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Evaluating the Impact of Copper Concentrations on Apple Variety Performance: Insights from a Callus Media System

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Abstract: Copper (Cu)-based fungicides are widely used in agriculture, but excessive Cu accumulation in soils can pose risks to crop performance, particularly in perennial fruit crops like apples. This study evaluated the physiological responses of three apple varieties—Braeburn, Fuji, and Cripps Pink—under Cu concentrations ranging from 0 to 20 mg Cu kg^{-1} using a callus media system. Key traits including the callus growth, fresh and dry weight, copper accumulation, tolerance index (TI), and bioaccumulation factor (BCF) were assessed. The results revealed that high Cu concentrations significantly inhibited the callus growth and biomass. Braeburn's growth was notably reduced to 0.27 times the control at 15 mg Cu kg^{-1}, and Fuji showed no growth at concentrations above 10 mg Cu kg^{-1}. Cripps Pink displayed greater tolerance, maintaining growth even at 20 mg Cu kg^{-1}. Copper accumulation peaked in Fuji at 4200 mg kg^{-1} at 20 mg Cu kg^{-1}, while Cripps Pink's BCF peaked at 120 under 15 mg Cu kg^{-1}. The TI for Cripps Pink consistently exceeded 100% across all Cu levels, contrasting with Fuji's TI, which dropped significantly at 15 mg Cu kg^{-1}. These findings suggest that maintaining soil Cu levels below critical thresholds is essential to preserve varietal performance and inform Cu management practices in apple cultivation.

Keywords: copper toxicity; apple; callus; copper accumulation; physiological responses; bioconcentration factor (BCF); translocation index (TI); copper-based fungicides; soil–plant systems

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1. Introduction

Apples accounted for over 21.5% of fresh fruit in horticultural exports in New Zealand in 2024 [1]. New Zealand exports approximately 360,000 metric tons of apples to 80 countries [1]. Apple exports have increased gradually since 1989, and a healthy apple orchard environment is undoubtedly crucial to the economy. Among apple varieties, the Royal Gala (21.9%) is New Zealand's largest volume variety, followed by Envy (15.5%), Rockit (10.8%), Dazzle (7.6%), Fuji (6.1%), Cripp's Pink (5.6%), and Braeburn (4.4%) [1]. Three apple varieties were selected in the present study.

The use of Cu-based fungicides for disease control on apples has always been viewed from two directions, as highly positive disease control benefits are counterbalanced by the risk of phytotoxicity to trees and soil and the contamination of fruit [2]. Two major apple pathogens, the fire blight pathogen *Erwinia amylovora* and the apple scab pathogen *Venturia inaequalis*, are susceptible to Cu [3]. These two diseases can devastate apple trees [4], and thus Cu-based fungicides have been used for many years in New Zealand. It has long been established that Cu can be toxic to plants in high concentrations. One of the typical toxicity symptoms of Cu is inter-venial chlorosis. Excessive amounts of Cu in orchard soils can impair the growth of roots and shoots [5], restrain photosystem II activity, inhibit protein synthesis and enzyme activities, reduce carbohydrate concentrations, and induce plant senescence and even death, resulting in lower fruit productivity [6–12].

Long-term use of Cu-based fungicides increases Cu accumulation in orchard soils [6,13,14]. Cu concentrations in apple orchard soils in China have been shown to have increased substantially due to the application of Cu, totaling more than 16 kg ha^{-1}

Cu per year and ranging from 2.5 to 9 mg Cu kg^{-1} [15]. The present study recorded a total Cu concentration in the soil of two apple orchards in New Zealand of >60 mg Cu kg^{-1}.

Previous studies have demonstrated that the Cu uptake, compartmentalization, and tolerance characteristics are highly variable among plant species and cultivars within the same area [16,17]. However, relatively little research is currently available on Cu accumulation and tolerance in apple trees, and most of it focused only on rootstocks. Rootstocks of horticultural crops regulate plant growth and development and affect Cu uptake, accumulation, and toxicity through ion exclusion or retention [4,14]. Progress has been made in demonstrating the physiological mechanisms of excess Cu uptake, accumulation, and tolerance in grapevine rootstocks [11,18] and citrus [14]. Excess Cu suppressed the plant biomass and root architecture, being less pronounced in *Malus prunifolia Borkh*. The other four apple rootstocks (*Malus asiatica Nakai.*, *Malus baccata Borkh*, *Malus hupehensis Rehd.* and *Malus micromalus Makino*) indicated relatively higher Cu tolerance [19]. The Cu concentration in the Delicious apple variety's tissues increased with heavy metals in the soil from the polluted area [20].

In vitro tissue culture provides an important tool for studying the physiological and biochemical mechanisms which operate in response to stress at the cellular level. Plant tissue culture allows experimental control of the heterogeneity and characterization of cell behavior under stress independent of regulatory systems which operate at the whole plant level [21]. Tissue culture can also be useful for enhancing metal tolerance through the in vitro selection of tolerant cell lines which can be regenerated into whole plants. A callus is unorganized cell mass growth, similar to wound-healing plant tissue [22]. A callus represents a proliferating mass of cells [23]. Callus culture potentially provides a simple way to understand the impacts of forms. The selection and characterization of various cell cultures tolerant to heavy metals have been documented [24,25].

The aim of the present study was to investigate the effects of varying copper concentrations on the physiological responses of apple varieties using a callus media system. The objectives were (1) to assess the influence of varying copper concentrations on the physiological traits of three apple varieties using a callus media system, (2) to determine the threshold at which copper concentrations begin to override varietal differences in physiological responses, and (3) to explore how findings from the callus media system may inform potential impacts on apple performance in soil–plant systems. In addition to addressing varietal responses to copper toxicity, this study contributes to sustainable agriculture by indicating practices which limit copper accumulation in soils. Understanding these dynamics is crucial for balancing effective disease control with long-term soil and crop health, aligning with sustainable development goals in agriculture.

2. Materials and Methods

2.1. Plant Materials

Three apple varieties (Braeburn, Fuji, and Cripps Pink) were obtained from three apple orchards in Hastings, New Zealand in November 2022 (Table 1). The three apple varieties were selected due to their rising export proportions in New Zealand [1]. Although they represented a smaller percentage of exports in 2024 (Fuji = 6.1%, Braeburn = 4.4%, and Cripps Pink = 5.6%), each has shown consistent growth in their export shares since 2018. Additionally, limited research exists on the effects of copper-based fungicides on these specific varieties, despite their increasing significance in the apple industry. Twenty shoot cuttings were randomly sampled in a single block in each orchard, and nutrient content tests were conducted (Table 2, in triplicate). Specimens were stored for two weeks in a laboratory incubator (5 ± 0.5 °C, 60% humidity) at Lincoln University until callus induction. Three apple orchards' soils were analyzed as background data (Table 3).

Table 1. Orchard history, management practices, and locations of three apple varieties.

Orchard	Variety	Cu Fungicide Spray Rates	Root Stock	Year Planted	Tree Spacing	Row Spacing	Locations
D	Fuji	1.8 kg ha yr^{-1}	MM793	2010	2.50	4.50	39°38′09.0″ S 176°43′48.8″ E
M	Cripps Pink	1.8 kg ha yr^{-1}	MM106	1992	2.45	5.00	39°36′38.8″ S 176°48′18.6″ E
W	Braeburn	1.8 kg ha yr^{-1}	MM106	1990	2.50	5.00	39°35′15.9″ S 176°52′35.2″ E

Table 2. Nutrient compositions of apple leaves from three orchards (mean ± s.d., n = 60).

Orchard Variety Determinant	D Fuji	M Cripps Pink	W Braeburn	Plant Range †
Nitrogen (%) §	3.1 ± 0.25 a ‡	2.7 ± 0.22 a	2.9 ± 0.05 a	2.1–3.8
Phosphorus (%) §	0.36 ± 0.02 a	0.30 ± 0.01 a	0.31 ± 0.02 a	0.18–0.5
Potassium (%) §	2.4 ± 0.30 a	2.2 ± 0.20 a	2.1 ± 0.03 a	1.7–2.7
Sulfur (%) §	0.40 ± 0.03 a	0.39 ± 0.03 a	0.33 ± 0.02 a	0.25–0.45
Calcium (%) §	1.1 ± 0.05 a	1.1 ± 0.04 a	1.0 ± 0.05 a	1–1.8
Magnesium (%) §	0.25 ± 0.01 a	0.23 ± 0.01 a	0.22 ± 0.01 a	0.2–0.3
Sodium (%) §	<0.01	<0.01	<0.01	-
Manganese (mg kg^{-1}) §	40 ± 0.80 a	34 ± 1.1 a	35 ± 3.5 a	34–72
Copper (mg kg^{-1}) ***	8.0 ± 0.44 b	14 ± 1.0 a	9.0 ± 0.35 b	7–20
Boron (mg kg^{-1}) §	27 ± 0.42 a	28 ± 0.56 a	29 ± 0.31 a	23–33
Zinc (mg kg^{-1}) §	29 ± 0.82 a	25 ± 0.98 a	26 ± 0.24 a	10–40
Iron (mg kg^{-1}) §	44 ± 0.73 a	45 ± 0.97 a	46 ± 0.37 a	40–90

*** Significant at the 0.001 probability level. † The plant range represents the desired plant nutrition concentration of determinants in the robust plants, referred from Eurofins (Eurofins Hawke's Bay Laboratory Service Centre, Hastings, New Zealand) ‡ Values with the same lower-case letters in a row are not significantly different at $p < 0.05$. § n.s. = not significant.

Table 3. Soil chemical properties of three apple orchards (mean ± s.d., n = 15). Data from Hill Laboratories (2020).

Orchard Variety Determinant	D Fuji	M Cripps Pink	W Braeburn
$pH_{1:2}$ § [1]	7.2 ± 0.15 a ‡	7.0 ± 0.09 a	7.1 ± 0.08 a
Total Cu (mg kg^{-1}) *** [2]	34 ± 1.6 b	126 ± 1.6 a	45 ± 2.8 ab
Olsen P (mg L^{-1}) ** [3]	35 ± 1.5 b	43 ± 2.1 a	23 ± 1.6 c
K (me $100g^{-1}$) ** [4]	1.0 ± 0.09 b	1.5 ± 0.04 a	0.53 ± 0.09 c
Ca (me $100g^{-1}$) ** [4]	23 ± 0.40 a	21 ± 0.79 a	14 ± 0.44 b
Mg (me $100g^{-1}$) ** [4]	2.8 ± 0.05 a	2.5 ± 0.08 a	1.2 ± 0.14 b
Na (me $100g^{-1}$) ** [4]	0.40 ± 0.03 a	0.13 ± 0.03 b	0.08 ± 0.01 b
CEC (me $100g^{-1}$) ** [5]	295 ± 1.6 a	27 ± 3.4 a	17 ± 2.0 b
Total Base Saturation (%) §	96 ± 3.4 a	94 ± 5.7 a	100 ± 2.6 a
Available Nitrogen (kg ha^{-1}) ** [6]	87 ± 3.3 c	122 ± 2.5 a	103 ± 9.3 b
Anaerobically Mineralizable N (g g^{-1}) ** [7]	82 ± 2.7 ab	93 ± 5.6 a	74 ± 1.6 b
Organic Matter (%) *** [8]	11 ± 0.83 a	5.6 ± 0.40 b	5.5 ± 0.20 b
Total C (%) *** [8]	6.6 ± 0.03 a	3.3 ± 0.04 b	3.1 ± 0.04 b
Total N (%) ** [6]	0.61 ± 0.04 a	0.32 ± 0.03 b	0.25 ± 0.02 b
C/N Ratio §	11 ± 0.49 ab	10 ± 0.39 ab	12 ± 0.32 a
Anaerobically Mineralizable N/Total N ratio **	1.4 ± 0.06 b	2.9 ± 0.17 a	3.0 ± 0.10 a

Table 3. *Cont.*

Orchard Variety Determinant	D Fuji	M Cripps Pink	W Braeburn
Volume Weight (g mL^{-1}) ** [9]	0.73 ± 0.02 b	0.87 ± 0.02 ab	0.91 ± 0.01 a
Soil Texture §	Loamy	Loamy	Loamy

** Significant at the 0.01 probability level. *** Significant at the 0.001 probability level. ‡ Values with the same lower-case letters in a row are not significantly different at $p < 0.05$. § n.s. = not significant. [1] 1:2 (v/v) soil: water slurry followed by potentiometric determination of pH. [2] GBC Avanta atomic absorption spectrophotometer (AAS). [3] Olsen P extraction followed by Molybdenum Blue colorimetric. [4] 1M neutral ammonium acetate extraction followed by ICP-OES. [5] Summation of extractable cations (K, Ca, Mg, and Na) and extractable acidity. [6] CN analyzer. [7] Anaerobic incubation at 40 °C for 7 days. [8] Loss on ignition (LOI). [9] The bulk density of dried, ground soil.

2.2. Sterilization

Ten young and healthy leaves were thoroughly rinsed under running tap water for 10 mins and then sterilized with 10% bleach for 30 seconds. The sterilized samples were rinsed three times using deionized water. The samples were placed in 75% (v/v) ethanol for 30 mins and rinsed three times again using deionized water in a laminar flow cabinet. The sterilized leaves were cut into 0.5 cm × 0.5 cm pieces in a laminar flow cabinet to form explants.

2.3. Media Preparation

The culture medium used was MS + vits (Murashige and Skoog + vitamin salts, Duchefa, Haarlem, Netherlands) [26] supplemented with 5 g L^{-1} agar, 20 g L^{-1} sucrose, 0.5% phyto-agar, and plant hormones (0.4 mg L^{-1} BA, 0.1 mg L^{-1} NAA, and TDZ). The pH of the medium was adjusted to 5.8 with 0.1 N HCl or 0.1 N KOH prior to autoclaving (121 °C and 1.06 kg cm^{-2} pressure for 15 mins). The autoclaved 1 L solution mix was poured into 20 plastic Petri dishes (90 mm in diameter and 15 mm high) under a sterile laminar flow cabinet and allowed to solidify. Media were stored in a laboratory refrigerator at 4 °C until the explants were placed onto the Petri dishes.

2.4. Callus Induction

The sterilized explants of three apple varieties (Braeburn, Fuji, and Cripps Pink) were transferred onto MS + vits for callus induction. Five explants were placed in a Petri dish and replicated five times for each variety (5 explants × 5 replicates × 3 varieties = 75 explants). The cultures were maintained in a thermostat at a temperature of 23 ± 2 °C for the first two days in the dark and then transferred to a 16 h light and 8 h dark photoperiod (provided by cool white fluorescent light) with a relative humidity level of 60–70% being maintained [27]. The explants were subcultured when calli were induced at the edge of the explants, moving small pieces of callus using a sterilized scalpel. Calli from explants were subdivided aseptically to be placed on the MS + vits media. Care was taken to use fragments derived from the same region of the original callus to reduce variability. The callus medium was replaced every three weeks to subculture for achieving replicated and homogenous growth calli.

2.5. Copper Exposure to Calli Using $CuSO_4 \cdot 5H_2O$ Solution

Visually homogenous calli originating from a single explant of each variety were subcultured three times and then used in this experiment. MS + vits media with the same hormones were used apart from concentrations of $CuSO_4 \cdot 5H_2O$. A stock solution of Cu was prepared by dissolving 3930 mg Cu^{2+} sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) in 1000 mL of deionized water [28], and then 1000 mg kg^{-1} of $CuSO_4 \cdot 5H_2O$ stock solution was used to amend the Cu concentration in the MS + vits media. $CuSO_4 \cdot 5H_2O$ was applied to create the following MS + vits media: 0.0006 (control, no addition), 1, 5, 10, 15, and 20 mg Cu kg^{-1}. Five calli were placed in one media and replicated five times in each variety

(5 explants × 5 replicates × 6 different $CuSO_4 \cdot 5H_2O$ doses × 3 varieties = 450 calli). The cultures were maintained in a thermostat at a temperature of 23 ± 2 °C in the dark, and a relative humidity level was maintained at 60–70%. The calli's growth was observed for 40 days.

2.6. Cu Analysis in Calli with Atomic Absorption Spectrophotometer

The dried callus material was ground into powder with a mortar and wet-digested in 65% nitric-perchloric acid (1 mL per 0.1 g of dry matter). The digested material was suspended in distilled water [29], and the total concentrations of Cu were determined by a GBC Avanta atomic absorption spectrophotometer (AAS) (GBC Scientific Equipment Pty Ltd., Melbourne, Australia).

2.7. Parameters' Calculation

2.7.1. Callus Growth

The calli's growth became lagged after 40 days. The callus size was marked at 25 days and 40 days on the back sides of the Petri dishes to calculate the callus growth until growing stopped. Pictures were taken to check the growth of each callus visually. The ImagJ software (64-bit Java 8) measured the area of the callus in each sample. Figure 1 explains the net callus growth at 25 and 40 days and the calculation of the total callus growth (mm^2).

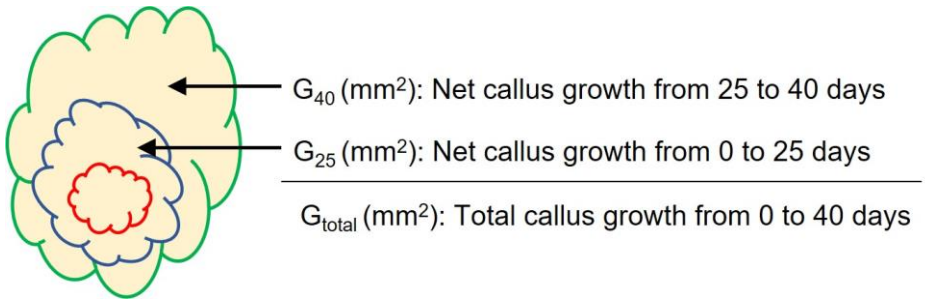

Figure 1. Description of callus growth (mm^2) and calculation.

2.7.2. Dry Weight (g)

After measuring the callus growth, 50 pieces of the calli were used to determine the dry weight (g). Calli were removed from the media and rinsed with DI water. Fresh calli were oven-dried (65 °C) and kept in a desiccator for one day. The dry weight (g) was measured after one week of dehydration.

2.7.3. Tolerance Index (TI, %)

After measuring the callus growth, 50 pieces of calli were used to determine the Cu resistance. The calli were removed from the media, rinsed with DI water, oven-dried at 70 °C for one week, and weighed before Cu analysis. The tolerance indices (TIs) were calculated using the following formula [30]):

$$TI\ (\%) = Callus_t / Callus_c \times 100 \quad (1)$$

where $Callus_t$ is the dried weight of the callus in the media with Cu concentrations of 1, 5, 10, 15, and 20 mg Cu kg^{-1} and $Callus_c$ is the dried weight of the callus in the media without Cu's addition (control; 0.006 mg Cu kg^{-1}).

2.7.4. Bioaccumulation Factor (BCF)

The bioaccumulation factor (BCF) was used to quantify the bioaccumulation effect of calli in the uptake of Cu from media. It indicates a plant's capacity to accumulate heavy metals [31]. The BCF was calculated as follows:

$$\text{BCF} = (\text{Cu concentration in calli}/\text{Cu concentration in media}) \times 100 \quad (2)$$

2.8. Statistical Analysis

In this research, various statistical methods were applied to evaluate the effects of copper concentration and apple variety on the physiological traits in a controlled callus media system. The Shapiro–Wilk test was initially used to assess the normality of the data. Two-way analysis of variance (ANOVA) was conducted to examine the effects of two independent factors: the media copper concentration and apple variety. The ANOVA was used to determine their individual and interactive effects on multiple dependent variables, including the callus copper concentration, growth parameters (G_{25}, G_{40}, and G_{total}), fresh weight (FW), dry weight (DW), tolerance index (TI), and bioconcentration factor (BCF). When the two-way ANOVA identified significant differences ($p < 0.05$), Tukey's honest significant difference (HSD) test was applied as a post hoc analysis. In addition, Pearson correlation analysis was performed to explore the relationships between various physiological traits and the copper concentration. The statistical results were presented using line plots, which visually represented the distribution of copper accumulation, growth, the fresh and dry weight, tolerance index, and bioconcentration factor across different copper concentrations and apple varieties. All statistical analyses were conducted using Python software 3.14.2.

3. Results

3.1. Callus Cu Accumulation

The copper concentration in the media resulted in a significant increase in callus copper accumulation across the three apple varieties (Figure 2). Braeburn and Fuji exhibited higher copper accumulation than Cripps Pink (Figure 3A). When exposed to 15 mg Cu kg^{-1}, the callus copper concentration in Braeburn and Fuji ranged from 0 to 103 mg kg^{-1}. At the higher concentration of 20 mg Cu kg^{-1}, Braeburn reached 3150 mg kg^{-1}, and Fuji reached 4200 mg kg^{-1}. Cripps Pink showed a more gradual increase in copper accumulation, reaching 118 mg kg^{-1} at 10 mg Cu kg^{-1}. At 15 mg Cu kg^{-1}, Cripps Pink's callus copper concentration reached 1800 mg kg^{-1}, and at 20 mg Cu kg^{-1}, it slightly declined to 1700 mg kg^{-1}.

3.2. Callus Growth

Copper exposure led to a reduction in callus growth (measured by G_{25}, G_{40}, and G_{total}) in all three apple varieties (Figure 2). Braeburn's callus growth was stimulated in the 5 mg Cu kg^{-1} media, with an increase of 1.43 times compared with the control (Figure 3B–D). However, growth was inhibited in the 15 mg Cu kg^{-1} media, reducing to 0.27 times that of the control, and there was no growth observed at 20 mg Cu kg^{-1}. Fuji exhibited no growth at copper concentrations above 10 mg Cu kg^{-1}. In contrast, Cripps Pink maintained some degree of growth at 20 mg Cu kg^{-1}, with G_{total} reduced to 0.81 and 0.72 times at 5 and 20 mg Cu kg^{-1}, respectively.

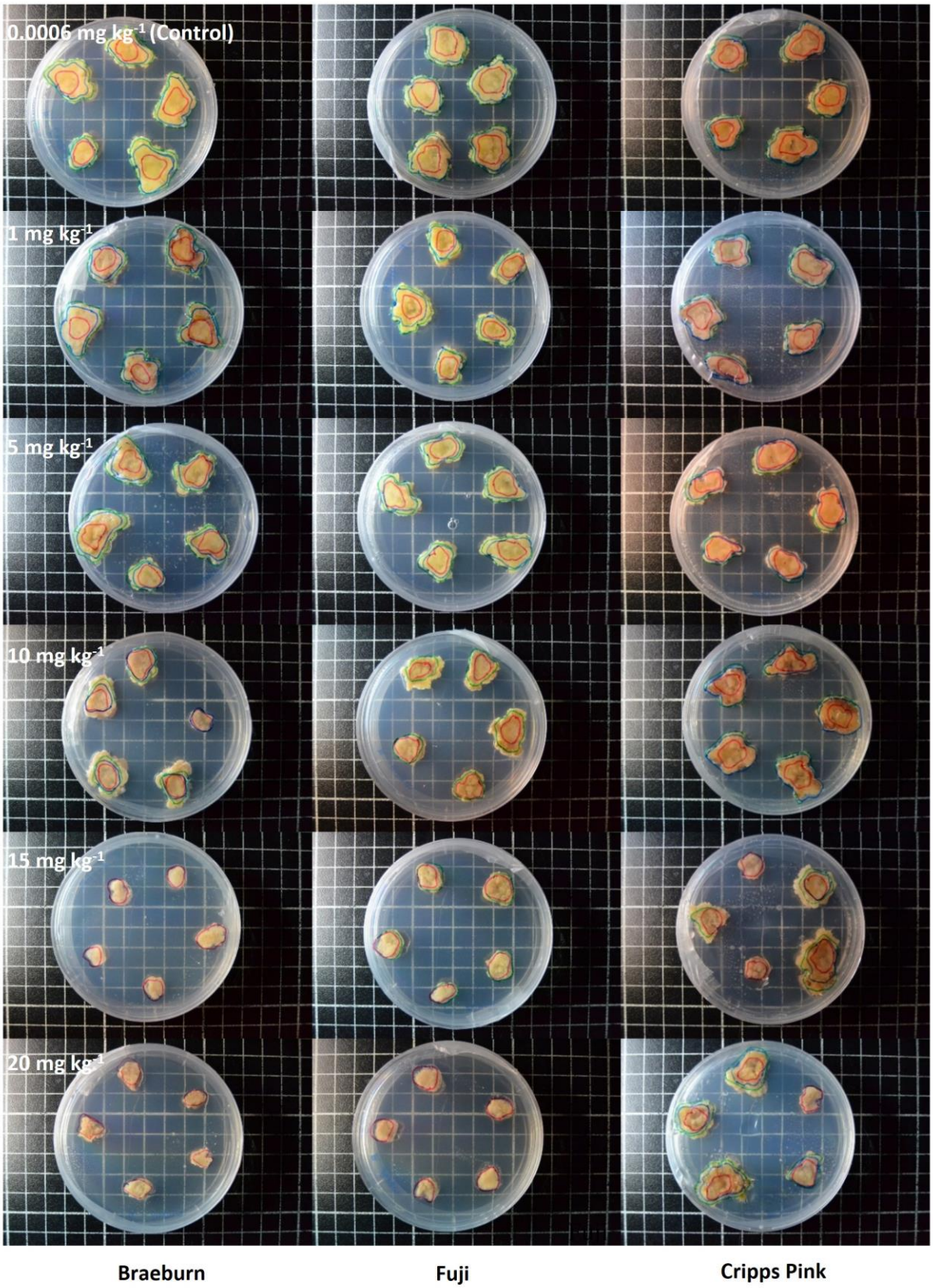

Figure 2. Visual comparison of callus growth in three apple varieties exposed to varying Cu concentrations over 40 days. Media Cu concentrations were 0.0006 (control), 1, 5, 10, 15, and 20 mg Cu kg^{-1}. Callus growth over time is marked by red (day 0), blue (day 25), and green (day 40) lines (n = 5).

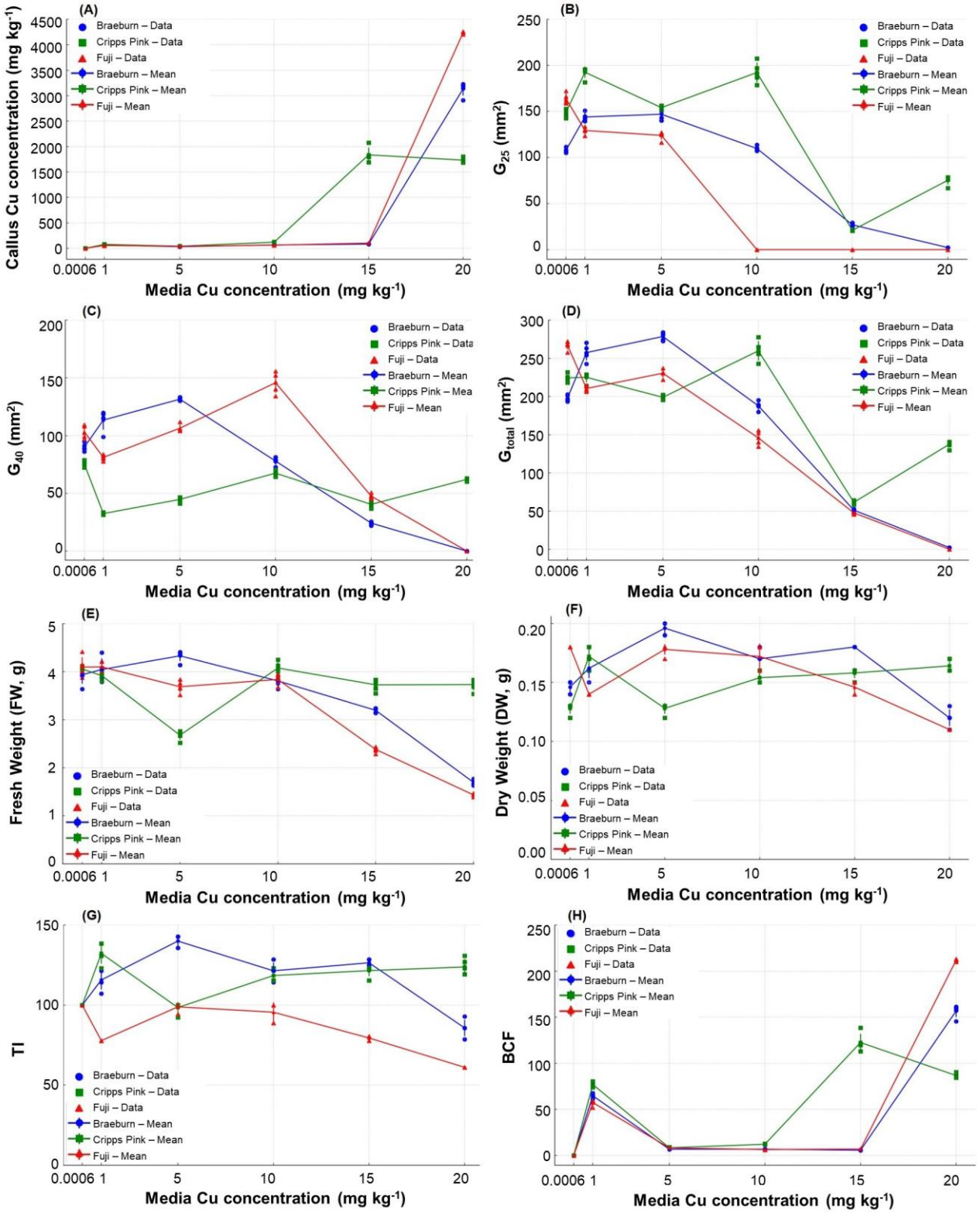

Figure 3. Line plots callus responses to Cu concentration (0.0006 (control), 1, 5, 10, 15, and 20 mg Cu kg^{-1}) in MS + Vit media (n = 5). (**A**) Callus Cu concentration (mg kg^{-1}). (**B**) G_{25} (mm^2). (**C**) G_{40} (mm^2). (**D**) G_{total} (mm^2). (**E**) Fresh weight (g). (**F**) Dry weight (g). (**G**) Tolerance index (TI, %). (**H**) Bioconcentration factor (BCF).

3.3. Fresh Weight and Dry Weight

The fresh weight (FW) and dry weight (DW) showed similar trends across the three varieties. At lower copper concentrations (up to 5 mg Cu kg^{-1}), the calli exhibited stable or increased growth. Braeburn maintained FW values of 3.2 g at 15 mg Cu kg^{-1}, but a sharp decline was observed at 20 mg Cu kg^{-1} (Figure 3E). FW reductions in Cripps Pink began at 5 mg Cu kg^{-1}, and it further decreased at higher copper concentrations. Fuji displayed no growth in terms of FW or DW at copper concentrations above 10 mg Cu kg^{-1}. In terms of dry weight, Braeburn and Fuji exhibited similar reductions in their DWs at concentrations of 15 mg Cu kg^{-1} and above (Figure 3F). Cripps Pink showed more stable DW values at lower concentrations but also exhibited a decline in DW at 20 mg Cu kg^{-1}.

3.4. Tolerance Index (TI) and Bioaccumulation Factor (BCF)

The tolerance index (TI) differed across the varieties, with Fuji exhibiting a marked decrease in TI at copper concentrations above 15 mg Cu kg^{-1} (Figure 3G). Cripps Pink maintained TI values above 100% at all copper concentrations tested. Braeburn's TI remained above 100% at most concentrations but dropped below this level at 20 mg Cu kg^{-1}. The bioaccumulation factor (BCF) increased with higher copper concentrations for all varieties. Fuji had the highest BCF at 20 mg Cu kg^{-1}, reaching 210, while Cripps Pink peaked at 15 mg Cu kg^{-1} with a BCF of 120 (Figure 3H).

3.5. Two-Way ANOVA Outcomes

The two-way ANOVA revealed significant effects from both the apple variety and media Cu concentration on all dependent variables, including the callus Cu concentration, G_{25}, G_{40}, G_{total}, fresh weight (FW), and bioconcentration factor (BCF) ($p < 0.05$). Additionally, the interaction between the variety and media Cu concentration was statistically significant for these traits ($p < 0.05$). For most of the dependent variables, the media Cu concentration had a stronger effect than the apple variety, as evidenced by the higher sum of squares (SS) and F values for the media Cu concentration compared with the variety (Table 4). However, for the translocation index (TI), the apple variety had a larger influence, with higher SS and F values (F = 619, $p < 0.05$).

Table 4. Two-way ANOVA for callus copper concentration, growth, and other physiological traits by apple variety and media copper concentration (n = 5).

	Original Dataset				**>10 mg Cu kg^{-1} Excluded Dataset**			
	Variety SS	**Media Cu SS**	**Variety F**	**Media Cu F**	**Variety SS**	**Media Cu SS**	**Variety F**	**Media Cu F**
Callus Cu Con.	530,839	10,722,184	120	9683	456	33,198	65	4730
G_{25}	58,225	283,710	1630	3177	8783	2071	230	54
G_{40}	11,475	78,359	427	1168	30,073	2792	983	91
G_{total}	18,042	617,339	271	3712	6393	312	97	5
FW	2.9	35	94	450	2.5	2.1	57	49
DW	0.002	0.013	33	80	0.006	0.002	103	34
TI	17,847	5618	619	78	5443	1219	222	50
BCF	1981	249,012	110	5525	343	39,429	67	7747

Callus Cu Con. = callus Cu concentration (mg Cu kg^{-1}); G_{25} = net callus growth from 0 to 25 days (mm^2); G40 = net callus growth from 25 to 40 days (mm^2); G_{total} = total callus growth from 0 to 40 days (mm^2); FW = fresh weight (g); DW = dry weight (g); TI = tolerance index; BCF = bioaccumulation factor.

In the ANOVA conducted after excluding media Cu concentrations above 10 mg kg^{-1}, the influence of the apple variety became more pronounced across several dependent variables, including the callus Cu concentration, G25, and FW, where the SS and F values for the variety increased, while the effect of the media Cu concentration diminished (Table 4).

4. Discussion

4.1. Callus Parameters as Indicators of Copper Toxicity

Braeburn and Fuji exhibited significant copper accumulation at 20 mg Cu kg^{-1}, suggesting higher susceptibility to copper toxicity relative to Cripps Pink, which demonstrated lower copper uptake (Figure 3A). These results indicate varietal differences in copper transport and regulation. Fuji's marked callus growth inhibition at concentrations above 10 mg Cu kg^{-1} suggests greater sensitivity (Figure 3B–D), contrasting with Cripps Pink's more gradual response, indicating potential tolerance mechanisms [32]. The tolerance index (TI) values highlighted varietal differences in copper sensitivity (Figure 3G). Fuji's sharp decrease in TI at 15 mg Cu kg^{-1} reinforced its vulnerability, while Cripps Pink's consistent TI above 100% across all concentrations indicated a higher degree of tolerance. This could be attributed to enhanced copper sequestration or antioxidant defenses [33]. The bioaccumulation factor (BCF) also revealed Fuji's higher copper translocation at 20 mg Cu kg^{-1}, while Cripps Pink exhibited a lower BCF despite high copper exposure (Figure 3H). This suggests that Cripps Pink may employ mechanisms to limit copper's toxic effects, such as vacuolar sequestration, which aligns with the findings for other metal-tolerant species [34].

4.2. Biomass Reduction and Sensitivity Thresholds

The reductions in fresh weight (FW) and dry weight (DW) at copper concentrations above 10 mg Cu kg^{-1} highlight the onset of significant copper stress, with Braeburn showing slightly better resilience at 15 mg Cu kg^{-1} compared with Fuji (Figure 3E,F). This suggests that varietal differences in tolerance thresholds existed but are overwhelmed at higher copper levels [35]. The observed biomass reduction supports the identification of 10 mg Cu kg^{-1} as a critical threshold for copper toxicity. Beyond this point, the physiological stress is sufficient to override varietal differences, leading to uniform reductions in growth and biomass, consistent with copper stress studies on other plant species [36].

4.3. High Copper Concentrations Override Varietal Differences

The two-way ANOVA results illustrated that high copper concentrations (>10 mf kg^{-1}) overrode the inherent genetic variability of the apple varieties (Table 4, left column). Under these conditions, copper stress induced a uniform physiological response which diminished the role of genetic diversity. This pattern aligned with previous findings, which showed that heavy metal stress can suppress genetic variability by imposing a dominant stress response across genotypes [32,35]. In this study, traits like the callus Cu concentration and BCF were more strongly influenced by the copper concentration in the media, reflecting the overriding effect of copper stress.

However, when media Cu concentrations above 10 mg kg^{-1} were excluded from the analysis (Table 4, right column), the influence of the apple variety became more pronounced. The increase in SS and F values for the variety indicated that under moderate copper conditions, the genetic differences between the apple varieties played a more significant role in determining the callus Cu concentration, G_{25}, and FW. This suggests that high copper levels can negate genetic advantages related to stress tolerance, leading to a convergence of physiological responses under extreme copper stress [36]. Interestingly, for the translocation index (TI), the genetic differences between apple varieties remained important even at lower copper concentrations, as evidenced by the larger SS and F values for the variety. This suggests that copper translocation within plant tissues is more tightly regulated by genetic factors, regardless of the external copper stress.

4.4. Extrapolation to Soil–Plant Systems

The callus experiment provided valuable insights into how copper stress may affect whole plants in soil–plant systems. Although the in vitro conditions used in this study differed from natural soil environments, where buffering mechanisms such as organic matter and microbial activity can mitigate the effects of copper toxicity, the results suggest that media copper concentrations above 10 mg Cu kg^{-1} may pose a risk to plant health.

The masking effect observed in callus systems, where copper toxicity overrides varietal differences, may also occur in soil environments, particularly in soils with low organic matter or poor buffering capacity. In such soils, high copper concentrations could lead to impaired root growth, reduced water and nutrient uptake, and overall decreased plant vigor, as seen in the callus experiment. These findings are consistent with studies which reported similar copper toxicity effects in soil-grown plants, where high soil copper levels led to reductions in plant growth, chlorosis, and stunted development [34].

Additionally, soil microbial activity could be negatively affected by elevated copper concentrations, further compounding the negative effects of copper on plant growth. Copper toxicity in soils can inhibit beneficial microbial processes, such as nutrient cycling and nitrogen fixation, leading to poorer soil health and reduced plant productivity. The bioaccumulation factor (BCF) observed in the callus experiment, particularly at high copper concentrations, suggests that in a soil–plant system, similar translocation of copper into plant tissues could occur, potentially leading to toxic levels of copper in plant biomass. This aligns with the findings of in vivo studies which showed copper accumulation in plant tissues to be a major concern in soils with high copper content [33].

4.5. Implications for Agricultural Practices and Soil Health

The results of this study highlight the importance of managing copper concentrations in agricultural soils to avoid copper toxicity and preserve varietal performance. As demonstrated by the callus experiment, copper concentrations above 10 mg Cu kg^{-1} resulted in significant growth inhibition and biomass reduction, particularly in more sensitive varieties such as Fuji. Maintaining copper levels below this threshold in soil is crucial to ensure that apple varieties can express their full genetic potential and maintain healthy growth.

The observed differences in copper tolerance between the apple varieties suggest that careful varietal selection should be considered in areas where soil copper concentrations are elevated. For instance, Cripps Pink's higher tolerance to copper suggests that this variety may be better suited for cultivation in copper-affected soils, while Fuji, with its higher sensitivity, may require stricter copper management practices. Furthermore, integrating organic amendments and improving the soil organic matter content could help mitigate copper toxicity by enhancing the soil's buffering capacity and promoting beneficial microbial activity [37].

4.6. Limitations and Future Research Directions

While the callus media system provides a controlled environment for studying copper toxicity, it is important to acknowledge its limitations in fully replicating soil–plant systems. In vitro conditions lack the complex interactions found in natural soils, such as root–microbe interactions, organic matter influences, and dynamic water and nutrient cycles. As a result, the extrapolation of these findings to field conditions should be approached with caution. Future research should focus on conducting in vivo experiments in soil–plant systems to validate the findings of this study and explore the effects of copper toxicity in more realistic agricultural settings. Additionally, further research is needed to investigate the long-term effects of copper toxicity on apple varieties in soil, including potential adaptations and tolerance mechanisms which may develop over time.

4.7. Balancing Copper Use and Crop Health: Implications for Sustainable Management

Based on the findings of this study and the current literature, several strategies may help address this issue. (1) For monitoring and threshold management, implementing regular soil and plant tissue testing to monitor copper accumulation can help manage its application more precisely, ensuring levels stay within thresholds which minimize crop toxicity while still providing effective disease control. (2) Regarding copper alternatives and reduced application rates, where feasible, integrating alternative fungicides or combining copper fungicides with biocontrol agents could reduce reliance on copper without compromising disease management. Additionally, reducing the application rates or frequency

based on disease pressure and environmental conditions may limit excess accumulation. (3) Varietal selection for copper tolerance could prove useful as this study highlighted the variability in copper tolerance among apple varieties, suggesting that more tolerant varieties like Cripps Pink may be better suited for orchards with elevated copper levels. Selecting varieties based on their tolerance to copper could help maintain crop health in soils with residual copper levels. (4) For soil amendments to increase buffering capacity, incorporating organic matter or using soil amendments, such as compost or biochar, can enhance the soil's buffering capacity, helping to immobilize copper and reduce its bioavailability to plants. This practice could mitigate copper toxicity in soils already exposed to copper-based fungicides. (5) Regarding future research directions, while this study focused on varietal tolerance differences, further research on physiological responses to copper in apples could identify traits for breeding or engineering more copper-tolerant cultivars. Additionally, long-term field studies could provide a better understanding of copper's cumulative effects and potential mitigation strategies.

5. Conclusions

This study demonstrated that high copper concentrations significantly affect key physiological traits, including callus growth, copper accumulation, the fresh and dry weight, and the bioconcentration factor (BCF). The two-way ANOVA revealed that the media Cu concentration had a dominant influence over varietal differences, particularly at concentrations above 10 mg kg^{-1}, where copper stress overrode the genetic variability between apple varieties. Under extreme copper stress, the physiological response became more uniform across varieties, with high copper levels suppressing the expression of genetic potential. In contrast, at moderate copper concentrations, the influence of genetic differences became more prominent. This indicates that lower copper levels allow varietal traits to play a more significant role in determining apple callus performance. Notably, the Cripps Pink variety exhibited greater copper tolerance compared with the Braeburn and Fuji varieties, suggesting its potential suitability for cultivation in environments with elevated copper exposure. Although this study was conducted in a controlled callus media system, the findings provide valuable insights into how copper stress may impact apple varieties in real-world soil–plant systems. The callus media system proved to be a reliable model for investigating copper toxicity, and the results suggest that maintaining copper levels below critical thresholds is essential for preserving varietal differences and optimizing apple performance. Future research should aim to validate these findings in soil–plant systems to further refine copper management practices in apple orchards. The findings of this study underscore the importance of sustainable soil management in agricultural systems which rely on copper-based fungicides. By highlighting copper toxicity thresholds and varietal responses, this research provides a basis for sustainable practices which minimize environmental degradation while maintaining agricultural productivity. These insights can guide the development of sustainability tools, such as soil monitoring frameworks, and inform policy measures to regulate copper levels in orchard soils. Ultimately, this work contributes to a sustainable agricultural model by balancing immediate crop protection needs with the long-term ecological resilience required for sustainable development.

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