The Interactive Effect of Plant Growth Regulators and Metal Stress Inducers on the Micropropagation of Giant Pumpkin (*Cucurbita maxima* L.)

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Abstract:

This study aims to improve the efficiency of micropropagation of *Cucurbita maxima* by examining the effect of growth regulators BA and 2,4-D and their interaction with stress inducers $K_2 Cr_2 O_7$ and $CoCl_2$ on MS medium using stem and leaf parts. This study was conducted in the Plant Laboratory / Department of Life Sciences / College of Science, University of Tikrit, and the results showed that the best bud regeneration was achieved using a moderate concentration (2) mg/L of BA with 1 mg/L of -2,4-D while unbalanced concentrations led to reduced or inhibited growth. $K_2 Cr_2 O_7$ enhanced the number of branches and leaves when combined with BA within certain limits, yielding the best results at a concentration of 4, with 13 branches, 13 leaves, and a height of 8 cm, while $CoCl_2$ showed an inhibitory effect. The results confirm the importance of hormonal balance in improving the efficiency of micropropagation in plant tissue culture technology.

Keywords: (*Pumpkin plant*), Murashige and Skoog medium, Potassium dichromate ($K_2 Cr_2 O_7$)

Introduction

The giant pumpkin (Cucurbita maxima) is a member of the Cucurbitaceae family and is a plant of significant economic and nutritional importance due to its large fruit size and high nutritional value, containing proteins, fats, sugars, fiber, and minerals, making it an important focus for plant studies. However, the propagation of pumpkins using traditional methods faces numerous challenges such as low germination rates and increased susceptibility to diseases, highlighting the need for developing modern agricultural techniques to enhance the production of healthy, microbe-free plants (1). This plant has also been used for centuries in folk medicine to treat various ailments, especially digestive system disorders and intestinal parasites, and these pharmacological effects are attributed to its chemical composition rich in active compounds. Plants exposed to stress conditions often produce various bioactive compounds known as secondary metabolites, and the levels of these compounds are influenced by non-traditional environmental conditions. Cucurbits are a natural source of a wide range of bioactive compounds such as carotenoids, tocopherols, phenols, terpenoids, saponins, sterols, fatty acids, functional carbohydrates, and polysaccharides (2). The pumpkin plant contains many active compounds that exhibit important pharmacological properties, including potent antioxidants and anti-cancer properties, in addition to its anti-diabetic effects. Pumpkin seed oil is also widely used in the treatment of benign prostatic hyperplasia, a common disease in elderly men (3). On the other hand, plant propagation using micropropagation techniques offers numerous advantages compared to traditional methods such as propagation by seeds, cuttings, or grafting, as these techniques allow for the production of large numbers of plants within a short period while ensuring they are free from viruses, insects, and diseases. Among the most important of these techniques is plant

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tissue culture, which is considered one of the most efficient methods for crop improvement and producing strains resistant to diseases and environmental stresses. Micropropagation technology also possesses a great capacity to produce high-quality plants and isolate strains with superior genotypes that are better adapted to different environmental conditions. Callus formation is one of the fundamental stages in plant tissue culture, as callus represents an unorganized parenchymatous mass of plant cells that can be induced from various plant parts such as leaves, stems, seeds, and nodes, when cultured in a suitable nutrient medium in the laboratory. This process requires precise surface sterilization of plant tissues using 70% ethanol or sodium hypochlorite solution, followed by thorough washing before culturing them in nutrient media containing plant growth regulators such as auxins and cytokinins. Callus growth can lead to the production of genetic copies that may differ from the mother plant due to the occurrence of somaclonal variation, which could contribute to the development of improved plant varieties of commercial importance (4). In this context, the concept of controlled mineral stress has emerged as one of the modern approaches that can influence the response of tissue-cultured plants, as this type of stress may interact with plant growth regulators, positively or negatively affecting the efficiency of micropropagation. The effect of different concentrations of potassium dichromate on plant growth has been studied, and it was observed that chromate significantly affects seedling growth and seed germination, and also stimulates the production of certain proteins associated with sugar metabolism processes. Understanding the factors affecting plant response to stress helps clarify the mechanisms that enable plant cells to protect themselves and tolerate stress, and this knowledge can be utilized to improve agricultural production and maintain environmental quality (5). A study by (6) also showed a significant increase in the total phenolic content in callus cultures when cobalt chloride was added to the MS medium at concentrations higher than the normal level. It was found that all cobalt concentrations (5, 25, and 50 micromolar) stimulated the production of phenolic acids, and the growth of callus in media containing $CoCl_2$ led to an increase in total phenol accumulation. The highest value for total phenolic content (8.40 mg/g dry weight) was recorded with the addition of 2 mg/L of $CoCl_2$, followed by the 1 mg/L concentration which recorded (7.88 mg/g dry weight), while increasing the concentration to 10 mg/L inhibited phenolic biosynthesis. This study aimed to evaluate the interactive effects of BA and 2,4-D with potassium dichromate ($K_2 Cr_2 O_7$) and cobalt chloride ($CoCl_2$) on the efficiency of shoot regeneration from stem and leaf explants of the giant pumpkin plant (Cucurbita maxima) under in vitro conditions.

2. Materials and Methods

1.2. Seed Sterilization

C. maxima pumpkin seeds were washed with distilled water for 3 minutes to remove any dirt or impurities. They were placed in a Petri dish containing (1-3% v/v) and washed for 2 minutes, rinsed again with distilled water, and immersed in a commercial solution (sodium hypochlorite (NaOCl) (1-3% v/v) diluted with distilled water for 2 minutes. After that, the seeds were washed with distilled water for 3 minutes to remove any harmful substances from the alcohol, commercial solution, and other materials.

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The seeds were then placed in a dish containing sterile filter paper and left to dry for a few minutes, after which they were ready for cultivation in the media (7).

2.2. Plant Growth Conditions

The medium was prepared by adding 4.9 g of MS medium and adding 30 g of sucrose. The medium was solidified by adding 8 g of agar (17). The pH of the MS medium was adjusted to 5.7-5.8. The planting tools and culture media were sterilized in an autoclave at 121°C and 15 bar pressure for 12 minutes and left for the medium to solidify. Then, *C. maxima* pumpkin seeds were cultured on solid MS medium. Plant seedlings grew in the growth chamber four days after planting under a photoperiod of 16 hours of light and 8 hours of darkness, under artificially active photosynthetic radiation with a light intensity of 3000 lux, a temperature of 24°C, and relative humidity of 70%. Growing plant parts (explants) were cut from C. maxima plants, from stems and leaves, using a scalpel to a length ranging between 1 and 2 cm, then cultured on solid MS medium containing different concentrations of hormones, namely BA and 2,4-D respectively, in MS medium (7).

2.3. Stimulation using Potassium Dichromate and Cobalt Chloride

Potassium dichromate was used at concentrations (1, 2, 4 mg/L) to stimulate the growth of plant explants. Cobalt chloride was also used at concentrations (1, 2, 4 mg/L). The cultured explants from C. maxima samples, along with untreated control samples, were incubated under specific conditions (growth chamber, 24°C). The growth period for C. maxima explants took four to five weeks

- **Experimental Design**
  The experimental design Using the SAS program, the Duncan's Multiple Range The level of significance ($P \leq 0.05$)

3. Results and Discussion

1.3 Effect of BA and 2,4-D Interaction on Stem Culture Differentiation

The results showed that the control treatment (without growth regulators) and the treatment containing a balance between auxin and cytokinin (2,4-D (1), BA (1)) achieved the highest rate of branch number, reaching 6.00 branches, with no significant differences observed between them. In contrast, increasing the BA concentration to 2 mg/L while keeping 2,4-D constant at 1 mg/L led to a significant decrease in the number of branches, reaching 5.00 branches.

Regarding the effect of the interaction on branch length (cm), the treatment (2,4-D (1), BA (2)) recorded the highest significant value for branch length, reaching 10.00 cm, significantly outperforming the control treatment (8.00 cm) and the balanced treatment (5.00 cm). Concerning the effect of the interaction on the number of leaves, the treatment (2,4-D (1), BA (1)) achieved a significant superiority in leaf number, recording 12.00 leaves compared to 7.00 in the control treatment and 6.00 in the treatment with the higher BA concentration. This is often attributed to hormonal imbalance/toxic effects of high cytokinin concentrations, which is known in tissue culture for the Cucurbitaceae family. The results obtained are similar to the study conducted by (8), which found that the success of cucurbit regeneration is linked to selecting the type and concentration of cytokinin and low doses of auxin, and that increasing cytokinin beyond the optimal level may reduce regeneration efficiency.

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Table (1) shows the interaction of different concentrations of BA and (1) mg/L of 2,4-D on the regeneration of C. maxima shoots from sterile stem explants on M.S. medium.

D-2,4 (1)*BA **mg/L**	**Number of branches**	**Branch length (cm)**	**Number of leaves**
D-2,4 (0), BA (0)	6.00 a	8.00 b	7.00 b
D-2,4 (1), BA (1)	6.00 a	5.00 c	12.00 a
D-2,4 (1), BA (2)	5.00 b	10.00 a	6.00 c
D-2,4 (1), BA (4)	0.00 c	0.00 d	0.00 d

2.3**Effect of BA and 2,4-D Interaction on Leaf Culture Differentiation**

From the table, we observe that the second and fourth treatments recorded a value of (0.00) for all studied indicators (number of branches, branch length, number of leaves). This indicates that these concentrations led to a complete inhibition of the organogenesis process from leaf explants. In the case of the low concentration, the stimulation was perhaps insufficient to initiate division, whereas in the case of the high BA concentration, the tissues may have reached a stage of "hormonal toxicity" or imbalance that prevents bud initiation . Significant superiority of the treatment: The treatment containing 1 mg/L 2,4-D and 2 mg/L BA recorded the highest significant values for all traits: the number of branches reached 7.00 branches, branch length achieved 12.00 cm, and the number of leaves reached 9.00 leaves .Comparison of results with the control treatment: Although the control treatment (without additives) showed growth (branch number 6.00 and length 8.00 cm), the addition of the hormonal interaction at this ratio proved superior. This was explained by (9) as the presence of a low dose of 2,4-D supporting tissue responsiveness, while BA directs differentiation towards bud formation and multiplication; moderate concentrations of BA are more efficient in stimulating shoot formation. (10) confirmed that the success of micropropagation depends on the "optimal dose" of cytokinin; high concentrations may reduce response, while moderate doses give the best shoot multiplication depending on the cultivar and type of explant.

Table 2. Interaction of different concentrations of BA and (1) mg/L of 2,4-D on the regeneration of C. maxima shoots from sterile leaf explants on M.S. medium.

D-2,4 (1)*BA **(mg/L)**	**Number of branches**	**Branch length (cm)**	**Number of leaves**
D-2,4 (0), BA (0)	6.00 b	8.00 b	7.00 b
D-2,4 (1), BA (1)	0.00 c	0.00 c	0.00 c
D-2,4 (1), BA (2)	7.00 a	12.00 a	9.00 a
D-2,4 (1), BA (4)	0.00 c	0.00 c	0.00 c

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3.3Effect of BA Interaction on Stem Culture Differentiation

The results showed that a concentration of 1 mg/L of BA is the most efficient concentration for stimulating the growth and development of vegetative shoots, recording the highest significant value for branch length, reaching 10.00 cm, outperforming the control treatment and other treatments. BA at low and balanced concentrations works to break apical dominance and stimulate cell elongation and vascular tissue differentiation, leading to increased length while maintaining stability in the number of branches (6.00) and number of leaves (7.00) (Song et al., 2024). An inverse relationship is observed between increasing BA concentration beyond a certain limit and growth rates, as the number of branches, their length, and the number of leaves decreased significantly and sharply when moving to concentrations of 2 and then 4 mg/L. Branch length decreased from 10.00 cm at concentration (1) to 5.00 cm and 4.00 cm at the higher concentrations, respectively .While the control treatment achieved good results in the number of branches (6.00) and number of leaves (7.00), this indicates the presence of sufficient endogenous cytokinin content in the stem explants used. However, the exogenous addition of BA at a concentration of 1 mg/L was necessary to improve the "branch length" trait. This is explained by the fact that increasing cytokinin above the optimal concentration may cause physiological disturbance (stress/growth abnormalities), resulting in fewer and shorter shoots. Recent studies in cucurbits confirm that the response to BA is often dose-dependent; moderate concentrations show the best growth, while higher concentrations may inhibit growth quality or reduce response (11).

Table 3. Different concentrations of BA on the regeneration of C. maxima shoots from sterile stem explants on M.S. medium.

BA **(mg/L)**	**Number of branches**	**Branch length (cm)**	**Number of leaves**
BA (0)	6.00 a	8.00 b	7.00 a
BA (1)	6.00 a	10.00 a	7.00 a
BA (2)	4.00 b	5.00 c	5.00 b
BA (4)	4.00 b	4.00 d	5.00 b

4.3Effect of BA on Leaf Culture Differentiation

The most notable observation in the table is the complete failure of regeneration (values of 0.00) at concentrations of 1 mg/L and 4 mg/L. The treatment at a concentration of 2 mg/L achieved a tremendous significant leap in all indicators Number of branches: Recorded 10.00 branches .Branch length Reached 10.00 cm .Number of leaves: Reached 12.00 leaves .The control treatment recorded moderate values (branch number 6.00 and length 8.00 cm). Medium concentrations of cytokinins are often the most efficient for inducing shoot formation (12) .This indicates that the low concentration may not reach the stimulation threshold necessary to initiate bud differentiation in leaf tissue, while the high concentration may cause physiological inhibition/tissue stress that weakens the viability of the explants or impedes differentiation pathways. (13) confirmed that experiments on plants from the same family (Cucurbitaceae) show that the response is often "dose-dependent," and a decrease or

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inhibition may occur when deviating from the optimal cytokinin concentration.

Table 4. Different concentrations of BA on the regeneration of C. maxima shoots from sterile leaf explants on M.S. medium

BA **(mg/L)**	**Number of branches**	**Branch length (cm)**	**Number of leaves**
BA (0)	6.00 b	8.00 b	7.00 b
BA (1)	0.00 c	0.00 c	0.00 c
BA (2)	10.00 a	10.00 a	12.00 a
BA (4)	0.00 c	0.00 c	0.00 c

5.3 Effect of Cobalt Chloride Interaction with BA on Stem Culture Differentiation

The data in Table (5) present the results of a unique experiment studying the effect of the interaction between cytokinin (BA) and the cobalt element ($CoCl_2$) as a potential stimulating factor on the regeneration of sterile stem explants of the giant pumpkin plant (C. maxima). These results show a clear negative response to the addition of cobalt chloride at its various concentrations. The control treatment recorded the highest significant values in all indicators (number of branches 6.00, branch length 8.00 cm, number of leaves 7.00). Regarding the inhibitory effect of the cobalt element, it is observed from the table that with a fixed BA concentration at 2 mg/L, at concentrations of 1 and 2 mg/L, all indicators decreased significantly compared to the control. While the length was 8.00 cm in the control, it decreased to 5.00 cm and then 3.00 cm. At a concentration of 4 mg/L: complete failure in growth and regeneration occurred (values of 0.00). Regarding the interaction with BA: it appears that the interaction between BA (2 mg/L) and cobalt was not synergistic. This indicates that $CoCl_2$ at the used doses caused inhibition/tissue stress or reduced differentiation efficiency, especially since cobalt is often used as an inhibitor and becomes a growth inhibitor when the concentration of cobalt chloride (1, 2, or 4 mg/L) is unsuitable (14). This suggests that the treatment stimulated the initiation of relatively more buds/leaves but restricted elongation, and its effect may be beneficial or harmful depending on the dose and stage. The results indicate that the best growth was achieved without $CoCl_2$ (control), while the presence of $CoCl_2$ with BA gave a weaker or varied response (increase in leaves/branches with marked shortness). This was confirmed by (15) that $CoCl_2$ here is not an enhancer of regeneration and micropropagation in pumpkin plants, and it may require testing a more precise concentration range in cucurbit tissue culture.

Table 5. Stimulation of plant explant growth using cobalt chloride and its interaction with different concentrations of benzyl adenine (BA) on the regeneration of C. maxima* shoots from sterile stem explants on M.S. medium.

BA, CoCl2 **(mg/L)**	**Number of branches**	**Branch length (cm)**	**Number of leaves**
BA (0), CoCl2(0)	6.00 a	8.00 a	7.00 a
BA (2), CoCl2(1)	3.00 c	5.00 b	4.00 c
BA (2), CoCl2(2)	5.00 b	3.00 c	5.00 b
BA (2), CoCl2(4)	0.00 d	0.00 d	0.00 d

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6.3 Effect of Potassium Dichromate Interaction with BA on Stem Culture Differentiation

These results show a remarkable ascending response pattern with increasing concentration of potassium dichromate. Effective synergistic response: Contrary to previous results with cobalt chloride, potassium dichromate showed a strong stimulating effect when interacting with 2 mg/L of BA. The treatment achieved the highest significant superiority in the number of branches (13.00) and number of leaves (13.00). Meanwhile, the treatment achieved maximum branch elongation reaching 12.00 cm. We observe a precise variation in the physiological response based on the concentration of the additive. The concentration of 1 mg/L of K_2 Cr_2 O_7 led to complete growth cessation (values of 0.00), which may be explained by a state of initial hormonal imbalance or tissue shock at the beginning of the interaction. These results match the study by (16, 17). This pattern is explained by the fact that stress from hexavalent chromium (Cr(VI)) may act as an elicitor that drives cells towards forming a greater number of shoots (defensive/regenerative response), but at the same time limits elongation due to toxicity effects and oxidative stress that weaken longitudinal growth and redirect resources towards survival and differentiation rather than elongation (16).

Table 6. Stimulation of plant explant growth using potassium dichromate and its interaction with different concentrations of benzyl adenine (BA) on the regeneration of C. maxima* shoots from sterile stem explants on M.S. medium.

BA , K2Cr2O7	Number of branches	Branch length (cm)	Number of leaves
BA (0), K2Cr2O7(0)	6.00 c	8.00 b	7.00 c
BA (2), K2Cr2O7(1)	0.00 d	0.00 c	0.00 d
BA (2), K2Cr2O7(2)	7.00 b	12.00 a	9.00 b
BA(2) , K2Cr2O7(4)	13.00 a	8.00 b	13.00 a

Figer (1)

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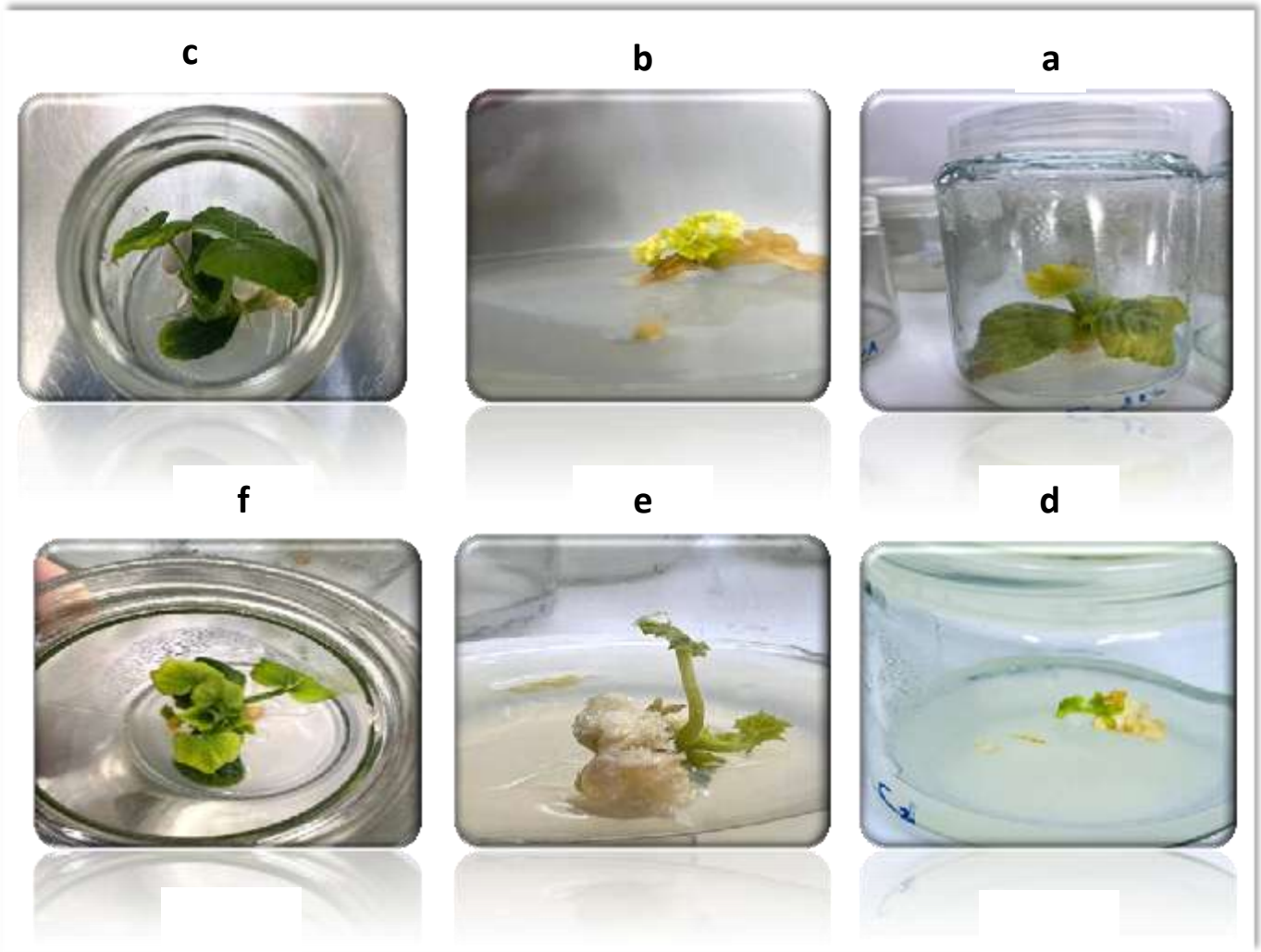

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Cultivation on cobalt	**A,b**
Plant without hormone (cultivation of seeds on a medium free of additives)	**c**
Cultivation of stem on Benzyladenine (BA) hormone	**D,e**
Germination on potassium dichromate	**f**

Conclusions

The plant growth regulator BA played the most prominent role in the differentiation and growth of explants. Stems excelled in their response to plant formation at a higher rate than leaves. Potassium dichromate was distinguished by its superior ability over cobalt chloride in stimulating branch formation, elongation, and leaf number.

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