STIMULATION OF HEAVY METAL TOLERANCE IN BROCCOLI (*BRASSICA OLERACEA*) IN VITRO

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Broccoli (*Brassica oleracea* var. *italica*) is highly recommended and important vegetable crop due to its flavor and the secondary metabolites that have anticancer activity. Contamination vegetable by heavy metals (HMs) considered one of major problems, causing a high risk to human health. This study investigated the impact of moringa seeds crude protein extract (CPE) used at four levels (10, 20, 30 and 40% w/v) on vegetative and biochemical parameters and the HMs removal percentages in broccoli plantlets cultured in Murashige and Skoog (MS) medium contaminated with full strength of HMs (Cd, Pb, Zn, Ni, Fe, Co, Mo, Cu, Cr mM/L) in vitro. The results indicated that the most efficient micropropagation protocol for broccoli was MSm8 (MS medium supplemented with 0.50 mg/L BAP + 0.50 mg/L KIN + 0.25 mg/L NAA) for multiplication. Inducing white true roots was more effective by MSr 4 (MS medium supplemented with 1.00 mg/L NAA. Adding moringa CPE had a positive impact on improving vegetative parameters in broccoli plantlets cultured in MS media contaminated with full strength of HMs and the maximum parameters were recorded at M2 (20%) of moringa CPE. Biochemical parameters also increased directly with increasing the moringa CPE levels, and the highest level (40%) of moringa CPE raised the scavenging rate of free radicals to (45%). Furthermore, the malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) quantities dropped significantly by 48.7% and 78.5%, respectively. In addition, the highest heavy metal removal percentages (Cd, Pb, Zn, Ni, Fe, Co, Mo, Cu, Cr mM/L) were 99.96, 80.01, 98.99, 99.82, 60.63, 99.90, 97.32, 99.61, and 80.22 mM/L, respectively, in comparison to the positive control in broccoli plantlets *in vitro*. Investigation postulated that moringa crude protein extract (CPE) had a positive effect in reducing the toxic effect of HMs on the plants grown in the contaminated areas.

Keywords: Brassicaceae, micropropagation, moringa, seeds crude protein extract, bioremediation

INTRODUCTION

Broccoli (*Brassica oleracea* var. *italica*) is a biennial plant that belongs to the family Brassicaceae, and it is an important edible green vegetable, with a stalk, large flowering head, and small leaves, and is regarded as a significant source of phytonutrients-natural substances and secondary metabolites that are good for human health, such as the compound glucoraphanin, which is converted into an anticancer agent called sulforaphane (Huang et al., 2011; Raiola et al., 2018 and Sahai and Kumar, 2020). Metals like zinc, iron, and copper are essential micronutrients required for a wide range of physiological processes in all plant organs through the activities of metal-dependent enzymes and proteins. However, they can also be toxic at elevated levels (Sepehri et al., 2018).

Phytoremediation is a green plant's ability to accumulate environmental contaminants, which is regarded as one of the cheapest and most eco-friendly technologies for cleaning up contaminated soil. It is mainly used for removing HM and soil radioactive elements (Sepehri et al., 2018). Cruciferous plants are among the most vulnerable to the absorption of some heavy metals (HMs) like cadmium (Cd), lead (Pb), manganese (Mn), zinc (Zn), nickel (Ni), copper (Cu), chromium (Cr), mercury (Hg) and arsenic (As), etc. They can be accumulated at high levels in the edible parts of the plant, which has highly toxic effects on the plant's vitality, inhibiting photosynthesis and transpiration, disturbing the metabolism of carbohydrates and causing secondary stresses such as oxidative and nutritional stress, which affect the plant's growth and development. In addition, there is a toxic cumulative effect on human health when consumed (Radwan and Salama, 2006 and Marzena et al., 2011).

Broccoli is in great demand due to its useful health properties based on the contents of many important vitamins, fiber, secondary metabolites and phenolic compounds. The great demand means that there is a need for high yields, which inevitably leads to exposure to environmental biotic and abiotic stresses, causing negative impacts on the final quantitative and qualitative yield (Albaladejo-Marico et al., 2024). In vitro micropropagation is a process that plays a very important role in the mass production of uniform plants. In vitro techniques have been applied to the Brassicaceae family from different points of view, resulting in somatic embryogenesis, regeneration and organogenesis (Singh, et al., 2022). In recent years, major research has used tissue culture techniques for crop improvement in the family Brassicaceae to enhance tolerance against environmental stress (Huang et al., 2011).

Despite the major efforts that have been made over recent years, HMs contamination in ecosystems has been increasing consistently worldwide, especially in developing countries, including Egypt. Meanwhile, demand for clean water resources for household, industrial, agricultural and commercial activities is increasing every day. Wastewater resulting from homes, industries

and the irrational use of pesticides in agriculture, as well as wastes used for de-acidifying soils; which contain inorganic compounds such as HMs; is often discharged into rivers, especially in arid and semiarid regions where clean water is scarce. Metals sorbed in soil and contaminating water can be transferred to plants, which are then consumed by livestock or humans, and accumulation can harm their health (Fu and Wang, 2011 and Ashraf et al., 2019).

A significant increase in the consumption of vegetables contaminated by HMs has been reported in recent years, which is a threat to human health. While the consumption of vegetables is an essential part of a balanced diet (Siegel et al., 2014 and Sepehri et al., 2018), they are important in the inhibition of free radicals and prevention of oxidative damage. However, metals such as Zn, Cu, Fe and Cr are essential to human health in trace amounts, but at high concentrations they have toxic and carcinogenic effects, causing health issues ranging from bone damage, nausea, vomiting, fibrosis, seizures, pulmonary cramps and skin irritation to damage to the kidneys, liver and central nervous system and even death. Hence, long-term consumption of polluted vegetables can lead to the accumulation of toxic levels of HMs, resulting in severe health problems (Ahmad and Goni, 2010; Li et al., 2014; Sharma et al., 2016; Khan et al., 2021 and Kiran et al., 2023).

Based on that, there is a need for safe, effective and economical ways to remove HMs by using biosorption from wastewater. Biosorption is defined as the biological ability of materials to adsorb HMs from wastewater, through a combination of metabolic mediation and physicochemical uptake (Sonal and Kuldip, 2022). Biological materials are considered a suitable solution with the following advantages: (i) readily available, (ii) little processing required, (iii) good adsorption capacity at low concentrations of metal, (iv) selective adsorption of HM ions and (v) easy regeneration (Liu, 2020 and Costa et al., 2021).

Moringa (*Moringa oleifera* Lam.) grows in tropical and subtropical zones. Previous studies indicate that crude protein extract of moringa seeds is one of the most effective biological materials that have a large adsorption capacity for removing HMs from wastewater. Moringa is easily cultivated and adapted in arid and semiarid regions and can contribute significantly to sustainable development. Most parts of the tree are edible, very beneficial to human health, with many applications and used as an excellent coagulant agent for HM removal, organic pollutant elimination and water purification (Krishnani and Ayyappan, 2006; Demirbas, 2008; Fu and Wang, 2011; Konada et al., 2017 and Ali and Seng, 2018). Moringa is a multipurpose tree and the seeds are rich in phytochemicals with antimicrobial activities. HM removal from aqueous solutions through biosorption has been well documented by using *Moringa oleifera* seed crude powder due to the presence of MoCP or coagulant protein, which acts as a polyelectrolyte cationic. The

soluble particles attach to the active agents in the water, binding them together and creating large flocs (Abd El-Kader et al., 2013; Saif et al., 2014; Lalhruaitluanga and Prasad, 2015 and Sepehri et al., 2018).
This study was conducted to investigate the potential of crude protein extract (CPE) from moringa seeds for reducing the accumulation and toxic effect of HM stress in in vitro propagated broccoli plants.

MATERIALS AND METHODS

Study was carried out at the Tissue Culture Unit Labs, Genetic Resources Department, Desert Research Centre, Cairo, Egypt.

1. Culture Medium

All experiments were conducted in vitro using MS medium (Murashige and Skoog, 1962) micro- and macro-elements (MS; Sigma, Germany). The pH of all media was adjusted to 5.8 using 1.0 M HCl or 1.0 M NaOH. Phytagel was used at 3-4% (w/v) and added after adjusting the pH. Media were distributed in culture tubes or jars and then sterilized for 21 min at 121°C (15-psi steam pressure for 30 min).

2. In Vitro Propagation of Broccoli

2.1. Explants preparation

Seeds of broccoli (*Brassica oleracea* var. *italica*) J1 from Australia were washed many times under running tap water and soaked for 30 min, and then the seeds were sterilized by dipping them in 70% ethanol for 5 sec, then immersed in 15% commercial NaOCl (3.25%) for 8-10 min. Finally, seeds were rinsed with distilled and sterile water three times for 10 min each.

2.2. In vitro seed germination

Sterilized seeds were placed under aseptic conditions in culture jars containing sterilized cotton and 30 ml of distilled water, with 15-20 seeds per jar. Seeds were incubated at 20±2°C under dark conditions for 4–6 days until they started germination.

2.3. In vitro multiplication and rooting culture

For the multiplication stage, sterilized plantlets (4.00–5.00 cm) were cultured in MS multiplication medium fortified with 30 g/L sucrose and different plant growth regulators (PGRs). Ten protocols were tested for multiplication and seven protocols for rooting (Table 1). One explant was placed per tube, with 8 replicates for each protocol and 2 tubes per replicate. The media were solidified with 3–4 g/L phytagel. Cultures were incubated at 22±2°C and 16 h light (2000 lux, fluorescent tubes) for 15-20 days to induce in vitro proliferated shoots. For mass multiplication of stock plants, we sub-cultured them 2-3 times at least on the optimal multiplication protocol medium before the rooting step (20-25 days). Survival percentage, number and length (cm) of shoots, number of leaves and number and length (cm) of roots were recorded for clusters.

Table (1). Plant growth regulator (PGR) protocols used in broccoli micropropagation in vitro.

PGR component			
MSm Protocols	**Multiplication (MSm)**	**MSr Protocols**	**Rooting (MSr)**
Protocols 1	MS free medium	Protocols 1	MS free medium
Protocols 2	0.5 mg/L BAB + 0.125 mg/L NAA	Protocols 2	0.25 mg/L NAA
Protocols 3	1 mg/L BAB + 0. 25 mg/L NAA	Protocols 3	0.50 mg/L NAA
Protocols 4	2 mg/L BAB + 0.50 mg/L NAA	Protocols 4	1.00 mg/L NAA
Protocols 5	0.5 mg/L KIN + 0.125 mg/L NAA	Protocols 5	0.50 mg/L IBA
Protocols 6	1 mg/L KIN + 0.25 mg/L NAA	Protocols 6	1.00 mg/L IBA
Protocols 7	2 mg/L KIN + 0.5 mg/L NAA	Protocols 7	2.00 mg/L IBA
Protocols 8	0.5 mg/L BAB + 0.5 mg/L KIN + 0. 25 NAA		
Protocols 9	0.5 mg/L 2iP + 0.5 mg/L GA_3		
Protocols 10	1 mg/L TDZ + 0.5 mg/L GA_3		

*MSm: MS medium fortified with PGRs for multiplication protocols; MSr: MS medium fortified with PGRs for rooting protocols; Calcium pantothenic acid (2.00 mg/L) was added to all treatments.

3. In Vitro HM Stress

Proliferated plantlets were separated into two-node explants and cultured in tubes containing 10 ml of MS multiplication medium contaminated with full strength of HM formula (Cd, Pb, Zn, Ni, Fe, Co, Mo, Cu, Cr mM/L). HM blanks (mM/L) were prepared according to Ahmed et al. (2020) and Afify and Amaal (2022) (Table 2). MS contaminated medium was used individually or combined with five levels of moringa crude protein extract (0, 10, 20, 30 and 40% w/v), in addition to the MS control medium (6 treatments, Table 3). One explant was cultured per tube and ten tubes were considered one treatment. Cultures were incubated for 6 weeks at 22±2°C under 16 h light (2000 lux, fluorescent tubes).

Table (2). The full-strength formula of heavy metals (HMs) used in the investigation by ICP.

Heavy metal elements	**Contaminated water with HMs blank (mM/L)**
Cd	6.672
Pb	0.005
Zn	11.848
Ni	1.107
Fe	0.044
Co	2.029
Mo	29.101
Cu	1.026
Cr	0.059

4. Preparation of Moringa Seed Protein Extract

Moringa (*Moringa oleifera* Lam.) crude protein was extracted from the seeds according to Ghebremichael et al. (2006). Seed husks were removed and the kernels were ground using an electric homogenizer. Seed oil was removed from the powder by mixing with 95% ethanol for 30 min. Solids were separated by centrifugation for 10 min at 4000 rpm and dried at room temperature overnight. A 5% (w/v) solution was prepared using distilled water from the dried samples. The solution was mixed for 30 mins and filtered using Whatman paper (no. 3) and 0.45 µm fiberglass. Seventy ml of moringa CPE was obtained from 100 g of seeds.

Table (3). MS multiplication protocol medium fortified with heavy metals (HMs) formula combined with different levels of moringa crude protein extract (CPE) (w/v) in vitro.

Treatments	Culture media components
MS control	MS medium free of PGRs
HM. M0	MS medium + HM
HM. M1	MS medium + HM + 10% CPE
HM. M2	MS medium + HM + 20% CPE
HM. M3	MS medium + HM + 30% CPE
HM. M4	MS medium + HM + 40% CPE

5. Biochemical Content Analysis

5.1. Sample preparation

Samples of three plants were randomly chosen from each in vitro treatment. Broccoli in vitro plantlets cultured in the MS multiplication medium with HM contamination were combined with moringa CPE levels for 45 days. Samples of vegetative plants were randomly taken in three replicates for each treatment. Half of the samples were stored in a deep freezer at -20°C, while the other half were dried in an oven at 65°C until a consistent weight was achieved. This was done for the required chemical analyses. Malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and radical scavenging capacity (RSC%) were measured. HMs were detected after the dried plants were digested using 30% H_2O_2 and concentrated sulfuric acid, as per Lowther (1980).

5.2. Determination of MDA

The color of adduct generated by the reaction between thiobarbituric acid (TBA) and MDA in the TBA assay was used to quantify lipid peroxidation in broccoli explants (Heath and, Packer, 1968 and Zhao et al., 1994). The supernatant was measured at 532 nm. The absorbance at 532 nm corrected by subtracting nonspecific absorption measured at 600 nm. The MDA-TBA concentration was calculated from the MDA standard curve and converted to µg/g fresh weight.

5.3. Determination of H_2O_2

H_2O_2 was determined in vitro based on potassium iodide oxidation in acidic media (Velikova et al., 2000). Spectrophotometric analysis was used to measure absorbance at 390 nm. The concentration of H_2O_2 was calculated using a standard curve and converted to µg/g fresh weight.

5.4. Antioxidant capacity in vitro assay

The 2,2-di-phenyl-1-picrylhydrazyl (DPPH) assay was measured using methanolic extract of fresh broccoli explants, combined with 2 ml of 0.004% DPPH in 80% methanol. About 2.5 ml of 80% methanol was used to homogenize 0.5 g of fresh broccoli explant. After mixing for 30 sec, the reaction mixture was kept at room temperature for 30 min. Absorbance was measured at 517 nm (Oktay et al., 2003). RSC% was calculated as:

$$RSC\ \% = [(A\ blank – A\ sample) / A\ blank] \times 100$$

Where A blank is the absorbance of the methanolic DPPH solution and A sample is the absorbance of the plant methanolic extract.

5.5. Assessment of HM levels

Using inductively coupled plasma mass spectrometry (ICP-MS), the levels of HM in tap water, treated water, and the digested solution of broccoli plant samples were determined (Ali et al., 2015).

6. Experimental Design and Statistical Analysis

All experiments were subjected to a completely randomized design. Recorded data were analyzed statistically using analysis of variance (ANOVA). Separation of means was performed to compare the differences between means, using Duncan's range test (1955) at a significance level of ($p \leq 0.05$).

RESULTS AND DISCUSSION

The present study was conducted to obtain the optimum micropropagation (shoot multiplication and rooting) medium for mass production of broccoli explants in vitro, and to study the impact of moringa crude protein extract levels on the reduction of accumulation and the toxic effect of HM stress on broccoli plants in vitro.

1. In Vitro Micropropagation

For shoot multiplication (Table 4), the results illustrated the effect of ten different MSm protocols of PGRs on in vitro broccoli explant shoot parameters/clusters after 20-0 days. The maximum survival percentage (96.67%) was recorded with 1.00 mg/L KIN + 0.25 mg/L NAA (protocol 6) medium, without any significant difference compared to the other treatments. Meanwhile, the highest mean shoot length (11.00 cm) was recorded in protocols 1, 5, 6 and 10. The highest mean number of shoots (5.00) and leaves (14.00) was achieved with 0.50 mg/L BAP + 0.50 mg/L KIN+0.25 mg/L NAA

(protocol 8) medium. Broccoli normally needs to be sub-cultured at least once every 2-3 weeks.

Table (4). Effect of ten different PGR media protocols on the vegetative parameters of broccoli explants/clusters in vitro after (20–30) days.

Protocols	Survival (%)	Shoot (number/cluster)	Shoot length (cm)	Leaves (number/cluster)	Notes
Protocol 1	90.00 a	1.00 b	11.00 a	9.67 bc	large leaves
Protocol 2	80.00 a	4.76 a	6.00 cd	9.00 bc	small leaves
Protocol 3	80.67 a	3.33 a	4.00 d	8.33b-d	small leaves
Protocol 4	91.67 a	4.71 a	6.00 cd	6.33 c-e	small leaves
Protocol 5	90.00 a	1.00 b	11.00 a	5.00 de	small leaves
Protocol 6	96.67 a	1.33 b	11.00 a	5.00 de	large leaves
Protocol 7	93.33 a	1.33 b	7.33 bc	5.00 de	small leaves
Protocol 8	93.33 a	5.00 a	9.67 ab	14.00 a	large leaves
Protocol 9	80.00 a	1.33 b	3.17 d	3.33 e	small leaves
Protocol 10	71.33 a	5.00 a	11.00 a	11.00 ab	medium leaves

*Means followed by the same letter within a column are at a significance level of ($p \leq 0.05$).

For in vitro rooting, to obtain true rooted (white root) plantlets, the effect of seven different MSr protocols of PGRs on in vitro broccoli explant rooting parameters/clusters after 20-25 days was observed and is shown in Table (5) and Fig. 2. In vitro explants reached 100% survival percentage in the MSr control medium (protocol 1) only, but without inducing any white true roots. The maximum white root percentage (100%) and mean root number (3.33) were recorded with 1.00 mg/L NAA (protocol 4) medium. Meanwhile, the highest mean root length (2.00 cm) was recorded with 2.00 mg/L IBA (protocol 7) MSr medium.

Table (5). Effect of seven different PGR media protocols on the rooting parameters of broccoli (explants/clusters) in vitro after (20-25) days.

Protocols	Survival (%)	True roots (%)	True roots (number/cluster)	True root length (cm)
Protocol 1	100.00 a	0.00 c	0.00 b	0.00 c
Protocol 2	80.00 ab	73.33 b	0.33 b	0.75 bc
Protocol 3	91.67 ab	100.00 a	1.67 ab	1.08 b
Protocol 4	93.33 ab	100.00 a	3.33 a	1.33 ab
Protocol 5	86.67 ab	86.67 ab	2.33 a	0.75 bc
Protocol 6	60.00 b	91.67 ab	2.67 a	1.88 a
Protocol 7	58.33 b	86.67 ab	3.00 a	2.00 a

*Means followed by the same letter within a column are at a significance level of ($p \leq 0.05$).

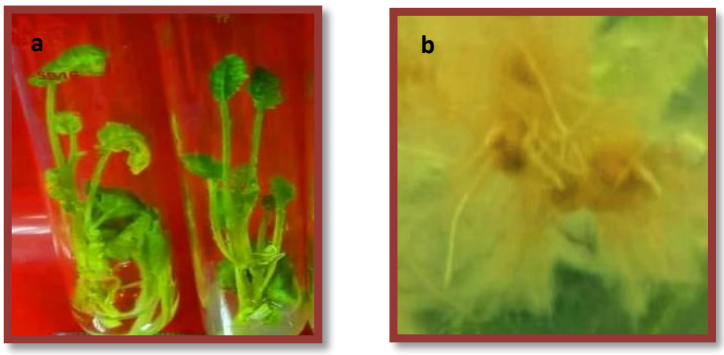

Fig. (1). a. In vitro shoots micropropagation of broccoli, and **b.** induced white true roots in broccoli planets in vitro.

Cytokinins such as 6-benzylaminopurine (BAP) and kinetin (KIN), in combination with auxins such as 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA), are widely used in tissue culture research (Garshasbi et al., 2012; Seyed et al., 2014 and Raghad et al., 2021). The results revealed that the most suitable media for broccoli multiplication were MSm8 (MS medium supplemented with 0.50 mg/L BAP+0.50 mg/L KIN+0.25 mg/L NAA) and MSm6 (MS medium supplemented with 1.00 mg/L KIN+0.25 mg/L NAA). Several studies have proven that BAP alone, or in combination with auxins, is optimal for *Brassica* shoot regeneration and multiplication (Gerszberg et al., 2015 and Thakur et al., 2023). Similar results were obtained in kale, red cabbage, cauliflower, broccoli and Savoy cabbage, where the highest stimulating effect on shoot multiplication was produced using BAP (Monika and Elwira, 2023). BAP with KIN alone or in combination with NAA is one of the most useful cytokinins for shoot regeneration in the Brassicaceae family, including broccoli (Ravanfar et al., 2011; Sharma et al., 2012; Gambhir et al., 2017 and Palvi et al., 2023). In the same trend, Asharf et al. (2014) reported that BAP effectively triggered shoot elongation and multiplication in *Brassica*. Similarly, in fourteen cabbage genotypes, adventitious shoot induction was higher on MS medium supplemented with 1 mg/L BAP (Pavlović et al., 2012). Broccoli was most effectively multiplied in medium supplemented with 1.5 mg/L BAP and 1.0 mg/L NAA (Azis et al., 2019). Nazirwan et al. (2018) showed that the highest broccoli shoot regeneration (70%) was obtained with MS medium containing 1 mg/L KIN+0.5 mg/L NAA. Also, Mollika et al. (2011) reported that the best regeneration for *Brassica juncea* was obtained with MS medium containing 2.0 mg/L BAP, 0.5 mg/L KIN, and 0.2 mg/L NAA.

The success of in vivo acclimatization depends on the production of true white roots in vitro. In our study, MSr4 (MS medium supplemented with 1.00 mg/L NAA) and MSr7 (MS medium supplemented with 2.00 mg/L IBA) were more effective in inducing true white roots from broccoli plantlets in

vitro. Lazzeri and Dunwell (1986) reported that high concentrations of NAA were more effective for rooting compared with IAA or IBA. Furthermore, NAA induced numerous thick white roots, whereas IAA produced only a few transparent, long roots in broccoli plantlets (Ravanfar et al., 2011; Adil and Abbasi, 2019 and Monika and Elwira, 2023). Similarly, Azis et al. (2019) found that MS medium supplemented with 2.0 mg/L NAA, followed by 0.5 mg/L NAA, gave the optimum true root initiation response. Meanwhile, Ogunsola and Ilori (2008) stated that MS medium supplemented with 1.0–2.0 mg/L IBA induced better rooting in broccoli.

2. In Vitro Hms Stress on Broccoli

2.1. The effect of moringa CPE levels on the vegetative parameters of broccoli under HMs stress

Effect of different levels of moringa extract (M 1-4) on vegetative parameters of broccoli explants cultured in the MS multiplication medium contaminated with the full strength of HM elements in vitro. Regarding survival percentage, the data presented in Table (6) and Fig. (2) show that it reached 100% in each of the MS media fortified with HM alone or with moringa extract at HM.M1 (10%) and HM.M2 (20%) levels. While the significantly lowest survival percentages (76.33% and 46.67%) were recorded at the highest levels of moringa extract HM. M3 (30%) and HM. M4 (40%), respectively. Unexpectedly, the shoot length recorded the highest mean value (10.33 cm) with the uncontaminated HM. M0 control medium, but the lowest mean value (4.33 cm) was also recorded in the MS medium with the highest levels of moringa extract HM. M3 (30%) and HM. M4 (40%) respectively. Whereas, the MS medium contaminated with HM plus moringa extract HM. M2 (20%) level gave the highest average values in each of shoot number (9.00) and leaf number (11) per cluster. However, the lowest mean values (4.67, 3.33) were recorded in the HM-contaminated medium (MS con.) only, without moringa CPE. The maximum white root number reached 1.00 root per cluster, only with the uncontaminated MS rooting medium, without any significant differences among the treatments. As for root length (cm), the highest mean value (1.75 cm) was recorded in HM. M3 (30%), while the lowest value (0.75 cm) was achieved in the MS contaminated medium with moringa extract HM. M1 (10%).

Regarding the above results, in general, adding moringa CPE at the first and second levels (10% and 20%) had a positive impact on improving broccoli vegetative parameters under HM stress conditions compared with both the MS M0 control medium and the MS contaminated medium. The maximum parameters were recorded with moringa CPE at level HM. M2 (20%) during micropropagation (multiplication, rooting) in vitro. Moringa seed extract contains high amounts of vitamins, minerals, and proteins (52%) plus essential amino acids, which induce the tolerance of plants under stress by triggering the enzymatic and/or non-enzymatic antioxidant systems,

thereby improving their growth rate and productivity (Khalofah et al., 2022). This result agreed with Abd El-Kader et al. (2013), who confirmed that the mean shoot and root number and length and leaf number significantly increased with increasing moringa CPE levels in rice (*Oryza sativa* L.) plantlets cultured in HM-contaminated MS medium. It has been reported that moringa leaf extract significantly increased tomato seedling vigor (Culver et al., 2012 and Vikashni et al., 2012), as well as growth in wheat (Afzal et al., 2008) and maize (Basra et al., 2011). Also, Muhammad et al. (2024) recorded that using moringa leaf extract improved growth parameters in wheat and also had positive impacts in all growing environments.

Table (6). Effect of moringa crude protein extract (CPE) levels on the biochemical parameters of broccoli plantlets cultured in MS medium contaminated with heavy metals in vitro.

Treatments	Parameters					
	Survival (%)	**Shoots (number/ cluster)**	**Shoot length (cm)**	**Leaves (number/ cluster)**	**Root (number/ cluster)**	**Root length (cm)**
MS control	91.67 ab	5.00 b	10.33 a	10.67 a	1.33 a	1.37 ab
HM. Mo	100.00 a	4.67 b	5.00 bc	3.33 c	1.00 a	1.33 ab
HM. M1	100.00 a	7.00 ab	4.50 c	8.33 ab	1.00 a	0.75 b
HM. M2	100.00 a	9.00 a	7.00 b	11.33 a	1.00 a	1.47 ab
HM. M3	76.33 b	7.33 ab	4.33 c	8.00 ab	1.00 a	1.75 a
HM. M4	46.67 c	5.00 b	4.33 c	7.00 b	1.00 a	1.50 ab

*Means followed by the same letter within a column are at a significance level of ($p \leq 0.05$).

Fig. (2). The effect of moringa crude protein extract (CPE) levels on vegetative parameters of broccoli under heavy metals (HMs) stress.

2.2. Effect of different levels of moringa CPE on biochemical parameters of broccoli plantlets cultured in MS medium contaminated with HMs in vitro

Broccoli in vitro plantlets were cultured in multiplication MS medium contaminated with the full strength of HM elements as a positive control (HM. M0), either individually or combined with four different levels of moringa CPE (10, 20, 30 and 40% w/v), as opposed to the MS control medium (negative control, MS control). Table (7) reveals an increase in the levels of MDA and H_2O_2. The estimated increase in MDA and H_2O_2 relative to the negative control was 2.61 and 4.94 times, respectively. The results also showed that when moringa extract was added to the growing medium containing wastewater, the levels of MDA and H_2O_2 in the leaves of broccoli plants were reduced. Increasing the percentage of moringa extract in the MS contaminated medium led to an increased reduction in MDA and H_2O_2 content in broccoli leaves. When 4% of moringa extract (HM. M4) was added, the MDA level in broccoli leaves decreased by the greatest proportion, 48.67%. Conversely, the addition of 1% moringa extract (HM. M1) led to a 19.28% decrease in the MDA level in broccoli leaves compared with the positive control (HM. M0). Concerning the positive control, the results also demonstrated that, in the agricultural setting, the highest percentage of H_2O_2 accumulation in broccoli leaves was 39.78% when adding 2% moringa extract (HM. M2), while the lowest percentage was 21.52% when adding 4% of the extract (HM. M4). In comparison to the negative control, the data presented in the same table indicated a 34.62% decrease in the percentage of free radical scavenging for broccoli plants cultivated under HM-contaminated conditions. Results also showed that increasing the amount of moringa extract in the culture medium increased the rate at which broccoli plants scavenged free radicals. In comparison to the positive control, the highest percentage of free radical scavenging (45.86%) was recorded when 4% moringa extract (HM. M4) was applied to the culture medium.

Table (7). Effect of moringa crude protein extract (CPE) levels on the biochemical parameters of broccoli plantlets cultured in MS medium contaminated with heavy metals in vitro.

Treatments	Physiological indices under Stress		
	MDA (µg/g FW)	H_2O_2 (µg/g FW)	RSC (%)
MS. Mo	2.46 ± 0.014 e	5.94 ± 0.13 e	63.82 ± 0.67 a
HM. M0	6.43 ± 0.012 a	29.36 ± 0.35 a	29.20 ± 0.11 e
HM. M1	5.19 ± 0.01 b	11.18 ± 0.29 b	34.06 ± 0.35 d
HM. M2	4.03 ±0.02 c	11.68 ± 0.22 b	31.87 ± 0.45 d
HM. M3	3.42 ±0.03 d	7.85 ± 0.16 c	40.35 ± 0.44 c
HM. M4	3.30 ± 0.015 d	6.32 ± 0.11 d	45.86 0.57 b

*Means followed by the same letter within a column are at a significance level of ($p \leq 0.05$).

The results of this study, which show that adding more moringa to the growing medium increased the rate of free radical scavenging and decreased

the amounts of H_2O_2 and MDA, are in full agreement with earlier research by Abd El-Kader et al. (2013) and Kaltsa et al. (2021). This could be because moringa seed extract acts as a natural coagulant (Suarez et al., 2003). Likewise, high amounts of several antioxidant substances, including ascorbic acid, vitamin E, phenolic acids, beta carotene, quercetin, and flavonoids, are also present in moringa seed extract (Lalas and Tsaknis, 2002 and Lakka et al., 2020). Moringa's antioxidants appear to aid in preventing cell damage (Jahan et al., 2018). Furthermore, cationic electrolytes like Ca^{2+} or Mg^{2+} found in moringa extract enhance its cross-linking process, making it highly effective in removing free radicals (Pedroso-Santana and Fleitas-Salazar, 2020). This suggests that it plays a very important role in protection against oxidative damage caused by Cd (Mobin and Khan, 2007 and Zhang et al., 2009). Additionally, the characteristics of moringa extract include its biodegradability, biocompatibility, and ease of application in the pre-made growing media (Kaltsa et al., 2021). According to earlier research, moringa seed extract was a great adsorbent for removing metal ions from the prepared agricultural medium (Sharma et al., 2006). Because moringa seed extract has high "oxygen-nitrogen" connections that attach to HMs, its chemistry reveals more potent antioxidants that prevent HM damage (Bhatti et al., 2007 and Ali and Seng, 2018).

2.3. Effect of the moringa CPE levels on the HMs elements purification percentages in vitro

The results of the protein extract of moringa seeds at several levels in eliminating HMs from broccoli plants cultured in MS medium contaminated with HM are given in Table (8). The findings demonstrated that the protein extract from moringa seeds reduced the levels of HM, including Pb, Zn, Cd, Ni, Fe, Co, Mo, Cu, and Cr, in broccoli leaves. An increase in the levels of moringa CPE in the MS HM contaminated culture medium correlated with a greater percentage of HM removal, except at the 4% concentration (HM. M4) for the minerals Zn and Mo, as indicated in the preceding table. The protein extract from moringa seeds removed the least amounts of HMs (iron and chromium), with iron removal rates ranging from 28.79% to 60.63% and chromium removal rates from 24.41% to 80.22%. Moringa CPE had the highest clearance rate (more than 99%) for the HMs Cu, Co, Ni, and Cd. The optimal level was 4% of moringa seed protein extract (HM. M4), which gave the highest removal percentages of HMs (Cd, Pb, Zn, Ni, Fe, Co, Mo, Cu, Cr Mm/L) at (99.96, 80.01, 98.99, 99.82, 60.63, 99.90, 97.32, 99.61, and 80.22 Mm/L), respectively, compared with the 3% level, in broccoli plantlets cultured in vitro.

Moringa extract is a simple, low-cost, and sustainable solution that is also available to the majority of the population, environmentally friendly, safe for human health, and thus more suitable for solving environmental problems in developing countries (Hsu and Kao, 2004; Khawaja et al., 2010 and Phiri,

2010). Many studies investigated the effect of moringa crude protein extracts on removing HMs from contaminated vegetable samples. Moringa seed protein is a more effective sorbent for removing HM contamination and volatile organic components in aqueous systems (Sharma et al., 2006). Masood et al. (2017) reported that the leaves contained higher concentrations of HM than the stem in celery, broccoli, and lettuce when irrigated with contaminated water. This result agreed with Abdeen (2016), who reported that using 2% concentration of moringa seed extract achieved the highest removal of Fe, Zn, Mn, and Cu (45.74, 46.15, 64.29 and 47.37%), compared with 3%. Furthermore, Elsergany (2023) found that moringa CPE at 200 mg/L was capable of removing Mo, Cu, Cd, Cr, and Co by 97.4, 66.5, 51.8, 50.3, and 45.8% from wastewater samples, respectively. The highest percentages of removal of Fe, Zn, Mn, and Cu caused by 2% extract of CPE were 45.74, 46.15, 64.29, and 47.37%, respectively.

Table (8). Effect of moringa crude protein extract (CPE) levels on the percentage of heavy metal removal in broccoli plantlets cultured in MS heavy metals–contaminated media in vitro.

Heavy metal elements	Removing percent			
	(10%) CPE	(20%) CPE	(30%) CPE	(40%) CPE
Cd	96.57	98.72	98.41	99.96
Pb	80.00	80.00	80.01	80.01
Zn	98.107	98.716	99.271	98.99
Ni	99.82	99.82	99.82	99.82
Fe	28.79	36.25	46.81	60.63
co	96.49	98.19	99.90	99.90
Mo	93.63	96.00	98.09	97.32
Cu	99.60	99.61	99.61	99.61
Cr	24.41	53.66	62.03	80.22

CONCLUSION

Heavy metal contamination is a major environmental and food safety concern, especially in developing counters. HMs are environmentally persistent and non-biodegradable in nature, and have great negative impacts on plant and animal production, due to their accumulation in different parts, causing a high risk to human health. Therefore, the application of different bioremediation techniques has become a necessity to reduce HM accumulation and the toxic pathways to food and human health.

Phytoremediation is an environmentally friendly solution to these problems, as it cleanses contaminated soil and water from HMs by using natural plant compounds. Some previous studies indicate that crude protein of moringa seeds is an excellent coagulant for the removal of HMs from contaminated soil and irrigation water in polluted areas.

This work explored the possibility of using moringa seed crude protein extract as a low-cost, sustainable, and environmentally friendly coagulant to eliminate and reduce the accumulation and harmful effects of HMs on plants growing in polluted areas.

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إستحثاث تحمّل إجهاد العناصر الثقيلة في البروكلي (*Brassica oleracea*) معمليًا

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يُعدّ البروكلي (*Brassica oleracea*) من المحاصيل النباتية المهمة جدًا بسبب نكهته المميزة ومحتواه من مركبات الأيض الثانوية ذات النشاط القوي المضاد للسرطان. ويُعدّ تلوث محاصيل الخضر بالمعادن الثقيلة مشكلة بيئية خطيرة، إذ تُعتبر من أبرز المشكلات التي تُشكّل خطرًا كبيرًا على صحة الإنسان. هدف هذا البحث هو دراسة تأثير مستخلص البروتين الخام للبذور (CPE) المستخدم بأربعة مستويات (١٠، ٢٠، ٣٠، و٤٠٪) على الصفات الخضرية والكيميائية الحيوية ونِسَب إزالة المعادن الثقيلة في نبات البروكلي النامي معمليًا. أشارت النتائج إلى أن البروتوكول الأكثر كفاءة في تكاثر البروكلي معمليًا كان بيئة MS8 من مواشيج وسكوج الأساسية والمضاف إليها ٠,٥٠ ملجم/لتر NAA +٠,٥٠ ملجم/لتر BAP + ٠,٢٥ ملجم/لتر KIN. كذلك كانت البيئة MS4 المزودة بـ ١,٠٠ ملجم/لتر NAA أكثر فعالية في تحفيز تكوين الجذور الحقيقية البيضاء. وأوضحت النتائج أن إضافة مستخلص البروتين الخام لبذور المورينجا (CPE) كان له تأثير إيجابي في تحسين الصفات النباتية للبروكلي النامي معمليًا على البيئات الملوثة بالمعادن الثقيلة (HM)، حيث سُجِّلت أعلى قيم للنمو عند استخدام مستخلص بروتين بذور المورينجا بتركيز M2 أي بنسبة ٢٠٪ من CPE. كما ازدادت قيم الصفات الكيميائية الحيوية مباشرةً بزيادة مستويات مستخلص البروتين البذري (CPE)، وسجّل أعلى مستوى (٤٠٪) من مستخلص المورينجا زيادة في معدل إزالة الشوارد الحرة بنسبة ٤٥٪، مما يشير إلى أن ارتفاع مستويات مستخلص المورينجا زاد من قدرة النباتات على مقاومة الإجهاد. علاوة على ذلك، انخفضت كميات MDA و H_2O_2 بشكل كبير بنسبة ٤٨,٧ و٧٨,٥٪على التوالي، مما أدى إلى انخفاض مؤشرات التلف الخلوي. كما سُجِّلت أعلى نسب إزالة للمعادن الثقيلة Cd، Pb، Zn، Ni، Fe، Co، Mo، Cu، Cr بمستويات ٩٩,٩٦، ٨٠,٠١، ٩٨,٩٩، ٩٩,٨٢، ٦٠,٦٣، ٩٩,٩٠، ٩٧,٣٢، ٩٩,٦١، و٨٠,٢٢ مم/لتر. وقد أثبت مستخلص المورينجا فعالية عالية جدًا في إزالة المعادن الثقيلة من أنسجة النبات، إذ وصلت نسب الإزالة لبعض المعادن مثل الكادميوم والكوبالت والنيكل إلى ما يقارب ١٠٠٪، مما يدل على قدرة النبات على سحب هذه الملوثات من الوسط. وفي الختام، وُجد أن لمستخلص بروتين المورينجا الخام(CPE) تأثيرًا إيجابيًا في تقليل التأثير السام للعناصر الثقيلة (HM)على النباتات المزروعة في البيئات الملوثة.