South African Journal of Botany
Volume 139, July 2021, Pages 230-240

Micropropagation of *Philodendron selloum*: Influence of copper sulfate on endophytic bacterial contamination, antioxidant enzyme activity, electrolyte leakage, and plant survival

M.K. Seliem [a], M.E. El-Mahrouk [b], A.N. El-Banna [c], Y.M. Hafez [d], Y.H. Dewir [b]

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Highlights

- Copper sulfate proved effective for eliminating endophytic bacteria in *Philodendron selloum in vitro* cultures.
- Moderate level of copper sulfate did not induce growth abnormalities and was optimal for shoot multiplication of *P. selloum.*
- RAPD molecular marker revealed genetic fidelity of the regenerated plantlets at all levels of copper sulfate.

Abstract

Endophytic microorganisms is a major constrain to the establishment and growth of tissue culture plants. We report the use of copper sulfate ($CuSO_4.5H_2O$) to eliminate the endophytic bacteria in *Philodendron selloum* in vitro cultures. Contaminated shoots were cultured onto Murashige and Skoog (MS) medium containing 5 mg/L BA and supplemented with different concentrations of copper sulfate at 0, 35, 70 and 140 mg/L for 6 weeks. Copper sulfate at 70 mg/L completely eliminated the endogenous bacteria without decline in plant growth. However, 35 mg/L copper sulfate was optimal for maximum shoot multiplication (25), survival percentage (100%) and growth of plants. Antioxidant enzymes activity of catalase, peroxidases, and polyphenol oxidase were increased because of copper sulfate treatments. Conversely, electrolyte leakage was decreased at low copper sulfate (≤70 mg/L). Randomly amplified polymorphic DNA (RAPD) analysis revealed that plantlets exposed to different levels of copper sulfate were not genetically different from control plants.

Keywords

Copper sulfate; Philodendron; Antioxidant enzymes; Endophytic; RAPD

1. Introduction

Plant micropropagation is the first step in biotechnological research for the development and improvement of ornamentals. However, prohibiting and averting microbial contamination of *in vitro* plants is crucial to successful micropropagation. Microbial contamination is a major factor in the loss of time, effort, and plant tissue culture. There are distinct methods of sterilization to eliminate contamination, such as chemical agents (liquid detergent, antiseptic agents, sodium hypochlorite, or mercuric chloride), antibiotics, autoclaving of media and instruments, UV sterilization, and improvement of cultural practices (Reed and Tanprasert 1995; Javed et al., 2017). Epiphytic microbes that are present because of inactive aseptic technique or insufficient sterilization of equipment can be managed by improving performance in the laboratory, but endophytic microbes in explants cause contamination in the culture (Vanden Houwe and Swennen 2000; Gangopadhyay et al., 2017).

Philodendron *selloum* (Araceae) is native to the tropical rain forests of Central and South America. It has large, shiny dark green leaves with deep lobes. The plant has a trunk as it matures, but the huge, drooping leaves usually hide it. Philodendron is one of the most important ornamental foliage plants, and is grown as a pot plant (Chenetal.2005). *In vitro* propagation is the best method other than conventional propagation for the production of Philodendron to meet the growing demand in both the local and export markets (Seenietal., 2001). Therefore, vegetative propagation through tissue culture technique of Philodendron is an alternative method for obtaining rapid clonal multiplication. There are some reports on *in vitro* propagation of Philodendron (Gangopadhyayetal., 2004; Kelieetal., 2004; Xiong2009; Chenetal., 2012; Hassanetal., 2016). However, the difficulty of establishing or maintaining aseptic culture is a definite factor in the tissue culture of aroid plants (Chenand Yeh,2007). Previous reports noted that many aroid plants have endogenous bacterial infections leading to the loss of vegetative parts, including Dieffenbachia (Deberghand Maene,1981), Anthurium (Geier,1990), and Aglaonema (Chenand Yeh,2007). In addition, various endophytic bacteria produce phytotoxins, which cause plant deterioration at high concentrations (Leifertetal.1989). Therefore, endophytic bacteria obstruct the aroid plants. Endophytic bacterial contaminants are difficult to detect because they have no visible symptoms and often remain inside the plant tissue (Vissetal., 1991). They centralize in the plant in cell sections and the intercellular spaces of cortical parenchyma (Gunsonand Spencer-Philips,1994). Surface sterilization does not remove endophytic bacteria (Reedetal., 1995). Thus, eliminating endophytic contaminants is more difficult (Buckleyetal.1995). To avoid internal bacterial contamination, several methods using antibiotic supplementation to culture media have been investigated (Asifetal.2013; Bohraetal.2013). However, the phytotoxic effect of antibiotics, which can cause growth retardation, limit their application (Shehataetal.2010; Silvaetal.2003). Antibiotic degradation by high temperature and time has made them ineffective against some bacteria. Additionally, exposure to concentrated sterilization solutions such as mercuric chloride and sodium hypochlorite is ineffective in endophytic bacteria because the solution does not penetrate tissue in sufficient quantities to kill bacteria (Javedetal., 2017).

Copper (Cu) is an essential microelement for the normal growth and development of plants, as it is needed for several physiological functions of plants including photosynthesis, electron transport, respiration, oxidative stress response, plant hormone signaling and cell wall biosynthesis, signal transduction, and cell wall lignification. (Ravenetal., 1999; Nas2004; Festaand Thiele 2011; Marschner2012; Palmerand Guerinot 2009). Cu ions play an essential role as cofactors in many enzymes such as Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, amino oxidase, laccase, plastocyanin, and polyphenol oxidase (Yruela2005). Copper is an important ingredient in many antifungal and antibacterial

compounds (Lamichhaneetal., 2018). It shows antimicrobial activity against a wide range of microorganisms (Faundezetal., 2004), including bacteria, fungi, and viruses (Grassetal.2011; Gyawalietal.2011; Noyceetal.2007). In addition, its compounds have relatively low cost and toxicity to mammals, thereby increasing their advantage over other chemicals for the control of foliar bacterial diseases. However, Cu is highly toxic at high concentrations, which reduces photosynthetic oxygen transformation in the chloroplasts (Maksymiecand Baszynski,1996) and increases reactive oxygen species formation, plasma membrane permeability, and leakage of potassium (K) ions from the roots (Weckxand Clijsters,1996; Chenetal., 2015) and induces iron (Fe) deficiency (Taylorand Foy,1985). In addition, the negative effects of copper on plants are varied and dependent on the species (Gharabietal., 2005). Previous reports have indicated that higher copper concentrations have a positive effect on the *in vitro* culture of several plants, including *Cucumis melo* (Garcia-Sogoetal.1991), *Nicotiana tabacum* (Purnhauserand Gyulai 1993; Gorietal., 1998), *Hordeum vulgare* (Dahleen1995; Choetal.,1998), *Oryza sativa* (Yangetal.,1999; Sahrawatetal., 1999; Amarasinghe2009), *Lepidium sativum* (Sabaetal.,2000), *Eleusine coracana* (Kotharietal., 2004), *Tinospora cordifolia* (Kumaretal.,2003), *Withania somnifera* (Sinhaetal., 2010), *Daucus carrota* (Kowalskaetal., 2012), and *Gymnema sylvestre* (Chungetal., 2019). In addition, some aroid plants, such as *Pistia stratiotes* (Olkhovychetal., 2016) and *Colocasia esculenta*, show tolerance of medium copper concentrations (Hilland Miyasaka,2000).

Excess copper concentration leads to rapid increases in hydrogen peroxide (H_2O_2) levels and the total activity of antioxidant enzymes (Liuetal., 2018). Various antioxidant enzymes, such as peroxidase (POX), polyphenol oxidase (PPO), and catalase (CAT), play important roles in reactive oxygen species (ROS) metabolism during exposure to high copper levels. A system designed from various antioxidant defense enzymes is used to reduce the concentrations of superoxide and H_2O_2 (Liuetal., 2018). Higher levels of copper can cause strong phyto-, cyto-, and genotoxic effects (Koplikuand Mesi 2015). Additionally, they can lead to chromosome stickiness, bridges and fragments, c-mitosis, and disintegrated nuclei. Excess copper leads to cellular and DNA damage, which interacts with DNA and nuclear proteins, inducing DNA damage and conformational changes (Koplikuand Mesi 2015; Javedetal., 2017). Therefore, it is important to confirm the genetic fidelity of regenerants produced on media supplemented with copper. RAPD is an important marker to evaluate the genotoxicity, genetic homogeneity, and true-to-type nature of *in vitro* plants (Javedetal., 2017; El-Mahrouketal., 2016). The objectives of this study were to study the effects of copper sulfate on shoot multiplication, antioxidant enzyme activity, endophytic contamination, and genotoxicity of regenerants.

2. Material and methods

2.1. Plant material

In vitro plantlets of *Philodendron selloum* were maintained on MS (Murashige and Skoog 1962) solid medium (30g L^{-1} sucrose+8.0 g L^{-1} agar) and kept at 25 ± 2°C and 40 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF: 16h/d) for four weeks before being used in this study.

2.2. Isolation of endophytic bacteria

The bacterial contaminants that appeared around the base of the *in vitro* grown explants were serially diluted and plated in triplicate on Luria Bertani (LB) agar media containing 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl in double-distilled water. The plates were incubated aerobically at 37°C for 24h. Individual pure colonies were screened on LB agar media.

2.3. DNA extraction and 16S rRNA-based bacterial identification

Bacterial*genomic DNA*was*isolated*from the overnight-grown Luria broth culture using a G-spin™ Genomic DNA Extraction Mini Kit (Cat. No. 17121, Intron Biotechnology DR, Korea) *according to the manufacturer's instructions*. After isolation of genomic DNA, amplification of 16S rDNA was performed using the universal primer sets 27F (5-AGA GTT TGA TCC TGG CTC AG-3) and 1492R (5-TAC GGT TAC CTT GTT ACG ACTT-3) in a thermal cycler (Veriti™eritiC CTT GTT ACG ACTT-3Applied Biosystems). The PCR reactions consisted of 4μL of dNTPs (1.0 mM each, Roche), 2 μL of 10×buffer (Roche), 0.2 μL of each primer (0.5 μg), 0.2μL of *Taq* polymerase (5 U/ μl), 1 μL of 30 ng template DNA, and sterile Milli-Q water in a final volume of 20 μL. The amplification conditions consisted of an initial denaturation at 95°C for 5min followed by 35 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5min. Products were electrophoresed in 1.5% agarose gel containing RedSafe dye in TAE buffer (40mM Tris-acetate, 20 mM glacial acetic acid, 1 mM EDTA, pH 7) at 80 V. The amplified bands were detected on a UV-trans-illuminator and photographed by a gel documentation system (UVITEC, UK) and then subjected to analysis by Phoretix program 1D Gel Analysis software version 4.01. The PCR fragments were purified using the Qiagen PCR Purification Kit (Qiagen, Hilden, Germany). The 16S rRNA gene was sequenced using both primers 27F and 1492R with the Big Dye Terminator Cycle Sequencing kit v1.1. Sequencing reactions were run on an 3500xL Genetic Analyzer (Applied Biosystems). The nucleotide sequences were compared with known taxonomic information at NCBI GenBank using the nucleotide BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) for identification.

2.4. Effect of copper sulfate on percentage of bacterial contamination

Isolated endophytic bacteria were inoculated onto MS medium containing different concentrations of copper sulfate ($CuSO_4$ $5H_2O$: 0.025, 35, 70, and 140 mg/L) to determine the contamination percentage. The medium was poured into sterilized Petri plates (9 cm) and incubated for 4 d. Different treatments were inoculated by loop. Every treatment had four replications. Inoculated plates were incubated at 28 °C for 3 d, and the bacterial contamination percentage was detected by the appearance of clonal growth.

2.5. Shoot multiplication and copper sulfate concentrations

Axillary shoots of about 2.0 cm were separated into pairs and used as explants. All explants were cultured in a cylindrical culture jar (375 mL capacity) containing 60 mL MS basal medium and supplemented with 5 mg/L BA. Different concentrations of copper sulfate ($CuSO_4$ $5H_2O$: 35, 70, and 140 mg/L) were added to the media before autoclaving to achieve the objective of the experiment. MS basal medium containing 0.025 mg/L $CuSO_4$ $5H_2O$ served as a control. All cultures were kept in a culture room at 25 ± 2 °C with a photoperiod of 16 h at a photosynthetic photon flux (PPF) density of 30 µmol m^{-2} s^{-1} supplied by cool white fluorescent lamps Number of shoots, number of leaves, and shoot fresh weight were recorded after six weeks of culture.

2.6. Assay of antioxidant enzymes

To determine the activity of the antioxidant enzymes, 0.5 g fully expanded leaves of *in vitro* plants were homogenized under liquid nitrogen with 1.5 mL of respective extraction buffer using a pre-chilled mortar and pestle. The homogenate was filtered through four layers of cheesecloth and centrifuged at 22,000×g for 20 min at 4 °C. The supernatant, which was re-centrifuged at 22,000×g for 20 min at 4 °C, was used for detection of the following antioxidants. Catalase (CAT; EC 1.11.1.6) activity was measured by following the consumption of H_2O_2 at 240 nm (Aebi, 1984). A 1 mL reaction mixture contained 20 µg total protein, 50 mM sodium phosphate buffer (pH 7.0), and 10 mM H_2O_2. The reaction was initiated by adding the protein extract. For each measurement, the blank corresponded to the absorbance of the mixture at zero time, and the actual reading corresponded to the absorbance after 1 min. One unit of CAT activity was defined as a 0.01 decrease in absorbance at 240 nm per mg protein per minute. Peroxidase (POX; EC 1.11.1.7) activity was determined according to the procedure proposed by Hammerschmidtetal.(1982). The reaction mixture consisted of 2.9 mL of 100 mM sodium phosphate buffer (pH 6.0) containing 0.25% (*v*/*v*) guaiacol (2-methoxy phenol) and 100 mM H_2O_2. The reaction was started by adding 100 µL of crude enzyme extract. Changes in absorbance at 470 nm were

recorded at 30 s intervals for 3 min. Enzyme activity was expressed as an increase in absorbance min^{-1} g^{-1} fresh weight. Polyphenol oxidase (PPO; EC 1.10.3.1) activity was determined according to the method described by Malikand Singh(1980). The reaction mixture contained 3.0 mL buffered catechol solution (0.01 M), freshly prepared in 0.1 M phosphate buffer (pH 6.0). The reaction was started by adding 100 µL of crude enzyme extract. Changes in the absorbance at 495 nm were recorded at 30 s for 3 min. Enzyme activity was expressed as an increase in absorbance min^{-1} g^{-1} fresh weight.

2.7. Assay of electrolyte leakage

Measurements were carried out as described by Szalaietal.(1996) and Whitlowetal.(1992), with some modifications according to Dewiretal.(2015). Leaf discs of *in vitro* plantlets of different copper sulfate treatments were placed individually into 25 mL deionized water (Milli-Q 50, Millipore, Bedford, Mass., USA). Flasks were shaken for 20h at ambient temperature to facilitate electrolyte leakage from the injured tissues. Initial electrical conductivity measurements were recorded for each vial using an Acromet AR20 electrical conductivity meter (Fisher Scientific, Chicago, Il., USA). Flasks were then immersed in a hot water bath (Fisher Isotemp, Indiana, PA) at 80°C for 1h to induce cell rupture. The vials were again placed on the Innova 2100 platform shaker for 20h at 21°C. Final conductivity was measured for each flask. Electrolyte leakage percentage was calculated as (initial conductivity/final conductivity)×100.

2.8. Chemical composition of *in vitro* plantlets

Plant samples (leaves, stems, and roots) were oven-dried at 70°C for 24h. Dry samples were ground to obtain a homogenous powder in a metal-free mill (IKa-Werke, M 20 Germany). Concentrated sulfuric acid (95%, 5 mL) was added to the sample (0.2g), and the mixture was heated for 10min on a sand hotplate. Then, 0.5 mL of perchloric acid was added, and heating was continued until a clear solution was obtained. The solution was left to cool before it was filtered and diluted to 50 mL with distilled water (Evenhuisand de Waard,1980). Phosphorus (P mg/L) was extracted according to the methods described by Murphyand Riley(1962) and detected colorimetrically in a spectrophotometer (GT 80+, UK). Soluble cations K^{+}, Ca^{2+}, and Mg^{2+} were estimated using the atomic absorption spectrometry method according to the USDA(2004). The Cu concentration (mg/kg dry weight) was determined using the methods of Pageetal.(1982) in plantlets based on atomic absorption spectrophotometry (Avanta E; GBC). Trace elements (Mn, Zn and Fe) were quantified by atomic absorption spectrometry (Avanta E; GBC), according to Pageetal.(1982)
.

2.9. Plant DNA extraction and RAPD polymerase chain reaction conditions

DNA was extracted from fresh leaves (oldest two leaves on the plant) of the mother plant and acclimatized plants (three plants every treatment) by acetyltrimethylammonium bromide according to Doyleand Doyle(1990). A polymerase chain reaction (PCR) was performed and repeated three times using four random decamer primers (Table1) (Al-Saghirand Abdel-Salam,2015; Joshietal., 2009). RAPD-PCR was carried out in presence of 1• Taq DNA polymerase buffer (10 mM Tris-HCl of pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 100 mM dNTPs, 5 pmol single random primers, 25 ng DNA template, and 0.5 unit of Taq DNA polymerase in a total volume of 25 mL. PCR amplification was performed in an automated thermal cycler (MJ Mini; Bio-Rad, Foster City, CA) programmed as follows: 95°C for 4min followed by 40 cycles of 1 min denaturation at 94°C, 30s annealing at 35°C, and 2 min polymerization at 72°C, followed by a final extension step at 72°C for 7 min. The amplification products were resolved by electrophoresis in 1.5% agarose gels in 0.5• Tris-borate-EDTA (TBE) buffer and documented on a Gel Documentation system (UVITEC CAMBRIDGE Company, Cambridge, UK).

Table 1. List of the primers and their nucleotide sequences.

Primer name	Sequence (5`→3`)
OPE-11	GAGTCTCAGG
OPD-07	TTGGCACGGG
OPD-12	CACCGTATCC
OPE-12	TTATCGCCCC
OPK-10	GTGCAACGTG

2.10. Estimation of genomic template stability

The polymorphic pattern generated by RAPD-PCR profiles by using the selected primers allowed the calculation of Genomic Template Stability (GTS, %) as follows: GTS%=(1-a/n)×100, where *a* is the average number of polymorphic bands detected in plants treated with different concentrations of copper sulfate and *n* is the number of total bands in the non-treated plants. Polymorphisms in RAPD profiles included the appearance of a new band and disappearance of a band compared to the control profile. To compare the sensitivity of genomic template stability, changes in these values were calculated as a percentage of their control.

2.11. Nucleotide sequence accession numbers

The 16S rDNA partial gene sequences generated from this study were deposited in the GenBank database under the accession number MT157396.

2.12. In vitro rooting

Shoot clusters of Philodendron were cultured on MS medium without PGRs and supplemented with the same copper sulfate concentrations for subsequent growth and elongation for four weeks. Shoots (> 2cm long) were separated individually and cultured on MS medium without PGRs and containing the same concentrations of copper sulfate for rooting. Plantlet length, number of leaves, number of roots, root length, and plantlet weight were recorded after four weeks of culture.

2.13. Acclimatization

Plantlets at the 4–6 leaf stage were transplanted into culture trays (72 wells) filled with a mixture of sterilized peat moss and perlite (1:1). The plantlets were covered with a clear plastic film during the first 15 d of culture in air-conditioned greenhouse. The environment in the greenhouse was adjusted to 25 ± 2°C air temperature, 60–70% relative humidity, and 100 μmol m^{-2} s^{-1} PPF. Survival percentage, plant length, number of leaves and roots/plant, root length, and plant weight were recorded after four weeks of culture.

2.14. Experimental design and statistical analysis

Experiments were set up in a completely randomized design, and each treatment had five replicates. Each replicate was represented by a culture jar containing three explants. Observations on shoot multiplication as well as *in vitro* rooting were recorded after six weeks of culture. Data were subjected to analysis of variance using SPSS software (version 20; IBM Corp., Armonk, NY). The mean separations were performed using Duncan's multiple range testing method, and significance was determined at $P \le 0.05$.

3. Results and discussion

3.1. Molecular identification of the isolated bacteria

The universal primers 27Fand 1492R were found to be suitable for amplification of the region of the bacterial 16s rRNA gene. The amplified fragment (Fig. 1) was the expected molecular size (1480 pb). The sequence of 16S rRNA of the bacterial strain was submitted to GenBank (GenBank accession number MT157396). A similarity search was performed by

using the BLAST program, which indicated close genetic relatedness with the rRNA sequence of *Bacillus pumilus* 16S ribosomal RNA gene, partial sequence, accession no. KY038573.1 (100% similarity) in the NCBI database. This higher identical value confirmed the isolated strain to be *Bacillus pumilus* (Fig.2). PCR amplification and sequencing of the rRNA locus provides a rapid and often specific method for bacterial identification. Sequencing of the 16S rRNA gene has been widely used to estimate relationships among bacteria, and more recently it has also become an important tool for identification of an unknown bacterium to the genus or species level (Amannetal., 1995).

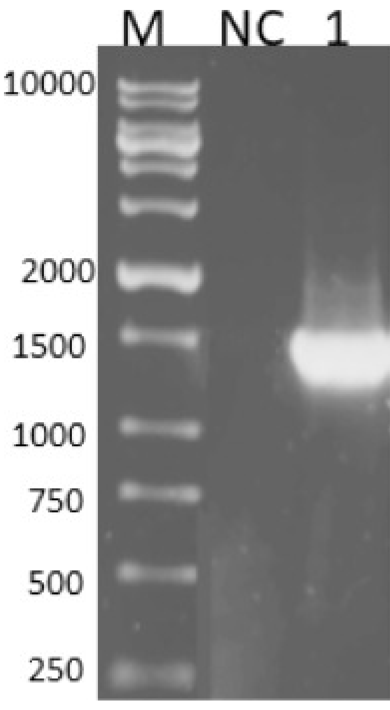

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Fig. 1. The 16S rRNA gene of the bacterial isolate amplified with 27F and 1492R primers. 1, PCR product ~1480 bp.; M, Sizer-1000 DNA marker; NC, negative control.

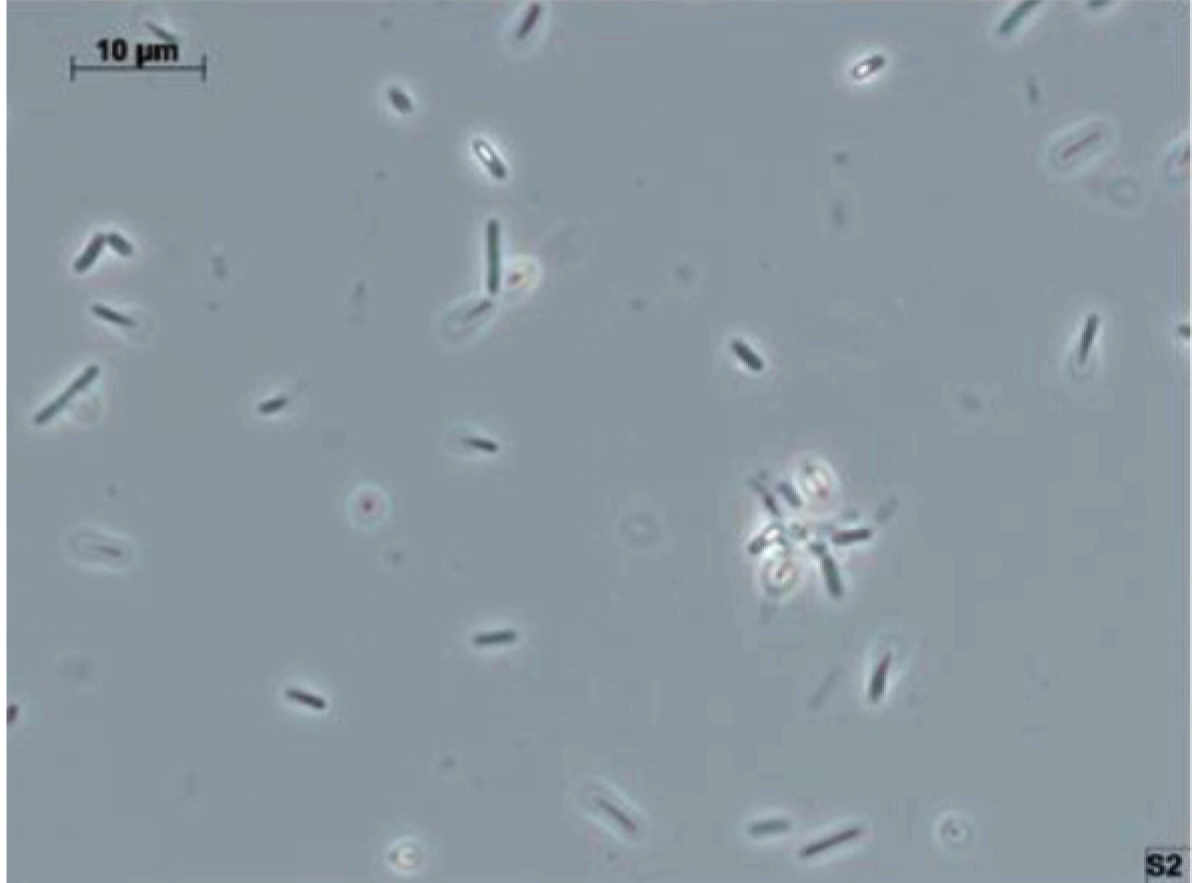

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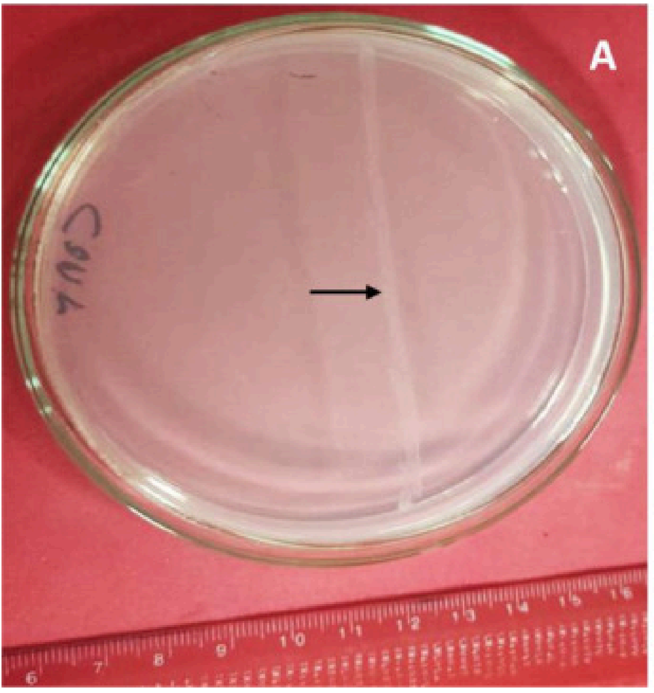
Fig. 2. Representative morphology of isolated bacteria from in vitro *Philodendron selloum*.

3.2. Effect of copper sulfate on bacterial contamination percentage

Addition of copper sulfate to the medium significantly reduced the contamination percentage (Table2 and Fig.3). Concentrations of 35, 70, and 140 mg/L copper sulfate limited the percentage of bacterial contamination to 25%, 0.0%, and 0.0%, respectively, as compared with the control, which showed 100% contamination. Therefore, 70 and 140 mg/L were the best treatments for inhibiting bacterial contamination. These results supported those of previous studies showing that copper is an important element in several antifungal and antibacterial compounds and plays a key role in inhibiting microbes (Lamichhaneetal., 2018). Furthermore, it has antimicrobial activity against a wide range of microorganisms (Faundezetal., 2004), including bacteria, fungi, and viruses (Grassetal.2011; Gyawalietal.2011; Noyceetal.2007).

Table 2. Effect of copper sulfate on contamination percentage and shoot multiplication of *Philodendron selloum* after 6 weeks.

Treatment (mg/L)	Contamination (%)	Number of shoots	Number of leaves/ shoot	Shoot fresh weight (g)
Control (0.025)	100.0 a	15 c	3.0	0.107 d
35	25.0 b	25 a	3.0	0.128 bc
70	0.0 c	19 b	3.0	0.132 b
140	0.0 c	6 d	3.5	0.152 a
Significant	**	**	N.S	**



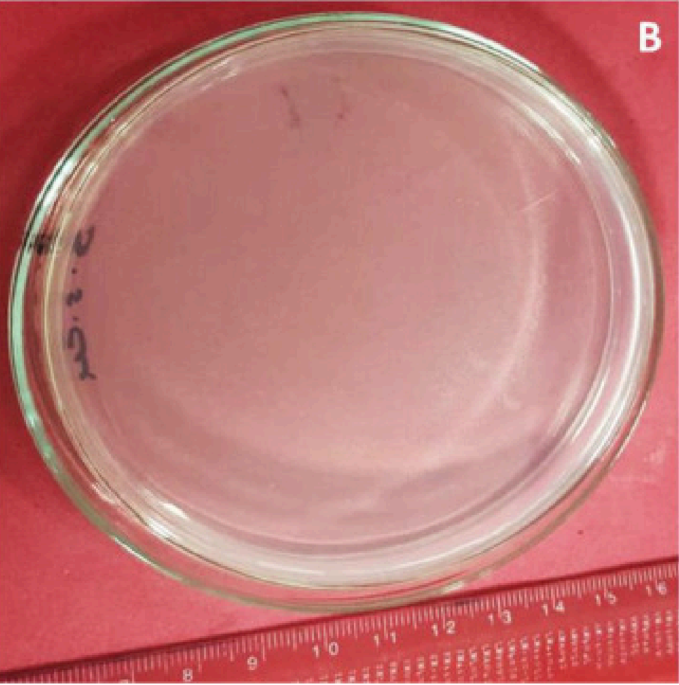


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Fig. 3. Effect of copper sulfate on contamination percentage; A) Control treatment showing bacterial contamination (arrow). B) 140 mg/L treatment showing contamination free.

3.3. Effect of copper sulfate on shoot multiplication

Copper sulfate at four concentrations affected the *in vitro* shoot multiplication of *P. selloum* (Table 2 and Fig. 4A). The treatment of 35 mg/L copper sulfate had the highest shoot number (25), followed by 19 shoots at 70 mg/L. The data showed that 140 mg/L had a negative effect on shoot number (6 shoots). The concentrations of 35 and 70 mg/L copper sulfate were the

best for shoot multiplication, with 25 and 19 shoots, respectively. There were no significant differences among treatments with regard to number of leaves. In contrast, shoot weight increased with increasing copper concentration. The heaviest shoots (0.152 and 0.132 g) were observed under 140 and 70 mg/L copper sulfate, respectively. Copper is an important microelement that is essential for many physiological and metabolic processes in plants (Festaand Thiele 2011; Marschner2012). It plays an essential role in several functions including transcription, protein trafficking machinery, oxidative phosphorylation, and iron mobilization (Yruela2005). Many studies have shown that some aroid species exhibit positive growth under medium copper levels (Olkhovychetal., 2016; Hill and Miyasaka 2000). However, the optimum required level of copper depends on the species (Clemens,2001). Previous studies found that MS medium supplemented with higher levels of copper (0.01 to 20 mg/L) significantly affects shoot multiplication (Javedetal., 2017: Fatimaetal., 2011; Prażakand Molas,2015; Ibrahimetal., 2016). Although treatment with 35 mg/L copper sulfate (13.92 mg/L Cu) is toxic for many *in vitro* plants, in this study it gave the best results for shoot multiplication in *P. selloum*. Shoot weight was dependent on the number of shoots in a *P. selloum* cluster. From there, shoot weight decreased with increasing shoot number. Treatment with 140 mg/L copper sulfate improved shoot weight but was toxic to the culture and yielded a significant reduction in shoot number; yellowing of leaves was also evident when compared with other treatments. A similar result was observed in taro plants grown at higher Cu^{2+} levels that had tip necrosis in older leaves, covering up to one-quarter of the most severely affected leaves (Hilland Miyasaka,2000). In contrast, *P. selloum* grown on media containing copper sulfate at 35 or 70 mg/L did not exhibit any symptoms with regard to vegetative growth. The range between useful and toxic concentration for plant species significantly differs, and accurate estimation is required when applying Cu to plants (Javedetal., 2017). Many species grow well at a Cu^{2+} level between 2 and 20 mg kg^{-1} of dry weight (Mengeletal., 2001). However, the critical toxicity of Cu in vegetative plant parts is greater than 20 to 30 μg g^{-1} dry weight in some plant species (Robsonand Reuter 1981). A previous report indicated that Arundo plants can tolerate up to 300 ppm of Cu without any adverse effect on biomass production (Pietrinietal., 2019). However, a significant reduction in shoot number under high Cu levels has been observed by many researchers (Fatimaetal., 2011; Javedetal., 2017). Metal toxicity has a negative effect on growth and biomass production (Ashfaqueetal., 2016; Atharand Ahmad 2002). Copper encourages ROS production (Lombardiand Sebastian 2005) and activates the antioxidant enzyme functions (Liuetal., 2018) to remove the excess ROS from the tissues, which causes oxidative stress (Dauphineeetal., 2017). Additionally, the uptake and transport of some essential metals such as iron (Fe) and Zinc (Zn) is reduced at high Cu levels (Kumaretal.2009), which may have a negative effect on shoot multiplication.

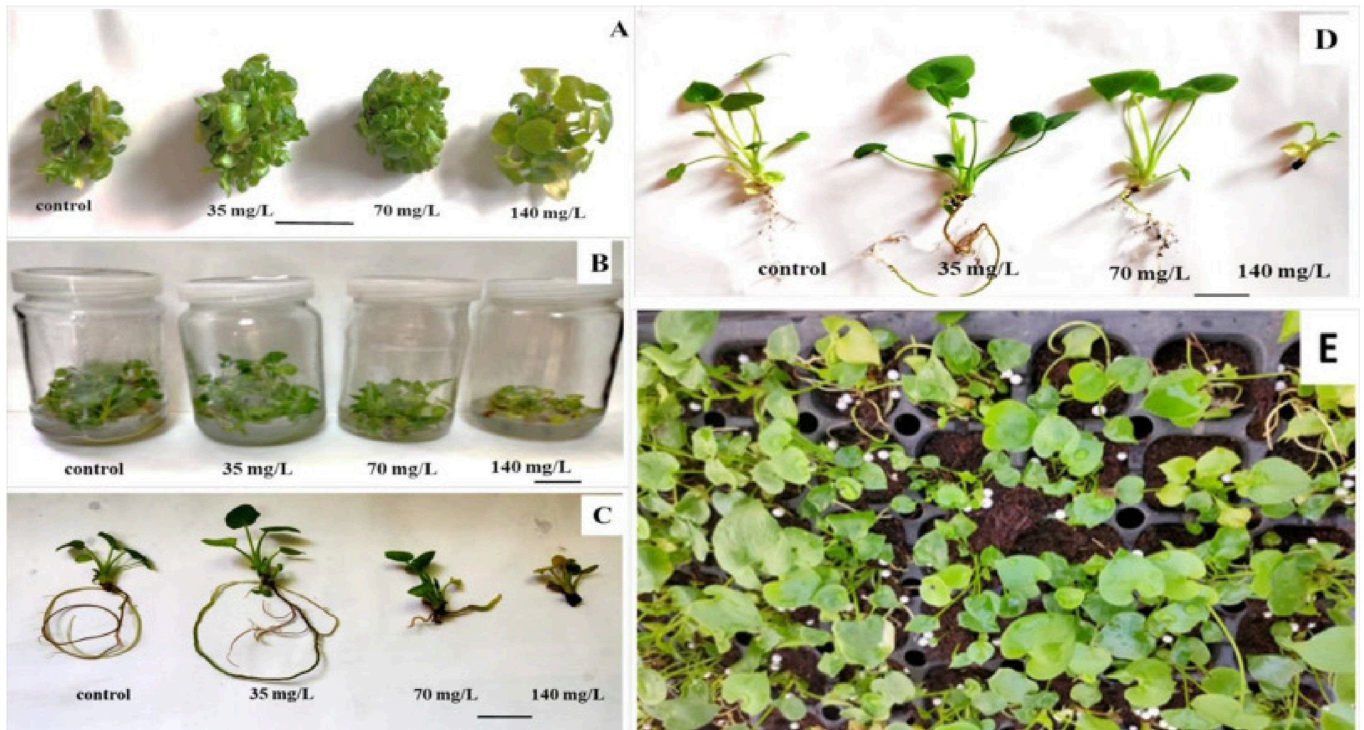

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Fig. 4. Effect of copper sulfate at 0, 35, 70, 140 mg/L on *in vitro* propagation of *Philodendroelloum*; A) shoot multiplication on MS (Murashige and Skoog) medium supplemented with 5 mg/L BA . B and C) *In vitro* rooting on free hormone MS medium. D) Acclimatized plants after three weeks. E) Acclimatized plants after four weeks in trays. Bar (2 cm)

3.4. Effect of copper sulfate on chemical composition of in vitro plantlets

Using copper sulfate at different concentrations negatively affected the mineral nutrient concentration of *in vitro P. selloum* (Table3). Excessive copper concentrations in the medium caused a reduction of P, Ca, K, Mg, Fe, and Mn concentrations in plant tissue. However, Zn concentrations were not significantly affected by increasing copper sulfate concentrations in the media. In addition, there was a linear increase in Cu accumulation with increasing copper sulfate in the medium. This result indicated that plantlets derived from the control medium had the highest concentrations of P, Ca, K, Mg, Fe, and Mn, at 1935.6 mg/Kg, 0.53%, 2.62%, 0.35%, 193.2 mg/Kg and 95.2%, respectively. Similar observations were reported by Linand Wu(1994) (on *Lotus purshianus L*) and Pietrinietal.(2019) (on *Arundo donax L*). On the other hand, the lowest Cu concentration (5 mg/Kg) was observed in the control medium

(normal level). The previous reports mentioned that excessive copper concentrations reduced mineral nutrient uptake and accumulation in plant tissue (Linand Wu,1994).

Table 3. Effect of copper sulfate on mineral content of *Philodendron selloum.*

Treatment (mg/L)	P (mg/kg)	Ca^{++} (%)	K+ (%)	Mg^{++} (%)	Fe^{++} (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Mn (mg/kg)
Control (0.025)	1935.6 a	0.53 a	2.62 a	0.35 a	193.2 a	5.00 c	41.2	95.2 a
35	1698.9 b	0.37 b	2.14 b	0.25 b	128.5 b	198.13 d	41.0	61.5 b
70	1462.0 c	0.28 c	2.19 b	0.16 c	124.2 b	294.38 b	40.2	62.0 b
140	904.2 d	0.20 d	1.37 c	0.10 d	104.0 c	717.5 a	40.5	48.0 c

3.5. Effect of copper sulfate on antioxidant enzyme activity and electrolyte leakage

The treatments showed significant increases in the activity of catalase (CAT), peroxidases (POX), and polyphenol oxidase (PPO). CAT activity was increased significantly as a result of copper sulfate treatment. However, 35 mg/L copper sulfate increased CAT activity significantly compared with the control. A large increase in CAT activity was observed under 70 and 140 mg/L of copper sulfate. CAT activity increased with copper sulfate concentration (Fig.5A). Similarly, POX activity was also increased significantly under copper sulfate treatment at several concentrations (35, 40, and 140 mg/L) as compared with the control treatments (Fig.5B). However, PPO activity was increased under 35 mg/L copper sulfate, increased slightly under 70 mg/L, and increased with high significance under 140 mg/L as compared to the control (Fig.5C). The obtained results are in agreement with previous findings that plants infected with phytopathogens and treated with non-traditional treatments show increased activity of antioxidant enzymes such as catalase, peroxidase, and polyphenol oxidase. Stimulation of antioxidant enzyme activities usually occurs as a consequence of elevated levels of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide, which are harmful to plant cells and cause oxidative burst. As a result, antioxidants are increased and neutralize the harmful effect of ROS (Linglanetal., 2008; Prasad2012; El-Bannaetal., 2018; Hafezetal., 2018; Abdelaaletal., 2020), thereby increasing plant disease resistance against pathogen attacks (Hafezetal., 2012; Omaraetal., 2019).

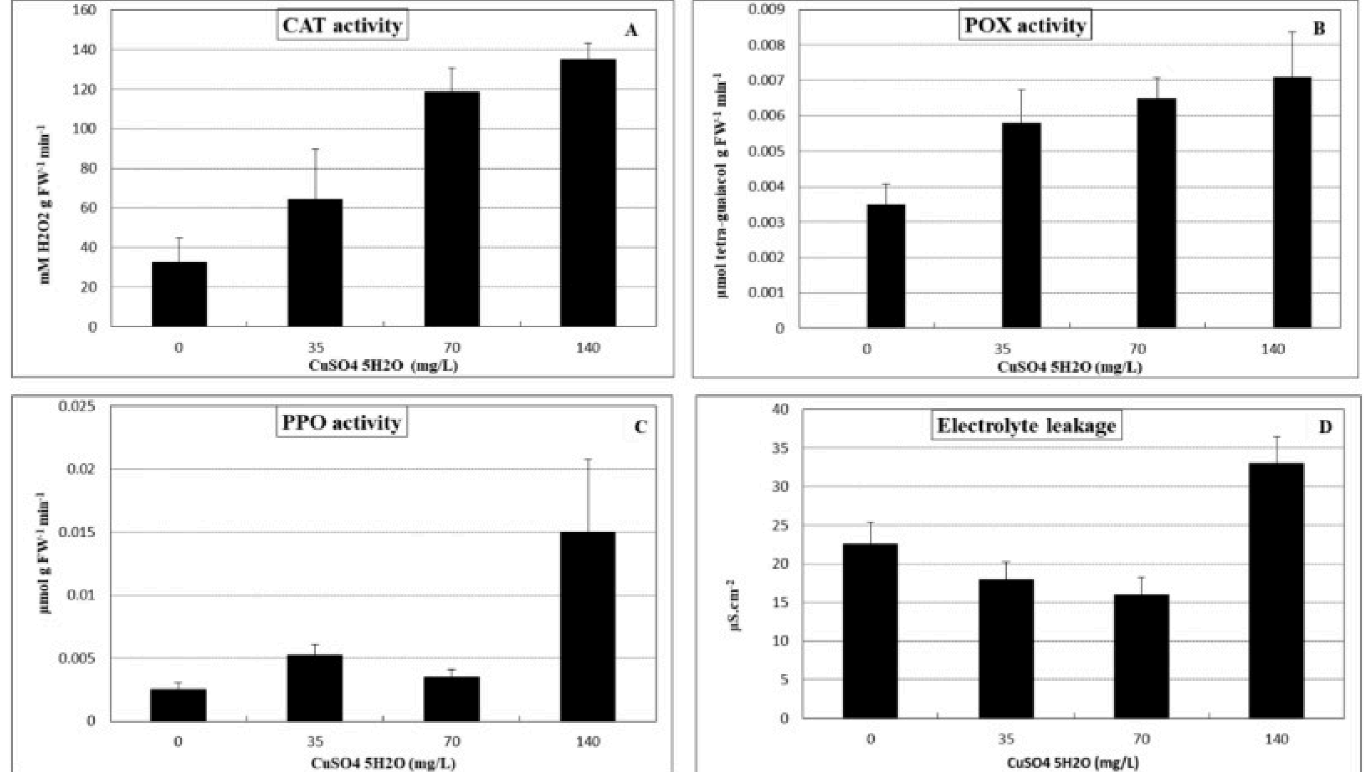

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Fig. 5. Effect of copper sulfate at 0, 35, 70, 140 mg/L on antioxidant enzymes activity and electrolyte leakage of *Philodendron selloum* shoots cultured on rooting medium (free hormone MS medium) after 10 weeks of culture.

The electrolyte leakage (EL) results showed that 35 and 70 mg/L copper sulfate conferred the highest significant reduction in EL as compared to the control. However, 140mg/L of copper sulfate yielded the highest increase in EL in the treatments after the control (Fig.5D). EL has been used to measure injuries in cell membranes in response to various stresses (Viczianetal., 2014). In our study, the increase in electrolyte leakage might have been due to the fact that the high concentration of copper sulfate (140 mg/L) dramatically increased the antioxidant enzymes, which were correlated with the high level of ROS. ROS can cause nucleic acid damage, protein and lipid denaturation, and cell death; therefore, the EL percentage was increased similarly to that of the untreated control. Application of the aforementioned treatments at 35 and 70 mg/L copper sulfate led to decreased electrolyte leakage and increased resistance to bacterial contamination (Rajetal., 2012).

3.6. Effect of copper sulfate on in vitro rooting

A marked effect of copper was recorded on the growth and plantlet rooting of *P. selloum* (Table 4 and Fig. 4B and C). The low copper level (35 mg/L) was found to be better for plantlet length, number of leaves and roots, root length, and plantlet weight, at 5.7 cm, 6, 5, 14 cm, and 0.557 g, respectively. In addition, plantlets grown on media supplemented with 0.025 and 35 mg/L had the best results for root length. Meanwhile, high levels of copper (140 mg/L) had a negative effect on plantlet growth. No roots appeared on plantlets derived from the 140 mg/L treatment. Although copper is an important element for normal plant growth and development at moderate levels, it is potentially toxic at high levels. Copper plays an essential role in various physiological processes and is used as a cofactor for several metalloproteins. However, excess copper inhibits plant growth and damages important cellular processes (Yruela, 2005). It is important for regulatory proteins and is essential in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism, and hormone signaling (Marschner, 2012; Raven et al., 1999). On the other hand, high copper levels are extremely toxic, causing symptoms such as stunting, leaf discoloration, and inhibition of root growth (Van Assche and Clijsters, 1990; Marschner, 2012). In our study, 140 mg/L copper sulfate yielded stunted plantlets with the fewest leaves and no roots. In addition, higher concentrations of copper sulfate reduced the growth rate of shoots as compared with other treatments. A reduction in growth and biomass production due to copper toxicity was observed in previous studies (Manios et al., 2003; Javed et al., 2017). In addition, excess Cu lowers the efficiency of essential processes such as photosynthesis, which have a negative effect on growth (Oustriere et al., 2017). Similar observations on growth and root morphology have been reported in *Colocasia esculenta* (Hill and Miyasaka, 2000), *Dendrobium kingianum* (Prażak and Molas, 2015), and *Allium cepa* (Koplikuand Mesi, 2015).

Table 4. Effect of copper sulfate on in vitro rooting of *Philodendron selloum* after 4 weeks.

Treatment (mg/L)	Shoot length (cm)	Number of leaves/plantlet	Number of roots/plantlet	Root length (cm)	Plantlet fresh weight (g)
Control (0.025)	4 b	4 b	2 b	12 b	0.307 b
35	5.7 a	6 a	5 a	14 a	0.557 a
70	4 b	4 b	3 b	3.5 c	0.348 b
140	1.3 c	4 b	0 b	0 d	0.159 c

Treatment (mg/L)	Shoot length (cm)	Number of leaves/plantlet	Number of roots/plantlet	Root length (cm)	Plantlet fresh weight (g)
Significant	**	**	**	**	**

3.7. Effect of copper sulfate on acclimatization

Significant side effects of copper sulfate have been observed on acclimatization and plant growth (Table5 and Fig.4 D and E). The addition of copper sulfate improved the acclimatization percentage except in the 140 mg/L treatment. The highest survival percentage (100%) was observed at 35 and 70 mg/L. High copper sulfate levels have a negative effect on *in vitro* rooting, which is reflected negatively in acclimatization percentage. In a study on *Spathiphyllum cannifolium*, Ibrahimetal.(2016) found that a high level of copper (20 mg/L) reduced the number and weight of roots. Our study found that plants generated on medium supplemented with 35 mg/L copper sulfate had good results with regard to plant length, number of leaves and roots, root length, and plant fresh weight, at 9.5 cm, 9, 8, 15.2cm, and 1.007g, respectively. Additionally, control and 70 mg/L had the same significant effect on plant length, number of roots and root length. Number of leaves and roots did not differ significantly between the control and 35 mg/L treatment. In contrast, high copper concentrations had an unfavorable effect on the growth of acclimatized plants as a response to excessive root zone Cu^{2+}. The same results were reported on *Spathiphyllum cannifolium* (Ibrahimetal., 2016) and *Colocasia esculenta* (Hilland Miyasaka,2000), where toxic root-zone Cu^{2+} levels inhibited root elongation.

Table 5. Effect of copper sulfate on growth and survival of *Philodendron selloum* after 4 weeks acclimatization.

Treatment (mg/L)	Survival (%)	Plantlet length (cm)	Number of leaves/plantlet	Number of roots/plantlet	Root length (cm)/plantlet	Plantlet fresh weight (g)
Control (0.025)	90 b	8.5 b	9 a	8 a	7.5 b	0.617 c
35	100 a	9.5 a	9 a	8 a	15.2 a	1.007 a
70	100 a	8.5 b	6 b	8 a	7.5 b	0.709 b
140	10 c	3.5 c	4 c	0 b	0 c	0.160 d

Treatment (mg/L)	Survival (%)	Plantlet length (cm)	Number of leaves/plantlet	Number of roots/plantlet	Root length (cm)/plantlet	Plantlet fresh weight (g)
Significant	**	**	**	**	**	**

3.8. Effect of $CuSO_4$ on genetic fidelity and genomic template stability

Five -mer primers were utilized to screen the genome for alteration in response to the $CuSO_4$ treatments. The primers yielded specific and stable banding patterns (Table6 and Fig.6). RAPD patterns generated by the copper sulfate-exposed plantlets were not clearly different from those obtained using control DNA for all copper sulfate concentrations. The number of total bands varied from 2 (OPE-12) to 11 (OPA-07). The tested primers produced only monomorphice bands, except primer Opd- 07, which produced four polymorphic bands. The differences in RAPD patterns refer to loss of normal bands and appearance of new bands as compared with the control. RAPD profiles of the randomly selected *in vitro* plants in comparison to the mother plant were almost identical, thus assuring a totally genetic fidelity-maintained protocol for this commercially important plant. Additionally, the GTS % for all treated plants was calculated (Fig.7). The GTS for untreated (control) seedlings was fixed as 100%. There was a non-significant ($P < 0.05$) difference in the GTS % of all treated plants; the average of genome stability was 95.5, 97.5, and 97.5 for $CuSO_4$ concentrations of 35, 70, and 140 mg/L, respectively. It can be concluded from the results that the $CuSO_4$ treatments did not significantly change the genome stability of *in vitro* philodendron plants. The RAPD method is sensitive and capable of detecting variations in plant genome profiles (Salamaetal.2019). The RAPD technique has been effectively utilized to detect genotoxic effects in several plants induced by various metals (Mattielloetal.2015; Ghoshetal.2019). RAPD primers were used to study the genotoxic effects of $CuSO_4$ for both control and treated *in vitro* plantlets. The appearance of new patterns can be explained by changes in the genomic DNA template stability due to mutations, large deletions, homologous recombination, or changes in priming sites leading to new annealing events (AlQuraidietal.2019). The absence of normal DNA bands can be characterized as DNA disintegration or rearrangement (Venkatachalametal.2017).

Table 6. RAPD band patterns generated from genomic DNA of control plant and copper sulfate –treated *Philodendron selloum in vitro* plantlets.

$CuSo_4$ (mg/L)	Replicates	Band profile	Primers					Total Bands	a+b
			OPD12	OPE12	OPE11	OPD07	OPK10		
	control	-	7	2	9	9	10	37	-
35	1	a	0	0	0	2	0	2	3
		b	0	0	0	1	0	1	
	2	a	0	0	0	0	0	0	2
		b	0	0	0	2	0	2	
	3	a	0	0	0	0	0	0	0
		b	0	0	0	0	0	0	
70	1	a	0	0	0	0	0	0	0
		b	0	0	0	0	0	0	
	2	a	0	0	0	0	0	0	2
		b	0	0	0	2	0	2	
	3	a	0	0	0	0	0	0	1
		b	0	0	0	1	0	1	
140	1	a	0	0	0	0	0	0	2
		b	0	0	0	1	0	1	
	2	a	0	0	0	0	0	0	1
		b	0	0	0	1	0	1	
	3	a	0	0	0	0	0	0	0
		b	0	0	0	0	0	0	
Total No. of bands per primer	7	2	9	11	10				

a= appearance of new bands, b= disappearance of normal bands, a+b =denotes polymorphic bands.

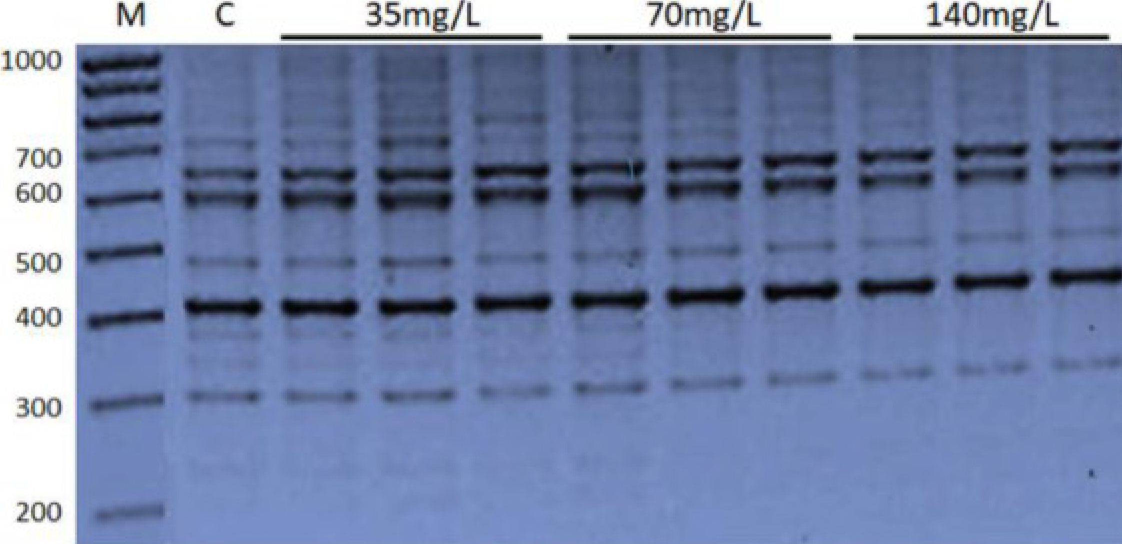

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Fig. 6. Representative RAPD profiles of Philodendron plants treated with 35,70 and 140 mg/L copper sulfate using primer OPA12. M: Molecular weight marker (1000bpSizer DNA ladder), C: control plant.

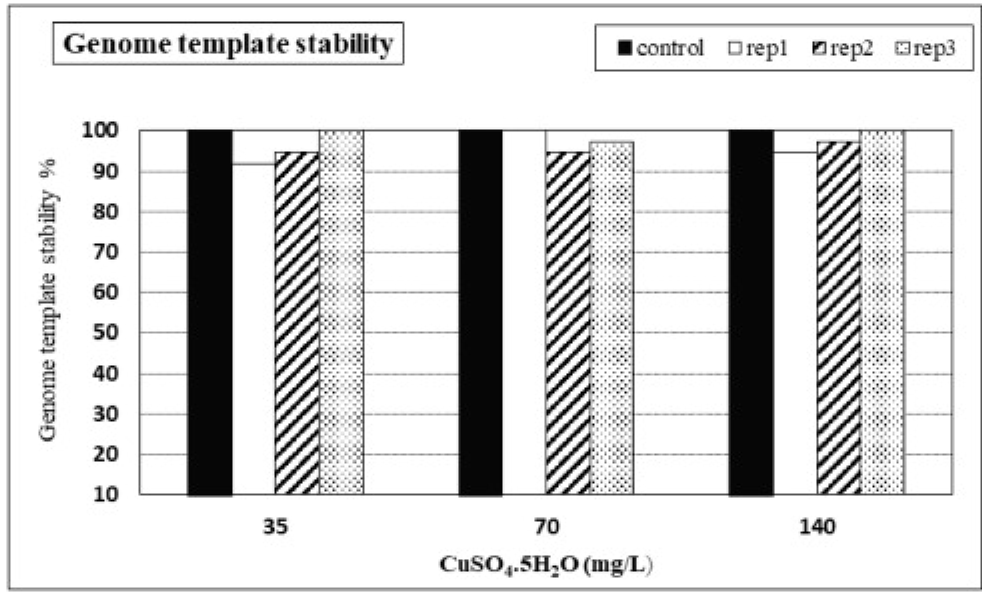

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Fig. 7. Genome template stability (%) in *in vitro* plants treated with different concentrations of $CuSO_4$. Three replicates for each treatment were used.

Conclusion

Copper sulfate was useful for elimination of endophytic bacteria of *Philodendron selloum in vitro* cultures. . No decline in plant growth or abnormalities were observed at low concentration of copper sulfate (35 mg/L) while high concentration at 140 mg/L was toxic. Therefore, it is recommended for improving the micropropagtion of *P. selloum*. High level of copper sulfate increased antioxidant enzymes activity. Copper sulfate did not induce genetic variations as revealed by RAPD analysis of regenerated plantlets.

Declaration of Competing Interest

There is no conflict of interest between authors of this manuscript

Acknowledgments

The authors wish to thank all members of PBHC, PPBL (ISO/17025) and the EPCRS Excellence Center (ISO/9001, ISO/14001 and OHSAS/18001), Fac. of Agric., Kafrelsheikh University, Egypt for their technical support.

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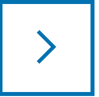

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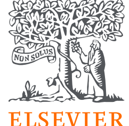

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