Influence of silver nitrate and copper sulfate on somatic embryogenesis, shoot morphogenesis, multiplication, and associated physiological biochemical changes in *Gladiolus hybridus* L.

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Abstract
Micronutrients are essential for in vitro plant tissue growth, and manipulating their levels in culture medium to promote in vitro growth and morphogenesis is essential in plant tissue culture experiments. In the present study, the effects of copper sulfate hydrate (CuSO₄·5H₂O, 0–15 μM), silver nitrate (AgNO₃, 0–60 μM), and their combinations on callus induction and various morphogenic processes at different treatment levels were studied in *Gladiolus hybridus*. AgNO₃ was found to be more efficient at promoting the parameters studied, with 40 μM being more efficient than other treatment levels tested. In the case of CuSO₄·5H₂O treatments, maximum efficiency was achieved with 7.5 μM. Results of the experiments on morphogenesis and associated physiological biochemical changes suggest that treatments that involved combining the two metals promoted morphogenesis to a greater degree by affecting physiological biochemical changes essential for the morphogenic parameters studied. This technique seems to be appropriate when applied to embryogenic tissue formation and somatic embryo differentiation.

Key Message
Treatment with silver nitrate, copper sulfate, and their combination at different stages of in vitro morphogenesis promoted transdifferentiation through influence on physiological biochemical changes essential for the developmental processes in *Gladiolus hybridus*.

Keywords
Ornamental plants · Plant growth regulators · Embryogenic callus · Silver nitrate · Copper sulfate · Antioxidants

Abbreviations
PCTOC  Plant cell, tissue, and organ culture
MS  Murashige and Skoog medium
PGRs  Plant growth regulators
2,4-D  2,4-Dichlorophenoxy acetic acid
BAP  N°-benzylaminopurine
NAA  Naphthalene acetic acid
IBA  Indole butyric acid
CuSO₄·5H₂O  Copper sulfate (hydrate)
AgNO₃  Silver nitrate
SE  Somatic embryogenesis
Chl  Chlorophyll
PS  Photosystem
FW  Fresh weight
DW  Dried weight
ROS  Reactive oxygen species
SOD  Superoxide dismutase
H₂O₂  Hydrogen peroxide
CAT  Catalase
APX  Ascorbate peroxidase
GPX  Guaiacol peroxidase

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Introduction

In vitro plant regeneration is influenced by culture medium nutrients. Micronutrients are indispensable to the composition of many enzyme molecules involved in physiological biological processes essential for plant development (Niedz and Evens 2007; Bidabadi and Jain 2020). Physiological molecular aspects of plant in vitro morphogenesis have been investigated in several species and varieties (Dalton 2020), but little is known about the effect of nutritional composition of culture media on morphogenic processes in plant tissue culture experiments (Bidabadi and Jain 2020). Pioneer studies on the importance of organic and inorganic nutrients to in vitro plant development were performed by Murashige and Skoog (1962). Since then, studies have highlighted the importance of the concentration of basal medium constituents for proper plant growth, development, and morphogenesis in plant tissue culture experiments (Preece 1995; Mahendra and Dutta Gupta 2004; Chimdessa 2020; Dalton 2020), with inorganic constituents offering variables for studying the influence of elemental composition on in vitro morphogenic potential of plants (Kothari-Chajer et al. 2008; Silvestri et al. 2019).

Micronutrients are regarded as indispensable components of enzyme molecules that serve as secondary messengers involved in regulating physiological biochemical pathways associated with the control of in vitro plant tissue growth (Maksymiec 1997; Niedz and Evens 2007). However, literature focuses on mineral uptake and their physiological nutritional role in plant development rather than morphogenesis (Isah 2015). Plants require metal ions in trace quantity for normal growth and development owing to the roles they play in nucleic acid metabolism and redox reactions as well as in the structural configuration of many enzyme molecules. Higher cellular concentration of metal ions may affect metabolic processes with resultant growth inhibition through cellular toxicity (Zenk 1996). Copper sulfate (CuSO₄) is among the components of MS medium that serve as a principal source of copper (Cu), a transitional metal having two oxidation states (Cu²⁺) under normal physiological growth condition, and serves as source of Cu⁺ essential for plant growth and development. It is regarded as the most crucial micronutrient for plant growth and development, but its concentration in growth medium needs optimization for enhanced performance (Lipman and Mackinney 1931; Arnon and Stout 1939). Owing to the broad-spectrum antimicrobial activity of Cu, its supplementation in in vitro growth medium of plants could promote multiplication with substantial reduction in infection due to endogenous latent microbes (Javed et al. 2017; Kim et al. 2017; Silvestri et al. 2019). Although optimum physiological requirements of Cu varies across plant species (Adrees et al. 2015), increased concentration promotes shoot morphogenesis through the crucial role it plays in metabolic processes in cells (Delhaize et al. 1985; Droppa and Horvath 1990; Javed et al. 2017). This may be achieved through enhancement of photosynthesis, respiration, antioxidant reactions, hormone biosynthesis, and perceptions essential to plant growth under in vitro conditions (Nomura et al. 2015; Peñarrubia et al. 2015). It has been estimated that about 50% of the Cu found in plants is associated with chloroplasts and bound to plastocyanin that functions in cells as a mediator of electron transfer between photosystem (PS) I and PS II (Weigel et al. 2003). While it is an integral part of photosynthetic machinery, present in thylakoids, high levels inhibit PS II and associated enzymes of chlorophyll (Chl) biosynthesis (Moustakas et al. 1994; Sanchez-Viveros et al. 2011). Inhibition of photosynthesis through Chl biosynthesis may result in damage to membrane permeability, disturbing cellular physiological metabolic processes (Shioi et al. 1978; Baszynski et al. 1982). Further, owing to its redox activity, higher concentration of free Cu ions may cause cellular toxicity that must be regulated during in vitro uptake through regulation of Cu transporters to economize deficiency via transcriptional and post-transcriptional regulation (Burkhead et al. 2009; Ravet and Pilon 2013). Hence, in compliance with the laws of minimum and physiological nutritional tolerance (Good 1931; Shelford 1913, 1931), an optimal and cellular tolerable range of copper concentration exists when supplemented above levels of standard culture medium formulations.

Because of its stability, availability, specificity, and solubility in water and as a potent inhibitor of ethylene, silver nitrate (AgNO₃) is also used in plant cell, tissue, and organ culture (PCTOC) experiments for the crucial role it plays in regulating plant physiological processes essential for in vitro plant morphogenesis. Among the physiological processes influenced by silver ions are polyamine biosynthesis, ethylene- and calcium-mediated biochemical pathways, and morphogenesis (Kumar et al. 2009a). Silver nitrate, or in the form of silver thiosulfate, has been reported to influence in vitro morphogenesis in many PCTOC experiments, but its mechanism of action is unclear (Eapen and George 1997; Bais et al. 2000, 2001; Kim et al. 2017). Evidence has suggested that its interfering effect on ethylene perception is due to water solubility and lack of cellular toxicity when an effective concentration is used (Beyer 1976a, b; Kumar et al. 2009a). An interactive effect of silver occurs through displacement of the active site of copper at receptor complex, causing an opposite effect that results in stable physiological processes essential for the enhancement of in vitro morphogenesis (Kumar et al. 2009a). In plant cells, Ag⁺ (dissolved in AgNO₃) may react with compounds (such
as DNA, RNA, amino acids, and lipopolysaccharides) to form silver nanoparticles that may form bond(s) with many biotic receptors to become geno- or cytotoxic (Ratte 1999; Park et al. 2010; Yin et al. 2011). As cells are sensitive to nontoxic low concentrations of Ag\(^+\), where the metal ions interfere with auxin and ethylene signaling, Ag\(^+\) has found increased use in PCTOC experiments for the regulation of plant developmental processes (Strader et al. 2009; Steinitz et al. 2010; Parimalan et al. 2011). Silver (nitrate) is also highly used as an inhibitor of ethylene, and its interactive effect with Cu may result in the displacement of Cu at the active site of receptors in cells (Ciardi and Klee 2001). Basal salts and vitamins present in PCTOC medium also contain chlorine, and addition of AgNO\(_3\) results in the formation of AgCl precipitate (Murashige and Skoog 1962; Caldas et al. 1990; Steinitz et al. 2010). Over time, silver in particulate form may be released into aqueous solution in the form of Ag\(^+\) for uptake in plant cells to improve cellular processes essential for plant growth and development in vitro, affecting overall biomass yield (Kittler et al. 2010; Tripathi et al. 2017). There has been increasing interest in the use of metal nanoparticles in nutrient medium used alone or in combination to promote morphogenesis in vitro plant culture (Kim et al. 2017; Malik et al. 2021).

**Gladiolus hybridus**, the eighth most valuable cut flower perennial bulbous plant belonging to Iridaceae and native to Africa is cultivated for its moderate-to-long vase life and beautiful spikes (Memon et al. 2016). Its estimated annual sales were reported to be around 370 million corms cultivated in over 130,000 ha (Narain 2004; Singh 2011) with the low propagation method, predominantly vegetative, using corms and cormlets, and seeds used only for raising hybrid varieties (Memon et al. 2016). Plant cell, tissue and organ culture techniques offer alternative propagation methods for mass production of *G. hybridus* en masse (Ziv et al. 1970; Memon et al. 2016); physiological growth requirements could be optimized by improving our knowledge on nutritional physiological factors regulating biomass production under in vitro culture conditions (Bidabadi and Jain 2020). In most reported experimental literature on the morphogenesis of plant species, only one culture medium formulation is used for the duration of study, a limitation that does not permit complete nutritional characterization of physiological optimum growth requirements of different in vitro developmental stages of cultures. Appraisal of the literature reports on in vitro studies on members of the genus *Gladiolus* revealed that substantial information is known about clonal propagation and biomass production (Memon 2012; Memon et al. 2012, 2016), but physiological growth requirements of nutrient media have not been investigated. In the present study, influence of copper sulfate hydrate (CuSO\(_4\)·5H\(_2\)O) and AgNO\(_3\) on callus induction, somatic embryogenesis (SE), shoot biomass production, and physiological biochemical changes associated with morphogenic processes of *G. hybridus* were assessed.

### Materials and Methods

#### Explant collection, surface sterilization, and culture establishment

Healthy *G. hybridus* corms were obtained from the Horticultural Society of India, Calcutta, West Bengal (India), and their outer coverings removed and washed with cetrimide for about 15 min, then under running tap water for 3 min. Thereafter, obtained corms were treated with 70% ethanol for 5 min and rinsed with sterile distilled water three times. Corms were surface-sterilized with mercuric chloride (0.05%) for 3 min and rinsed with sterile distilled water three to four times to remove traces of HgCl\(_2\). Surface-sterilized corms were longitudinally sliced into 5–10-mm explant pieces before being cultured on solid MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D; 4.52 μM), sucrose (3%) and inositol (100 mg L\(^{-1}\)) for callus induction under dark condition of incubation. pH of medium was adjusted to 5.7 before autoclaving at 10,321.35 pa and 121 °C for 15 min. The induced callus cultures were proliferated under illumination condition on control MS medium supplemented with 2,4-D (4.52 μM), BAP (2.22 μM), and BAP + NAA (8.88 + 1.35 μM) until brownish embryogenic callus formation characterized by near-globular or elongated and cylindrical structures on callus surface was observed (Mujib et al. 2017; Khajuria et al. 2021). The number of embryos induced on the callus surface and their proliferation increased with culture duration, but data were taken after 4 weeks of cultivation for each. Proliferated somatic embryos were transferred to maturation medium supplemented with GA\(_3\) (2.60 μM) where the embryos elongated before germination on medium added with BAP (2.22 μM). Recovered plantlets were transferred to medium supplemented with IBA (4.84 μM) for efficient root formation before acclimatization experiment. Effect of CuSO\(_4\)·5H\(_2\)O (0.1, 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0 μM), AgNO\(_3\) (5.0, 10, 20, 30, 40, 50 and 60 μM), and their combination on callus induction, embryogenic tissue formation and its proliferation, embryo induction, proliferation, maturation, germination, shoot morphogenesis, and rhizogenesis of microshoot (shoot generated on medium without metal supplementation) was assessed using solid MS medium supplemented with various plant growth regulators (PGRs). Rooted plantlets were acclimatized in potted mixtures before transfer to the herbal garden (Jamia Hamdard, New Delhi, India) under field condition.
Fresh stock solutions of CuSO₄ and AgNO₃ were prepared by dissolving the salts (gram dried weight in mg) in distilled water, and adjusting total concentration in medium volume during medium preparation. All cultures were incubated at room temperature (25 ± 1 °C) and 16 h photoperiod provided by cool white fluorescent lamps of 100 μmol m⁻² s⁻¹ photon flux density (Phillips, India) (Lambardi et al. 1993).

**Statistical analysis**

Experiments were performed in an arranged complete randomized block design (CRBD) and generated data from triplicate experiments (consisted of eight replicates per treatment and repeated twice), analyzed using SPSS ver. 21 (USA). Results are presented as mean ± standard error of the replicated experiments. Significant differences between the treatments were assessed by analysis of variance (ANOVA) followed by a Tukey’s range test assessment, with \( p \leq 0.05 \) considered significant.

**Results and discussion**

**Shoot morphogenesis and somatic embryogenesis**

Callus induction was achieved from corm piece explants within 2 weeks cultivation under dark condition of incubation (Fig. 1a) on solid MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D, 4.52 μM), and for which a maximum of 72.66% efficiency was achieved. Induced callus cultures were proliferated (under illumination condition) on solid MS medium supplemented with the same PGR for two subcultures, with intervals of 3–4 weeks cultivation (Fig. 1a, b). Hard, compact, brownish, and semi-brown callus cultures were observed within the proliferated callus mass (Fig. 1c). The different callus masses were separated to observe their differential morphogenesis on control MS medium and when supplemented with 2,4-D (4.52 μM), N⁶-benzylaminopurine (BAP, 2.22 μM) and BAP + naphthalene acetic acid (NAA, 8.88 + 1.35 μM) combinations.

**Callus induction frequency (%)**

\[
\text{Callus induction frequency (\%)} = \frac{\text{Number of explants cultured}}{\text{Number of explants transformed into callus}} \times 100.
\]

**Embryogenic potential per culture tube** (number of embryos g⁻¹ of callus)

\[
= \frac{\text{Number of embryos per culture tube} \times \text{number of embryos in g}^{-1} \text{of sample} \times \text{total weight of embryos per culture tube g}}{\text{Initial inoculum density (g)}}.
\]

**Relative growth rate of cultures (%)**

\[
= \frac{\text{Percentage of the initial embryogenic callus transferred in culture tube}}{\text{Number of SEs per gram after 28 days cultivation}}.
\]

**Shoot forming † capacity**

\[
= \frac{\text{Average number of shoots per regenerating callus mass (g}^{-1}) \times \text{Percentage of regenerating explant-formed callus}}{100}.
\]

**Physiological biochemical analysis of tissues**

Tissue biomass was harvested from cultures and prepared for analysis experiments; physiological biochemical analysis of the various in vitro generated tissues was carried out as described previously (Isah and Umar 2018; Isah 2019). Analysis of Chl \( a \) and \( b \), total Chl, and carotenoid (Car) content of the shoot culture was carried out as reported by Arnon (1949) and Wellburn (1994). Glycinebetaine content of the shoots was analyzed using calorimetric method (Grieve and Grattan 1983) as modified by Lokhande et al. (2010). In the case of embryogenic tissue, induced embryos, proliferated embryos, and mature and germinated embryos, biochemical parameters of proline (Bates et al. 1973), total soluble sugar (Watanabe et al. 2000) as modified by Lokhande et al. (2010), lipid peroxidation via malondialdehyde (MDA) content analysis (Alexieva et al. 2001), superoxide dismutase (SOD) assay (Becana et al. 1986), catalase (CAT) activity (Cakmak and Marschner 1992), ascorbate peroxidase (APX) activity (Nakano and Asada 1981), and guaiacol peroxidase (GPX) activity (Hemeda and Klein 1990) were respectively assayed.
Fig. 1  a Callus induction from corm explants;  b embryogenic tissue formation and proliferation;  c–i embryo induction, proliferation, and maturation;  j, k mature somatic embryo germination;  l–n shoot morphogenesis from callus;  o, p mature somatic embryo germination;  q–s mature embryo germination in singularity form;  t, u multiple shoot morphogenesis;  v–x rhizogenesis on medium supplemented with CuSO$_4$·5H$_2$O, AgNO$_3$, and CuSO$_4$·5H$_2$O + AgNO$_3$.  y treatment of the shoots with 60 μM AgNO$_3$ on root morphogenesis medium;  z acclimatized Gladiolus hybridus plants after 4 weeks cultivation (bars: a–k 2.0, l–z 1.5 mm), arrowheads show somatic embryo induction or shoot morphogenesis.
After 4 weeks cultivation, in some of the callus masses, direct shoot differentiation were observed in medium supplemented with BAP, but at low frequency (Fig. 1d, e, i, m). In medium supplemented with the various PGRs, somatic embryo differentiation was similarly observed at variable frequency, with the highest induction on 2,4-D (4.52 μM) where 80.15% somatic embryo differentiation was observed. This was followed by BAP + NAA (8.88 + 1.35 μM), which...
produced 72.87% somatic embryos. The least differentiation was observed with BAP (2.22 μM), which recorded 16.23% efficiency in embryo induction (b–d). The embryos were oval to roundish and elliptical, in some cases with cotyledonary apex and yellowish radicle primordia at polar end (Fig. 1b–d). Embryos were proliferated on medium supplemented with BAP and NAA combinations to obtain embryos at different stages of development and for which 14.63% embryo mass proliferation efficiency was achieved after 4 weeks cultivation (Fig. 1b–d). Mature somatic embryos were obtained when cultivated on medium supplemented with gibberellic acid (GA3) level (2.60 μM), where 54.67% and 63.85% maximum embryo maturation was recorded after 4 and 8 weeks cultivation, respectively. Mature somatic embryos germination frequency was similarly assessed on medium supplemented with BAP (2.22 μM) and for which maximum of 42.77% embryo germination was achieved after 6 weeks culture. The embryos had a weak root system and, as such, were cultured on medium supplemented with indole butyric acid (IBA, 2.42 μM) for 2 weeks to facilitate complete root development before being cultured again on medium supplemented with GA3 to facilitate complete embryo germination into plant (c–p).

**Influence of copper sulfate and silver nitrate on in vitro morphogenesis**

**Indirect somatic embryogenesis**

The growth and morphogenesis of plant tissue cultures is influenced by composition of culture medium, and appropriate nutrient levels in growth medium could serve as a substitute to PGRs in promoting tissue growth and morphogenesis (Preece 1995; Dahleen and Bregitzer 2002; Silvestri et al. 2019). To study the influence of CuSO4·5H2O and AgNO3 (Preece 1995; Dahleen and Bregitzer 2002; Silvestri et al. 2019). In the present study and on callus induction medium, AgNO3 proved efficient at promoting callus biomass production from cormic explants, with 97.37% efficiency achieved on medium supplemented with 40 μM CuSO4·5H2O, where a maximum of 84.72% was recorded for medium supplemented with 7.5 μM. Combinatorial treatment (AgNO3 40 μM + CuSO4·5H2O 7.5 μM) produced a maximum of 98.26% callus biomass induction frequency over other concentration treatment levels tested, suggesting that the combinatorial dosage was more efficient than the other treatments tested (Fig. 2A). Concentration of nutrients in culture medium through interplay with supplemented PGRs has been shown to influence callus induction in several plant in vitro culture systems (DeFos- sard 1974; Ramage and Williams 2002). Influence of AgNO3 on callus growth is also species and genotype dependent; it showed no effect on callus production in Zea mays (Vain et al. 1989) but promoted callus growth in Triticum aestivum (Wu et al. 2006). In Ragi and Kodo crop callus cultures, Cu concentration in growth medium up to 50 times the normal culture medium level improved callus induction, its fresh weight (FW), and plant regeneration, while cultures cultivated on medium devoid of Cu could not regenerate shoot, suggesting its importance in plant regeneration (Kothari-Chajer et al. 2008). In the case of Ocimum basilicum, callus induction, SE, and plant regeneration were promoted by moderate levels of CuSO4 supplemented in growth medium, while higher levels proved toxic to the cultures and resulted in reduction of the parameters studied (Ibrahim et al. 2018). AgNO3 (0.6–5.9 μM) promoted callus induction, and two-threefold plant regeneration was achieved; subsequently, the treatments’ effect efficiency on callus mass proliferation was assessed (Kothari-Chajer et al. 2008). AgNO3 at 40 μM promoted callus proliferation to a greater degree than CuSO4·5H2O, which had its maximum at 7.5 μM, where 2.59 ± 0.15 g and 1.24 ± 0.18 g callus mass accumulation were respectively achieved. With combinatorial treatment (AgNO3 40 μM + CuSO4·5H2O 7.5 μM), maximum callus mass was achieved after 4 weeks cultivation, with 3.11 ± 0.11 g callus mass (Fig. 2B).

The inorganic nutrients in MS medium supplemented with CuSO4·5H2O and AgNO3 also offer variables for studying the nutritional physiological role of metals in the morphogenetic potential of callus cultures in G. hybridus. Irrespective of PGRs supplemented in culture medium, callus proliferation and in vitro morphogenesis can be improved through salt concentration and composition modification in medium (Ramage and William 2002; Sakhanokho et al. 2008; Silvestri et al. 2019). In some species, embryogenic response occurs at low frequency and embryos may develop
Fig. 2 Influence of silver nitrate and copper sulfate on in vitro morphogenesis in *G. hybridus*
abnormally—with fused or altered number of cotyledons (Nic-Can et al. 2015; Garcia et al. 2019). However, supplementing culture medium with higher levels of AgNO$_3$ (10 mg L$^{-1}$) was found to promote embryogenic tissue formation and differentiation, while lower levels (1.0 mg L$^{-1}$) promoted asynchrony of embryos, abnormal embryo differentiation, and higher number of mature embryos formation (Kong and Yeung 1995; Kong et al. 2012). In the present study, solid MS medium supplemented with 40 μM AgNO$_3$ induced a maximum of 1.28 ± 0.16 g callus mass within 4 weeks cultivation, compared with 7.5 μM CuSO$_4$, which exhibited a maximum of 0.94 ± 0.18 g embryogenic callus mass accumulation. Treatment of the callus cultures with the two metals promoted maximum embryogenic callus mass production of 1.74 ± 0.14 g with AgNO$_3$ 40 μM + CuSO$_4$·5H$_2$O 7.5 μM supplementation, suggesting that this concentration is the most efficient for maximum embryogenic tissue formation from proliferated callus mass.
(Fig. 2C). CuSO$_4$ and AgNO$_3$ treatments have been shown to promote embryogenic callus and plant regeneration in many plant species (Purnhauser and Gyulai 1993; Nirwan and Kothari 2003; Kothari et al. 2004; Joshi and Kothari 2007). Embryogenic callus formation, proliferation, embryo differentiation, and germination were influenced by AgNO$_3$ supplementation in cultivation medium of *Picea glauca* (Kang and Yeung 1995), *Hedychnium muluense* (Sakhanokho et al. 2009), *Phoenix dactylifera* (Diab 2017; Roshanfekrrad et al. 2017; Abdolvand et al. 2018), and *Coffea* species in a genotype-dependent manner (Fuentes et al. 2000; Giridhar et al. 2004; López-Gómez et al. 2016; Rojas-Lorz et al. 2019), while it influenced shoot morphogenesis in *Brassica napus* (Uliaie et al. 2008; Cristea et al. 2012). AgNO$_3$ at 10 mg L$^{-1}$ improved the frequency of embryogenic callus formation from immature embryo-induced callus cultures of wheat (Wu et al. 2006).

For somatic embryo induction, embryogenic tissues were treated with concentrations of AgNO$_3$, CuSO$_4$·5H$_2$O, and their combination using solid embryo induction medium. Maximum somatic embryo differentiation was achieved on medium supplemented with AgNO$_3$ (40 μM), where 95.72% embryo production with an average of 26.52 ± 0.17 embryo induction, compared with supplementation with CuSO$_4$·5H$_2$O, where 7.5 μM exhibited the greatest embryo production efficiency with 16.31 ± 0.12 embryos, representing 89.09% embryo production efficiency. However, compared with singular treatment of the metals, their combination yielded a maximum number of embryo induction of 28.79 ± 0.18, representing 97.31% success in embryo induction from the embryogenic tissues (Fig. 2D, E). The obtained somatic embryos at different stages of development were proliferated on medium supplemented with BAP and NAA (8.88 + 1.35 μM). AgNO$_3$ at moderate levels (up to 50 μM) promoted SE in *Coffea canephora* (Kumar et al. 2007) and *Hedychnium bousigonianum*, with the production of up to 88 embryos g$^{-1}$ callus, while higher levels inhibited embryo differentiation from embryogenic callus cultures (Sakhanokho et al. 2009). In *Daucus carota* callus cultures, 5–7 days exposure to AgNO$_3$ (10–20 μM) was sufficient in inducing SE at high frequency with twofold enhanced embryo formation achieved relative to control cultures (Roustan et al. 1990). Improvement in SE and plant regeneration were achieved from immature embryo-derived callus cultures when the cultivation medium of *Oryza sativa* genotypes was supplemented with 2,4-D (9.0 μM L$^{-1}$) in combination with CuSO$_4$ (10–50 μM L$^{-1}$) (Sahrawat and Chand 1999).

To elucidate the influence of metals on embryo proliferation, somatic embryos were treated with various concentrations levels; maximum efficiency of embryo proliferation was achieved with medium supplemented with AgNO$_3$ at 40 μM, where 24.16 ± 0.14 embryos were produced, representing 96.38% embryo formation frequency, compared with CuSO$_4$·5H$_2$O (7.5 μM) treatment, which produced 18.21 ± 0.16 embryos, representing 89.21% efficiency. However, combination of the metals gave the best results, 26.52 ± 0.10 embryos, which represents 97.53% efficiency (Fig. 2F, G). For maturation treatment of the embryos, 40 μM AgNO$_3$ proved more efficient with production of 70.38% and 85.21% after 4 and 8 weeks of culture, respectively, while 7.5 μM CuSO$_4$·5H$_2$O produced a maximum of 63.48% and 70.24%, respectively. Combination of the two metals in the cultivation medium produced 75.21% and 87.39%, respectively, with 40 μM AgNO$_3$ + 7.5 μM CuSO$_4$·5H$_2$O treatment (Fig. 2H, I). In embryo germination experiments, maximum germination of mature somatic embryos was achieved on solid MS medium supplemented with 40 μM AgNO$_3$, with 65.22% germinated embryos, compared with 7.5 μM CuSO$_4$·5H$_2$O, which had 54.38% germinated embryos after 6 weeks cultivation. Combination of the two metals proved more efficient at facilitating somatic embryo germination, with the maximum achieved with medium supplemented with AgNO$_3$ 40 μM + CuSO$_4$·5H$_2$O 7.5 μM, where 68.34% embryo germination frequency was recorded (Fig. 2J). In other reported studies, embryogenic callus formation, somatic embryo differentiation, and germination were promoted when AgNO$_3$ was supplemented in different cultivation-stage media of *O. sativa*, with 3 mg L$^{-1}$ being the most efficient concentration compared with other levels tested, while 5 mg L$^{-1}$ was the most effective for plant regeneration from the callus cultures (Ghobeishavi et al. 2015).

**Shoot morphogenesis and multiplication**

Indirect shoot morphogenesis from callus cultures also offer alternatives for developing transgenic plants of horticultural importance (Rego and de Faria 2001; de Almeida et al. 2015). However, there are few reports on callus cultures of ornamental plants, and even in cases reporting indirect shoot morphogenesis, it occurs at low frequency (Rout et al. 2006; de Almeida et al. 2015). In the present study, callus cultures that showed shoot morphogenesis potential were separated and cultivated on solid MS medium supplemented with BAP (2.22 μM) and CuSO$_3$, AgNO$_3$, or their combination (Fig. 1I, m), to study their possible influence on shoot morphogenesis and multiplication (Fig. 1q–u). High levels of Cu in growth medium of plants has found to be toxic to cell growth, potentially leading to tissue growth inhibition in cultures (Yruelsa 2005) and a reduction in shoot length (Verma et al. 2011). In the present study, treatment of callus cultures with various AgNO$_3$ concentrations promoted maximum shoot morphogenesis on medium supplemented with 40 μM compared with other concentrations tested after 6 weeks cultivation, with production of 4.53 ± 0.13 shoots.
In the case of callus cultivation with CuSO₄-supplemented medium, 7.5 μM proved most efficient, with the production of 2.74 ± 18 shoots after 6 weeks cultivation. Combination of the two metals at different concentrations gave the best results in medium supplemented with AgNO₃ 40 μM + CuSO₄·5H₂O 7.5 μM, where 5.35 ± 0.23 shoot morphogenesis was achieved (Fig. 2K). Copper is required at higher concentration for improved plant regeneration from callus cultures (Dahleen 1995), and significant positive effect of Cu (1–100 μM) on shoot morphogenesis has been reported for wheat, triticale, and Nicotiana (Purnhauser and Gyulai 1993) as well as in studies with Secale, sorghum, wheat, and Eleusine (Popelka and Altpeter 2001; Nirwan and Kothari 2003; Kothari et al. 2004; Tahilian and Kothari 2012; Dalton 2020). Murashige and Skoog (1962) medium supplemented with Cu at 1.0 μM showed inhibitory effects on shoot regeneration in the in vitro cultures of Foeniculum vulgare, but optimum number of shoots were regenerated when not supplemented in growth medium of the plants, while other concentrations promoted morphogenesis to a variable degree (Dwivedi et al. 2020). Shoot bud differentiation and elongation was promoted by CuSO₄ treatment of callus cultures in Capsicum annum, with the highest response achieved during second-stage subculture on medium supplemented with 30-fold higher levels of the metal over the standard MS medium concentration, while for AgNO₃ treatment, 18.0 μM was the optimal level (Joshi and Kothari 2007). It promoted callus induction and shoot morphogenesis from callus cultures of Solanum lycopersicum in an explant type-, genotype-, and concentration-dependent manner (Shah et al. 2014), while in Zinnia species and Anthurium andraeanum genotypes, AgNO₃ up to 2.0 mg L⁻¹ promoted shoot morphogenesis, multiplication, and quality, accompanied with a reduction in time needed for rooting and acclimatization of micropropagated plants (Anantasaran and Kanchanapoom 2008; Cardoso 2019). Multiple shoot morphogenesis, elongation, and bud enlargement in the same species and in Vigna mungo were enhanced under AgNO₃ treatments (Hyde and Phillips 1996; Mookkan and Andy 2014). In banana tissue cultures, Tamimi and Othman (2020) reported a significant increase in shoot multiplication on medium supplemented with CuSO₄·5H₂O at various concentrations, with 6 mg L⁻¹ being the most efficient, for which a twofold increase in shoot morphogenesis and elongation was recorded.

Because the shoots showed potential for multiple shoot differentiation, the experiment was extended to the possible influence of these metals on multiple shoot morphogenesis. AgNO₃ concentrations proved more efficient in multiple shoot differentiation, with maximum production achieved on solid medium supplemented with 40 μM, where 19.57 ± 0.12 shoots were produced. For CuSO₄, 7.5 μM proved the most effective, with the production of 14.72 ± 0.19 shoots after 6 weeks cultivation (Fig. 2L). Combination of the two metals further enhanced multiple shoot differentiation, with the maximum produced by AgNO₃ 40 μM + CuSO₄·5H₂O 7.5 μM combinations, where 23.54 ± 0.17 shoots were produced after 6 weeks cultivation. In studies with other species, multiple shoot morphogenesis was achieved from shoot-tip explants on medium supplemented with AgNO₃ without PGR supplementation, and when in combination with BAP, with the highest response achieved when 11.77 μM of AgNO₃ was supplemented in the cultivation medium, while combination with kinetin (Kin) could not evoke a morphogenic response in the cultures (Ozudogru et al. 2005).

Rhizogenesis

For rhizogenesis on the optimized root morphogenesis solid MS medium, i.e., supplemented with IBA (2.42 μM), influence of the two metals on root morphogenesis was studied at the various concentrations (Figs. 1v–y, 2M). Maximum root morphogenesis was achieved on AgNO₃-supplemented medium compared with CuSO₄ treatments. AgNO₃ at 40 μM was the most efficient, producing 13.36 ± 0.13 roots per microshoot, compared with CuSO₄·5H₂O treatments, where 7.5 μM was the most efficient, with 10.42 ± 0.15 roots recorded after 6 weeks cultivation. Combination of the two metals proved more efficient than singular treatment, suggesting a synergistic response to the various treatments, with the highest achieved on medium supplemented with AgNO₃ 40 μM + CuSO₄·5H₂O 7.5 μM (Fig. 2M). Stimulatory effect of AgNO₃ on root morphogenesis has been reported in several plant species, including Coffea arabica (Giridhar et al. 2003), Routa aquatica (Sunandakumari et al. 2004), Gentiana lutea (Petrova et al. 2011), Musa acuminate (Tamimi 2015), P. cineraria (Venkatachalam et al. 2017), and in Viola canescens (Khajuria et al. 2021). Also, requirement of Cu for increased
lateral root formation has been demonstrated with seedlings of *Eucalyptus camaldulensis* (Dunn et al. 1997). However, high levels of Cu in growth medium may cause symptoms such as chlorosis and brown necrosis that may impact root morphogenesis, architecture, and biomass production (Marchsner 1995; Lequeux et al. 2010), although we did not observe these symptoms in our cultures in the present experiment.

**Influence of silver nitrate and copper sulfate on physiological biochemical changes associated with somatic embryogenesis and shoot morphogenesis**

### Changes during somatic embryogenesis

Cellular stress is known to trigger increased production of reactive oxygen species (ROS), which subsequently activate the expression of genes encoding antioxidative (enzyme) systems as cellular defensive strategy essential for SE (Gressel and Galun 1994; Arora et al. 2002; Zavattieri et al. 2010; Ochatt and Revilla 2016). Overproduction of ROS could result in direct cellular damage or formation of secondary toxic substances, for which plants have evolved antioxidant protection systems of SOD, CAT, APX, GPX, and MDA (Larson 1988; Kairong et al. 1999; Das and Roychoudhury 2014). Cu facilitates morphogenesis through its role in ROS generation and as a cellular signaling molecule, reducing the rate of infection in cultures (Lamb and Dixon 1997; Javed et al. 2017). Copper-mediated ROS production is facilitated through binding to antioxidant enzymes to reduce their chelating power or reduction in uptake and transport of metals such as zinc and iron (Baker and Walker 1990; Lombardi and Sebastiani 2005). However, higher cellular levels of Cu could promote generation of free radicals that may cause damage to proteins and other biomolecules in cells (Kumar et al. 2009b). Because Cu ions reduce the ability of antioxidants to chelate active oxygen species and induce oxidative stress essential for SE, enzymes are expressed at higher levels to maintain cellular homeostasis (Haliwell and Gutteridge 1984). Similarly, AgNO₃ through its ions plays modulatory roles in antioxidant defense systems that, depending on species or genotype, activate stress responses essential for in vitro morphogenesis (Al Ramadan et al. 2021). In the present study, influence of CuSO₄·5H₂O, AgNO₃ and their combinations at different concentrations on the expression of antioxidant systems during SE was studied throughout the stages of embryogenic tissue formation and proliferation, somatic embryo induction, proliferation, maturation, and germination on solid MS medium supplemented with various PGRs and varying concentration.

SOD, a copper-containing metalloenzyme, plays a critical role in scavenging of free radicals in cells, and its highest activity is associated with chloroplasts’ photosynthetic reaction centers connected to cytochrome oxidase (Asada et al. 1977; Shkolnik 1984). It plays a prominent role in embryogenic competence acquisition and expression and at high concentration results in the generation of H₂O₂. In the present study, assessing the expression of SOD activity during embryogenic competence acquisition, CuSO₄·5H₂O at 7.5 μM was efficient at promoting SOD activity, with 6.83 μKat mg⁻¹ protein recorded during formation of embryogenic tissue. This increased to 7.58 μKat mg⁻¹ during embryo differentiation but fell to 6.78 μKat mg⁻¹ for proliferation and 5.13 μKat mg⁻¹ in germinating embryos, compared with 5.38, 5.98, 5.45 and 5.13 μKat mg⁻¹ protein in the control cultures, respectively. With AgNO₃ treatment, maximum activity of the enzyme was noted with 40 μM treatment, where 7.92 μKat mg⁻¹ protein was recorded in embryogenic tissue. It increased further to 8.72 μKat mg⁻¹ during embryo differentiation but decreased to 7.42 μKat mg⁻¹ for proliferation, and was lowest during germination, having 6.33 μKat mg⁻¹ (Fig. 3A1–4). AgNO₃ + CuSO₄·5H₂O supplementation in the cultivation medium at different concentrations promoted SE as well as SOD expression; the activity was high during embryogenic tissue formation with 8.55 μKat mg⁻¹ protein. It increased to 8.99 μKat mg⁻¹ during somatic embryo induction but decreased to 7.93 μKat mg⁻¹ during proliferation, and was lowest during somatic embryo germination, where 6.39 μKat mg⁻¹ protein was recorded (Fig. 3A1–4). This suggests that the promortory role of the metals on SE is associated with the expression of SODs at differential levels, commensurate with stage of somatic embryo development through a gradual reduction in enzyme expression level.

Low intracellular levels of H₂O₂ could promote cell growth and increased SE accumulation through the role it plays as cellular messenger, inducing gene expression essential for SE (Cui et al. 1999). However, during later stages, excessive accumulation of H₂O₂, due to the low activity of CAT and other peroxidases, may result in tissue browning and cell necrosis (Laukkannen et al. 1999; Kong et al. 2012; Nic-Can et al. 2015). CAT is largely associated with the removal of high levels of H₂O₂ in cells (Klapheck et al. 1990) and reduction in its activity, with low accumulation of H₂O₂ promoting SE (Cui et al. 1999). With CuSO₄ treatment, expression of CAT was highest...
when 7.5 μM was supplemented, with 6.11 μKat mg⁻¹ protein recorded during embryogenic tissue formation. This decreased to 5.24 μKat mg⁻¹ during somatic embryo induction, 4.72 μKat mg⁻¹ for proliferating embryos, and 3.21 μKat mg⁻¹ in germinating somatic embryos. In controls, CAT expression was 3.54 μKat mg⁻¹ during embryogenic tissue formation, 3.39 μKat mg⁻¹ for somatic embryo induction, 2.98 μKat mg⁻¹ for proliferation, and 2.43 μKat mg⁻¹ in germinating embryos (Fig. 3B1–4). With AgNO₃ treatment, highest CAT activity was noted with 40 μM treatments, where 6.99 μKat mg⁻¹ protein was recorded during embryogenic tissue formation. This decreased to 6.97 μKat mg⁻¹ during somatic embryo differentiation, 6.15 μKat mg⁻¹ in proliferating embryos, and 4.42 μKat mg⁻¹ protein during germination. Combination of the two metals at different concentrations promoted SE, accompanied with decrease in CAT activity; activity of the enzyme was high during embryogenic tissue formation, for which 7.76 μKat mg⁻¹ protein was recorded. It showed a further decrease as somatic embryo differentiation advanced, with 6.97 μKat mg⁻¹ during embryo formation, 6.67 μKat mg⁻¹ for proliferation, and 4.69 μKat mg⁻¹ protein for germination, suggesting a gradual decrease in CAT expression during SE in G. hystereus influenced by the levels of CuSO₄, AgNO₃, and their combination (Fig. 3B1–4). Increase in activity of CAT was observed with up to 10 μM treatment of Erythrina variegata in vitro cultures with CuSO₄·5H₂O. Higher concentrations were insignificant, while SOD expression was high in all the treatments tested, and showed a dose-dependent trend in activity (Javed et al. 2017).

APX, derived from ascorbic acid in cells, is involved in the reduction of H₂O₂ to water and at the same time oxidizes ascorbate to MDA. Activity of the enzyme was higher in AgNO₃-treated cultures than those treated with CuSO₄·5H₂O: in the case of AgNO₃-treated cultures, highest activity was noted in 40 μM treatment with 4.62 μKat mg⁻¹ protein recorded during embryogenic tissue formation. This decreased to 4.29 μKat mg⁻¹ during embryo differentiation, 3.94 μKat mg⁻¹ for proliferation, and 2.94 μKat mg⁻¹ protein during germination compared with the control, with 2.74, 2.23, 1.85, and 1.64 μKat mg⁻¹ protein, respectively. For CuSO₄·5H₂O, higher activity of the enzyme was observed in cultures treated with 7.5 μM, where 3.86 μKat mg⁻¹ protein was recorded in embryogenic tissue. This decreased to 3.58 μKat mg⁻¹ during embryo differentiation, 2.98 μKat mg⁻¹ for proliferating embryos, and 2.17 μKat mg⁻¹ protein for germinating somatic embryos (Fig. 3C1–4). Combinatorial treatment of the various tissues with the two metals produced highest tissue mass differentiation and other responses accompanied with increased enzyme activity, suggesting a synergistic response. Combinatorial treatment of AgNO₃ 40 μM + CuSO₄·5H₂O 7.5 μM gave maximum response and APX activity with 4.75 μKat mg⁻¹ protein recorded during embryogenic tissue formation that subsequently decreased to 4.28 in differentiating embryos. During proliferation of the somatic embryos, it decreased to 4.12 μKat mg⁻¹ and then to 3.34 μKat mg⁻¹ protein in germinating embryos compared with the control cultures (Fig. 3c1–4). Gupta and Datta (2004) reported changes in the activities of SOD, CAT, and peroxidase during the first 14 days of SE in Gladiolus. According to their study, activities of SOD peaked during the period when somatic embryos appeared as “tiny beads,” but decreased with further development of the embryos, similar to the results of our study in the control and metal-treated cultures of the various embryo tissues analyzed; however, they used different culture duration (Fig. 3A–C).

GPX also plays a role in cellular detoxification of H₂O₂ accumulated due to the activity of SOD to tolerable levels essential for morphogenesis; it showed higher activity in AgNO₃-treated cultures compared with those treated with CuSO₄·5H₂O. However, combinatorial treatments were more efficient, suggesting their differential influence on morphogenesis at variable levels supplemented in culture medium (Fig. 3D1–4). In the case of CuSO₄·5H₂O treatments, maximum activity was noted with medium supplemented with 7.5 μM cultures, where 2.41 μKat mg⁻¹ protein was recorded during embryogenic tissue formation. This decreased to 2.18 μKat mg⁻¹ during somatic embryo differentiation, 2.14 μKat mg⁻¹ for proliferation, and 1.59 μKat mg⁻¹ protein during mature embryo germination, compared with 1.85, 1.57, 1.46, and 1.28 μKat mg⁻¹ protein of the respective control cultures (Fig. 3D1–4). In the case of AgNO₃ treatments, 40 μM proved the most efficient, with the production of 2.93 μKat mg⁻¹ protein GPX activity during embryogenic tissue formation and 2.65 μKat mg⁻¹ for somatic embryo differentiation, while proliferating embryos had 2.59 μKat mg⁻¹ and germinating embryos showed 2.24 μKat mg⁻¹ protein after 4 weeks cultivation. Combined treatment of AgNO₃ 40 μM + CuSO₄·5H₂O 7.5 μM produced a maximum of 3.27 μKat mg⁻¹ protein GPX activity during embryogenic tissue formation, which decreased to 2.97 μKat mg⁻¹ during somatic embryo differentiation and further to 2.75 μKat mg⁻¹ for somatic embryo proliferation, while the lowest was recorded in germinating somatic embryos with 2.52 μKat mg⁻¹ protein GPX activity, suggesting decreased expression of the enzyme during the various developmental stages to scavenge free radicals and other cellular toxic stress agents.

In vitro morphogenesis of SE is also associated with high cellular stress, generating free radicals at higher levels in cells through the activity of SODs, which may also result in membrane damage (Zavattieri et al. 2010). Among parameters that can be used to study the extent of membrane damage due to the cellular oxidative stress that causes lipid peroxidation by polyunsaturated fatty acids hydroperoxidation
Fig. 3 Influence of CuSO₄·5H₂O and AgNO₃ on the expression of antioxidant systems and pigment molecules during in vitro morphogenesis (somatic embryogenesis and shoot morphogenesis) in *G. hybridus*
Fig. 3 (continued)
Fig. 3 (continued)
is MDA activity in plant cells (Baille et al. 1996). Excessive accumulation of Cu$^{2+}$ in growth medium may also cause a range of toxic effects, including inhibition of photosynthetic rate, which damages plasma membrane permeability and may result in metabolic disorder(s) due to Cu toxicity to plant species and varieties under cultivation (Gori et al. 1998; Macnair et al. 2000). In the present study, CuSO$_4$·5H$_2$O, AgNO$_3$, and their combined treatments enhanced the activity of MDA at various stages of morphogenesis commensurate with the enhancement of tissue transdifferentiation observed due to the influence of the metals. However, the combinatorial treatments were more efficient than the singular metal treatment (Fig. 3E1–4). For CuSO$_4$·5H$_2$O, 7.5 μM treatment was the most efficient, where 15.49 μKat mg$^{-1}$ protein MDA activity was recorded during embryogenic tissue formation. This decreased to 15.36 μKat mg$^{-1}$ during somatic embryo differentiation and 14.59 μKat mg$^{-1}$ for its proliferation, while germinating embryos showed 11.15 μKat mg$^{-1}$ protein MDA activity. Treatment with AgNO$_3$ levels gave the best results with 40 μM; a maximum of 18.16 μKat mg$^{-1}$ protein MDA activity was recorded during embryogenic tissue formation but decreased to 17.85 μKat mg$^{-1}$ during somatic embryo differentiation. This was further decreased during embryo proliferation with 17.48 μKat mg$^{-1}$ MDA activity and 15.52 μKat mg$^{-1}$ in germinating embryos compared with the control cultures, where 9.78, 9.21, 8.46, and 8.24 μKat mg$^{-1}$ protein MDA activity was respectively recorded. To further assess the possible synergistic effect of the two metals on promoting the morphogenic process and possible involvement of MDA activity, an assay was carried out after cultures were treated with AgNO$_3$ + CuSO$_4$·5H$_2$O combination for 4 weeks. High activity of the enzyme was observed during embryogenic tissue formation, where 19.36 μKat mg$^{-1}$ protein was recorded. This was decreased to 18.91 μKat mg$^{-1}$ during somatic embryo differentiation, then to 18.21 μKat mg$^{-1}$ in proliferating embryos, while germinating embryos had 16.95 μKat mg$^{-1}$ protein MDA activity, suggesting possible high membrane damage in tissues during SE due to high cellular accumulation of hydroxy, alkoxy, and peroxy radicals, triggering activity of the enzyme to overcome membrane damage at various stages of morphogenesis that could not be ameliorated by high expression of SOD, CAT, APX, and GPX (Fig. 3E1–4). This may also be ascribed to the inability of peroxidase activity to catalyze H$_2$O$_2$ detoxification, which resulted in production of high cellular levels of toxic hydroxyl radicals from H$_2$O$_2$ due to the production of superoxide radicals (Chen and Schopfer 1999; Piqueras et al. 2002). Lower expression of peroxidase, polyphenol oxidase, and indole-3-acetic acid (IAA) oxidase activities has been observed in plants showing Cu deficiency that were supplemented with the metal in nutrient cultivation medium (Marschner 1995).

Changes during shoot morphogenesis

To study the influence of CuSO$_4$·5H$_2$O, AgNO$_3$, and their combinations on shoot morphogenesis-associated biochemical changes in G. hybridus, different concentration of the two metals were tested and parameters of Chl, Car, glycinebetaine, and proline content evaluated from harvested shoot cultures (Fig. 3f, g). Exposure to high concentration of the metals may inhibit the electron transport chain by blocking photosynthetic electron transport in PS II oxidizing or reducing sites of the photosystem, which may result in ROS generation and oxidative stress to plants (Fernandes and Henrques 1991; Dudev and Lim 2008; Huang et al. 2020; Pontes et al. 2020), with an overall effect on plant physiological parameters associated with photosynthetic quantum and other adjustment.
responses in metabolic processes (Souza et al. 2019; Hameed et al. 2021). In the present study, Chl $a$ content of the shoot cultures was highest in 7.5 μM treatment with CuSO$_4$·5H$_2$O, where 0.198 ± 0.12 mg g$^{-1}$ FW was accumulated. With AgNO$_3$ treatment, maximum production of the pigment molecule was recorded when 40 μM was supplemented, with 0.29 ± 0.11 mg g$^{-1}$ FW Chl $a$. Combination of the two metals at varied concentrations yielded a maximum production of 0.32 ± 0.14 mg g$^{-1}$ FW Chl $a$ in AgNO$_3$ 40 μM + CuSO$_4$·5H$_2$O 7.5 μM treatment cultures (Fig. 3F1). In the case of Chl $b$, a maximum of 1.18 ± 0.13 mg g$^{-1}$ FW was recorded for 7.5 μM CuSO$_4$·5H$_2$O treatment, while AgNO$_3$ at 40 μM proved more effective, with a production of 1.34 ± 0.12 mg g$^{-1}$ FW Chl $b$ (Fig. 3F2). Combined treatment yielded a maximum of 1.42 ± 0.10 with 40 μM AgNO$_3$ + 7.5 μM CuSO$_4$·5H$_2$O levels, suggesting that this concentration is the most effective at promoting Chl biosynthesis essential for plant growth. For total Chl content of the shoot cultures, maximum was recorded with 7.5 μM CuSO$_4$·5H$_2$O treatments, where 1.92 ± 0.12 mg g$^{-1}$ FW Chl was recorded, compared with the higher content of 1.34 ± 0.13 mg g$^{-1}$ achieved with 40 μM AgNO$_3$ treatment of the shoot cultures (Fig. 3F3). Combination treatments of the two metals further enhanced total Chl accumulation to 1.52 ± 0.11 mg g$^{-1}$ FW recorded with AgNO$_3$ 40 μM + CuSO$_4$·5H$_2$O 7.5 μM combination levels. CuSO$_4$·5H$_2$O at 7.5 μM was also the most effective at Car accumulation, with 0.616 ± 0.12 mg g$^{-1}$ FW recorded. AgNO$_3$ at 40 μM proved more efficient at enhancement of Car production in the shoot cultures, with the production of 0.73 ± 0.17 mg g$^{-1}$ FW, while combination treatment with the two metals yielded maximum accumulation with AgNO$_3$ 40 μM + CuSO$_4$·5H$_2$O 7.5 μM levels, with the production of 0.743 ± 0.14 mg g$^{-1}$ FW (Fig. 3F4). This suggests that supplementing Cu in the culture medium at moderate levels enhanced leaf Chl content, while higher levels inhibited its biosynthesis owing to a possible effect on photosynthetic machinery of thylakoids, where it forms an integral functional unit of PS II (Hameed et al. 2021). Maximum shoot morphogenesis, elongation, leaf expansion, and Chl content was achieved with shoot-tip explants of M. acuminata when cultivated on medium supplemented with AgNO$_3$ (up to 10 mg L$^{-1}$) over control cultures and other ethylene inhibitors tested (Tamimi 2015). Inhibition of ethylene action due to supplementation of AgNO$_3$ in growth medium, serving as a source of silver ions, has been shown to promote leaf Chl content (Perl et al. 1988; Ehsanpour and Jones 2001). In Solanum tuberosum shoot cultures, treatment with AgNO$_3$ up to 10 μM promoted shoot morphogenesis and Chl $a$, Chl $b$, and total Chl content of leaves, while higher levels (above 15 μM) decreased the morphogenic response and total Chl content of leaves (Kaur and Kumar 2020). In Nicotiana tabacum control and Cu-tolerant tissue cultures, Chl $a$ and Chl $b$ concentration showed no significant variation in accumulation, but the ratio of Chl $a$/Chl $b$ varied, suggesting its tolerance to the concentrations tested through proper chloroplast development (Gori et al. 1998). At high CuSO$_4$ supplementation in MS medium, improved shoot morphogenesis was achieved in Stevia rebaudiana in vitro cultures and was associated with an increase in Chl content, but higher levels proved detrimental to the cultures (Jain et al. 2009). Treatment of banana shoot cultures with CuSO$_4$·5H$_2$O promoted high Chl accumulation in leaves, with the highest in medium supplemented with 6.0 mg L$^{-1}$ (Tamimi and Othman 2020). In E. variegata tissue cultures, moderate levels of copper (10 μM) promoted shoot morphogenesis through the number of shoots induced per explant and their length, while higher concentrations (15–20 μM) proved toxic to the parameters, with yellowing leaves and changes in antioxidant systems (Javed et al. 2017), possibly due to the essential role that Cu plays as a micronutrient required for physiological metabolic enzyme processes of photosynthesis, respiration, and hormone perception (Hansch and Mendel 2009; Nomura et al. 2015; Rahmati Ishka and Vatamaniuk 2020).

Proline, an osmoprotectant that serves as a storage sink of carbon, nitrogen, and free radicals scavenger, showed highest accumulation in medium supplemented with 7.5 μM CuSO$_4$·5H$_2$O, with a production of 158 ± 0.19 μM mg$^{-1}$ FW compared with the maximum of 227 ± 0.15 in AgNO$_3$ treatment of 40 μM. Combination of the two metal salts further enhanced proline accumulation in the shoot cultures, with the highest production in AgNO$_3$ 40 μM + CuSO$_4$·5H$_2$O 7.5 μM treatment cultures, where 239 ± 0.16 μM mg$^{-1}$ FW was recorded (Fig. 3G1). Glycinebetaine production in the shoot cultures was enhanced by the CuSO$_4$·5H$_2$O treatments, with its highest accumulation achieved in medium supplemented with 7.5 μM, with 178 ± 0.21 μM mg$^{-1}$ FW production. AgNO$_3$ proved more effective at promoting the accumulation of glycinebetaine compared with CuSO$_4$·5H$_2$O treatments, with the maximum production observed in medium supplemented with 40 μM, where 209 ± 0.18 μM mg$^{-1}$ FW was accumulated by the shoot cultures, while combined treatment with the two metals resulted in the highest production of 224 ± 0.17 μM mg$^{-1}$ FW in AgNO$_3$ 40 μM + CuSO$_4$·5H$_2$O 7.5 μM treatment levels (Fig. 3G2). Reduction in the morphogenic response observed with higher levels of the metals, in the present study, can be explained by their possible triggering effects on the Fenton reaction which generates hydroxyl radicals known to cause lipid peroxidation as well as DNA and protein damage (Drazkiewicz et al. 2004). In a study with AgNO$_3$, CuSO$_4$, and their nanoparticles, Malik et al. (2021) reported their differential promotory influence on callus induction, embryogenic tissue formation, and plant regeneration in T. aestivum genotypes in a concentration-dependent
manner, with the synthesized nanoparticles being more efficient than the two metals employed.

**Conclusion**

Results of the present study suggest that supplementing CuSO₄ (5H₂O), AgNO₃, or their combination in the in vitro growth medium of *G. hybrida* has a concentration-dependent promotory influence on callus induction, embryogenic tissue differentiation, SE, shoot morphogenesis, and multiplication. Supplementing in vitro growth medium with these metals also influences tissue growth during the various morphogenic stages by influencing the physiological biochemical changes essential for morphogenic processes. This can be ascribed to the role they play in influencing the different physiological biochemical pathways and changes essential for in vitro developmental processes.

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**Declarations**

**Conflict of interest** The authors declare no conflicts of interest.

**Ethical approval** The research work was carried out in compliance with the ethical standards that do not involve the use of humans.

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