phenolics on cell suspension culture of *Vitis vinifera* [55]. Additionally, the flavonoid content in callus culture and the phenolic content in suspension culture of *Iphiona mucronata* were increased when elicited with Phe [56]. Finally, Phe enhanced flavonoid and phenolic accumulation in *in vitro* regenerated leaves of *Coleus aromaticus* [57].

The co-application of SA and PEG with Pro or Phe yielded complex and varied outcomes in terms of secondary metabolite production. In certain cases, such as the combination of 100 mg/l Phe with 100 mg/l SA, there was a significant increase in flavonoid synthesis, suggesting a synergistic effect. However, other combinations either failed to elicit any

substantial changes or even resulted in a reduction in flavonoid content. Notably, the pairing of Pro or Phe with SA generally led to enhanced flavonoid and phenolic levels compared to the application of Pro or Phe alone, especially at higher concentrations, indicating a potential additive or synergistic interaction. The effect of salicylic acid alone or in combination with Phe on production of phenolic compounds was supported previously [54]; revealed that combination between SA (40 µM) and Phe (100 µM) could obtain the greatest total phenolics content in adventitious root cultures of *Rubia tinctorum*. Additionally, SA alone could enhance the amount of total flavonoids and phenolics [58]–[60]. These suggested that SA may enhance the stimulatory effects of Pro and Phe on secondary metabolite biosynthesis, possibly through synergistic interactions. PEG, used as an osmotic stress agent, exhibited variable effects on flavonoid and phenolic production when combined with Pro or Phe. Some combinations led to either an increase or a decrease in flavonoid and phenolic levels compared to the control. These results were supported with other previous studies such as [61]–[63] which showed that PEG could enhance total flavonoids and phenolics. Also PEG alone enhanced production of some phenolic compounds as quercitrin [64], rosmarinic acid, caffeic acid and salvianolic acid [65]. These indicated that the interaction between PEG and amino acids may modulate secondary metabolite biosynthesis through complex mechanisms. Overall, the results suggested that exogenous substances such as Pro, Phe, SA, and PEG could significantly modulate flavonoid and phenolic production. Further research is warranted to elucidate the underlying molecular mechanisms governing these effects and optimize treatment conditions for maximal secondary metabolite production. These findings had implications for agricultural practices and the pharmaceutical industry, as manipulating the levels of these amino acids in plants could potentially increase their flavonoid content, leading to crops with enhanced nutritional or medicinal value.

As the highest significant flavonoid content and phenolic content were observed in shoots treated with PheSAE and ProE, respectively, chemical profiling using LC-ESI-MS/MS of these extracts compared to CE was provided **(Table S1)**. This is the first comprehensive chemical study of *in vitro* culture of vetiver whose results were supported with previous and similar chemical studies [4].

5. Conclusion

In vitro plantlets are attractive models to study the biosynthesis of natural products and the tools that boost their production. In the current work, *in vitro* plantlets of *C. zizanioides* was established. The effect of different elicitors on shoot induction, multiplication was studied and propagated to the yield of total phenolics and total flavonoids contents. Phe, SA, Pro and PEG were shown to have crucial roles on the shoots growth and the yield of metabolites. However, LC-MS/MS profiling revealed a very similar chemical profile across different treatments. The presented preliminary results would pave the way for more deep and comprehensive studies about the biosynthesis and production of natural products in *C. zizanioides*.

6. Conflicts of interest

There are no conflicts to declare.

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