International Journal of
Plant Biology

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Influence of High Concentrations of Copper Sulfate on In Vitro Adventitious Organogenesis of *Cucumis sativus* L.

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Abstract: Copper (Cu) is an essential plant micronutrient. This report is the first to assess the effects of high copper sulfate ($CuSO_4$) levels on in vitro callus and shoot formation of cucumber. Four-day-old cotyledon explants from the inbred line Wisconsin 2843 and the commercial cultivars Marketer and Negrito were used. Murashige and Skoog (MS)-derived callus and shoot induction medium containing 0.5 mg L^{-1} indole-3-acetic acid (IAA) and 2.5 mg L^{-1} 6-benzylaminopurine (BAP) was supplemented with $CuSO_4$ (0.2–5 mg L^{-1}). The response on callus-derived shoots showed that the optimal concentration of $CuSO_4$ was 8- to 200-fold greater than in standard MS medium. Shoot frequency (SF) and shoot number (SN) were assessed, and Marketer > Negrito > Wisconsin 2843, where 1, 0.2 and 5 mg L^{-1} $CuSO_4$ produced the highest results, respectively. SF and SN increased 6- and 10-fold in Wisconsin 2843 and twice in the other cultivars. All explants formed calluses, and in two of the three cultivars, callus extension was significantly affected by $CuSO_4$ application. SN showed a strong relationship with $CuSO_4$ levels and no association with callus extension. The results show that specific $CuSO_4$ concentrations higher than in standard MS medium increase adventitious cucumber shoot organogenesis.

Keywords: copper sulfate; morphogenesis; cotyledon explant; callus; shoot regeneration; cucumber; Cucurbitaceae

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Citation: Miguel, J.F. Influence of High Concentrations of Copper Sulfate on In Vitro Adventitious Organogenesis of *Cucumis sativus* L. *Int. J. Plant Biol.* **2023**, *14*, 974–985. https://doi.org/10.3390/ijpb14040071

Academic Editor: Adriano Sofo

Received: 15 September 2023
Revised: 18 October 2023
Accepted: 20 October 2023
Published: 24 October 2023

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1. Introduction

Copper (Cu) is an essential redox-active transition element active in many physiological processes in plants [1]. This heavy metal micronutrient plays a critical role as a structural component of numerous proteins and a cofactor for many enzymes. Those include phytohormone receptors, ascorbate oxidase, copper-containing amine oxidases, Cu/Zn superoxide dismutases (SODs), cytochrome c oxidase, laccase, plastocyanin, and polyphenol oxidases. Cu is involved in a wide range of processes, such as photosynthetic electron transport, mitochondrial respiration, metabolism of carbohydrates, nitrogen, and cell walls, oxidative stress response, disease resistance, pollen viability, water permeability of xylem vessels, and hormone signaling (for reviews, see [2–7]). Either deficient or in excess, it can cause disorders in plant growth and development [2]. Its deficiency leads to defects in photosynthesis, reduced respiration, chlorosis, and wilting of leaves [8], among others. Due to its relatively low mobility in plants, young organs are usually the first to develop Cu-deficiency symptoms [4]. Its role as a redox agent makes Cu an essential element but also potentially toxic, as Cu ions can catalyze the production of damaging free radicals [2,9]. The redox cycle between Cu^{2+} and Cu^{+} can catalyze the formation of extremely toxic hydroxyl radicals (·OH) from the reaction between superoxide $\cdot O^{2-}$ and H_2O_2, and damage lipids, polypeptides, proteins, nucleic acids, and other biomolecules [2,10]. Some common symptoms of its toxicity include low photosynthesis efficiency, disturbed protein complexes, damage to membrane permeability, chlorosis and necrosis, stunting, and root malformation [2,11]. While copper at high levels acts as a pro-oxidant and increases the generation of reactive oxygen species (ROS), at lower levels, it protects plants from the

harmful effects of ROS [12]. These effects are largely determined by plant species/ecotypes, copper concentration, speciation, bioavailability, and exposure time [2,12].

Inorganic macro- and micronutrient levels widely used in plant tissue culture media are based on those of MS [13] mineral solution developed for tobacco. For instance, it contains copper as $CuSO_4$ at 0.025 mg L^{-1}. However, for many of its micronutrients, a clear optimal level was not evident. Furthermore, tissue culture of non-tobacco species may require a different concentration of its micronutrients for optimal response [14].

Heavy metals such as copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn) are essential plant micronutrients that are required in small but critical amounts for its normal growth and development [15,16]. Copper, at much higher levels than in standard MS medium, has been reported to benefit in vitro culture and morphogenesis.

Cucumber (*Cucumis sativus* L.) belongs to the Cucurbitaceae family. This species is an economically important vegetable crop and a model plant for sex determination and vascular biology studies [17]. According to the Food and Agriculture Organization of the United Nations (FAO), in 2021, the world production of cucumbers, including gherkins, ranked third among vegetable crops with 93,528,796 tons [18]. Crop improvement of cucumber for resistance/tolerance against major biotic and abiotic stresses is difficult by conventional breeding due to its narrow genetic base, a genetic variability of only 3–8% [19], and several crossing barriers with related species [20]. Genetic engineering and plant transformation techniques provide opportunities to overcome these limitations. Despite the number of publications on genetic transformation of cucumber (for reviews, see [21–25]), with Agrobacterium-mediated gene transfer as the most popular method, its efficiency remains low [23–25]. One of the main limitations in obtaining transgenic cucumber plants is related to regeneration, i.e., the low morphogenic response in explants of certain genotypes, and the reduced regeneration rate resulting from the usual transformation steps [24]. A reliable plant genetic transformation system demands an efficient and stable regeneration process [21–23].

In vitro regeneration of cucumber can be achieved by using different cell and tissue culture techniques. Regeneration via organogenesis and somatic embryogenesis has been described in this species, and commonly used explants include cotyledons [26–29], cotyledonary nodes [26,30–32], hypocotyls [27,33–35], shoot tips [36–39], and leaves [27,37,40,41]. Regeneration of cucumber has also been reported from protoplasts [42–44] and suspension cultures [45–47]. Cucumber regeneration is largely dependent on genotype, explant type, and culture medium [24,26,34]. Although in vitro plant regeneration of cucumber has been extensively reported, a high regeneration system for this species is below the expected [23].

Genome editing and genetic transformation are valuable tools for functional genomics and genetic improvement of cucumber and other crop plants. However, the efficiencies of transformation and regeneration are critical for their successful application.

A positive effect of high copper levels has been reported in in vitro culture and micropropagation of different plant species, such as melon [48], watermelon [49], carrot [50], Indian ginseng [51], tobacco [52], tetraploid and hexaploid wheat [52,53], triticale [52], barley [14], indica rice [54], sorghum [55], bamboo [56], and date palm [57]. Nonetheless, negative effects of excessive exposure to this micronutrient have also been reported (e.g., Garcia-Sogo et al. [48] in melon, Purnhauser and Gyulai [52] in tobacco, and Ghaemi et al. [53] in tetraploid wheat). Copper also has a broad spectrum of antimicrobial efficacy. In a recent study by Silvestri et al. [58], high levels of $CuSO_4$ reduced bacterial contamination and maintained high in vitro multiplication. In in vitro plant regeneration, more attention should be paid to the micronutrient composition of culture media, particularly in species/genotypes with regeneration limitations, such as in cucumber. A literature review revealed no previous studies on the effect of high concentrations of copper ion on in vitro adventitious regeneration of cucumber.

Given copper's involvement in diverse plant physiological processes, including hormone signaling and oxidative stress protection, which could significantly affect in vitro

plant regeneration, along with its positive outcomes in other species, the present work aimed to evaluate the influence of different concentrations of $CuSO_4$ on in vitro adventitious organogenesis, using cotyledon as explants from one inbred line and two commercial cultivars of cucumber.

2. Materials and Methods

2.1. Plant Material and In Vitro Regeneration

Cucumber seeds from the inbred line Wisconsin 2843 (kindly provided by Dr. C.E. Peterson, Michigan State University, East Lansing, MI, USA) and cultivars Marketer and Negrito (Semillas Fitó S.A., Barcelona, Spain) were used as starting material. Wisconsin 2843 is a breeding population developed to provide multiple disease resistance, variable fruit characteristics, and other qualities, such as gynoecious sex expression, non-bitter fruit, and genes for parthenocarpy [59]. Marketer and Negrito are medium-cycle commercial slicing cultivars, monoecious flowering habit, and dark-green cylindrical fruits. Marketer is of good quality for the fresh market. Negrito is very productive, but susceptible to various pests and diseases, which can be a major problem under greenhouse conditions [24,60].

Mature seeds were decoated and surface-sterilized by immersion in a diluted commercial bleach (5% *w/v* sodium hypochlorite) with 0.1% (*v/v*) 7X-O-matic (MP Biomedicals, Irvine, CA, USA) for 30 min. The seeds were then rinsed three times with sterile distilled water for 5, 10 and 15 min, respectively. After sterilization, the seeds were germinated in the dark (150 mm × 25 mm test tubes) on solid germination medium (GM, [61]) consisting of full-strength MS [13] basal medium supplemented with 1% (*w/v*) sucrose, and 0.8% (*w/v*) agar (Agar industrial; Pronadisa, Hispanlab, S.A., Alcobendas, Spain), and its pH was adjusted to 5.7 before autoclaving at 115 °C for 30 min. After 1 to 2 days, when the radicle emerged and curved into the medium, the test tubes were transferred to a plant growth chamber (Figure 1e) maintained at 26 ± 2 °C with a 16 h photoperiod under cool-white fluorescent lamps (Gro-Lux, Sylvania Inc., Wilmington, MA, USA) at a photon fluence rate of 90 µmol m^{-2} s^{-1}. The same incubation conditions were used in the subsequent in vitro culture steps. Cotyledons from four-day-old seedlings were used as explant sources by excising transversely 1–2 mm behind their proximal and distal ends. The experimental assessments are based on naked-eye observations.

Cotyledonary explants were cultured for 3 weeks in 300 mL glass jars with the abaxial surface in contact with the callus and shoot induction medium, consisting of MB3 basal medium [62] containing MS basal salts, 3% (*w/v*) sucrose, 0.1 g L^{-1} myo-inositol, 1 mg L^{-1} thiamine-HCl, and Staba vitamins [63], and by adding 0.5 mg L^{-1} IAA, 2.5 mg L^{-1} BAP, and $CuSO_4$ as cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) at 0.0, 0.2, 1.0 and 5.0 mg L^{-1}, in addition to its standard concentration in MS (0.025 mg L^{-1}). The plant growth regulators (PGRs) used were previously selected by Miguel [24] for the genotypes used in this investigation. The medium was solidified with 0.8% (*w/v*) agar (Industrial, Pronadisa) and its pH was adjusted to 5.7 before autoclaving. At the end of this phase, callus regeneration frequency (%) (CRF) and callus extension index (CEI) were evaluated. The CRF (mean ± SE) is the frequency of explants with callus on the cut surface (proximal and distal edges); the CEI (mean ± SE) corresponds to arbitrary values (from 0 to 4) on the extent of callus on the explant cut surface, where 0 = absence of callus, 1 = traces of callus, 2 = callus on less than half, 3 = callus on half or more, and 4 = callus covering the full extension.

Adventitious buds and shoot primordia were then transferred to 300 mL glass jars with shoot development and elongation medium where auxins were not included, and BAP was substituted by 0.2 mg L^{-1} kinetin, as suggested by unpublished data from V.F. Moreno. After 2 weeks of culture, the response on shoot regeneration frequency (SRF) and shoot number index (SNI) was determined. The SRF (mean ± SE) indicates the percentage of cultivated explants with at least one formed shoot. The SNI (mean ± SE) is assigned arbitrary values (ranging from 0 to 3) representing the number of shoots per explant, where 0 = absence, 1 = one shoot, 2 = two shoots, and 3 = three or more shoots. Index choice reflects the challenge in quantifying multiple shoot formation in cucumber's primary callus.

Only shoots with normal appearance were considered. Individualized shoots were further rooted on hormone-free MB3 basal medium [62] for 3 to 4 weeks. Plantlets with well-developed root systems were transferred to plastic pots containing peat and vermiculite (3:1, *v/v*) as subtract, initially covered with clear plastic bags, and acclimatized in the plant growth chamber (Figure 1e) under the same conditions as described above. They were then transferred and established in the greenhouse (Figure 1f).

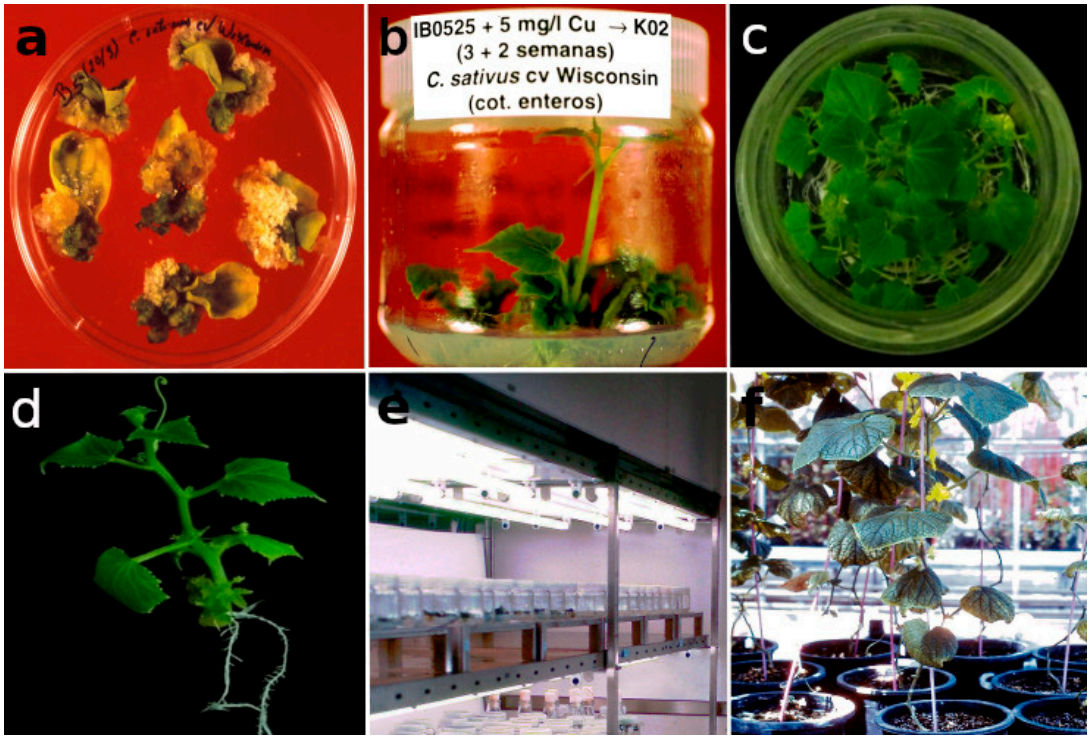

Figure 1. In vitro regeneration from cotyledon explants in cucumber (*C. sativus* L.) inbred line Wisconsin 2843. (**a**) Explants after 3 weeks on callus and shoot induction medium (CSIM) supplemented with 0.5 mg L^{-1} IAA, 2.5 mg L^{-1} BAP, and without extra $CuSO_4$ (control). Adventitious bud and shoot formation from callus induced on several explants; (**b**) regeneration of plantlets after 2 weeks on shoot development and elongation medium containing 0.2 mg L^{-1} kinetin from a previous culture in CSIM supplemented with 5 mg L^{-1} $CuSO_4$; (**c**) rooting of shoots on MB3 basal medium; (**d**) plantlet after 3–4 weeks' culture on rooting medium; (**e**) all cultures and acclimatization of rooted plantlets were conducted in a growth chamber; (**f**) micropropagated plants at the flowering stage established in the greenhouse.

2.2. Data Analysis

The experimental design was a two-way 3 × 4 factorial arrangement on a completely randomized design, with at least 11 replicate jars per treatment with 6 explants per jar. Treatment means comparison was assessed by non-linear regression analysis. Count data variables, i.e., callus extension index (CEI) and shoot number index (SNI), were submitted to generalized Poisson regression and negative binomial regression analyses, respectively. Logistic regression was performed for data collected as binary values. Relationships between SNI (response variable) and the predictors (CEI, $CuSO_4$ concentration) were analyzed using generalized Poisson regression. All statistics were analyzed using R statistical software version 3.4.4 [64]. The R packages stats [64], MASS [65], and VGAM [66] were used to perform logistic regression, negative binomial regression, and generalized Poisson regression, respectively. Dispersion tests were performed using the AER package [67] to test the suitability of applying Poisson generalized linear models to the count data. Binary data were tested for overdispersion by fitting the model twice, i.e., firstly using a binomial family, and then a quasibinomial family, and by using the pchisq function [68] from the

stats package, indicating a lack of overdispersion. Multicollinearity between predictors was assessed by calculating the generalized variation inflation factor (GVIF; [69]) for each predictor using the vif function in the car package [70], indicating that multicollinearity was not a problem in the present analyses (GVIFs < 1.24). The rockchalk package [71] was used to combine levels not significantly different from each other in the analysis of the association between variables. Model performance was evaluated using the Akaike information criterion (AIC; [72]) and Bayesian information criterion (BIC; [73]), and the best-fitting model was selected based on the lowest AIC and BIC values. The Wald test was used in regression to test for significance. A probability value of $p < 0.05$ was the level of significance for all analyses.

3. Results

3.1. Frequency and Extension of Callus

The callus formation (see Figure 1a, inbred line Wisconsin 2843) occurred in the first 2 weeks of culture, starting from the cut edges of the primary explants. All treatments recorded 100% callus regeneration frequency (Table 1). The callus extension index (CEI) showed significant differences in two of the three cultivars of cucumber for $CuSO_4$ concentration. The response in CEI, ranked from highest to lowest, varied as follows: for Marketer, from 2.60 ± 0.06 (0.2 mg L^{-1} $CuSO_4$) to 2.91 ± 0.05 (0 mg L^{-1} $CuSO_4$); for Negrito, with no significant differences, from 2.52 ± 0.07 (1.0 mg L^{-1} $CuSO_4$) to 2.70 ± 0.06 (0.2 mg L^{-1} $CuSO_4$); and for Wisconsin 2843, from 2.03 ± 0.02 (5.0 mg L^{-1} $CuSO_4$) to 2.52 ± 0.06 (1.0 mg L^{-1} $CuSO_4$). These results are between the arbitrary values of 2 (callus on less than half the cutting zone) and 3 (callus on half or more of the cutting zone). In virtually all cases, the order of magnitude of the CEI response of cultivars to the $CuSO_4$ concentration was different. In the control group, calluses of the Wisconsin 2843 line contrast with the lighter and more friable calluses of the Marketer and Negrito cultivars. With the addition of high concentrations of $CuSO_4$, the calluses generally became more compact and greenish.

Table 1. Effect of adding different concentrations of $CuSO_4$ to the induction medium on in vitro callus and shoot regeneration from cotyledon explants of three cultivars of *C. sativus* L. Values are expressed as mean ± standard error of the mean.

Cultivar	$CuSO_4$ $5H_2O$	Callus Frequency (%) *	Callus Extension Index ‡	Shoot Frequency (%) §	Shoot Number Index †
	mg L^{-1}				
Wisconsin 2843	0.0	100.00	2.38 ± 0.06 ^{ab}	2.78 ± 1.95 ^{b}	0.03 ± 0.02 ^{b}
	0.2	100.00	2.19 ± 0.05 ^{b}	5.56 ± 2.72 ^{b}	0.07 ± 0.04 ^{b}
	1.0	100.00	2.52 ± 0.06 ^{a}	4.55 ± 2.58 ^{b}	0.06 ± 0.04 ^{b}
	5.0	100.00	2.03 ± 0.02 ^{c}	16.67 ± 4.42 ^{a}	0.31 ± 0.09 ^{a}
Marketer	0.0	100.00	2.91 ± 0.05 ^{a}	30.30 ± 5.70 ^{b}	0.35 ± 0.07 ^{b}
	0.2	100.00	2.60 ± 0.06 ^{b}	37.50 ± 5.75 ^{ab}	0.54 ± 0.09 ^{ab}
	1.0	100.00	2.67 ± 0.06 ^{ab}	50.00 ± 5.93 ^{a}	0.76 ± 0.11 ^{a}
	5.0	100.00	2.85 ± 0.05 ^{ab}	26.76 ± 5.29 ^{b}	0.37 ± 0.09 ^{b}
Negrito	0.0	100.00	2.54 ± 0.06 ^{a}	25.00 ± 5.14 ^{b}	0.31 ± 0.07 ^{b}
	0.2	100.00	2.70 ± 0.06 ^{a}	42.42 ± 6.13 ^{a}	0.61 ± 0.10 ^{a}
	1.0	100.00	2.52 ± 0.07 ^{a}	16.67 ± 4.62 ^{b}	0.20 ± 0.06 ^{b}
	5.0	100.00	2.59 ± 0.06 ^{a}	21.88 ± 5.21 ^{b}	0.31 ± 0.08 ^{b}

*,‡ Data were scored after 3 weeks on the callus and shoot induction medium. §,† Data were scored after 2 weeks on the shoot development and elongation medium. ‡ Generalized Poisson regression, §logistic regression, and † negative binomial regression were used for data analysis. Means in the same column followed by different letters are significantly different (Wald test, $p < 0.05$).

3.2. Frequency and Number of Shoots

Groups of adventitious buds emerged between 1.5–3 weeks of culture, particularly at the proximal end of the explant, which at 2–4 weeks could give rise to shoot primordia. The in vitro regeneration process is shown in Figure 1a–d. Both shoot regeneration frequency (%) (SRF) and shoot number index (SNI) revealed significant differences in all the three cultivars of cucumber for $CuSO_4$ concentration (Table 1). For Wisconsin 2843, both SRF and SNI tended to increase with increasing concentration of $CuSO_4$; the highest SRF (16.67 ± 4.42) and SNI (0.31 ± 0.09) were obtained for the medium supplemented with 5.0 mg L^{-1} $CuSO_4$, resulting in 6- and 10.3-fold higher values, respectively, than the control. In the case of Marketer, both variables increased with increasing concentration of $CuSO_4$ up to 1.0 mg L^{-1}, but thereafter decreased significantly as the concentration increased; the maximum SRF (50.00 ± 5.93) and SNI (0.76 ± 0.11) were 1.7- and 2.2-fold greater, respectively, when compared to the control. In relation to Negrito, both variables increased with 0.2 mg L^{-1} of $CuSO_4$, but thereafter decreased significantly as the concentration increased; the highest SRF (42.42 ± 6.13) and SNI (0.61 ± 0.10) were 1.7- and 2-fold higher, respectively, than the control. All cultivars revealed significant differences between the medium with the highest response and the control. From the above results, the performance of the cultivars, ranked from highest to lowest, was as follows: Marketer > Negrito > Wisconsin 2843. The percentage of rooted shoots ranged from 92% to 100%, with Wisconsin 2843 showing the highest values.

3.3. Relationship between Variables

Relationships between the variables were assessed through multiple regression analysis (Table 2), with shoot number index (SNI) as the response variable and callus extension index (CEI) and $CuSO_4$ concentration as the predictors. No relationship was observed between SNI and CEI in any of the three cucumber cultivars tested. A strong relationship was found between SNI and $CuSO_4$ concentration (Wisconsin 2843: $p = 0.0027$; Marketer: $p = 0.0016$; Negrito: $p = 0.0002$).

Table 2. Factors associated with shoot number index (SNI) in generalized Poisson regression for three cultivars of *C. sativus* L.

	Wisconsin 2843					Marketer					Negrito				
Factor	**Estimate**	**Std. Error**	**z Value**	**Pr(>|z|)** ‡		**Estimate**	**Std. Error**	**z Value**	**Pr(>|z|)**		**Estimate**	**Std. Error**	**z Value**	**Pr(>|z|)**	
(Intercept): 1	0.61	0.22	2.73	0.00	**	0.14	0.09	1.51	0.13		0.18	0.10	1.75	0.08	
(Intercept): 2	−1.06	1.41	−0.75	0.45		0.28	0.50	0.55	0.58		0.30	0.60	0.50	0.62	
CEI §	−0.28	0.68	−0.41	0.68		−0.23	0.18	−1.26	0.21		−0.34	0.22	−1.53	0.13	
$CuSO_4$ †	−1.42	0.47	−3.00	0.00	**	−0.58	0.18	−3.16	0.00	**	−0.86	0.23	−3.78	0.00	***
DF	560					558					532				

** $p < 0.01$, *** $p < 0.001$; ‡ The Wald test was used for significance. § Callus extension index. † Concentration of $CuSO_4$ on MS-derived callus and shoot induction medium. Levels not significantly different from each other were combined. The reference levels for Wisconsin 2843, Marketer and Negrito cultivars were set to 5, 1 and 0.2 mg L^{-1} $CuSO_4$, respectively.

4. Discussion

The purpose of this work was to evaluate the effect of different concentrations of $CuSO_4$ on the organogenic response of several cucumber cultivars. Cooper is an essential plant micronutrient that is required at low concentrations. For instance, in the MS [13] mineral solution, copper is added as $CuSO_4$ at a concentration of 0.025 mg L^{-1}. Different studies on in vitro plant regeneration showed that MS basal medium could be optimized for a specific genotype by adjusting the concentration of some of its micronutrients (e.g., [14,54,74]).

Essential heavy metals like copper (Cu) are vital micronutrients for plant growth and development. Excessive levels lead to toxicity, causing oxidative stress, enzyme inhibition, and cellular damage, and disrupting the balance of essential and non-essential elements, impacting enzyme and protein function and metabolisms [16].

In the present study, we observed that all explants formed calluses, the response on callus extension was dependent on the genotype and in two of the three cultivars on the level of $CuSO_4$ in the medium. Callus regeneration was generally observed in both proximal and distal zones of the cotyledon, whereas shoot-buds were formed almost exclusively from the proximal part. This is in general agreement with other studies in cucurbits and other plants in which the proximal part of the cotyledon, adjacent to the cut edge of the explant, is the most active site for the regeneration of organized structures: cucumber [75,76], melon [77], horned melon [78], watermelon [79], squash [80], bottle gourd [81], soybean [82], geranium [83]. Cotyledon polarity on adventitious shoot regeneration is most likely the key factor for the absence of association between the number of shoots and the extension of calluses, obtained for all cultivars.

The results on shoot regeneration showed dependence on the genotype and on the level of $CuSO_4$ in the medium, indicating an interaction between the two factors. For both frequency (SRF) and number of shoots (SNI), the optimal concentration of $CuSO_4$ was 8- to 200-fold higher than the control (0.025 mg L^{-1}—the standard MS concentration) and was cultivar-specific. Differences in the ability for callus formation and regeneration have been extensively reported among cucumber genotypes (e.g., [34,84]; see mini-review by [23]) and within other species. Likewise, when high concentrations of copper were applied, differences have been reported within rice [54], and barley [14], with the optimal concentration of the ion being cultivar-specific.

The positive effects of high $CuSO_4$ concentrations on cucumber's in vitro shoot organogenesis align with those obtained in other cucurbits. Garcia-Sogo et al. [48] observed increased callus-derived shoot-buds in melon with $CuSO_4$ levels from 0.1 to 5 mg L^{-1} on MS-based media. Watermelon regeneration improved with 0.1 to 1.0 mg L^{-1} $CuSO_4$ [49]. Copper's positive impact extends beyond cucurbits: it enhanced embryoid production at 2 to 10 mg L^{-1} in tetraploid wheat [53] and shoot/root regeneration at 0.125 to 25 mg L^{-1} in wheat, triticale, tobacco, and barley [14,52,85]. Date palm callus cultures benefited from 0.5 mg L^{-1} $CuSO_4$ for shoot regeneration [57], and bamboo's nodal explants showed optimal regeneration with fivefold higher $CuSO_4$ concentrations than in MS medium [56].

Silvestri et al. [58] reported that the application of $CuSO_4$ at 1.25–2.5 mg L^{-1} in in vitro multiplication of hazelnut (*Corylus avellana* L.) significantly reduced bacterial contamination and maintained a high bud sprouting. Copper has a broad spectrum of antimicrobial efficacy, yet plant response differs by species [58].

The enhancement of adventitious shoot formation with the application of high concentrations of $CuSO_4$ was accompanied by an increase in the qualitative response, i.e., greener and more vigorous shoots. Likewise, the presence of high levels of $CuSO_4$ on adventitious regeneration of melon [86], Finger [74,87] and Kodo millet [74] improved the quality of response.

Some heavy metals, including copper (Cu), have been documented to stimulate morphogenesis [52,74,87,88].

Despite several studies reporting positive effects of high copper levels in plant morphogenesis, the underlying basis for these outcomes remains unclear. According to Purnhauser and Gyulai [52], some Cu enzymes might play a major role in plant regeneration, since Cu^{2+} is a component or activator of many important enzymes that participate in electron transport, protein and carbohydrate biosynthesis, polyphenol metabolism, and so forth.

The present results on the effect of copper on callus extension and the number and development of organized structures (adventitious buds and shoots) suggest that this metal ion can modulate genes related to regeneration and/or alter the level or activity of endogenous growth regulators. Copper's potential influence on these processes aligns with previous findings indicating that plant hormone biosynthesis and signaling are influenced by the copper concentration [89]. This interaction highlights the complex regulatory mechanisms through which copper impacts plant regeneration and development.

Furthermore, adverse effects generated by in vitro culture, such as those caused by excessive ethylene, reactive oxygen species (ROS), and (poly)phenols, can be mitigated by

increased copper levels. In a study by Miguel JF (in preparation), Wisconsin 2843 exhibited proportionally higher regeneration response with silver nitrate treatment, an ethylene inhibitor, compared to the cultivars Marketer and Negrito. High ethylene levels have been reported to hinder regeneration [90,91]. Considering the role of copper in mitigating stress and excess ethylene, it is plausible that in the present investigation, Wisconsin 2843 responded favorably to higher levels of copper to counteract elevated ethylene concentrations. The role of copper micronutrient in protecting against the harmful effects of ROS is known [12]. (Poly)phenols, which can harm in vitro culture and regeneration [92,93], decreased when exposed to higher copper levels, while stimulating growth [94].

The level of copper required for optimal in vitro regeneration in this investigation varies among genotypes, confirming that cucumber regeneration is largely genotype-dependent [23]. In Wisconsin 2843, the presence of multiple disease-resistance genes [59] may be behind the proportionally greater regeneration benefits observed at the higher copper level (5 mg L^{-1}) compared to the other cultivars of Marketer and Negrito. The reported link between disease resistance and metal tolerance [95] could explain their ability to thrive at higher copper concentrations. In addition, the up/downregulation of copper homeostasis could enhance regeneration, potentially resulting in variations among cultivars.

Other studies may indirectly suggest the importance of copper in plant morphogenesis. The respiration rate in cells under callus proliferation and cell division is normally higher [57], and enhanced levels of copper may be required, since it plays a key role in the respiration process [2]. Rapid cell division and differentiation require adequate amounts of precursors for cell wall biosynthesis [96], where copper metalloenzymes play an important part [97]. The molecular and physiological mechanisms associated with this essential but potentially toxic heavy metal should be further investigated to understand its effect on in vitro plant regeneration.

Three main points were achieved with the present research, which can be useful for several biotechnological approaches and explored in other cultivars and species to improve their regeneration efficiency. Firstly, the evaluated levels of copper effectively enhanced in vitro regeneration of one relevant inbred line and two commercial cultivars of cucumber. This finding can be explored in other cultivars and species, with special emphasis on those difficult to regenerate, such as cucumber. Secondly, workable regeneration efficiencies are essential for the successful application of various biotechnological approaches. Our study yielded significant outcomes applicable to large-scale micropropagation, including rapid production of disease-free and genetically uniform plants, germplasm conservation, somaclonal variation studies, and crop improvement through modern tools. Cucumber faces challenges in conventional breeding for resistance against various stresses, such as temperature fluctuations, drought, salinity, diseases (e.g., Fusarium, anthracnose, angular leaf spot, cucumber green mottle mosaic virus (CGMMV)), and pests (e.g., nematodes). These hurdles highlight the need for genetic transformation and advanced gene editing tools, essential not only for crop improvement but also for better understanding cucumber's genetic basis. Lastly, micronutrient composition of mineral solutions such as MS should be reviewed for particular species/cultivars, especially those difficult to regenerate, to maximize their potential.

This report is the first to assess the effect of high concentrations of copper ion on in vitro adventitious regeneration of cucumber. From the present study, it is concluded that optimized $CuSO_4$ levels in MS basal medium effectively enhanced shoot organogenesis in one inbred line and two commercial cultivars of cucumber. These findings can be further used for large-scale micropropagation of this species and in genetic transformation studies, and could help improve the regeneration efficiency of transformed cells.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in Harvard Dataverse at https://doi.org/10.7910/DVN/UWONNZ, reference UNF:6:FzVB+1KBa MW5YdrNYe/d6g==.

Conflicts of Interest: The author declares no conflict of interest.

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