Nuclear magnetic resonance characterizes metabolic differences in *Cymbopogon schoenanthus* subsp. *proximus* embryogenic and organogenic calli and their regenerated shoots

Asmaa Abdelsalam1 · Kamal Chowdhury2 · Arezue Boroujerdi3 · Ahmed El-Bakry1

Received: 18 May 2021 / Accepted: 18 November 2021 / Published online: 1 December 2021
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Abstract
NMR-based metabolic profiling of polar extracts of somatic embryogenic and organogenic cultures of the medicinal plant *Cymbopogon schoenanthus* subsp. *proximus* were studied. Regeneration through somatic embryogenesis was done on media containing 2,4-D and BAP while organogenesis was achieved on media containing NAA and BAP. Fifty-two metabolites were identified in embryogenic calli (EC), organogenic calli (OC), regenerated embryogenic shoots (ES), and organogenic shoots (OS). Chemometrics and cluster analysis were used to depict the correlation between the groups investigated. Metabolic profiles revealed unique metabolites in ES (serine and lactate) and in OS (2-hydroxyisobutyrate, tyrosine, histamine and homoserine). Quantitative differences as manifested by relative concentrations through heat map and fold change analyses were significant in the following comparisons: calli type, embryogenic tissues, organogenic tissues, and shoot type. In a comparison of calli types, proline, asparagine and arginine were upregulated in EC and sucrose was upregulated in OC. When comparing embryogenic tissues, monosaccharides were upregulated in EC, while proline, pyroglutamate and 4-aminobutyrate were upregulated in ES. Upon comparison of organogenic tissues, trigonelline increased by 17-fold in OS; however, monosaccharides were upregulated in EC. Finally, when comparing shoot types, 4-aminobutyrate, betaine and proline were upregulated in ES, while mono- and disaccharides were upregulated in OS. The embryogenic system was characterized by accumulation of stress related metabolites (proline); however, glycolate was identified only in the organogenesis system. The present work contributes to the understanding of the metabolic characteristics and differences between the two regeneration systems as manifested by profiles of EC, OC, and their regenerated shoots.

Key Message
Metabolic characteristics of two regeneration systems (somatic embryogenesis and organogenesis) of *Cymbopogon schoenanthus* were examined using NMR-based profiles of calli and their regenerated shoots, revealing clear qualitative and quantitative differences between the two systems at the studied developmental stages.

Keywords In vitro regeneration · Medicinal plants · Metabolomics · Multivariate analysis · NMR spectroscopy

Abbreviations
EC   Embryogenic calli
OC   Organogenic calli
ES   Embryogenic shoots
OS   Organogenic shoots
NMR  Nuclear Magnetic Resonance
BAP  6-Benzylamino purine
NAA  Naphthaleneacetic acid
2,4-D 2,4 Dichlorophenoxy acetic acid
MSB5  Murashige and Skoog 1962; Gamborg et al. 1968
**Introduction**

*Cymbopogon schoenanthus* subsp. *proximus* is a perennial grass that grows wild in southern areas of the Egyptian desert. The plant is used in traditional medicine for treatment of diabetes, inflammation, parasitic infection, and renal spasms (Boulos 1983). Previous studies have reported the antioxidant and antimicrobial activities of the plant extract (Selim 2011; Hashim et al. 2016). Crude extract of the plant is used in Proximol® drug production which is prescribed for ureteric stones, excess uric acid and urate crystals in urine and urinary tract infection cases. Previous phytochemical investigations have reported that the plant extract is rich in mono- and sesquiterpene metabolites (El Tahir and AbdelKader 2008).

In vitro propagation of plants can be achieved through different methods (Kumar and Reddy 2011). De novo morphogenesis through somatic embryogenesis and organogenesis are the most common methods for the regeneration and micropropagation in the genus *Cymbopogon* (Bhattacharya et al. 2009; Dey et al. 2010). Different methods for regeneration in *C. schoenanthus* subsp. *proximus* have been successfully achieved, including somatic embryogenesis, direct organogenesis, and de novo organogenesis (El-Bakry and Abdelsalam 2012, Abdelsalam et al. 2017a, 2018).

Differences between embryogenic and non-embryogenic calli of many plant species have been previously reported, e.g. in sugarcane, carnation and *Oryza sativa* (Nieves et al. 2003; Karami and Ostad-Ahmadi 2008; Vega et al. 2009). Both tissue systems have shown different morphological, anatomical, and histological characteristics, as well as phytochemical composition. The metabolomic analysis of the embryonic and non-embryogenic calli of a number of plant species using NMR spectroscopy has been discussed before (Parrilla et al. 2018; Mahmud et al. 2014). However, no previous studies have addressed the difference between the embryogenic and organogenic calli at the metabolomic level. In our previous work we described the metabolome differences between shoots regenerated through somatic embryogenesis and direct and indirect organogenesis (Abdelsalam et al. 2017b).

Therefore, the present study was carried out to compare and analyze the metabolome of embryogenic and organogenic calli of the medicinal important herb, *C. schoenanthus* subsp. *proximus* using NMR spectroscopy. Furthermore, the changes in the metabolic profiles that take place during regeneration through somatic embryogenesis and the organogenesis processes were studied through comparing the metabolites of each of the embryogenic and organogenic calli and their respective regenerated shoots.

**Materials and methods**

**Plant material**

*Cymbopogon schoenanthus* mature plants were collected from the botanical garden at Aswan University, Egypt. Mature inflorescences were stored in the dark at 25 °C inside paper bags. The plant material was taxonomically identified by Prof. Hasnaa Hosni, Professor of Plant taxonomy, Botany department, Cairo University, Egypt. A voucher plant specimen was placed in the Herbarium of the Faculty of Science, Helwan University, Egypt.

**Seed sterilization**

Mature seeds were separated from the inflorescence 24 h before culture. Healthy seeds were rinsed with tap water for 15 min and soaked in distilled water for 5 min. The seed was surface sterilized first using 95% ethanol for 1 min and then by dipping in 1.05% sodium hypochlorite solution for 20 min with stirring. Under aseptic conditions, and after removing the sodium hypochlorite solution, the seeds were washed 3 times in sterile distilled water, each for 15 min.

**Embryogenic callus induction and plant regeneration via somatic embryogenesis**

Somatic embryogenesis protocol was carried out as described by El-Bakry and Abdelsalam (2012). Embryogenic calli were induced by culturing surface sterilized seeds on Murashige and Skoog media (1962) with B5 vitamins (Gamborg et al. 1968) (MSB5) containing 3% sucrose and supplemented with 4 mg/L 2,4-Dichlorophenoxy acetic acid (2,4-D) and 0.5 mg/L 6-benzyl amino purine (BAP). Sixty seeds were cultured at 5 seeds per plate. After 4-weeks, embryogenic calli (EC) were separated into two groups. The first group consisted of 9 explants collected randomly from different plates, immediately frozen in liquid nitrogen and stored at − 80 °C. The second group of explants were subcultured onto media of the same composition and growth regulator concentrations for an additional 4 weeks. Then, EC including somatic embryos (SE) were transferred to MSB5 medium supplemented with 1 mg/L 2,4-D and 0.125 mg/L BAP for another 4 weeks to allow for SE maturation and germination. Ten weeks after initial seed culture, somatic embryo-derived shoots (ES) were formed. ES were moved to a medium containing 0.2 mg/L BAP for further shoot elongation for 2 weeks. Finally, ES were harvested, immediately frozen in liquid nitrogen, and stored at − 80 °C until extraction.
Organogenic callus induction and plant regeneration via de novo organogenesis

To induce organogenic calli (OC), surface sterilized seeds were cultured on MSB5 medium containing 3% sucrose and supplemented with 0.5 mg/L BAP and 4 mg/L 1-Naphthaleneacetic acid (NAA) (Abdelsalam et al. 2018). Ten petri dishes were cultured at 5 seeds per plate. After 4 weeks of seed culture, 9 organogenic calli were collected randomly from 9 petri dishes, immediately frozen in liquid nitrogen, and stored at − 80 °C.

To regenerate organogenic shoots (OS), OC were subcultured into magenta boxes containing 50 mL medium of the same composition and growth regulator concentrations. After eight weeks of initial seed culture OS were collected, frozen directly in liquid nitrogen, and stored at − 80 °C until extraction.

For all tissue culture experiments, the pH of the medium was adjusted to 5.7 before adding 2 g/L phytagel, then sterilized at 120 °C and 1 kg cm−2 pressure for 20 min.

All cultures were incubated at 25 °C under cool white fluorescent light (3000 lx) for 8 h light photoperiod for calli (first eight weeks in case of EC and first 4 weeks in case of OC) followed by 16 h photoperiod for embryo maturation, germination and shoot growth from all cultures.

Sample preparation and metabolite extraction

Six to nine replicates from each callus and shoot type were collected carefully, immersed directly in liquid nitrogen, and then stored at − 80 °C for at least 4 h before lyophilization. Dry tissues were ground to powder and homogenized. Twenty mg from each replicate was used for metabolite extraction. The metabolites were extracted according to Kim et al. (2010) using a constant volumetric ratio of 2:2:1 methanol:chloroform:water based on dry mass and water loss ratio (Bligh and Dyer 1959; Wu et al. 2008). The polar layer was removed from the extract and dried under vacuum for 24 h.

NMR sample preparation and data collection

Each dried polar extract was re-suspended in a constant volume (620 μL) of NMR buffer (1 mM deuterated Trimethylsilylpropanoic acid (TMSP-d4), 100 mM sodium phosphate buffer and 0.1% sodium azide, in 99.9 atom % D2O). NMR spectroscopic data (1D and 2D) were collected at 25 °C on a 700 MHz Bruker Avance™ III spectrometer (Billerica, MA). Predefined NMR experimental parameters as well as data processing were followed as previously reported (Abdelsalam et al. 2017b).

NMR data analysis

Metabolites were identified from the polar fraction of the calli and shoots by comparing 1H data with the Chenomx NMR Suite library of compounds (Chenomx Inc., Edmonton, Alberta, Canada). Confirmation of Chenomx assignments were achieved by comparing 1H-13C HSQC data with standard 1H-13C HSQC data available online at MMCD (Madison Metabolomics Consortium Database) (Cui et al. 2008) and with those reported in the literature.

Statistical analyses (principal components analysis (PCA), fold change analysis, and cluster analyses) were carried out using MetaboAnalyst 4.0 (Chong et al. 2019) based on bucket tables created by AMIX software (Bruker, Billerica, MA) with 95% confidence intervals. The spectral region was 0.5–10.0 ppm with 0.01 ppm bucket widths and advance bucketing; water regions (4.7–4.8 ppm) were excluded from the spectra. The spectral bins were normalized to total intensity. Cluster analyses were carried out using Ward’s linkage as a clustering algorithm and Euclidean distance as a similarity measuring.

Results

As a part of our ongoing research on the medicinal herb C. schoenanthus subsp. proximus, here we present and discuss the metabolic differences between embryogenic and organogenic calli. We also identified metabolome variation in somatic embryogenesis and organogenesis stages by comparing the metabolic profiles of EC and OC with their respective regenerated shoots, ES and OS, respectively.

Somatic embryogenesis stages are shown in Fig. 1. Embryogenic calli were induced by culturing the wild mature seeds on MSB5 medium supplemented with 2,4-D and BAP. The growing calli were dark yellow in color, highly granular, compact, and friable in texture (Fig. 1A). Distinct globular somatic embryos with clear suspensor regions developed within 5-weeks (Fig. 1B). Embryos matured to scutellar stage and then germinated to first leaf sheath after 6 and 8 weeks from initial seed culture (Fig. 1C, D). Bipolar structure somatic embryos developed after tissue transfer to medium supplemented with ¼ growth regulator concentrations (Fig. 1E). After subculturing on MSB5 with 0.2 mg/L BAP, embryogenic shoots continued to grow (Fig. 1F, G), elongated to 2–3 cm length and 1–3 mm width (Fig. 1H, I), and developed roots (Fig. 1I).

The different stages of organogenesis are illustrated in Fig. 2. Four-week-old organogenic calli were transparent, glossy, non-granular, and non-friable (Fig. 2A, B). Unipolar organogenic shoots were initiated after 5 weeks from seed culture (Fig. 2 C), continued to grow (Fig. 2 D, E, F),
Fig. 1 Different stages of somatic embryogenesis in *C. schoenanthus*: A Embryogenic culture induction and growth at 4-weeks; B 5-week-old culture showing globular somatic embryos with a clear suspensor region; C 6-weeks embryogenic callus with somatic embryos in the scutellar stage; D 8-weeks mature somatic embryos showing sheath of the first embryonic leaf (EL); E Bipolar mature embryo at 9 weeks showing embryogenic shoots (ES) and embryogenic root (ER); F, G Embryogenic shoots growing at 10 and 14 weeks respectively; H 16-weeks embryogenic shoots I Rooting embryogenic plantlets after 18 weeks in culture

Fig. 2 Different stages of de novo organogenesis in *C. schoenanthus*: A, B and C Organogenic callus induction at 2, 4 and 5 weeks showing the induction and growth of shoot primordia; D 6-weeks organogenic calli showing growth of first leaf; E, F Shoot growth and development at 7 and 8 weeks; G Rooting at 10-weeks and growth of regenerated organogenic plantlets
and completely developed and elongated to 3–5 cm after 10 weeks (Fig. 2G).

Metabolic profiling of the polar extract from calli and shoots was performed using 1D 1H and 2D 1H-13C HSQC experimentally collected NMR data and NMR databases. A total of 52 compounds were annotated in a spectral region of δ 0.5–10 ppm (Table 1). The identified compounds belong to various classes of chemicals including amino acids, amines, carbohydrates, organic acids, carboxylic acids, phenols, and alkaloids. The molecular formula, chemical shifts and coupling constants of the identified metabolites are listed in the supplementary data Table 1.

The metabolome heterogeneity between different morphogenic calli and between morphogenic calli and their corresponding regenerated shoots was accomplished through the unsupervised PCA, heat map cluster analysis, hierarchical cluster analysis as well as fold change.

**Metabolite differences between EC and OC**

A total of 41 compounds were identified in EC and OC polar extracts. Except for glycolate, which was only found in OC, all 41 metabolites were present in both callus types (Table 1).

Score plots, constructed using 2D and 3D pairwise PCA, illustrated a clear separation between EC and OC samples (Fig. 3A, B) due to variations in metabolite concentrations. The metabolites responsible for this separation and their relative concentrations were identified using heat map correlation dendrogram and fold change analyses. Heat map correlation (Fig. 3C, Table 2) shows eight metabolites, out of the top twenty-five, significantly increased in EC in comparison with OC (4-aminobutyrate, arginine, asparagine, glucose-6-P, betaine, malonate, proline and glycine). Fold change analysis (Fig. 3D) showed that the concentrations of 4-aminobutyrate, proline and malonate increased 2.9-fold, 2.7-fold and 2.4-fold respectively in EC compared to OC. Amino acids arginine and asparagine increased 1.8-fold and 1.2-fold respectively in EC. On the other hand, myo-inositol and sucrose were higher by 1.5-fold and 1.1-fold respectively in OC. In addition, threonine and galactarate increased 1.6-fold and 1.9-fold in OC.

**Metabolite difference between EC and ES**

Six metabolites (4-hydroxybenzoate, 4-pyridoxate, galate, lactate, lysine and serine) were identified in ES but not detected in EC (Table 1). These metabolites differentiate ES from EC.

PCA of EC and ES showed that the calli samples were grouped together and completely separated from the shoot samples (Fig. 4A, B). As indicated by heat map correlation analysis (Fig. 4C), ES showed higher intensities of several

| Compound Name       | EC  | OC  | ES  | OS  |
|---------------------|-----|-----|-----|-----|
| 1 2-Hydroxyisobutyrate | –   | –   | √   | –   |
| 2 4-Aminobutyrate    | √   | √   | √   | √   |
| 3 4-Hydroxy benzoate | –   | –   | √   | –   |
| 4 4-Pyridoxate       | –   | –   | √   | –   |
| 5 Acetate            | √   | √   | √   | √   |
| 6 Alanine            | √   | √   | √   | √   |
| 7 Arginine           | √   | √   | √   | √   |
| 8 Asparagine         | √   | √   | √   | √   |
| 9 Aspartate          | √   | √   | √   | √   |
| 10 Betaine           | √   | √   | √   | √   |
| 11 Choline           | √   | √   | √   | √   |
| 12 Citrate           | √   | √   | √   | √   |
| 13 cis-Aconitate     | √   | √   | √   | –   |
| 14 Dimethylamine     | √   | √   | √   | √   |
| 15 Ethanolamine      | √   | √   | √   | √   |
| 16 Format            | √   | √   | √   | √   |
| 17 Fructose          | √   | √   | √   | √   |
| 18 Fumarate          | √   | √   | √   | √   |
| 19 Gallate           | –   | –   | √   | –   |
| 20 Galactarate       | √   | √   | √   | √   |
| 21 Glucose           | √   | √   | √   | √   |
| 22 Glucose-1-P       | √   | √   | √   | √   |
| 23 Glucose-6-P       | √   | √   | √   | √   |
| 24 Glutamine         | √   | √   | √   | √   |
| 25 Glutamate         | √   | √   | √   | √   |
| 26 Glutarate         | √   | √   | √   | √   |
| 27 Glycine           | √   | √   | √   | √   |
| 28 Glycolate         | –   | –   | √   | –   |
| 29 Histamine         | –   | –   | √   | –   |
| 30 Homoserine        | –   | –   | √   | –   |
| 31 Isobutyrate       | √   | √   | √   | √   |
| 32 Isoleucine        | √   | √   | √   | √   |
| 33 Isovalerate       | √   | √   | √   | √   |
| 34 Lactate           | –   | –   | √   | –   |
| 35 Leucine           | √   | √   | √   | √   |
| 36 Lysine            | –   | –   | √   | –   |
| 37 Malate            | √   | √   | √   | √   |
| 38 Malonate          | √   | √   | √   | √   |
| 39 Myo-inositol      | √   | √   | √   | √   |
| 40 Phenylalanine     | √   | √   | √   | √   |
| 41 Proline           | √   | √   | √   | √   |
| 42 Pyroglutamate     | √   | √   | √   | √   |
| 43 Pyruvate          | √   | √   | √   | √   |
| 44 Serine            | –   | –   | √   | –   |
| 45 Succinate         | √   | √   | √   | √   |
| 46 Sucrose           | √   | √   | √   | √   |
| 47 Threonine         | √   | √   | √   | √   |
| 48 Trans-aconitate   | √   | √   | √   | √   |
| 49 Trigonelline      | √   | √   | √   | √   |
amino acids (asparagine, betaine, glutamate, proline and pyroglutamate). While the monosaccharides (glucose, glucose-1-P, glucose-6-P and fructose) and other amino acids (arginine, threonine and leucine) were up regulated in ES. Fold change analysis (Fig. 4D) showed that the concentration of proline and pyroglutamate increased 13-fold and 11.6-fold, respectively, in ES in comparison with EC. Also, 4-aminobutyrate, asparagine and betaine increased tenfold, sevenfold, and 8.7-fold, respectively, in ES. Glucose and fructose production was enhanced more than 18-fold and myo-inositol increased 4.4-fold in EC compared to ES.

**Metabolite differences between OC and OS**

The metabolites 2-Hydroxyisobutyrate, 4-hydroxybenzoate, 4-Pyridoxate, gallate, histamine, homoserine, lysine and tyrosine were found exclusively in OS and not in OC; hence, these metabolites differentiate OS from OC. (Table 1).

In Fig. 5A and B, OC and OS are clearly distinguished in 2D and 3D scores plots. Based on heat map correlation (Fig. 5C, Table 2), OS was characterized by higher concentrations of sucrose and amino acids e.g. alanine, asparagine, leucine, and threonine. While monosaccharides (glucose, glucose-6-phosphate, and fructose) and the amino acid arginine were accumulated in OC. Trigonelline was also upregulated in OS to a higher level than in OC. Fold change analysis (Fig. 5D) demonstrated that threonine, glutamine, and valine were upregulated 14-fold, 13-fold, and 11-fold, respectively, in OS in comparison with OC. Also, trigonelline increased 17-fold in OS. On the other hand, the concentration of glucose and fructose were upregulated 3.5-fold and 2.9-fold, respectively, in OC.

**Metabolite differences between ES and OS**

Qualitatively, ES can be discriminated from OS, as indicated by Table 1. ES is distinguished by the presence of cis-aconitate, lactate, malonate, and serine, while OS is characterized by the presence of 2-hydroxyisobutyrate, glycolate, histamine, homoserine, and tyrosine metabolites.

PCA score plots showed a clear distinction between ES and OS based on metabolite concentration (Fig. 6A, B). Heat map dendrogram (Fig. 6C) presents the relative metabolite concentrations between ES and OS, where metabolites, 4-aminobutyrate, betaine and proline have been upregulated in ES. On the other hand, carbohydrates (sucrose, glucose, glucose-1-P, glucose-6-P and fructose) were accumulated in OS. Fold change analysis (Fig. 6D) showed that metabolites 4-aminobutyrate, asparagine and proline were upregulated 14.4-fold, 8.3-fold, and 4.5-fold, respectively, in ES. In OS, glucose, fructose, and glucose-6-P were elevated 2.1-fold, 6.6-fold, and 7.6-fold, respectively.

**Metabolite characteristic of morphogenic calli and de novo regenerated shoots**

To study the metabolome correlations between shoots and calli, multivariate analysis was carried out. The unsupervised PCA analysis showed that EC, OC, ES and OS are separated into four groups in both 2D and 3D scores plots (Fig. 7A, B). Similar results were obtained from hierarchical cluster dendrogram (Fig. 7C), which showed two main clusters: shoots from embryogenesis and organogenesis were grouped in one cluster, while EC and OC were clustered with no incorporation observed between treatments.

The metabolic profiles of callus tissues and the de novo regenerated shoots revealed some significant results. There are four metabolites (4-hydroxybenzoate, 4-Pyridoxate, gallate and lysine) which distinguished shoot tissues (ES and OS) from calli tissues (EC and OC) (Table 1). ES was characterized by the unique presence of serine and lactate metabolites in comparison with OS and calli (EC and OC). While 2-hydroxyisobutyrate, tyrosine, histamine and homoserine were detected only in OS. Interestingly, glycolate is observed in the organogenic system (OC and OS) but not in the embryogenic system (EC and ES). The metabolites cis-aconitate and malate were not found in OS, however they were detected in ES and both types of calli (EC and OC).

The embryogenic system was characterized by accumulation of the stress-related amino acids 4-aminobutyrate and proline. The organogenic system showed upregulation of sucrose and myo-inositol.

**Discussion**

Somatic embryogenesis and organogenesis are well known morphogenetic pathways in cell and tissue differentiation leading to plant regeneration and multiplication. Previous studies considered the morphological and histological variation between the two regeneration systems (Anzidei et al. 2000; Pathi et al. 2013). A number of studies clarified some of the genetic control and gene expression aspects,
Fig. 3  Metabolite variables associated with callus type (EC = embryogenic calli and OC = organogenic calli). A and B Pairwise PCA analysis. The ovals in the 2D scores plot indicate 95% Hotellings confidence intervals. PC1 and PC2 explain a total of 76% of the variance and with PC3, 91.8% of total variance. C Heat-map dendrogram shows a relative abundance of metabolites in the polar extract of morphogenic calli. Color scale is relative to the abundance of each metabolite. Each row represents a metabolite, and each column represents a callus sample. D Fold change analysis (fold change threshold = 1) shows the change in relative concentration of significant metabolites.
in particular, during somatic embryogenesis (Chugh and Khurana 2002). Little attention, so far, has been given to metabolome variation during morphogenesis.

The present work explores the metabolomes of both morphogenetic pathways in order to understand similarities and differences among both pathways.

Metabolite differences between EC and OC

The concentration of the monosaccharide glucose-6-P increased in EC in comparison with OC, in contrast to the disaccharide sucrose. Sucrose is a non-reducing disaccharide that is cleaved by the action of invertase into its building monohexosides. Early stages of somatic embryogenesis were shown to have a boosted invertase activity (Iraqi and Tremblay 2001; Konradova et al. 2002) which might explain the recorded increase of glucose-6-P in EC versus OC. Glucose can be utilized by the plant after phosphorylation to glucose-6-P by hexokinase enzymes (Granot et al. 2013). Glucose-6-P has many functions in the plant cell. It can be converted to UDP-glucose which is vital for polysaccharides and cell wall biosynthesis. Also, it plays an essential role in glycosylation reactions of different compounds, for example terpenoids and flavonoids (Kleczkowski et al. 2010). Moreover, Dyson et al. (2014) reported that glucose-6-P is essential in controlling the transition from the heterotrophic status to photosynthetic status in plants, in addition to, its importance for zygotic embryo germination in Arabidopsis (Yang et al. 2019).

EC is characterized by a significant accumulation of the amino acids 4-aminobutyrate, arginine, asparagine, betaine, and proline. The overproduction of these amino acids in plants under stress conditions has been reported in different studies. For example, 4-aminobutyrate and proline have been reported to accumulate in Phlox subulata plant under drought stress (Xiong et al. 2021), and arginine and asparagine were accumulated in date palm and wheat under stress conditions (Al Kharusi et al. 2020; Curtis et al. 2018). Also, betaine and proline concentrations increased in rice plant under abiotic stress (Ahmed et al. 2021). The accumulation of such osmo-protectant amino acids in EC in comparison with OC can be attributed to 2 reasons. First, embryogenic calli are known to develop under stress conditions resulting from the use of 2,4-D on the culture medium, a known stress factor during somatic embryogenesis (Shariatpanahi et al. 2006). Also, the transition from somatic cell to somatic embryo was found to activate the expression of stress-associated genes (Jin et al. 2014; Salvo et al. 2014). The second reason may be that the accumulation of the amino acids proline and arginine in EC may arise from the essential role of these metabolites in the somatic embryogenesis process. Proline has an important function in somatic embryo maturation in conifer and strawberry plants (Feirer 1995; Gerdakaneh et al. 2011). Arginine is a precursor of polyamines in plants through arginine decarboxylase enzyme. Polyamines are necessary in somatic embryogenesis and their decrease leads to reduction in the embryogenesis process (Bertoldi et al. 2004; Minocha et al. 2004). In other studies, high concentrations of arginine and asparagine have been reported in embryogenic calli of Boesenbergia rotunda, Silybum marianum and Brachypodium distachyon compared to non-embryogenic calli (Ng et al. 2016; Khan et al. 2014; Mamedes-Rodrigues et al. 2018).

Metabolite differences between EC and ES

In the present study, ES showed higher concentrations of sucrose, while the monosaccharides glucose and fructose were upregulated in EC. Higher concentration of hexoses along with lower concentration of sucrose in EC when compared to ES could refer to the important role of the simple sugars in cell proliferation and differentiation processes needed for transition from EC to plantlet stage. These metabolite variations during embryogenesis have been reported in several preceding studies (Borisjuk et al. 1998; Hill et al. 2003; Hudec et al. 2016). Carbohydrates are considered as a signal for gene expression during plant growth, developmental and floral transition (Weber et al. 1997; Eveland and Jackson 2012).

The concentrations of the amino acids asparagine, betaine, glutamate, phenylalanine, proline, and pyroglutamate increased in ES compared to EC (in contrast to arginine). The accumulation of glutamate in shoots compared to calli may be because the enzymes responsible for their biosynthesis (glutamate synthases) are present in different isoforms in plant leaves (Hirel and Lea 2002; Forde and Lea 2007) where glutamate is a precursor of chlorophyll biosynthesis in plants (Reinbothe and Reinbothe 1996;
Fig. 4 Metabolite variables associated with embryogenic tissue (EC = embryogenic callus and ES = embryogenic shoots). A and B Pairwise PCA analysis. The ovals in the 2D scores plot indicate 95% Hotellings confidence intervals. PC1 and PC2 explain 86.8% of the total variance and with PC3, 92.5% of total variance. C Heat-map dendrogram shows a relative abundance of metabolites. Color scale is relative to the abundance of each metabolite in the polar extract. Each row represents a metabolite, and each column represents a replicate. D Fold change analysis (fold change threshold = 1) shows the change in relative concentration of significant metabolites.
Fig. 5 Metabolite variables associated with organogenic tissue (OC = organogenic callus, OS = organogenic shoots). A and B Pair-wise PCA analysis. The ovals in the 2D scores plot indicate 95% Hotelling’s confidence intervals. PC1 and PC2 explain 87.5% of the total variance and with PC3, 92.4% of total variance. C Heat-map dendrogram shows a relative abundance of metabolites. Color scale is relative to the abundance of each compound. Each row represents a metabolite, and each column represents a sample of callus or shoot. D Fold change analysis (fold change threshold = 1) shows the change in relative concentration of significant metabolites.
Fig. 6 Metabolite variables associated with shoot type (ES = embryogenic shoots, OS = organogenic shoots). A and B Pairwise PCA analysis. The ovals in the 2D scores plot indicate 95% Hotellings confidence intervals. PC1 and PC2 explain 88.1% of the total variance and with PC3, 92.3% of total variance. C Heat-map dendrogram shows a relative abundance of metabolites. Color scale is relative to the abundance of each compound. Each row represents a metabolite, and each column represents a sample of shoot. D Fold change analysis (fold change threshold = 1) shows the change in relative concentration of significant metabolites.
Yaronskaya et al. (2006). Moreover, Cangahuala-Inocente et al. (2014) suggested that the decrease in amino acid levels in early stages of somatic embryogenesis may be due to their necessity in cell differentiation to complete the development process. Proline also showed a higher concentration in ES which may be attributed to stress resulting from long-term culturing conditions. Its role in cell protection during long-term stress has been reported by Kishor and Sreenivasulu (2014).

The accumulation of arginine in EC may be because it is a precursor of many compounds such as urea, nitric oxide, and polyamines. These metabolites have a regulatory role in cell development and early seedling germination (Feirer 1995; King and Gifford 1997; Llebrés et al. 2018).

![Fig. 7 Multivariate analysis, A 2D and B 3D scores plots for embryogenic callus = EC; organogenic callus = OC; embryogenic shoots = ES shoots; organogenic shoots = OS. The ovals in the 2D scores plot indicate 95% Hotellings confidence intervals. PC1 and PC2 explain 58.9% of the model variance, and with PC3, 72.3% of the total variance. C Hierarchical cluster analysis (HCA) dendrogram shows correlations between embryogenic and organogenic calli and their regenerated shoots based on metabolite variation](image-url)
Metabolite differences between OC and OS

The present study showed higher concentrations of sucrose, alanine, asparagine, leucine, and threonine in OS, while the monosaccharides glucose, glucose-6-phosphate, and fructose, and the amino acid arginine were accumulated in OC. OS accumulated sucrose as an autotrophic tissue carrying out photosynthesis. Autotrophic green cells are capable of carrying out different metabolic pathways and could result in an increase in amino acid concentrations. Hildebrandt et al. (2015) reported that the accumulation of amino acids in growing photosynthetic tissues is due active protein synthesis. Also, some amino acids have a known role being restricted in chloroplast and green tissue. For example, asparagine was found to play a role in the photosynthesis process of pea leaves (Ta et al. 1986). Furthermore, amino acids alanine, aspartate, glutamate, threonine, and glycine can be synthesized in leaves during photosynthesis from intermediates synthesized during the carbon reduction pathway (Bassham 1964; Kirk and Leech 1972). Palma et al. (2010) reported high concentrations of the amino acids asparagine, glutamine and valine in differentiating callus of the Vanilla planifolia plant when compared to undifferentiated calli.

In the present study, trigonelline was found in all tissues studied (both calli and shoots). Its concentration in OS was higher than its concentration in OC. In our previous work (Abdelsalam et al. 2017b) we reported the presence of trigonelline in wild plants and in in vitro regenerated shoots, but not in greenhouse shoots. Here, trigonelline has been recognized in both types of calli. Trigonelline is a pyridine alkaloid synthesized through the methylation of nicotinic acid and known to possess anticancer activity (Chen and Wood 2004). This alkaloid was reported to accumulate in leaves of many plants including Coffea arabica (Ashihara 2006). Plant cell cultures have been reported to produce large amounts of trigonelline when compared to ex vitro cultivated plants. For example, trigonelline accumulated 12- to 13-fold higher concentrations in Trigonella foenum-graecum callus cultures than in plant roots and shoots (Radwan and Kokate 1980). In addition, Beygi et al. 2021 used osmotic stress and elicitors to enhance the trigonelline biosynthesis in Trigonella foenum-graecum calli. In our previous study (Abdelsalam et al. 2021), the concentration of trigonelline increased tenfold when using methyl jasmonate in the tissue culture media in comparison to the control.

Metabolite differences between ES and OS

In our previous work (Abdelsalam et al. 2017b) we described the metabolome variation between embryogenic shoots and indirect de novo organogenic regenerated shoots using fold change analysis. In the present work, metabolome difference between ES and OS was visualized using heat map dendrogram. Herein, metabolites 4-aminobutyrate, betaine and proline accumulated in ES. These osmo-protectant metabolites have been upregulated in ES because of stress created in ES due to long-term culturing conditions. This suggestion agrees with Graner et al. 2019, where they described the effect of long-term culture stress in the Neoregelia johanonis plant. In addition, during the later stages of embryogenesis, several genes, such as LATE EMBRYO ABUNDANT (LEA), are highly expressed (Olvera-Carrillo et al. 2010). These proteins have a role in the development of somatic embryos as well as in stress response (Gulzar et al. 2020).

Mono- and disaccharides were downregulated in ES in comparison with OS in the present work. Also, the concentrations of amino acids alanine, asparagine and threonine increased in OS. Sugars are known to have an important function in somatic embryogenesis at various stages (Lipavská and Konrádová 2004). Down regulation of sugar concentration during somatic embryogenesis in comparison with zygotic embryogenesis has been reported in Acca sellowiana (Pescador et al. 2008). Bartos et al. observed the decrease in soluble sugars and amino acids including asparagine and glutamine during cotyledonary stages in Coffea arabica.

Characteristic metabolites in morphogenetic calli and in their de novo regenerated shoots

In this study, ES were characterized by the presence of serine and lactate metabolites. The most important biosynthetic pathway of serine is the glycolate pathway which is restricted to autotrophic tissue (Bauwe et al. 2010; Häusler et al. 2014). This may explain the absence of serine from the different callus tissues. Also, the essential role of serine under stress conditions (Ho and Saito 2001) and embry development (Yamaoka et al. 2011; Ros et al. 2014) may explain the presence of this amino acid in ES, but not in OS. The presence of lactate also has been correlated to hypoxic stress in Arabidopsis (Dolferus et al. 2008).

The present data shows that tyrosine was detected only in OS. It is an aromatic amino acid reported to control organogenesis in tobacco callus (Skooq 1971). We suggest that tyrosine was not detected in either EC and OC because of the high activity of cell proliferation and growth during this stage to produce organs. This condition was reported to increase phenylalanine biosynthesis rather than tyrosine from their common precursor chorismate (Schenck and Maeda 2018). Our findings suggest that tyrosine was not detected in ES because these shoots are stressed by long-term culture conditions, which may induce tyrosine breakdown. Tyrosine degradation under stress conditions was documented by Frelin et al. (2017). Tyrosine is a precursor of a number of metabolites which possess many physiological
functions in plants like betalain which has a role in pollination and seed dispersal in plants (Belhadj et al. 2017). This isoquinoline alkaloid plays a defensive function against herbivores and plant infections (Sato et al. 2007), and it is used as human drug (Schenck and Maeda 2018).

Both shoot types (ES and OS) were characterized by the presence of lysine. Lysine is an essential amino acid with high nutritional value (Fornasier et al. 2003). The presence of lysine in shoots but not in calli may be because many of the enzymes that participate in their biosynthesis are known to be located in the plastid (Bryan 1990).

Both OC and OS showed the presence of glycolate which was absent from both EC and ES. Glycolate is metabolized during the photorespiration process. It can be converted into various metabolites like glycine, serine and glycerate (Tolbert 1979). The effect of 2,4-D on glycolate metabolism through activation of glycolate oxidase has been reported in pea leaves (McCarthy-suarez 2011). Our data may suggest that the presence of 2,4-D in the culture medium of somatic embryogenesis may increase the metabolism of glycolate to glycine in EC and to serine in ES.

The embryogenic system presented shows the upregulation of stress related amino acids as 4-aminobutyrate and proline in comparison with the organogenic system. Karami and Saidi (2010) identified stress-related genes and proteins that are linked to somatic embryogenesis.

**Conclusion**

Morphogenetic calli whether embryogenic or organogenic, were found to be metabolically distinct from the green autotrophic shoots, both qualitatively and quantitatively. When compared to shoots, calli had higher concentrations of hexoses such as glucose, fructose, glucose-6-P, and the amino acid arginine. Regenerated shoots showed a number of key metabolites (sucrose, alanine, asparagine and trigonelline) which were found characteristic to the autotrophic, metabolically active plant tissue. Embryogenic calli was clearly different from organogenic calli: the former accumulated higher concentrations of amino acids such as 4-aminobutyrate, betaine and proline. On the other hand, organogenic calli has uniquely produced glycolate. Such metabolite variations may be attributed to the difference in culture spans and growth regulators used for induction in each case. Embryogenic shoots obtained from calli induced on 2,4-D and regenerated after a relatively prolonged culture span showed marker metabolites such as serine and lactate. Organogenic shoots obtained from calli induced using NAA and regenerated after shorter culture span were characterized by the presence of histamine, homoserine and tyrosine. Stress-related amino acids were shown to accumulate in the embryogenic system in comparison with organogenic system.

Our findings suggest that shoots regenerated through de novo organogenesis are more physiologically developed and metabolically active than their somatic embryogenesis counterparts. The present study should be considered a first step towards understanding the metabolic activity of shoots regenerated in vitro through different morphogenetic pathways. Also, metabolites that characterize different types of morphogenetic calli were identified as an expression of their metabolism under culture conditions, involving stress conditions, and during their growth and development.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02202-3.

**Acknowledgements** This work was funded by the Culture Affairs and Missions Sector, Ministry of Higher Education in Egypt; South Carolina IDEA Networks of Biomedical Research Excellence (SC-INBRE, 2 P20 GM103499); National Science Foundation Historically Black Colleges and Universities Undergraduate Program, Division of Human Resources Division (NSF HBCU-UP, HRD-1332516); and National Science Foundation Major Research Instrumentation, Division of Biological Infrastructure (NSF MRI, DBI-1429353).

**Author contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by AA. The NMR data analysis and metabolite identification were revised by AB. The first draft of the manuscript was written by AA. AE made extensive critical revisions to the initial draft, concentrating on crucial themes and the manuscript’s core goal. The final manuscript was revised by all authors.

**Availability of data and materials** All data generated or analyzed during this study are included in this published article and are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose. Asmaa Abdelsalam, Kamal Chowdhury, Areeza Boroujerdi and Ahmed El-Bakry declare that they have no conflict of interest.

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

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