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In vitro Performance of Two Banana Cultivars Under Copper Stress

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Abstract

Environmental pollution is one of the most issues directly or indirectly affecting human life. Heavy metals are considered as the most environmental pollutants affecting plant growth and productivity. Thus, herein the toxicity of copper on *in vitro* growth and development of two banana commercial cultivars i.e. 'Grand Nain' and 'Williams-Zeef' was assessed. Six concentrations of cupric sulfate were used in banana proliferation medium, i.e. 0, 100, 200, 500, 1000 and 1500ppm. Results clearly showed the harmful effect of copper on banana performance reflected by a significant reduction in survival rate, leaf number, fresh and dry weights as well as a complete inhibition of root formation at higher concentrations. Furthermore, excess of copper was able to reduce photosynthetic pigments contents (chlorophyll-A, -B and carotenoids). The two banana cultivars were affected differently under copper levels. In general, 'Williams-Zeef' was more tolerant than 'Grand Nain' as exposed by less reduction in both morphological and physiological characteristics. *In vitro* screening protocol used in this investigation was easy, simple, inexpensive and reduces time and space which are regularly needed to achieve such tasks. With which it was easily to figure out the harmful effect of copper and clearly differentiate between the tested banana cultivars. The results of this study could help in banana improvement by selecting the proper genotypes to be used in copper-contaminated soils, after increasing the number of screened cultivars.

Keywords*: Musa, Environmental pollution, In vitro screening, Heavy metals*

1. Introduction:

Banana (*Musa* spp.) is one of the most important fruit crops cultivated in tropical and subtropical regions of the world. It contributes to the food security of millions of people in the developing countries (Bakry *et al*., 2009). It is grown in every humid tropical region and constitutes the 4th largest fruit crop worldwide. Banana production occupies an important share in the total fruit of Egypt. The annual production of banana by about 120 countries is about 106.7 million tons (FAO, 2015). In Egypt, the total cultivated area of banana in 2009 was about 2700 ha (about 6480 fed.) with a production of about 1,100,000 tons (FAO, 2009).

Banana is one of the best choices for new reclaimed lands such Sinai and Western desert. However, it faces several abiotic stresses including drought, salinity and heavy metals. Heavy metal pollution is one of the most troublesome environmental problems faced by plant species. Copper poses serious problems due to its widespread industrial and agricultural use (Fernandes and Henriques, 1991). It is an essential micronutrient for normal plant growth and constitutes of the protein component of several enzymes in plants, mainly those participating in electron flow, catalyzing redox reactions in mitochondria، chloroplasts, cell wall and cytoplasm of plant cells (Lolkema, 1985). However، exposure to high concentration of copper can cause a broad range of deleterious effects such as inhibition of photosynthesis, damage to plasma membrane permeability as well as other metabolic disturbances, either in field plants (Lanaras *et al.,* 1993؛ Ouzounidou *et al*., 1993) or *in vitro* grown plants (Gori *et al.,* 1998; Romeu–Moreno and Mas, 1999). The content of total and available copper in the Egyptian soils is in increase due to irrigation with sewage water in some areas and the intensive use of pesticides, which have a noticeable content of heavy metals. Copper status in Egyptian lands varied depending on geographic location, however it exceeded 100ppm in some areas (El-Sayed, 1988). This concentration could be increased by 6.78 times in soils irrigated with sewage water as compared to non-irrigated soils (Selem *et al.,* 2000).

Plant tissue culture technique provides an easy controlled system for stress screening. These *in vitro* systems decrease the environmental factors affecting screening and provide controlled tool with symmetrical balance of stress application (Errabii *et al*., 2006). Moreover, tissue culture-based screening has the simplicity to manipulate large number of plants and stress treatments with a limit space and in a short time (Misra *et al.,* 2002; Abouzaid *et al.,* 2016; Elazab and Youssef, 2017)

In vitro assessment of copper toxicity on plant growth and development has been achieved in several plant species, including *Nicotiana tabacum* (Gori et al., 1998), tomato (El-Aref and Hamada, 1998), *Citrus reticulata* (Chakravarty and Goswami, 2000), *Alianthus altissima* (Gatti, 2008) and date palm (AL-Mayahi, 2014). However, a limit number of investigations is available on the effect of copper toxicity on banana (Nassar, 2004; Deo and Nayak, 2011). Therefore, the aim of the present study was to assess the *in vitro* performance of two banana commercial cultivars, i.e. 'Grand Nain' and 'Williams-Zeef' under different levels of copper.

2. Materials and Methods:

2.1. Plant Materials and *in vitro* conditions

In vitro regenerated plantlets of 'Grand Nain' and 'Williams-Zeef' banana commercial cultivars (*Musa acuminata* Colla, subgroup Cavendish) were obtained from the private Zamzam Tissue Culture Laboratory, Cairo, Egypt. Plantlets were subcultured three times at an interval of 30 days. The proliferation culture media consisted of the full strength MS medium (Murashige and Skoog, 1962) supplemented with 22 µM 6-benzyleaminopurine (BAP), 30.0 g/L sucrose and solidified with 2.0 g/L gelrite. The cultures were incubated at 26°C ± 1°C under 16 hr light regime.

2.2. Copper treatment

After the third subculture, banana plantlets were transferred to full MS medium with the following treatments: 100, 200, 500, 1000 and 1500ppm $CuSO_4.5H_2O$ with a control of copper free medium. All media were supplemented with 30.0 g/L sucrose and 2.0 g/L gelrite. Cultures were incubated as described above.

2.3. Morphophysiological evaluation

After 5 weeks of treatment, plantlets were harvested, and some measurements were recorded on proliferated plantlets. Those included survival percentage, leaf number (LN), root number (RN), fresh (FW, g) and dry weight (DW, g) and copper uptake (mg/g DW). In addition, photosynthetic pigments contents (mg/g FW), i.e. chlorophyll-A, -B and carotenoids were estimated spectrophotometrically according to Lichtenthaler (1987).

2.4. Data analysis

A complete randomized design was used to perform the experiment. Three replicates with 4 jars each were used per treatment. Analysis of variance was performed utilizing MSTAT-C statistical program (Nissen, 1984). Means were compared using Duncan's least range test at 1% probability level.

3. Results and Discussion

Copper is an essential micronutrient for normal plant growth and metabolism. It plays a vital role in many metallo enzymes, photosynthesis-related plastocyanin, and membrane structure (Li and Xiong, 2004). However, when it exists in a high concentration, copper inhibits plant growth and seed germination, induces chlorophyll degradation, and interferes with photosystem activity (Caspi *et al*., 1999).

In the present study, the toxic effect of copper on *in vitro* performance of the two banana cultivars (i.e. 'Grand Nain' and 'Williams-Zeef') was evaluated. Results showed that copper affected all the morphological and physiological characteristics in both cultivars. However, 'Williams-Zeef' was – in general – more tolerant than 'Grand Nain', reflected by lower reduction in the most evaluated parameters. Analysis of variance showed highly significant differences due to the different copper concentrations compared to control in all studied traits (Table 1). On the other hand, among the morphological traits, the two cultivars showed highly significant effect in all evaluated characteristics except leaf and root numbers.

Regarding the morphological traits, the survival rate was affected in both cultivars in a different manner compared to control, No difference was detected due to 100ppm copper in both cultivars. Particularly, in 'Grand Nain', the significant reduction in survival rate began from the concentration 500ppm in which it reached 64.3%. Moreover, the highest level of copper (1500ppm) was enough to reduce the survival rate with 68.7% compared with control (Table 2). On the other hand, although the survival rate was gradient-affected in 'Williams-Zeef' through the successive increased levels of copper, No significant differences were observed in all treatments compared to control. This result clearly reflected the difference of the two cultivars in their response to the toxic effect of copper.

Table 1. Analysis of variance of the *in vitro* performance of two banana cultivars under different levels of copper.

Trait	Source of variance	df	Means of squares
Survival rate	Cultivars (A)	1	0.283**
	Copper concentrations (B)	5	0.239**
	A×B	5	0.047*
	Error	24	0.016
Leaf number	Cultivars (A)	1	0.124 NS
	Copper concentrations (B)	5	19.890**
	A×B	5	0.542*
	Error	24	0.200
Root number	Cultivars (A)	1	0.281 NS
	Copper concentrations (B)	5	37.320**
	A×B	5	0.336 NS
	Error	24	0.411
Fresh weight	Cultivars (A)	1	0.412**
	Copper concentrations (B)	5	2.116**
	A×B	5	0.010 NS
	Error	24	0.034
Dry weight	Cultivars (A)	1	0.004**
	Copper concentrations (B)	5	0.012**
	A×B	5	0.001 NS
	Error	24	0.00001
Cu uptake	Cultivars (A)	1	5.048**
	Copper concentrations (B)	5	202.227**
	A×B	5	9.156**
	Error	24	0.606
Chlorophyll-A	Cultivars (A)	1	0.010*
	Copper concentrations (B)	5	0.490**
	A×B	5	0.027**
	Error	24	0.002
Chlorophyll-B	Cultivars (A)	1	0.024**
	Copper concentrations (B)	5	0.015**
	A×B	5	0.001*
	Error	24	0.00001
Carotenoids	Cultivars (A)	1	0.001 NS
	Copper concentrations (B)	5	0.076**
	A×B	5	0.003**
	Error	24	0.00001

*, **: significant and highly significant at 0.05 and 0.01 levels of probability, respectively. NS: non-significant.

The number of leaves (LN) and roots (RN) in the two evaluated cultivars was almost the same at control. Copper treatments affected LN and RN significantly in both cultivars starting from the lowest concentration (100ppm) with a reduction of 45.49 and 53.03% in 'Grand Nain' and 'Williams-Zeef' respectively (Table 2). Moreover, copper levels were enough to prevent rooting completely in both cultivars initiated from 200ppm (Table 2). In addition, all evaluated concentrations of copper significantly affected the fresh and dry weight in both cultivars. However, no significant differences were observed between the two cultivars, which indicate that both cultivars showed the same response to copper in these traits. The effect of copper toxicity on *in vitro* growth of the two banana cultivars is shown in Figure (1).

Table 2. Averages of some morphological characteristics of two banana cultivars under different levels of copper.

Trait	Cultivar	Copper concentration (ppm)					
		Control	100	200	500	1000	1500
SR (%)	GN	$100±0.00^{A}$	$100±0.00^{A}$	$86.70±13.33^{AB}$	$64.3±15.67^{BC}$	$46.7±7.13^{CD}$	$31.3±8.67^{D}$
	Zeef	$100±0.00^{A}$	$100±0.00^{A}$	$94.3±5.67^{AB}$	$87.7±6.23^{AB}$	$82.0±1.00^{AB}$	$71.3±4.33^{ABC}$
LN	GN	$5.65±0.24^{A}$	$3.08±0.37^{B}$	$1.67±0.35^{CDE}$	$0.64±0.16^{EF}$	$0.47±0.07^{F}$	$0.31±0.09^{F}$
	Zeef	$5.11±0.39^{A}$	$2.40±0.20^{BC}$	$1.80±0.32^{CD}$	$1.04±0.23^{DEF}$	$1.14±0.23^{DEF}$	$1.02±0.21^{DEF}$
RN	GN	$6.75±0.38^{A}$	$0.39±0.24^{B}$	$0.00±0.00^{B}$	$0.00±0.00^{B}$	$0.00±0.00^{B}$	$0.00±0.00^{B}$
	Zeef	$5.61±1.17^{A}$	$0.47±0.24^{B}$	$0.00±0.00^{B}$	$0.00±0.00^{B}$	$0.00±0.00^{B}$	$0.00±0.00^{B}$
FW	GN	$2.23±0.26^{A}$	$0.98±0.08^{B}$	$0.87±0.04^{B}$	$0.72±0.15^{B}$	$0.76±0.01^{B}$	$0.68±0.05^{B}$
	Zeef	$2.05±0.14^{A}$	$0.61±0.05^{B}$	$0.64±0.07^{B}$	$0.56±0.06^{B}$	$0.56±0.04^{B}$	$0.54±0.02^{B}$
DW	GN	$0.180±0.01^{A}$	$0.083±0.00^{C}$	$0.080±0.00^{D}$	$0.073±0.01^{E}$	$0.05±0.00^{F}$	$0.04±0.00^{G}$
	Zeef	$0.14±0.01^{B}$	$0.05±0.01^{F}$	$0.05±0.00^{F}$	$0.05±0.01^{F}$	$0.05±0.00^{F}$	$0.04±0.00^{H}$
Cu uptake	GN	$1.04±0.04^{I}$	$4.35±0.21^{H}$	$6.41±0.35^{F}$	$11.11±0.63^{D}$	$13.00±0.05^{C}$	$19.73±0.43^{A}$
	Zeef	$4.35±0.24^{H}$	$4.57±0.29^{GH}$	$6.22±0.91^{FG}$	$9.05±0.56^{E}$	$9.13±0.42^{E}$	$17.82±0.46^{B}$

Values represent means ± standard error, SR: survival rate, LN: leaf number, RN: root number, FW: fresh weight, DW: dry weight, GN: 'Grand Nain' and Zeef: 'Williams-Zeef'. Different letters in the same trait indicate significance based on Duncan's test (α = 0.05, n = 3).

The effect of *in vitro* toxicity of copper on banana growth and development has been studied in a limit number of investigations. In this regard, in accordance with our results high level of $CuSO_4$ (100 μM) showed toxic effect on banana leaves and completely inhibited root formation (Nassar, 2004). Moreover, the effect of different concentrations of copper on growth and development of *Musa acuminata* has been assessed (Deo and Nayak, 2011). Matching with the findings of the current study, the authors found that higher level of copper (100 μM) showed toxic effect on banana dry matter, shoot and root growth, as well as it completely inhibited root formation.

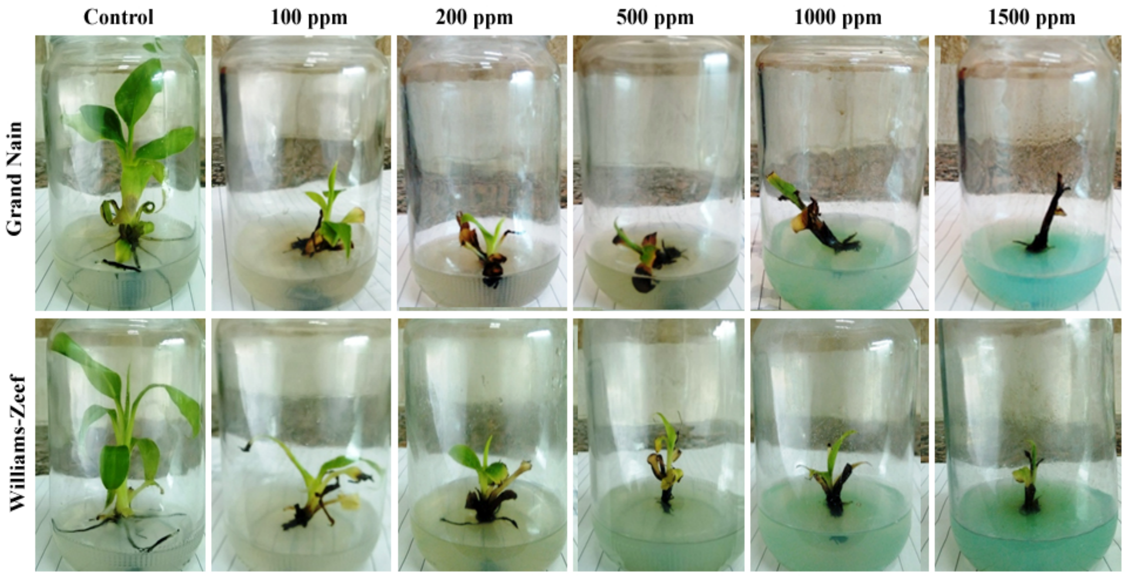

Figure (1): *In vitro* performance of two banana cultivars under different concentrations of copper.

Additionally, the toxic effect of copper has been assessed *in vitro* in several plant species. In this regard, Gori *et al.,* (1998) reported that *in vitro* copper treatment with a concentration higher than 50µM was enough to inhibit callus growth and shoot regeneration in *Nicotiana tabacum*. Moreover, copper was toxic to tomato explants at 100µM, it caused a reduction in callus growth and shoot regeneration. In addition, copper reduced the expression of some enzymatic bands of alcohol dehydrogenase and esterase (El-Aref and Hamada, 1998). Matching with our findings, Chakravarty and Goswami, (2000) reported that, high copper concentrations affected root growth more than shoot growth and inhibited the increase in fresh and dry weight of *in vitro* cultures of *Citrus reticulata*. Gatti, (2008) studied the *in vitro* tolerance of *Alianthus altissima* to some heavy metals including copper, they reported that shoot growth was reduced at higher copper concentrations. Furthermore, in date palm, relative low concentration of copper (8µM) was enough to reduce several morphological traits including shoot formation, number of shoots per callus, rooting percentage, number of roots and length of plant and root. In addition, other physiological characteristics were also reduced including the content of carbohydrates, total protein, total phenol and chlorophyll (AL-Mayahi, 2014).

Copper uptake was measured in the present study in both cultivars after the different treatments. Results showed that, the concentration of copper in plant tissue through all treatments was higher in 'Grand Nain' than that in 'Williams-Zeef' (Table 2). This result may explain the different performance of the two cultivars and their different response under copper treatments.

Furthermore, the content of photosynthesis related pigments (i.e. chlorophyll-A, -B and carotenoid) was detected in both cultivars under control and different copper levels. Results herein clearly showed the harmful effect of copper on the content of these pigments. In this regard, analysis of variance showed significant differences between the two banana cultivars in all photosynthesis traits except carotenoid content (Table 1). In addition, ANOVA exhibited highly significant differences between copper different levels and control. The more concentration of copper the more reduction was found in chlorophyll-A and -B (Figure 2). Copper treatment under all its concentrations significantly affected the carotenoid content in both cultivars compared with control. Interestingly, among copper levels, the concentration of 500ppm gave the highest content of carotenoids (Figure 2). In addition, 'Williams-Zeef' – generally – showed more tolerance than 'Grand Nain' reflected by less reduction in the content of photosynthesis related pigments under different copper concentrations (Figure 2).

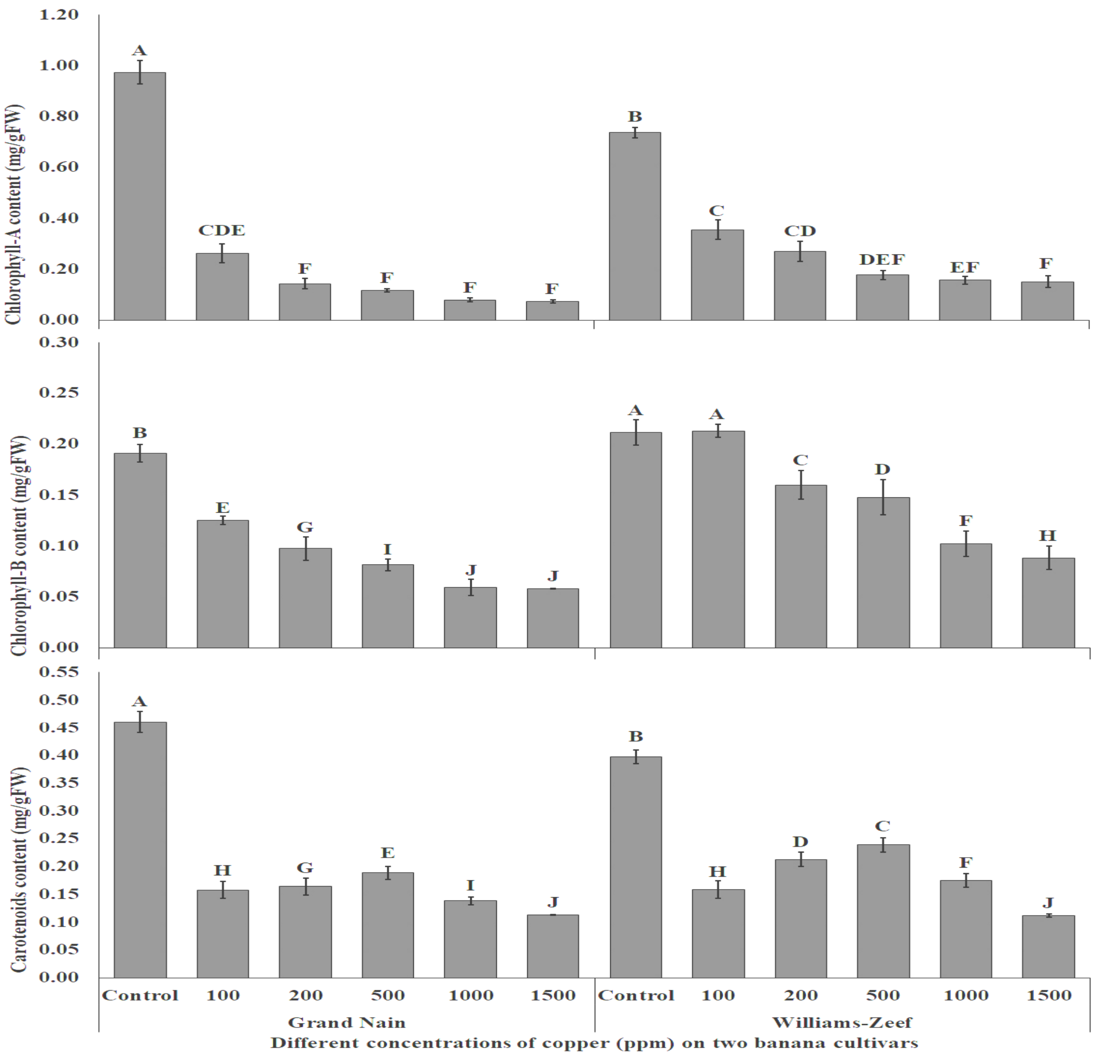

Figure (2): Averages of photosynthetic pigments content; upper: chlorophyll-A, middle: chlorophyll-B and lower: carotenoids, of two banana cultivars under different copper concentrations. Different letters in the same trait indicate significance at 0.01 probability level, Duncan's test (n = 3).

Excess copper inhibits many enzymes and interferes with several aspects of plant biochemistry, including photosynthesis, pigment synthesis, and membrane integrity (Fernandes and Henriques, 1991). Matching with our findings, copper induces chlorophyll degradation, and interferes with photosystem activity (Caspi *et al*., 1999). In addition, photosynthetic pigments contents in banana were increased under lower levels of copper; however, they were significantly reduced under high concentration, compared to control (Deo and Nayak, 2011). Unlike our results, Gori et al., (1998) did not found any differences in chlorophyll contents or chloroplast structure in tobacco due to *in vitro* copper treatments as compared with control. However, they used lower concentration (100µM) than what has been used in the current study.

In conclusion, this investigation clearly showed the harmful and toxic effect of copper on banana *in vitro* growth and development. Excess of copper in culture medium was able to reduce survival rate, leaf number, fresh and dry weight and completely inhibited root formation. Moreover, photosynthetic pigments contents were also reduced significantly due to copper toxicity. Although both cultivars tested in the present study have been affected by copper stress, 'Williams-Zeef' was in general more tolerant than 'Grand Nain'. Unlike previous investigations, the extreme high concentrations of copper used herein were helpful to differentiate between the two banana cultivars and draw a good explanation about how banana tolerate copper stress *in vitro*. These findings could help in selecting tolerant genotypes of banana after increasing the number of screened cultivars to choose the proper for copper contaminated soil.

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تقييم أداء صنفين من الموز معملياً تحت ظروف إجهاد النحاس

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الملخص

يعتبر التلوث البيئي أحد أهم العوامل التي تؤثر سلباً على حياة الانسان بطريقة مباشرة أو غير مباشرة. وتعتبر العناصر الثقيلة أكثر الملوثات البيئية تأثيراً على نمو وانتاجية النبات. لذا، ففي هذه الدراسة تم تقييم تأثير سمية عنصر النحاس معملياً على نمو وتطور صنفين من الموز (جراند نان وويليامز زيف). حيث تم استخدام ست تركيزات من كبريتات النحاس في البيئة الغذائية (صفر، ١٠٠، ٢٠٠، ٥٠٠، ١٠٠٠ و١٥٠٠ جزء في المليون). أظهرت النتائج بشكل واضح التأثير الضار للنحاس على أداء نباتات الموز معملياً حيث انعكس ذلك على انخفاض معنوي في معدل البقاء وعدد الأوراق والوزن الطازج والجاف كما تم تثبيط تكون الجذور كلياً تحت التركيزات المرتفعة. بالإضافة لذلك، فقد سبب مستوى النحاس في البيئة على تقليل محتوى الكلوروفيل بنوعيه والكاروتينويد. وتأثر صنفي الموز بشكل مختلف لمستويات النحاس، حيث أظهر الصنف ويليامز زيف درجة تحمل أعلى من الصنف جراند نان واتضح ذلك من قلة درجة تأثر الصفات المورفولوجية والفسيولوجية له تحت الاجهاد. ويعتبر البروتوكول المستخدم في هذه الدراسة لتقييم الموز تحت إجهاد النحاس بسيط وسهل وغير مكلف كما أنه يوفر الوقت والمساحة المطلوبة عادة لإجراء مثل هذا النوع من التقييمات. حيث أمكن باستخدامه اظهار التأثير الضار للتركيزات العالية من النحاس كما أظهر بوضوح الفرق بين صنفي الموز في درجة تحملهما للإجهاد. ويمكن الاستفادة من نتائج هذه الدراسة في انتخاب الطرز الوراثية المتحملة من الموز والمناسبة للزراعة في الأراضي الملوثة بتركيزات عالية من النحاس بعد فحص عدد كبير من الأصناف.