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THE ROLE OF COLCHICINE AND HARMEL SEED EXTRACT IN IMPROVING DROUGHT TOLERANCE AND INCREASING SECONDARY COMPOUNDS OF STEVIA *IN VITRO*

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ABSTRACT

The aim of this study was to determine the appropriate concentration of harmel (*Peganum harmala*), extracts that could raise the tolerance of stevia to drought, and to increase secondary compounds of the alkaloid compounds contained in this extract that could induce genetic variations. In addition, the study aimed to determine the appropriate concentration of colchicine that promotes drought tolerance, as it could amplify genetic material (ploidy), thus increasing desirable traits such as drought tolerance and the number of secondary compounds. Four concentrations of harmel seeds extract (0, 25, 50 and 75 ml/L) and four concentrations of colchicine (0, 2, 4 and 6 ml/L) were used, with five replicates per treatment, in a Completely Randomized Design (CRD) design and factorial experiments, estimated plant content of secondary compounds using High-performance liquid chromatography (HPLC). The data revealed that the higher average number of leaves, dry weight and secondary compounds recorded at 75 ml/L of harmel seeds extracted with 6 ml/L of colchicine, was 22.6 shoots/plant and, 301.6 mg, respectively. Subsequently, the plants that achieved the highest averages in the measured traits were treated with harmel and colchicine, then exposed to Poly Ethelene Glycol (PEG) concentrations (0, 5, 10 and 15 g/L) to increase and determine the tolerance of plants to drought stress. The data revealed that plants exposed to a certain level of harmel extract and colchicine exhibited plant relative growth rate (PRGR) and index of tolerance (intol), particularly at a concentration of 5 g/L of PEG was 43.6 mg/day and 1.208, respectively. The study concluded that harmel extract could improve drought stress tolerance. It is a natural extract that is safe for the environment and available at low prices, which is one of the goals of sustainable development, compared to colchicine, which is an unsafe compound and working with it is risky.

Key words: HPLC, Index of tolerance, PEG, Plant extract, Steviol, Tissue culture

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INTRODUCTION

The sugar leaf plant (*Stevia rebaudiana*) is related to the *Asteraceae* family. It is a perennial herbal plant that lives for 3-5 years and reaches a height of 50-60 cm, at which point it is harvested [1]. It is a promising natural sweetener and sugar crop in the world and in Iraq, possessing superior nutritional qualities and numerous health benefits, and serving as a suitable alternative to sugar [2]. Stevia is a plant-based sweetener and an alternative to all artificial sweeteners that are harmful to health. It is a primary and essential substitute for natural sweeteners such as cane sugar and sugar beet. Iraq is suffering from a severe drought crisis due to global warming and climate change, which necessitates finding drought-resistant varieties. Colchicine and *Peganum harmala* seed extract act as bio stimulants to stimulate stress responses in the stevia plant [3]. Enhancing drought tolerance mechanisms such as increased production of antioxidants and cellular protection compounds and leading to an increase in desirable secondary compounds such as stevioside due to the stimulation of biosynthetic pathways, which raises plant quality and the quantity of the final product, especially in *in vitro* culture and harsh conditions [4]. Natural plant extracts are among the factors that can improve plant growth and have the potential to induce genetic changes [5]. Therefore, it was important to study these extracts and determine their effect on tissue-cultured plants, and to ascertain whether they could replace chemical and physical mutagens, such as the extract of harmel seeds (*Peganum harmala*), which contain many glycosidic compounds [6]. Important alkaloids, defined as compounds found within plants that are basic and contain one or more atoms of nitrogen in their hydrocarbon ring, are beneficial as plant growth regulators, defensive agents against other organisms (most are bitter), or in maintaining ionic balance in plants, replacing inorganic bases [7]. Harmel seed extract also contains several alkaloids, such as beta-carboline alkaloids including harmaline and harmine, quinazoline alkaloids, pegamine alkaloids, and quinazoline [8]. All these compounds contain nitrogen in their composition, which is involved in the building of amino acids and nucleic acids, thus causing genetic variations such as genetic variations that increase the yield [9].

Polyploidy, defined as the duplication of the number of chromosomes in an organism's cells, has played a significant role in the evolution of vascular plants [10]. It has also played a pivotal role in increasing agricultural production, and recently, improved crops of economic importance have been developed using chemical agents such as colchicine or physical agents such as radiation [11]. Various species studied to show that the anatomical and physiological changes resulting from natural or artificial polyploidy can raise tolerance to biotic and abiotic stress in addition to disease resistance that has a positive effect on plant growth and total yield [12, 13]. Colchicine is characterized by its ability to duplicate chromosomes in plant cells to

produce artificial mutations characterized by high yields and improved characteristics in flowers and fruits of some economically important medicinal plants [14]. It also maximizes secondary metabolites, including steviol glycosides in stevia leaves, as this substance inhibits the formation of essential spindle fibers [15]. The process of chromosome division during the anaphase phase, which leads to the chromosomes remaining in the center of the cell, and then the genetic material duplicating.

The study conducted by Mutawakkil [16] confirms that treatment with harmel leaf extract led to an increase in the mutational index and a rise in the percentage of mitotic cells. As a result of research and experiments, harmel plant extracts have effects on improving plant growth. Al-Qubai and Tikka [17] revealed in their study that chromosome behavior became abnormal in samples treated with a 0.05% aqueous harmel extract for 24 hours. This abnormality was observed in prophase (kneading and uncoiling), metaphase (kneading, scattering, and chromosomal fractures), anaphase (stellate), and telophase (removal of a portion of the genetic material and polynuclear compared to normal mitosis). Furthermore, genetic material abnormalities, chromosomal fractures, changes in the position of genetic material, and morphological abnormalities in the dividing pea cells were also noted. Therefore, this study aimed to determine the appropriate levels of harmel extract and colchicine that have the potential to increase vegetative growth, drought stress tolerance, and increase secondary compounds in the Spanti variety of stevia.

MATERIALS AND METHODS

Explant material Stevia plant, cultivar Spanti, was obtained from the Horticulture Department of the Ministry of Agriculture in Baghdad. The growing tips of this plant were used as a source of plant parts. These plant parts were sterilized by immersion in a 16% sodium hypochlorite solution for 5 minutes [18], then washed by sterilized distilled water three times to remove the remaining sterile solution [19].

Tissue culture establishment

The removed shoots tips were cultured in Murashige and Skoog culture media [20] containing 3% sucrose. Plant growth regulators added to the media, including (NAA) at a fixed 0.5 mg/L and (BA) at 1.0 mg/L. Plants cultured are incubated in a chamber used for growth study at $25 \pm 2^{\circ}\text{C}$, using light cycle about 16 hours and 8 hours in dark for 4 weeks.

Treatment with harmel seed extract

Harmel seeds were brought from local markets, and the method according to Singh [21] was followed in preparing the harmel seed extract where 100 g of fine harmel powder was prepared, diluted to 1000 ml of distilled water and heated to 60°C . The solution soaked for 24 hours for better extraction of the important ingredients in the

harmel powder, the mixture was filtered, after which the filtrate was evaporated until 100 ml (concentration 10%). The following concentrations (0, 25, 50 and 75 ml/L) were then added to a nutrient medium, and the shoots tips were cultured in it.

Colchicine treatment

Colchicine powder 250 mg was diluted to 1000 mL distilled water and stirred until completely dissolved. The following volumes were withdrawn: (0, 2, 4 and 6 ml/L) [22], were added to the food medium, and the shoot tips were planted in it.

PEG treatments

Four concentrations of PEG (0, 5, 10 and 15%) [23] were added to the culture medium, and stevia plants were grown in them. The plants that gave the best measure of the number of leaves produced were those treated with harmel extract and colchicine, and the cultured plant incubated at of $25 \pm 2^{\circ}\text{C}$ and using light of 1000 lux to 16 hours of lighting and 8 hours of dark [24]. Observations were taken after four weeks of cultivation.

Acclimatization of tissue cultures

Tissue cultures were transferred to containers containing a 1:2 mixture of peat moss and perlite [25]. The samples were incubated in a greenhouse for eight weeks under high humidity conditions to maintain viability and minimize water loss, allowing them to acclimate to external environmental conditions. The percentage of plants that successfully acclimatized was 91%.

Plant relative growth rate (PRGR)

The relative plant growth rate was calculated according to Farshadfar *et al.*, [26]: $\text{PRGR} = \frac{W_2 - W_1}{W_1}$. Where W_1 is initial weight of plant and W_2 is weight of plant after four weeks.

Index of tolerance (INTOL)

Index of tolerance was estimated according to Farshadfar *et al.*, [26] $\text{INTOL} = \frac{\text{PRGR treatment}}{\text{PRGR control}}$ Where PRGR = relative growth rate.

Estimation of steviol compounds

Stevia glycoside standards kit containing dulcoside A, steviobioside, stevioside, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside F, ruboside was purchased from Chromadex (P/N KIT-00019568- 010, Irvine CA). Acetonitrile and water (HPLC grade) were supplied by Fischer Scientific (Fair Lawn, NJ), and Pharm Co. (Brookfield, CT). Phosphoric acid 49% - 51%; (HPLC grade) was purchased from Sigma-Aldrich (Bellfonte, PA) whereas Sodium phosphate (monobasic) analytical grade was gained from Scientific Strategies (Oklahoma, OK). The solvents degassed for fifteen minutes before they used. The HPLC performed method was an isocratic binary solvent mobile phase system using 32:68 mixture of

acetonitrile and 10 mmol/L sodium phosphate buffer pH= 2.63 like Joint FAO/WHO Expert Committee on Food Additives (JECFA) [27]. Each standard solution of steviol glycoside 1-9 was separately prepared at 10 mg/ml following the method of the Chaturvedula and Zamora [28]. The steviol glycosides (1, 3-4, 6-9) solutions were achieved by using phosphate buffer. Steviolbioside (2) and rebaudioside B (5) solutions were diluted using methanol. After that, all standard solutions of steviol glycosides were prepared to set the concentration at 0.25 mg/L of each individual compound in the mixture. Agilent (Wilmington, DE) 1100 HPLC System. Reversed phase High-performance liquid chromatography (RP-HPLC) method was conducted the column was washed using 50 mL of 90% azomethane before sampling for accuracy of their retention times. Injected 5 μ L of steviol glycoside mixture (1-9) at 20°C, 40°C, 60°C, and 79°C. All steviol glycosides were detected at the wavelength of 210 nm. The two compounds rebaudioside A (1) and stevioside (3) were injected once at the beginning and once at the end of the sequence for consistency [29].

Statistical design and analysis

The experiment was conducted using a factorial trial arrangement and a completely random design (CRD) using five replicates of treatment. Means were checked using the LSD test at a probability level of 0.05, using GenStat 2007 software [30].

RESULTS AND DISCUSSION

Effect of harmel extract and colchicine on growth of vegetative shoots number in stevia

The results in Table 1 indicate that the number of vegetative shoots was 18.8 shoot/plant at 75 ml/L of harmel extracts, compared to 8.5 shoot/plant in the control treatment. The highest shoot count was also recorded at 6 ml/L of colchicine, reaching 17.4 shoot/plant, compared to 9.9 shoot/plant in the control treatment. Regarding the interaction between harmel (75 ml/L) and colchicine (6 ml/L) recorded a higher rate, reaching 22.6 shoot/plant, compared to the control treatment in which 4.6 shoot/plant was recorded as in Figure 1 and 2.



Figure 1: Stevia plants grown in nutrient medium containing concentrations of harmel extract



Figure 2: Stevia plants grown in nutrients medium containing concentrations of colchicine

Effect of harmel extract and colchicine on dry weight in stevia

The results in Table 2 indicate that the dry weight was 207.1 mg at a concentration of 75 ml/L of harmel extract, compared to 144.0 mg in control treatment. The highest dry weight was also recorded at a concentration of 6 ml/L of colchicine, reaching 206.1 mg, compared to 146.2 mg in the control treatment. Regarding the interaction between harmel (75 ml/L) and colchicine (6 ml/L) recorded a higher rate, reaching 301.6 mg, compared to the control treatment in which 140.2 mg was recorded.

Effect of PEG on (PRGR) in stevia

Table 3 showed that the highest growth rate was 43.7 in the harmel and 40.3 mg/day colchicine treatments at concentrations of 75 ml/L and 6 ml/L respectively, compared to the control treatment, which recorded a lower rate of 27.8 mg/day, the higher growth rate recorded at 5 g/L of PEG, reaching 43.6 mg/day, respectively, compared to the 15 g/L, which recorded a decrease in growth rate at 31.3 mg/day. Regarding the interaction between harmal, colchicine, and PEG concentrations, the higher growth rate recorded at 5 g/L polyethylene glycol (PEG) for the harmel and colchicine treatments at concentrations of 75 ml/L and 6 ml/L, reaching 50.4 and 47.4 mg/day respectively, compared to the control treatment in PEG 15 g/L which recorded a decrease in growth rate at 20.0 mg/day.

Effect of PEG on INTOL in Stevia

Table 4 showed that the INTOL was 1.200 and 1.108 in the harmel and colchicine treatments at concentrations of 75 ml/L and 6 ml/L respectively. Compared to the control treatment, which recorded a lower rate of 0.693, the higher growth rate recorded at 5 g/L of PEG, reaching 1.208, respectively, compared to the 15 g/L, which recorded a decrease in growth rate is 0.867. Regarding the interaction between harmal, colchicine, and PEG concentrations, the higher growth rate recorded at a 5 g/L PEG for the harmel and colchicine treatments at concentrations of 75 ml/L and 6 ml/L, was 1.091 and 0.953 respectively. Compared to PEG 15 g/L which recorded a decrease in growth rate at 0.554 as in Figure 3.



Figure 3: Stevia plants grown in a nutrient medium containing different concentrations of PEG

Effect of harmel extract and colchicine on estimation of steviol compounds

Analysis of HPLC was performed on a concentration of 60 ml/L of harmel extract and a concentration of 7.5 ml/L of colchicine to estimate the secondary compounds

in the stevia plant, which recorded the highest values for the number of buds and dry weight. The HPLC results showed the superiority of harmel extract in estimating the secondary compounds in 5 g/L of PEG compared to colchicine, as shown in Figure 4.

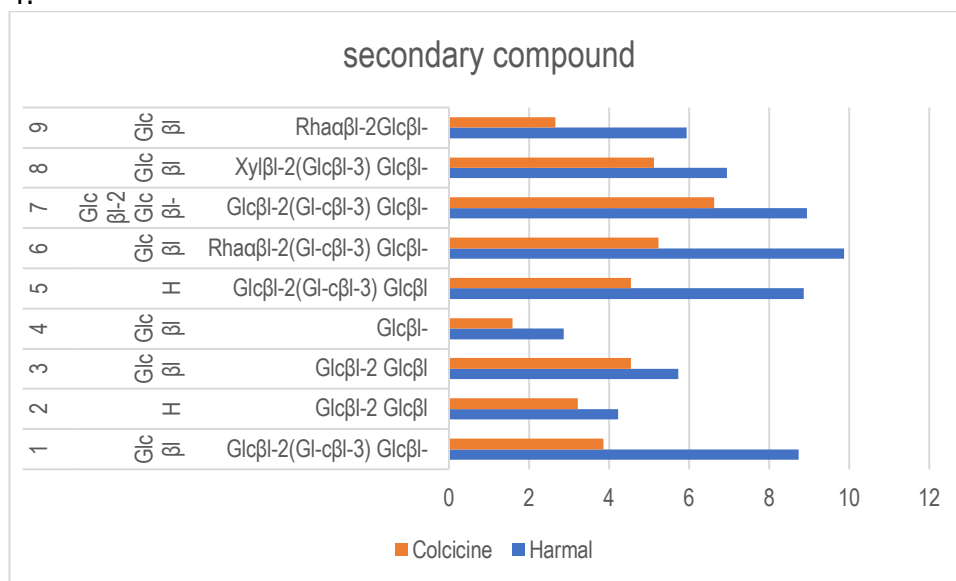


Figure 4: Estimation of steviol compounds

The progress of the harmel seed extract in leaf count, dry weight, and secondary metabolites compared to treatment of control may be assigned to the presence of active substances and compounds such as alkaloids (harmalin, harmine, and harmalol), flavonoids, and tannins in this extract, which enhance plant growth [31, 32], these results confirm the findings of Singh [14].

The increase in the number of leaves, dry weight, and secondary compounds in colchicine treatments may be because colchicine could double the number of chromosomes in cells, thus causing these cells to divide and grow larger to maintain a constant cytoplasm-nucleolus ratio. The increase in size signals an increase in protein production and consequently leads to an increase in vegetative growth characteristics [33, 34].

The potent effect of harmel seed extract, which is characterized by its high content of nitrogen-containing alkaloids, is because nitrogen is involved in the synthesis of amino acids that form proteins and are then encoded by nucleic acids [35, 36]. Sajjad *et al.* [37] indicated the necessity of using low concentrations of colchicine in seed treatment, ranging from 0.1% to 0.8%, as high doses cause plant malformations and reduce the production of tetraploid plants. Therefore, it is advisable to use colchicine at the lowest possible concentrations, given that colchicine is highly toxic to plants. Colchicine, in particular, has been identified as having the ability to double the number of chromosomes, thus inducing genetic variations in plant cells, It is known

that the average occurrence of mutations in plant cells increases with the increased use of mutagens, as genetic variation in plant cells occurs whether it results from the use of physical, chemical, or natural mutagens such as harmel seed extract [38].

The decrease in average leaf count and dry weight of high PEG levels may be attributed to decreased division of cells [39, 40], reduced volume of cytoplasm, cell swelling loss and imbalance of nutrients because of water decreasing and absorption of nutrient [41].

An electrical potential increase and content of cellular water decreases accompanied by stress increase or perhaps stress increase leads to physical damage of metabolism mechanism of carbon because of continuous stress exposure or maybe because content of cytoplasm of the plant cell becomes denser [42, 43].

Cytoplasm volume decreases when there is a lack of water in the cell. The increase in secondary metabolites is also attributed to the fact that plants tend to produce secondary metabolites when subjected to drought stress [44, 45, 46].

CONCLUSION AND RECOMMENDATIONS FOR DEVELOPMENT

It is concluded that adding harmel extracts at four concentrations showed an improvement in the number of leaves, an increase in dry weight, and an accumulation of the nine secondary compounds found in the stevia plant, which are of particular importance when exposed to drought stress. This is in comparison to adding four concentrations of colchicine, which is expensive and unsafe to use because it is a carcinogenic compound and working with it is risky. This reinforces the importance of plant extracts in improving vegetative growth characteristics and increasing drought tolerance, especially since harmel seeds can be easily obtained and are readily available. It is very cheap, and the extraction method is simple and safe to use compared to colchicine, which is expensive and unsafe to use because it is a carcinogenic compound and working with it is risky. It is also recommended to use the alcoholic extract of harmel rather than the aqueous one, because harmel extract contains many compounds that are insoluble in water but soluble in alcohol such as ethanol. Therefore, this must be considered when extracting harmal. It is also recommended to use harmel extract in field agriculture by spraying concentrations of harmel extract on plants at the seedling stage, especially on the tops of the plants, and studying its effect on yield.

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the Ministry of Science and Technology for estimation of secondary compounds in stevia plants using the HPLC technique.

Conflict of Interest

There is no conflict of interest.

Table 1: The harmel extract and colchicine effect on number of growing vegetative shoots in stevia (shoot/explant)

colchicine conc. ml/L	Harmel conc. ml/L				Average
	0	25	50	75	
0	4.6	8.2	11.2	15.6	9.9
2	7.2	9.4	13	17.4	11.8
4	10.8	12.4	13.8	19.4	14.1
6	11.2	17.6	18.2	22.6	17.4
LSD0.05	18.2				6.5
Average	8.5	11.9	14.1	18.8	
LSD0.05	9.3				
Variation source	d.f.	s.s.	m.s.	v.r.	F pr.
Harmel	3	171.84	57.279	47.98	<.001
Colchicine	3	7.938	2.646	2.22	0.095
Harmel. Colchicine	9	70.812	7.868	6.59	<.001
Residual	64	76.4	1.194		
Total	79	326.99			
s.e.	1.093				
cv%	17.4				

Table 2: The harmel extract and colchicine Effect on dry weight (mg) in stevia

colchicine conc. ml/L	Harmel conc. ml/L				Average
	0	25	50	75	
0	140.2	146	147.6	151.0	146.2
2	142.2	160.4	168.6	181.2	163.1
4	146.4	174.2	190.2	194.4	176.3
6	147.2	175.2	200.2	301.6	206.1
LSD0.05	154.7				59.2
Average	144.0	164.0	176.7	207.1	
LSD0.05	61.4				
Variation source	d. f.	s. s.	m. s.	v. r.	F pr.
Harmel	3	42330	14110	14661	<.001
Colchicine	3	26530	8844	9189.1	<.001
Harmel. Colchicine	9	40300	4477	4651.7	<.001
Residual	64	61.6	0.9625		
Total	79	109200			
s.e.	0.981				
cv%	0.6				

Table 3: Effect of PEG on (PRGR) (mg/day) in stevia

PEG conc. g/L	0	Harmel conc. ml/L			colchicine conc. ml/L			Average
		25	50	75	2	4	6	
0	36.1	38.1	41.1	44.6	36.5	39.1	41.3	39.6
5	30.1	42.8	48.3	50.4	40.7	45.5	47.4	43.6
10	25.0	35.2	38.1	40.2	33.4	35.1	38.2	35.0
15	20.0	31.3	33.4	39.4	29.3	31.4	34.4	31.3
LSD0.05	29.8							11.3
Average	27.8	36.9	40.2	43.7	35.0	37.8	40.3	
LSD0.05	15.9							
Variation source			d. f.	s. s.	m. s.	v. r.	F pr.	
Treatment			6	736.71	245.57	12.92	<.001	
PEG			3	56.16	18.72	0.98	0.406	
Treatment. PEG			18	2952.5	328.05	17.26	<.001	
Residual			112	1216.7	19.01			
Total			139	4962.1				
s.e.			4.360					
cv%			14.5					

Table 4: Effect of PEG on (INTOL) in stevia

PEG conc. g/L	0	Harmel conc. ml/L			colchicine conc. ml/L			Average
		25	50	75	2	4	6	
5	0.834	1.186	1.338	1.396	1.127	1.260	1.313	1.208
10	0.693	0.975	1.055	1.114	0.925	0.972	1.058	0.970
15	0.554	0.867	0.925	1.091	0.812	0.870	0.953	0.867
LSD0.05	0.567							0.333
Average	0.693	1.009	1.106	1.200	0.955	1.034	1.108	
LSD0.05	0.543							
Variation source		d. f.		s. s.	m. s.	v. r.	F pr.	
Treatment		6		0.8208	0.2736	4.62	0.005	
PEG		2		1.2783	0.4261	7.19	<.001	
Treatment. PEG		12		4.5977	0.5109	8.62	<.001	
Residual		84		3.7909	0.0592			
Total		104		10.488				
s.e.		0.2434						
cv%		16.3						

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