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

Tasiu Isah (✉ taasmore@yahoo.co.uk)

Jamia Hamdard <https://orcid.org/0000-0002-2112-530X>

Qurratul .

Hamdard University New Delhi

Shahid Umar

Hamdard University New Delhi

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Abstract

Micronutrients are essential for *in vitro* plant tissue growth, and studying their culture medium levels manipulation to promote growth and morphogenesis is essential in plant tissue culture experiments. In the present study, influence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0–15 μM), AgNO_3 (0–60 μM) and their combinations on callus induction and various morphogenic processes at different treatment levels were studied in *G. hybridus*. AgNO_3 was more efficient in promoting the parameters studied, with 40 μM been more efficient over other treatment levels tested. In the case of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ treatments, maximum efficiency was achieved with 7.5 μM over other concentration treatment of the tissues analysed. Results of the experiments on morphogenesis and associated physiological biochemical changes suggests that combined treatment with the two metals promoted morphogenesis at higher levels through influence on physiological biochemical changes essential for the morphogenic parameters studied. Application of the technique on embryogenic tissue formation and somatic embryos differentiation seems of appropriate application when using metals for the experiment.

Introduction

In vitro plant regeneration is influenced by culture medium nutrients with micronutrients regarded indispensable composition of many enzyme molecules involved in physiological biological processes essential for plant development (Maksymiec 1997; Niedz and Evens 2007). Physiological molecular aspects of the plant *in vitro* morphogenesis have been investigated in several species and varieties (Ramage and Williams 2002; Dalton 2020). However, limited information is known about the influence of nutritional composition of culture medias to morphogenic processes in plants. Pioneer studies on the importance of organic and inorganic nutrients to *in vitro* plant development were performed by Murashige and Skoog (1962). Since then, it has become increasingly understood that constituents of basal medium and accuracy in their concentration are important for proper plant growth, development and morphogenesis in plant tissue culture experiments (Preece 1995; Mahendra and Dutta Gupta 2004; Dalton 2020).

Inorganic constituents of nutrients in MS medium formulation offer variables for studying the influence of elemental composition on *in vitro* morphogenic potential of plants (Kothari-Chager et al. 2008; Silvestri et al. 2018). Micronutrients are regarded indispensable components of enzyme molecules, and 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 reports in this respect is on minerals uptake and their physiological nutritional role in plant development rather than morphogenesis.

Metal ions needed by plants in trace quantity for normal growth and development play roles in nucleic acid metabolism and redox reactions or as structural configuration of many enzyme molecules. Higher cellular concentration of the metal ions may affect metabolic processes, resulting in growth inhibition through cellular toxicity (Zenk 1996). Copper sulphate (CuSO_4) is among the compositions of MS

medium that serve as principal source of copper (Cu), a transitional metal having two oxidation states (Cu^{2+}) under normal physiological growth condition. It is regarded the most crucial micronutrient essential for plant growth and development but, the concentration in growth medium needs optimization for enhanced performance (Lipman and Mackinney 1931; Arnon and Stout 1939). Because of the broadspectrum anti-microbial activity of Cu, its supplementation in the *in vitro* growth medium of plants could promote multiplication with substantial reduction in infection frequency due to endogenous latent microbes (Javed et al. 2017; Kim et al. 2017; Silvestri et al. 2019). Although optimum physiological requirement of the Cu varies with plant species (Clemens 2001), increased concentration is promotory to 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 in photosynthesis, respiration, antioxidants reactions, hormones biosynthesis, and for perceptions essential for plant growth under the *in vitro* condition (Nomura et al. 2015; Penarrubia et al. 2015). It was estimated that about 50% of the copper found in plants is associated with chloroplast and bound to plastocyanin that function in cells as mediator of electron transfer between photosystems (PS) I and II (Weigel et al. 2003). While it is an integral part of photosynthetic machinery, been 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 the photosynthesis through Chl biosynthesis may result in damage to membrane permeability with overall effect on cellular physiological metabolic processes disturbance (Shioi et al. 1978; Baszynski et al. 1982). Further, due to its redox activity, higher concentration of free copper 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) optimal and cellular tolerable range of copper concentration exists when supplemented above the standard culture medium formulation levels.

Because of its stability, availability, specificity and solubility in water and as potent inhibitor of ethylene, silver nitrate (AgNO_3) is 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 the silver ions includes polyamines biosynthesis, ethylene and calcium-mediated biochemical pathways and morphogenesis (Kumar et al. 2009a). Silver nitrate or in the form of silver thiosulphate, has been reported to influence *in vitro* morphogenesis in many tissue culture experiments but, mechanism of the promotion is unclear (Eapen and George 1997; Bais et al. 2000, 2001; Kim et al. 2017). Evidences have suggested its interfering effect on ethylene perception due to water solubility and lack of cellular toxicity when effective concentration is used (Beyer 1976a, b; Kumar et al. 2009a). Interactive effect of the silver occurs through displacement of the active site of copper at receptor complex to cause opposite effect that results into stable physiological processes essential for enhancement of *in vitro* morphogenesis (Kumar et al. 2009a). In the plant cells, Ag^+ (dissolved in AgNO_3) 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). Therefore, cells are sensitive to non-toxic low concentration of Ag^+

for which the metal ions interfere with auxin and ethylene signaling to make its increased use in plant tissue culture experiment 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 inhibitor of ethylene and its interactive effect on Cu may result in the displacement of Cu in the active site of receptors in cells (Ciardi and Klee 2001). Basal salts and vitamins present in the PCTOC medias also contain chlorine and addition of AgNO_3 to them results in the formation of AgCl precipitate (Murashige and Skoog 1962; Caldas et al. 1990; Steinitz et al. 2010). Over time, the silver in its particulate form may become 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 the *in vitro* condition that in the overall reflects biomass yield (Kittler et al. 2010; Liu et al. 2010). There have been increasing interest on the use of metal nanoparticles in nutrients medias in singularity or combinations to promote morphogenesis in the *in vitro* culture of plants (Kim et al. 2017; Malik et al. 2021).

Gladiolus hybridus, the 8th 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 was reported to be around 370 million corms cultivated in over 130,000 ha (Narain 2004; Singh 2011) with the low propagation method predominantly been vegetative using corms and cormlets, and seeds used only for raising hybrid varieties (Memon et al. 2016). Plant tissue culture techniques offer alternative propagation method for mass production of *G. hybridus enmasse* (Ziv et al. 1970; Memon et al. 2016) in particular, when physiological growth requirements could be optimized with the help of knowledge on nutritional physiological factors regulating biomass production under the *in vitro* culture conditions (Bidabadi and Jain 2020). However, in most reported experimental literatures on the morphogenesis studies of plant species, only one medium is used for duration of a study, a limitation that does not permit complete nutritional characterisation of the physiological optimum growth requirement of different *in vitro* developmental stages of cultures. Although studies were conducted on different applications of PCTOC for enhanced biomass production in members of the genus *Gladiolus* (Memon 2012; Memon et al. 2012, 2016), physiological growth requirement of nutrients medium have not been investigated. In the present study, influence of copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and AgNO_3 on callus induction, somatic embryogenesis (SE), shoot biomass production and physiological biochemical changes associated with the morphogenic processes of *G. hybridus* were assessed.

Materials And Methods

Explants collection, surface sterilization and cultures establishment

Healthy *G. hybridus* corms were obtained from the Horticultural Society of India Calcutta West Bengal (India), its outer coverings removed, washed with cetrimide for about 15 mins and then under running tap water for 3 mins. Thereafter, obtained corms were treated with 70% ethanol for 5 mins and rinsed with sterile distilled water three (3) times. Corms were surface sterilized with mercuric chloride (0.05%) for 3

mins and rinsed with sterile distilled water 3–4 times to remove traces of the HgCl_2 . Surface sterilized corms were longitudinally sliced into 5–10 mm explant pieces before cultured on solid MS medium supplemented with 2,4-D ($4.52 \mu\text{M}$), sucrose (3%) and inositol (100 mg L^{-1}) for callus induction. Media pH were adjusted to 5.7 before autoclaved at 10321.35 pa and 121°C for 15 mins. The induced callus cultures were proliferated on control MS medium amended with 2,4-D ($4.52 \mu\text{M}$), BAP ($2.22 \mu\text{M}$) and BAP + NAA ($8.88 + 1.35 \mu\text{M}$) until brownish embryogenic callus formation characterised by near globular or elongated and cylindrical structures on callus surface was observed (Mujib et al. 2017). The number of embryos induced on the callus surface and their proliferation increased with increase in the culture duration but, data were taken after 4 weeks cultivation for each. Proliferated somatic embryos were transferred to maturation medium amended with GA_3 ($2.60 \mu\text{M}$) where the embryos elongated before germination on medium added with BAP ($2.22 \mu\text{M}$). Recovered plantlets were transferred to medium supplemented with IBA ($4.84 \mu\text{M}$) for efficient root formation before acclimatization experiment. Effect of CuSO_4 (0.1, 2.5, 5.0, 7.5, 10.0, 12.5, and $15.0 \mu\text{M}$), AgNO_3 (5.0, 10, 20, 30, 40, 50 and $60 \mu\text{M}$) and their combinations on callus induction, embryogenic tissue formation and its proliferation, embryos induction, proliferation, maturation and germination, shoot morphogenesis and rhizogenesis of micro shoot (shoot generated on medium without metals amendment) using solid MS medium supplemented with the various PGRs were carried out. Rooted plantlets were acclimatized in potted mixtures before transferred to the herbal garden (Jamia Hamdard New Delhi, India) field condition.

Fresh stock solutions of the CuSO_4 and AgNO_3 were prepared by dissolving gram dried weight (mg) of the salts in distilled water, and total concentration in medium volume added during media preparation. All cultures were kept under cultures incubation room temperatures of $25 \pm 1^\circ\text{C}$ and 16 h photoperiod provided by cool white fluorescent lamps of $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photon flux density (Phillips, India).

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of explants cultured}}{\text{No. of explants transformed into callus}} \times 100$$

$$\text{Embryogenic potential per culture tube (Number of embryos g}^{-1} \text{ of callus)} =$$

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

$$\text{Relative growth rate of cultures (\%)} = \frac{\text{Percentage of the initial embryogenic callus transferred in culture tube}}{\text{Number of SEs per gram after 28 days cultivation}}$$

$$\text{Shoot forming capacity} = \text{Average number of shoots per regenerating callus mass (g}^{-1}) \times \frac{\text{Percentage of regenerating explant-formed callus}}{100} \text{ (Lambardi et al. 1993)}$$

Physiological biochemical analysis of tissues

The various tissue biomass were harvested from cultures and prepared for the analysis experiments; details of the methods for physiological biochemical analysis of the various *in vitro* generated tissues were carried out as described earlier (Isah and Umar 2018; Isah 2019). Chlorophylls a and b, total chlorophyll (Chl) and carotenoids (Cars) content of the shoot cultures were carried out as reported by Arnon (1949) and Wellburn (1994). Glycinebetaine content of the shoots were analyzed using calorimetric method (Grieve and Grattan 1983) as modified in Lokhande et al. (2010). In the case of embryogenic tissue, induced embryos, proliferated embryos, mature and germinated embryos, biochemical parameters of proline (Bates et al. 1973), total soluble sugars (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 Marscher 1992), ascorbate peroxidase (APX) activity (Nakano and Asada 1981) and guaiacol peroxidase (GPX) activity (Hemeda and Klein 1990) were respectively measured.

Statistical analysis

Experiments were performed in an arranged complete randomized block design (CRBD) and generated data from triplicate experiments (consisted of 8 replicates per treatment and repeated twice) were analysed using SPSS ver. 21 (USA). Results are presented as means with 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 at probability levels of $p \leq 0.05$.

Results And Discussion

Shoot morphogenesis and somatic embryogenesis

Callus was induced from corm explants within two weeks cultivation (Fig. 1a) on solid MS medium amended with 2,4-Dichlorophenoxy acetic acid (2,4-D) (4.52 μM) where maximum of 72.66% callus induction efficiency was achieved. Induced callus cultures were proliferated on solid MS medium amended 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 sectorized to observe their differential morphogenesis on control MS medium and when amended with 2,4-D (4.52 μM), N⁶-Benzylaminopurine (BAP) (2.22 μM) and BAP + Naphthalene acetic acid (NAA) (8.88 +1.35 μM). After four weeks cultivation, in some of the callus masses, direct shoot differentiation were observed on medium added with BAP but at low frequency (Fig. 1d–e, l–m). On medium amended with the various PGRs, somatic embryos differentiation were similarly observed at variable frequency, with the highest induction been on 2,4-D (4.52 μM) where 80.15% somatic embryos differentiation were observed. This was followed by BAP + NAA (8.88 +1.35

μM) that produced 72.87% somatic embryos and least with the BAP (2.22 μM) that recorded 16.23% efficiency in the embryos induction (b–d). The embryos were oval to roundish, elliptical, in some cases with cotyledonary apex and yellowish radicle primordia at polar end (Fig. b–d). Embryos were proliferated on medium added with BAP and NAA combinations to obtain embryos at different stages of development and for which 14.63% embryos mass proliferation efficiency was achieved after four weeks cultivation (Fig. b–d). Mature somatic embryos were obtained when cultivated on medium added with Gibberelic acid (GA₃) level (2.60 μM) where 54.67% and 63.85% maximum embryos maturation were recorded after four and eight weeks cultivation, respectively. Mature somatic embryos germination frequency was similarly assessed on medium amended with BAP (2.22 μM) for which maximum of 42.77% embryos germination was achieved after six weeks culture. The embryos had weak root system and as such, were cultured on medium added with indole butyric acid (IBA) (2.42 μM) for two weeks to facilitate complete roots development before cultured again on medium added with GA₃ to facilitate complete embryos germination into plant (c–p).

Influence of copper sulphate and silver nitrate on the *in vitro* morphogenesis

Indirect somatic embryogenesis

Growth and morphogenesis of plant tissue cultures is influenced by medium composition, and appropriate nutrients levels in the growth medium could serve as substitute to PGRs in promoting tissue growth and possible morphogenesis (Preece 1995; Dahleen and Bregitzer 2002; Silvestri et al. 2018). To study the influence of CuSO₄ and AgNO₃ on morphogenic processes of indirect SE pathways, shoot morphogenesis, multiplication and rhizogenesis, various concentrations of the salts were supplemented in MS medium amended with PGRs. Generally, on dry weight (DW) basis, about 2–20 mg Kg⁻¹ Cu content in growth medium is considered beneficial to plant growth while 20–30 μg g⁻¹ per dried weigh (DW) of leaves constitute toxicity levels in some plant species (Robson and Reuter 1981). As such, each plant species has its tolerance and minimum range of Cu physiological utilisation because Cu is an important constituent of enzymes such as ascorbate, cytochrome, diamine and quinol oxidases, phenolase, SODs and fraction 1 protein involved in protein, carbohydrate biosynthesis and electron transport whose expression is essential in plant regeneration by *in vitro* culture (Purnhauser and Gyulai 1993). AgNO₃ supplementation in growth medium of plants also serves as source of silver ions that may interact with polyamines to promote SE and organogenesis (Zhang et al. 2001) through the S-adenosyl methionine inhibition by the ethylene produced in cultures that ultimately promote polyamines biosynthesis (Roustan et al. 1990; Parimalan et al. 2010). In the present study and on callus induction medium, AgNO₃ proved efficient in influencing callus biomass production from cormic explants with 97.37% efficiency achieved on medium added with 40 μM over CuSO₄·5H₂O treatments where maximum of 84.72% was recorded on medium supplemented with 7.5 μM. Combinatorial treatment (AgNO₃ 40 μM + CuSO₄·5H₂O 7.5 μM) produced maximum of 98.26% callus biomass induction frequency over other concentrations tested, suggesting the combinatorial dosage as the most efficient over the other treatments tested (Fig. 2A). Concentration of nutrients in culture medium through interplay with supplemented PGRs have been shown to influence callus induction in several *in vitro* culture systems of plant species (DeFossard 1974;

Ramage and Williams 2002). Influence of AgNO_3 to callus growth is also species and genotype dependent; it showed none 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, increase in Cu concentration in growth medium up to 50 times the normal culture medium levels improved callus induction, its fresh weight (FW) and plant regeneration while cultures cultivated on medium devoid of Cu could not regenerate shoot, suggesting the essentiality in plant regeneration (Kothari-Chajer et al. 2008). In the case of *Ocimum basilicum*, SE and plant regeneration were promoted by moderate levels of CuSO_4 supplemented in growth medium while higher proved toxic to the cultures, and resulted in reduction on the parameters studied (Ibrahim et al. 2018). For AgNO_3 (0.6–5.9 μM), promoted plant regeneration was achieved by 2–3 times and subsequently, efficiency of the treatments were tested on induced callus mass proliferation (Kothari-Chajer et al. 2008). AgNO_3 at 40 μM proved more efficient on callus proliferation over $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ that 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 but, combinatorial treatment (AgNO_3 40 μM + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 7.5 μM) had 3.11 ± 0.11 g callus mass as the best treatment results after four weeks cultivation (Fig. 2B).

Inorganic nutrients in MS medium of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and AgNO_3 also offer variables for studying nutritional physiological role of metals in morphogenic potential of callus cultures in *G. hybridus*. Irrespective of PGRs amended in the culture medium, callus proliferation and *in vitro* morphogenesis can be improved through medium salts concentration and composition modification (Ramage and William 2002; Silvestri et al. 2018). In some species, embryogenic response occurs at low frequency and embryos formed may turn out abnormal in structures - 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, overcomes abnormal embryos differentiation and higher number of mature embryos formation (Kong et al. 2012). In the present study, for the influence on embryogenic tissue formation from proliferated callus cultures, AgNO_3 was equally efficient in inducing maximum of 1.28 ± 0.16 g callus mass within four weeks cultivation on solid MS medium added with 40 μM over that of CuSO_4 that had 7.5 μM as the most efficient with 0.94 ± 0.18 g embryogenic callus mass accumulated. Treatment of the callus cultures with the two metals promoted maximum embryogenic callus mass production of 1.74 ± 0.14 g on AgNO_3 40 μM + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 7.5 μM amended levels, suggesting the concentration as the most efficient for maximum embryogenic tissue formation from proliferated callus mass (Fig. 2C). CuSO_4 and AgNO_3 treatments have promoted 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, embryos differentiation and germination were influenced by AgNO_3 amendment in the cultivation medium of *Picea glauca* (Kang and Yeung 1995), *Hedychium muluense* (Sakhanokho et al. 2009), *Phoenix dactylifera* (Diab 2017; Roshanfekrra et al. 2017; Abdolvand et al. 2018) and *Coffea* species in genotype-dependent manner (Fuentes et al. 2000; Giridhar et al. 2004; Lope-Gomez 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).

In the case of somatic embryos induction, obtained embryogenic tissues were treated with concentrations of AgNO_3 , $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and their combination using solid embryo induction medium. Maximum somatic embryos differentiation was achieved on medium amended with AgNO_3 (40 μM) where 95.72% embryos production with average of 26.52 ± 0.17 embryos induction over $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ where 7.5 μM was the most effective on embryos induction efficiency with 16.31 ± 0.12 embryos, representing 89.09% embryos production efficiency. However, compared to singular treatment of the metals, their combination yield maximum number of embryos induction of 28.79 ± 0.18 , representing 97.31% success in embryos induction from the embryogenic tissues (Fig. 2D–E). Obtained somatic embryos at different stages of development were proliferated on medium supplemented with BAP and NAA ($8.88 + 1.35 \mu\text{M}$). AgNO_3 at moderate levels (up to 50 μM) promoted SE in *Coffea canephora* (Kumar et al. 2007) and *Hedychium bousigonianum*, with the production of up to 88 embryos g^{-1} callus while higher levels inhibited embryos differentiation from embryogenic callus (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 2-fold enhanced embryos formation achieved relative to control cultures (Roustan et al. 1990). Improvement on SE and plant regeneration were achieved from immature embryo-derived callus when cultivation medium of *Oryza sativa* genotypes were supplemented with 2,4-D (9.0 $\mu\text{M L}^{-1}$) in combination with CuSO_4 (10–50 $\mu\text{M L}^{-1}$) (Sahrawat and Chand 1999).

To figure the influence of metals on embryos proliferation, somatic embryos were treated with various concentration levels. Maximum efficiency on embryos proliferation was achieved on medium added with AgNO_3 at 40 μM where 24.16 ± 0.14 embryos were produced, representing 96.38% embryos formation frequency recorded over the maximum for $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (7.5 μM) treatment that had 18.21 ± 0.16 embryos, representing 89.21% efficiency. However, combination of the metals gave the best results of 26.52 ± 0.10 embryos that represents 97.53% efficiency (Fig. 2F–G). For maturation treatment of the embryos, AgNO_3 at 40 μM levels proved more efficient with the production of 70.38% and 85.21% after four and eight weeks culture respectively, while $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at the maximum of 7.5 μM treatment produced 63.48% and 70.24% respectively. Combination of the two metals in the cultivation medium produced 75.21% and 87.39% respectively at 40 μM AgNO_3 + 7.5 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ treatment of the embryos (Fig. 2H–I). In embryos germination experiments, maximum germination of mature somatic embryos was achieved on solid MS medium amended with 40 μM AgNO_3 treatment levels with 65.22% germinated embryos over the maximum of 7.5 μM levels of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ that had 54.38% germinated embryos after six weeks cultivation. Combination of the two metals proved more efficient in facilitating somatic embryos germination, with maximum achieved on AgNO_3 40 μM + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 7.5 μM amended medium where 68.34% embryos germination frequency were recorded (Fig. 2J). In other reported studies, embryogenic callus formation, somatic embryos differentiation and germination were promoted when AgNO_3 was amended in different cultivation-stage medias of *Oryza sativa*, with 3 mg L^{-1}

been the most efficient concentration over other levels tested while 5 mg L^{-1} for plant regeneration from callus cultures (Ghobeishavi et al. 2015).

Shoot morphogenesis and multiplication

Indirect shoot morphogenesis from callus cultures offer alternatives for developing transgenic plants of horticultural importance (Rego and de Faria 2001; de Almeida et al. 2015). However, such reports from callus cultures of ornamental plants are very low, and even in cases where reported 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 sectored and cultivated on solid MS medium supplemented with BAP ($2.22 \mu\text{M}$) and CuSO_4 , AgNO_3 or their combination amendments (Fig. 1l–m) to study their possible influence on shoot morphogenesis and multiplication (Fig. 1q–u). High levels of Cu in growth medium of plants had been reported toxic to cell growth that lead to tissue growth inhibition in cultures (Yruela 2005), more particular on shoot length (Verma et al. 2011). In the present study, treatment of callus cultures with the various AgNO_3 concentrations promoted maximum shoot morphogenesis on medium supplemented with $40 \mu\text{M}$ over other concentrations tested after six weeks cultivation, with the production of 4.53 ± 0.13 shoots. In the case of CuSO_4 supplemented medium callus cultivation, $7.5 \mu\text{M}$ proved most efficient with the production of 2.74 ± 18 shoots after six weeks cultivation. Combination of the two metals at different concentrations gave the best results on medium added with AgNO_3 $40 \mu\text{M}$ + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ $7.5 \mu\text{M}$ where 5.35 ± 0.23 shoots morphogenesis was achieved (Fig. 2K). Copper is required at higher concentration for improved plant regeneration from callus cultures (Dahleen 1995). Significant positive effectiveness of Cu (1 – $100 \mu\text{M}$) on the shoot morphogenesis had been reported in wheat, triticale and *Nicotiana* (Purnhauser and Gyulai 1993) and in several studies with Secale, Sorghum, Wheat and *Eleusine* (Popelka and Altpeter 2001; Nirwan and Kothari 2003; Kothari et al. 2004; Tahiliani and Kothari 2012; Dalton 2020). MS medium supplemented with Cu at $1.0 \mu\text{M}$ showed inhibitory effects on shoot regeneration in the *in vitro* cultures of *F. vulgare* but, optimum number of shoots were regenerated when not supplemented in growth medium of the plants, while other concentrations promoted the morphogenesis at variable degree (Dwivedi et al. 2020). Shoot bud differentiation and elongation was promoted by CuSO_4 treatment of callus cultures in *Capsicum annuum* with the highest response achieved during second stage subculture on medium supplemented with 30 times higher levels of the metal over the standard MS medium concentration while for AgNO_3 treatments, $18.0 \mu\text{M}$ was the optimal levels (Joshi and Kothari 2007). It promoted callus induction and shoot morphogenesis from callus cultures of *Solanum lycopersicum* in explant type, genotype and concentration dependent manner (Shah et al. 2014) while in *Zinnia* species and *Anthurium andraenum* genotypes, AgNO_3 up to 2.0 mg L^{-1} promoted shoot morphogenesis, multiplication and quality accompanied with 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 AgNO_3 treatments on the basis of culture stage and subcultures of the cultivated plant tissues (Hyde and Phillips 1996; Mookkan and Andy 2014). In banana tissue cultures, Tamimi and Othman (2020) reported significant increase in shoot multiplication on medium

supplemented with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ at varied concentration levels with 6 mg L^{-1} as the most efficient for which two-fold 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. AgNO_3 concentrations proved more efficient in multiple shoot differentiation, with the maximum production achieved on solid medium added with $40 \text{ }\mu\text{M}$ for which 19.57 ± 0.12 shoots were produced over other concentrations tested. For CuSO_4 , $7.5 \text{ }\mu\text{M}$ 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 shoots differentiation, with the maximum produced by AgNO_3 $40 \text{ }\mu\text{M}$ + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ $7.5 \text{ }\mu\text{M}$ combinations where 23.54 ± 0.17 shoots were produced after six weeks cultivation and over other treatments tested. In reported studies with other species, multiple shoot morphogenesis had been achieved from shoot-tip explants on medium amended with AgNO_3 without PGRs supplementation and when in combination with BAP, with the highest response achieved when $11.77 \text{ }\mu\text{M}$ of the AgNO_3 was amended in the cultivation medium, while combination with kinetin could not evoke morphogenic response of the cultures (Ozudogru et al. 2005). AgNO_3 treatment of *Prosopis cineraria*, *Brassica napus*, *Passiflora giberti*, *Hevea brasiliensis* and *Mucuna pruriens* promoted vigorous dark-green multiple shoot morphogenesis with maximum achieved on medium supplemented with $1\text{--}2 \text{ mg L}^{-1}$ where average of up to 5 shoots/explant were recorded (Sirisom and Te-Chato 2012; Roh et al. 2012; Faria et al. 2017; Venkatachalam et al. 2017; Alam and Anis 2019)

Rhizogenesis

For rhizogenesis on the optimized root morphogenesis solid MS medium, i.e supplemented with IBA ($2.42 \text{ }\mu\text{M}$), influence of the two metals on root morphogenesis was studied at the various amended concentration levels (Fig. 1v–y; 2M). Maximum roots morphogenesis was achieved on AgNO_3 amended medias over that of the CuSO_4 treatments. AgNO_3 at $40 \text{ }\mu\text{M}$ was the most efficient, producing 13.36 ± 0.13 roots per micro shoot over that of the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ treatments where $7.5 \text{ }\mu\text{M}$ was the most efficient with 10.42 ± 0.15 roots recorded after six weeks cultivation. Combination of the two metals proved more efficient over singular treatment, suggesting synergistic influence response to the various treatments, with the highest achieved on medium added with AgNO_3 $40 \text{ }\mu\text{M}$ + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ $7.5 \text{ }\mu\text{M}$ over other concentrations tested (Fig. 2M). Stimulatory effect of AgNO_3 on root morphogenesis have been reported in several plant species such as *Coffea arabica* (Giridhar et al. 2003), *Routa aquatica* (Sunandakumari et al. 2004), *Gentiana lutea* (Petrova et al. 2011), *Musa acuminata* (Tamimi 2015) and *Prosopis cineraria* (Venkatachalam et al. 2017). Also, requirement of Cu for increased lateral roots formation had been demonstrated from the seedlings of *Eucalyptus camaldulensis* (Dunn et al. 1997). However, high levels of Cu in growth medium may cause some symptoms such as chlorosis and brown necrosis that may impact root morphogenesis, architecture and biomass production (Marschner 1995; Lequeux et al. 2010).

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

Changes during somatic embryogenesis

Cellular stress is known to trigger elevation in the production of reactive oxygen species (ROS) that subsequently activate the expression of genes encoding antioxidative enzyme systems as a cellular defensive strategy essential for SE (Gressel and Galum 1994; Arora et al. 2002; Zavattieri et al. 2010; Ochatt and Revilla 2016). Overproduction of the ROS could result into direct cellular damage or formation of secondary toxic substances (Benson 2000) that plants have evolved antioxidant protection systems of SOD, CAT, APX, GPX and MDA for overcoming such challenges (Larson 1988). The role of Cu in ROS generation and for serving as cellular signaling molecules in elevated levels of ROS have been known through its facilitation of morphogenesis, with a reduction in rate of cultures infection (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 Sebastian 2005). Higher cellular levels of the 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 reduces the ability of antioxidants to chelate active oxygen species and induce oxidative stress essential for SE, the enzymes are expressed at higher levels to maintain cellular homeostasis (Halliwell and Gutteridge 1984). Similarly, AgNO₃ through its ions play modulatory roles on antioxidant defense systems that on the basis of species or genotype activates 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 concentration levels on the expression of antioxidant systems during SE were studied through the stages of embryogenic tissue formation and proliferation, somatic embryos induction, proliferation, maturation and germination on solid MS medium amended with various PGRs and their concentration.

Superoxide dismutase, a copper-containing metallo-enzyme, plays critical role in scavenging free radicals in cells and its highest activity is associated with chloroplasts' photosynthetic reaction centers connected to the cytochrome oxidase (Asada 1977; Shkolnik 1984). It plays prominent role in embryogenic competence acquisition and expression through the activity at high levels that results in generation of H₂O₂. In the present study, for the expression of SOD activity during embryogenic competence acquisition, CuSO₄.5H₂O at 7.5 µM was efficient in promoting activity of the enzyme at higher levels with 6.83 µKat mg⁻¹ protein recorded during formation of the embryogenic tissue. It increased to 7.58 during embryos differentiation, 6.78 for proliferation and 5.13 in germinating embryos compared to the 5.38, 5.98, 5.45 and 5.13 µKat mg⁻¹ protein of the control cultures, respectively. In the case of AgNO₃ treatment, maximum activity of the enzyme was noted with 40 µM treatment where 7.92 µKat mg⁻¹ protein were recorded in embryogenic tissue. It increased further to 8.72 during embryos differentiation but, become reduced to 7.42 for proliferation and least during germination, having 6.33 (Fig. 3A₁₋₄). Combination of AgNO₃ + CuSO₄.5H₂O amendment in the cultivation medium at different concentration levels promoted SE accompanied with SOD expression; the activity was high during embryogenic tissue formation with 8.55 µKat mg⁻¹ protein. It increased to 8.99 during somatic embryos induction, 7.93 during its proliferation and lowest during somatic embryos germination where 6.39 µKat mg⁻¹ protein was recorded (Fig. 3A₁₋₄). This suggests the promotory role of the metals on SE to be

associated with the expression of SODs at differential levels commensurate with stage of somatic embryos development through gradual reduction in expression levels of the enzyme.

Low intracellular levels of H_2O_2 could promote cell growth and its increased accumulation for SE through the role it plays as cellular messenger that induce gene expression essential for the SE (Cui et al. 1999). However, during later stages, excessive accumulation of H_2O_2 , due to the low activity of CAT and other peroxidases may result into tissue browning and cell necrosis (Laukkannen et al. 1999; Kong et al. 2012; Nic-Can et al. 2015). Catalase is largely associated with the removal of high levels of H_2O_2 in cells (Klapheck et al. 1990) and reduction in its activity with low accumulation of H_2O_2 promotes SE (Cui et al. 1999). In CuSO_4 treatment of the cultures, expression of CAT was highest with 7.5 μM over other concentrations tested with 6.11 $\mu\text{Kat mg}^{-1}$ protein recorded during embryogenic tissue formation. It decreased to 5.24 during somatic embryos induction, 4.72 for proliferating embryos and 3.21 in germinating somatic embryos compared to the 3.54 in the control of embryogenic tissue, 3.39 for somatic embryos induction, 2.98 for proliferation and 2.43 $\mu\text{Kat mg}^{-1}$ protein of the germination (Fig. 3B₁₋₄). For AgNO_3 treatment, highest CAT activity was noted with 40 μM treatments where 6.99 $\mu\text{Kat mg}^{-1}$ protein was recorded in embryogenic tissue formation. It decreased to 6.97 during somatic embryos differentiation, 6.15 in proliferating embryos and 4.42 $\mu\text{Kat mg}^{-1}$ 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 $\mu\text{Kat mg}^{-1}$ protein was recorded. It showed further decrease with advancement in somatic embryos differentiation with 6.97 during embryos formation, 6.67 for proliferation and 4.69 $\mu\text{Kat mg}^{-1}$ protein for the germination, suggesting gradual decrease in CAT expression during SE in *G. hybridus* influenced by CuSO_4 , AgNO_3 and their combination treatment levels (Fig. 3B₁₋₄). Increase in activity of CAT was observed with up to 10 μM treatment of *Erythrina variegata in vitro* cultures with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and its higher concentrations were insignificant while SOD expression was high in all the treatments tested, and showed dose-dependent trend in activity (Javed et al. 2017).

Ascorbate peroxidase, derived from ascorbic acid in cells, is involved in the reduction of H_2O_2 to water and at the same time oxidizes ascorbate to MDA. Activity of the enzyme was higher in AgNO_3 treated cultures over that of the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$: in the case of AgNO_3 treated cultures, highest activity was noted in 40 μM treatment with 4.62 $\mu\text{Kat mg}^{-1}$ protein recorded during embryogenic tissue formation. It decreased to 4.29 during embryos differentiation, 3.94 for proliferation and 2.94 $\mu\text{Kat mg}^{-1}$ protein during germination compared to the control of 2.74, 2.23, 1.85 and 1.64 $\mu\text{Kat mg}^{-1}$ protein, respectively. For $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, higher activity of the enzyme was observed in cultures treated with 7.5 μM where 3.86 $\mu\text{Kat mg}^{-1}$ protein was recorded in embryogenic tissue. It decreased to 3.58 during embryos differentiation, 2.98 for proliferating embryos, and 2.17 $\mu\text{Kat mg}^{-1}$ 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 activity of the enzymes, suggesting their

synergistic influence response. Combinatorial treatment of AgNO_3 40 μM + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 7.5 μM gave maximum response and APX activity with 4.75 $\mu\text{Kat mg}^{-1}$ 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 and then to 3.34 $\mu\text{Kat mg}^{-1}$ protein in germinating embryos compared to 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 as "tiny beads" appeared but, become reduced with further development of the embryos, similar to results of our study in the control and metal treated cultures of the various embryo tissues analyzed but, variable on culture duration (Fig. 3A–C).

Guaiacol peroxidase that also plays role in cellular detoxification of H_2O_2 accumulated due to the activity of SOD to tolerable levels essential for morphogenesis, showed higher activity in AgNO_3 treated cultures over the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. However, combinatorial treatments were more efficient, suggesting their differential influence to morphogenesis at variable levels supplemented in culture medium (Fig. 3D₁₋₄). In the case of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ treatments, maximum of the activity was noted on medium supplemented with 7.5 μM cultures where 2.41 $\mu\text{Kat mg}^{-1}$ protein was recorded during embryogenic tissue formation. It decreased to 2.18 during somatic embryos differentiation, 2.14 for the proliferation and 1.59 $\mu\text{Kat mg}^{-1}$ protein during mature embryos germination when compared to 1.85, 1.57, 1.46 and 1.28 $\mu\text{Kat mg}^{-1}$ protein of the respective control cultures (Fig. 3D₁₋₄). In the case of AgNO_3 treatments, 40 μM proved the most efficient with the production of 2.93 $\mu\text{Kat mg}^{-1}$ protein GPX activity during embryogenic tissue formation, 2.65 for somatic embryos differentiation while proliferating embryos had 2.59 and germinating embryos showed 2.24 $\mu\text{Kat mg}^{-1}$ protein after four weeks cultivation. Combined treatment of AgNO_3 40 μM + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 7.5 μM produced maximum of 3.27 $\mu\text{Kat mg}^{-1}$ protein GPX activity during embryogenic tissue formation, decreased to 2.97 during somatic embryos differentiation and further to 2.75 for somatic embryos proliferation while lowest was recorded in germinating somatic embryos with 2.52 $\mu\text{Kat mg}^{-1}$ protein GPX activity, suggesting decreased expression of the enzyme during the various developmental stages to scavenge SOD-generated free radicals and other cellular toxic stress agents.

In vitro morphogenesis of SE is associated with high cellular stress that generates free radicals at higher levels in cells through the activity of SODs which may result in membrane damages (Zavattieri et al. 2010). Among parameters that can be used to study extent of membrane damage due to the cellular oxidative stress that causes lipid peroxidation by polyunsaturated fatty acids hydroperoxidation, is the activity of MDA in plant cells (Bailly et al. 1996). Excessive accumulation of Cu^{2+} in the growth medium may cause range of toxic effects that includes inhibition of photosynthetic rate that damages plasmamembrane 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, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, AgNO_3 and their combined treatments enhanced the activity of MDA at various stages of morphogenesis commensurate with the enhancement on tissue differentiation observed due to influence of the metals. However, the most efficient been combinatorial treatments over the singular metal

treatment (Fig. 3E₁₋₄). For CuSO₄.5H₂O, 7.5 μ M treatment was the most efficient where 15.49 μ Kat mg⁻¹ protein MDA activity was recorded during embryogenic tissue formation. It decreased to 15.36 during somatic embryos differentiation, 14.59 for its proliferation while germinating embryos showed 11.15 μ Kat mg⁻¹ protein MDA activity. Treatment with AgNO₃ levels gave the best results with 40 μ M; maximum of 18.16 μ Kat mg⁻¹ protein MDA activity recorded during embryogenic tissue formation but, decreased to 17.85 during somatic embryos differentiation. This was further decreased during embryos proliferation with 17.48 MDA activity and 15.52 in germinating embryos compared to the control cultures where 9.78, 9.21, 8.46 and 8.24 μ Kat 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, the assay was carried out after cultures were treated with AgNO₃ + CuSO₄.5H₂O combination for four weeks. High activity of the enzyme was observed during embryogenic tissue formation where 19.36 μ Kat mg⁻¹ protein was recorded. This was decreased to 18.91 during somatic embryos differentiation, then to 18.21 in proliferating embryos while germinating embryos had 16.95 μ Kat mg⁻¹ protein MDA activity, suggesting possible high membrane damage imposed to tissues during SE due to high cellular levels of hydroxy, alkoxy and peroxy radicals accumulation that trigger activity of the enzyme to overcome membrane damage at various stages of morphogenesis that high expression of SOD, CAT, APX and GPX could not ameliorate (Fig. 3E₁₋₄). It can also be ascribed to inability of peroxidases activity in catalyzing H₂O₂ detoxification that resulted in production of high cellular levels of toxic hydroxyl radicals from H₂O₂ 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 had been observed in plants that showed Cu deficiency but, supplemented with the metal in nutrients cultivation medium (Marschner 1995).

Changes during shoot morphogenesis

To study the influence of CuSO₄.5H₂O, AgNO₃ and their combinations on shoot morphogenesis-associated biochemical changes in *G. hybridus*, different concentration of the two metals were tested and parameters of Chl, Cars, glycinebetaine and proline content evaluated from harvested shoot cultures (Fig. 3F–G). Exposure to high concentration of the metals may inhibit 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 Henrique 1991; Dudev and Lim 2008; Huang et al. 2020; Pontes et al. 2020) with 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, Chla content of the shoot cultures was highest in 7.5 μ M treatment with CuSO₄.5H₂O where 0.1981 ± 0.12 mg g⁻¹ FW were accumulated over the control and other concentrations tested. For AgNO₃ treatment of the cultures, maximum production of the pigment molecule was recorded in 40 μ M treatment with 0.29 ± 0.11 mg g⁻¹ FW Chla. Combination of the two metals at varied concentration levels yield highest production of 0.32 ± 0.14 mg g⁻¹ FW Chla in AgNO₃ 40 μ M + CuSO₄.5H₂O 7.5 μ M treatment (Fig. 3F₁). In the case of Chlb, maximum of 1.18 ± 0.13

mg g⁻¹ FW was recorded in 7.5 µM CuSO₄.5H₂O treatment over other concentrations tested while AgNO₃ at 40 µM proved more effective with the production of 1.34 ± 0.12 mg g⁻¹ FW Chlb (Fig. 3F₂). Combined treatment assessment yield maximum of 1.42 ± 0.10 in 40 µM AgNO₃ + 7.5 µM CuSO₄.5H₂O levels, suggesting the concentration as the most effective in promoting Chl biosynthesis essential for plant growth. For total Chl content of the shoot cultures, maximum was recorded in 7.5 µM CuSO₄.5H₂O treatments where 1.192 ± 0.12 mg g⁻¹ FW Chl was recorded compared to the higher of 1.34 ± 0.13 achieved with 40 µM AgNO₃ treatment of the shoot cultures and over other treatments tested (Fig. 3F₃). Combination treatments of the two metals further enhanced total Chl accumulation to 1.52 ± 0.11 mg g⁻¹ FW recorded with AgNO₃ 40 µM + CuSO₄.5H₂O 7.5 µM combination levels. In the case of Cars production, CuSO₄.5H₂O at 7.5 µM was equally more efficient in their accumulation with 0.616 ± 0.12 mg g⁻¹ FW recorded in the treatment over other amended concentration levels of the metal. AgNO₃ at 40 µM proved more efficient in Cars production enhancement in the shoot cultures with the production of 0.73 ± 0.17 mg g⁻¹ FW while combination treatment with the two metals yield maximum accumulation with AgNO₃ 40 µM + CuSO₄.5H₂O 7.5 µM levels, with the production of 0.743 ± 0.14 mg g⁻¹ FW over other combination treatments tested (Fig. 3F₄). This suggests that supplementing Cu in the culture medium at moderate levels enhanced leaves Chl content, while higher inhibited its biosynthesis due to 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, leaves expansion and Chl content was achieved with shoot tip explants of *Musa acuminata* when cultivated on medium supplemented with AgNO₃ (up to 10 mg L⁻¹) over control cultures and other ethylene inhibitors tested (Tamimi 2015). Inhibition of ethylene action due to supplementation of AgNO₃ in growth medium to serve as source of silver ions have promoted increase in leaves Chl content (Perl et al. 1988; Ehsanpour and Jones 2001). In *Solanum tuberosum* shoot cultures, treatment with AgNO₃ up to 10 µM promoted shoot morphogenesis Chla, Chlb 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, Chla and Chlb concentration showed no significant variation in accumulation but, the ratio of Chla/Chlb varied, suggesting its tolerance to the concentrations tested through proper chloroplast development (Gori et al. 1998). At high CuSO₄ amendment in MS medium, improved shoot morphogenesis was achieved in *Stevia rebaudina in vitro* cultures and was associated with increase in Chl content but, higher levels proved detrimental to the cultures (Jain et al. 2009). Treatment of banana shoot cultures with CuSO₄.5H₂O promoted high Chl accumulation in leaves with the highest been on medium added with 6.0 mg L⁻¹ (Tamimi and Othman 2020). In *E. variegata*, 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 leaves yellowing prevalent in cultures and changes in antioxidant systems (Javed et al. 2017), possibly due to essential role that Cu play as micronutrient needed for physiological metabolic enzyme processes of photosynthesis, respiration and hormones perception (Hansch and Mendel 2009; Nomura et al. 2015; Rahmati Ishka and Vatamaniuk 2020).

Proline, an osmoprotectant that serve as storage sink of carbon, nitrogen and free radical scavenger, showed highest accumulation in 7.5 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ medium treatment cultures, with the production of $158 \pm 0.19 \mu\text{M mg}^{-1}$ FW over the maximum of 227 ± 0.15 in AgNO_3 treatment of 40 μM and other concentrations tested. Combination of the two metal salts further enhanced proline accumulation in the shoot cultures with the highest production been on AgNO_3 40 μM + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 7.5 μM treatment cultures where $239 \pm 0.16 \mu\text{M mg}^{-1}$ FW was recorded over other treatments tested (Fig. 3G₁).

Glycinebetaine production in the shoot cultures was enhanced by the $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ treatments with its highest accumulation achieved on medium supplemented with 7.5 μM where $178 \pm 0.21 \mu\text{M mg}^{-1}$ FW production over other concentrations tested. AgNO_3 proved more effective in promoting the accumulation over $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ treatments, with the maximum production been on medium added with 40 μM where $209 \pm 0.18 \mu\text{M mg}^{-1}$ FW was accumulated by the shoots while combined treatment with the two metals resulted in highest production of $224 \pm 0.17 \mu\text{M mg}^{-1}$ FW in AgNO_3 40 μM + $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 7.5 μM treatment level of the cultures and over other levels tested (Fig. 3G₂). Reduction in the morphogenic response observed with higher levels of the metals in the present study can be explained by the possible triggering effect they had on Fenton reaction that generates hydroxyl radicals which are known to cause lipid peroxidation, 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 *Triticum aestivum* genotypes in concentration-dependent manner, with the synthesized nanoparticles been more efficient over use of the two metals employed.

Conclusion

Results of the present study suggests that supplementing CuSO_4 ($5\text{H}_2\text{O}$), AgNO_3 or their combination in the *in vitro* growth medium of *G. hybridus* have promotory influence on callus induction, embryogenic tissue differentiation, SE, shoot morphogenesis and multiplication in concentration dependent manner. Supplementing the metals to *in vitro* growth medium influences tissue growth during the various morphogenic stages through influence on the physiological biochemical changes essential for the morphogenic processes. This can be ascribed to the role they play in influencing different physiological biochemical pathways and changes essential for the *in vitro* developmental processes.

Abbreviations

Plant Cell Tissue and Organ Culture

PCTOC

Murashige and Skoog medium

MS

Plant growth regulators

PGRs

2,4-Dichlorophenoxy acetic acid

2,4-D
N⁶-Benzylaminopurine
BAP
Naphthalene acetic acid
NAA
Indole butyric acid
IBA
Copper sulphate (hydrate)
CuSO₄
Silver nitrate
AgNO₃
Somatic embryogenesis
SE
Chlorophyll
Chl
Photosystems
PS
Fresh weight
FW
Dried weight
DW
Reactive oxygen species
ROS
Superoxide dismutase
SOD
Hydrogen peroxide
H₂O₂
Catalase
CAT
Ascorbate peroxidase
APX
Guaiacol peroxidase
GPX
Malondialdehyde
MDA
Complete randomized block design
CRBD

Declarations

Conflict of interest statement

Authors declare that conflict of interest does not exist in the manuscript contents.

Ethics approval and consent to participate

The research work was carried out in compliance with ethical standard that did not involve the use of humans.

Consent for publication

Authors declare having consent to publish the manuscript contents.

Availability of data and material

Available on reasonable request to corresponding author.

Competing interests

None exist

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Authors' contributions

TI conceived the research idea, performed all *in vitro* culture experiments but physiological and biochemical changes analysis experiment together with **Q**, while **TI**, **Q** and **SU** wrote, edited and approved the manuscript contents.

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Figures

Figure 1

a Callus induction from corm explants, **b** embryogenic tissue formation and proliferation, **c-i** embryos induction, proliferation and maturation, **j-k** mature somatic embryos germination, **l-n** shoot morphogenesis from callus, **o-p** mature somatic embryos germination, **q-s** mature embryos germination in singularity forms, **t-u** multiple shoot morphogenesis, **v-x** rhizogenesis on medium amended with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, AgNO_3 and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O} + \text{AgNO}_3$, **y** treatment of the shoots with $60 \mu\text{M}$ AgNO_3 on root morphogenesis medium, **z** acclimatized *Gladiolus hybridus* plants after 4 weeks cultivation. *Arrowheads show somatic embryos induction or shoot morphogenesis.*

Figure 2

Influence of silver nitrate and copper sulphate on *in vitro* morphogenesis in *G. hybridus*.

Figure 3

Influence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and AgNO_3 on the expression of antioxidant systems and pigment molecules during *in vitro* morphogenesis (somatic embryogenesis and shoot morphogenesis) in *G. hybridus*.