INFLUENCE OF EXOGENOUSLY APPLIED GLUTATHIONE AND GIBBERELLIC ACID IN A BEAN UNDER SALT STRESS IN VITRO CONDITIONS

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ABSTRACT

This stady was aimed to investigate effect of treatment with 0, 0.25 and 0.50 mM of Glutathione (GSH) and 0, 1.0 and 2.0 mg L^{-1} of Gibberellic acid (GA_3) in enhancing the growth of calli cultures was tested under normal and critical concentrations of sodium chloride (NaCl). The experiments were conducted during October, 2022 to June 2023 at the plant tissue culture laboratory in Center of Desert Studies, University of Anbar. The experiments designed according to the complete randomized design (CRD). The critical limit of NaCl in calli cultures was determined after 35 days of cultivation in MS medium. The results showed that the concentration of 200 mM caused the highest significant decrease in the fresh weight (FW) and relative fresh weight (RFW) of these cultures, which amounted to 457 mg and -0.086, respectively. However, it did not differ significantly with what was produced by the concentration with 150 mM. As for the salinity experiment, the combination of 0 mM + 0.5 mM + 2.0 mg L^{-1} for each of NaCl, GSH, and GA_3, respectively, was the most consistent in improving the study indicators. This combination achieved the highest significant average for FW, dry weight (DW), RFW, and the effectiveness of Catalase (CAT) which amounted to 973.7 mg, 52.8 mg, 0.947, and 13.119 U mg^{-1} protein, respectively. However, the combination with the combination of 0 mM + 0.5 mM + 1.0 mg L^{-1} for each of NaCl, GSH and GA_3, respectively, were recorded the lowest significant level of Malondialdehyde (MDA) content, which amounted to 1.951 µM g^{-1} FW.

KEYWORDS: Calli cultures, abiotic stress, growth, physiology, biochemistry.

تأثير الجلوتاثيون وحامض الجبرليك المُضاف خارجياً في الفاصوليا النامية تحت ظروف الأجهاد الملحي خارج الجسم الحي

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المستخلص

هدفت الدراسة الحالية الى تحديد مدى تأثير المعاملة بالتركيز 0 و 0.25 و 0.50 ملي مول من Glutathione (GSH) والتركيز 0 و 1.0 و 2.0 ملغم لتر$^{-1}$ من Gibberellic acid (GA_3) في تعزيز نمو مزارع الكالس تحت التراكيز الطبيعية والحرجة لكلوريد الصوديوم (NaCl). أجريت التجارب خلال الفترة من تشرين الأول 2022 ولغاية حزيران 2023 في مختبر زراعة الانسجة النباتية التابع لمركز دراسات الصحراء – جامعة الانبار. تم تصميم التجارب وفقاً للتصميم العشوائي الكامل (CRD). تم تحديد الحد الحرج لملح NaCl في مزارع الكالس بعد 35 يوم من الزراعة في وسط MS. بينت النتائج بأن التركيز 200 ملي مول قد تسبب في إحداث أعلى إنخفاض معنوي الوزن الطري والوزن الطري النسبي لتلك المزارع بلغ 457 ملغم و 0.086- على التوالي. ولم يختلف بشكل معنوي مع ما انتجه التركيز 150 ملي مول. أما بالنسبة لتجربة الملوحة فقد كانت التوليفة 0 ملي مول+0.5 ملي مول+2.0 ملغم لتر$^{-1}$ لكل من ملح NaCl وGSH و GA_3 على التوالي، الأكثر إنسجاماً في تحسين مؤشرات الدراسة، فقد حققت أعلى متوسط معنوي للوزن الطري والوزن الجاف والوزن الطري النسبي وفعالية إنزيم Catalase (CAT) بلغ 973.7 ملغم و 52.8 ملغم و 0.947 و 13.119 U ملغم$^{-1}$ بروتين على التوالي.. في حين أظهرت تلك التوليفة مع التوليفة المتضمنة 0 ملي مول+0.5 ملي مول+1.0 ملغم لتر$^{-1}$ لكل من ملح NaCl وGSH و GA_3 على التوالي، في تسجيل أدنى مستوى معنوي لمحتوى Malondialdehyde (MDA) بلغ 1.951 مايكرومول غم$^{-1}$ وزن طري.

الكلمات المفتاحية: خارج الجسم الحي، الاجهاد اللاأحيائي، النمو، الفسيولوجية، الكيموحيوية.

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INTRODUCTION

The bean *(Phaseolus vulgaris* L.) is one of the important crops known for its benefits in the fields of nutrition and health for the human body as it is characterized by a high content of protein, complex carbohydrates, dietary fibers, and some vitamins and minerals. It has the ability to treat many health problems such as cardiovascular diseases, diabetes, obesity, and tumors. This therapeutic ability is derived from its content of Polyphenolic compounds (27). The bean plant is classified as salt-sensitive (4), as the effect of salinity on it is not restricted to a lack of irrigation water only, but also extends to the occurrence of an imbalance in the ionic balance within the plant tissues, which may develop into the occurrence of Oxidative damage and cause damage to the bio-cellular molecules (3, 5). Plant tissue culture technology is the most suitable for overcoming the problems resulting from salt stress because it is accurate, simple, economic, and is highly applicable compared to traditional agriculture. Thus, it contributes to accelerating the production of plants that are tolerant to salt stress, as well as understanding the mechanisms of tolerance and the accompanying physiological and biochemical changes, which enhance the increased tolerance of different plant species (26, 34). Various chemical compounds play an important role in the growth and development of plants by regulating many biological processes. Therefore, treatment with these compounds is considered one of the successful solutions which would confront the various environmental stresses plants are exposed to during their life cycle (15, 25). One of these compounds is Glutathione, also called γ-glutamyl-cysteinylglycine (GSH). It is a polypeptide compound that plays an important role in responding to abiotic stresses. It is characterized by its antioxidant properties that maintain the balance among the various components of the antioxidant system. Among its other important physiological functions is the protection of cellular membranes, lipids and biochemical contents against hydrogen peroxide (H_2O_2) formed as a result of oxidative stress (Peroxidation). Thus, it works to increase the plant's adaptation to stress conditions, as well as increasing the activity of enzymatic and non-enzymatic antioxidants under the influence of salt stress, and increasing protein synthesis and nucleic acids, DNA and RNA (13, 28). Gibberellic acid (GA_3) is described as one of the plant growth regulators. Alone or in combination with other chemical compounds, it could be play a prominent role in improving plant biological processes, as it improves plant cell growth and development by reducing the oxidative damage through controlling the activities of antioxidant enzymes. It is also characterized by its ability to induce the gene expression responsible for enhancing plant cell tolerance to abiotic stress conditions, including salinity stress (18). This study was aimed to determine the critical salt dose for *Phaseolus vulgaris* L. callus cultures, at which the fresh weight (FW) of the callus begins to decrease. The study also aimed at testing the effect of different concentrations of GSH and GA_3 on callus cultures growing under normal and salt stress conditions by influencing some growth, physiological and biochemical characteristics of tissue cultures and the extent to which these enhance the tissue's tolerance to salinity stress.

MATERIALS AND METHODS

Preparation of the nutrient medium: The experiment was carried out in the Plant Tissue Culture Laboratory at the Center for Desert Studies/University of Anbar using the Sun Ray brand. The standard MS medium. The medium was prepared by dissolving 4.54 g of MS and 30 g of sucrose. Then, the pH was adjusted to 5.7±0.1, then agar was added at an amount of 7.0 g L^{-1}.

Sterilization: The tools, distilled water, and nutrient medium were sterilized in an Autoclave device at 121°C for 15 minutes. Seeds were washed with running water for 30 minutes, then they were inserted into the planting booth and were sterilized using 70% ethanol with continuous stirring for one minute, after which they were washed with sterile distilled water to remove any traces of alcohol. Then, NaOCl was used at a concentration of 4% for 10 min. The seeds were then washed with sterile distilled water five times in a row to remove any traces of the sterilizing substance. Then, the seeds were planted in tubes containing nutrient medium. The grown bottles were placed inside the

growth chamber under 25 °C ±1 and a lighting intensity of 1000 lux for 16 h of light and 8 h of darkness.

Calli cultures Induction: Calli cultures were obtained through hypocotyl peduncle induction. The explant was grown in a nutrient medium containing 4.54 g of MS, 30 g of sucrose, and 7.0 g of agar, in addition to containing plant growth regulators 2.0 mg L^{-1} of 2,4-D and 1.5 mg L^{-1} of BA. The cultures were incubated in the growth chamber at a temperature of 25°C±1, according to the physical conditions previously described.

Determining the critical salt limit: A portion of callus tissue growing in MS medium 0.5 g was taken. It was grown in a nutrient medium containing 0, 50, 100, 150, and 200 mM of NaCl concentrations. The critical salt limit was determined based on the FW and the RFW of callus samples growing in solutions after 35 days of cultivation in MS medium.

Salinity stress tolerance treatments: To determine the possibility of the effect of GSH and GA_3 on the tolerance of callus cultures to salinity stress, callus tissue was grown at 8 weeks of age in a prepared nutrient medium consisting of MS 4.54 gm L^{-1} with sucrose 30 gm L^{-1} and agar 7.0 g L^{-1}. The nutrient medium also contained two concentrations of NaCl, namely 0, and the critical salt limit obtained with concentrations of 0, 0.25 and 0.50 mM of GSH and the concentrations of 0, 1.0, and 2.0 mg L^{-1} of GA_3. The cultures were incubated in a growth chamber under 25 °C±1 and 1000 lux lighting for 8/16 days with alternating light and darkness. Data on some growth parameters were recorded 35 days after planting, including:

Fresh weight (FW): The FW of callus samples was determined.

Dry weight (DW): The callus samples were dried in an oven at 60 °C for 72 h, after which the DW was recorded.

Relative fresh weight (RFW): The RFW was calculated by the initial fresh weight of the cultured callus tissue (FWi) and its final fresh weight (FW_f) according to the following equation:

$$RFW = \frac{FW_f - FWi}{FWi}$$

Browning intensity (BI): The BI of callus cultures was estimated based on the method proposed by Sahraroo et al. (29), depending on the degree of the brown color observed in callus samples.

Estimation of ion element content

The content of callus cultures of sodium (Na^+) and potassium (K^+) was determined according to the method proposed by Seki et al. (34), using a photoelectric flame photometer.

K^+/Na^+ Ratio

Na^+ to K^+ ratio in callus samples was calculated.

Estimation of hydrogen peroxide (H_2O_2) content: The H_2O_2 content was determined using the spectrophotometric method described by Alexieva et al. (2). The absorbance of the solution was recorded at 390 nm.

Estimation of malondialdehyde (MDA) content: Lipid peroxide analysis was performed according to the method described by Neamah and Jdayea (25) by measuring the amount of MDA resulting from thiobarbituric acid (TBA) reaction.

Estimating Catalase (CAT) activity

The enzymatic extraction from callus samples was performed to determine the antioxidant enzymatic activity (9). The activity of CAT enzyme was determined according to the decrease in H_2O_2 based on absorbance reading every 30 sec for 3 min at 240 nm.

Experimental design and statistical analysis

All experiments were designed according to the Complete Randomized Design with five replications. Data for each experiment were statistically analyzed using Genestat program, version 12. The LSD value ($p < 0.05$) was extracted to compare between the means of the treatments.

RESULTS AND DISCUSSION

Determining the critical salt limit for bean calli cultures: The results have shown a significant effect of the NaCl treatments. Hence, despite the positive effect in improving the FW of the callus tissue when NaCl was added to the MS culture medium at a concentration of 50 mM, amounting to 1.987 g, this increase decreased with the continuous increases in the concentration of the NaCl treatment. Accordingly, the lowest average values were at the two concentrations of 150 and 200 mM, which recorded the lowest FW reaching 0.512 and 0.457 g, respectively. However, they did not differed significantly

(Figure 1). The results have also indicated that the relative growth index of the FW of the callus tissue was significantly affected by the treatment with NaCl. Hence, the 50 mM concentration achieved an increase in the RFW by 2.974, followed by the 100 mM concentration by 0.903, while the 150 and 200mM concentrations caused the lowest significant decrease in the index reaching 0.024 and -0.086, respectively, yet they did not differ significantly (Figure 2). The apparent increases in the FW of callus cultures and its relative coefficient was due to the positive role played by the low levels of NaCl (Figures 1 and 2). This result could be explained based on the findings of the study by Hongqiao et al. (14), which showed that Na^+ and Cl^- ions under low levels work to increase the absorption of some important nutrient elements such as C, S, Zn, and Cu, as well as improving salt stress-responsive gene expression at low concentrations, which contributes to cysteine (Cys) biosynthesis to prevent salinity-induced oxidative stress (24). Na^+ is described as an indispensable element for most plant species. It is useful for plant cells suffering from K+ deficiency, as it could be partially replace the functions of K^+ by acting as an osmoticum and enzyme activator (31). On the other hand, Cl^- is an essential nutrient element for plants which participates in the regulation of osmosis and maintenance of turgor pressure (7). It also functions in chloroplasts as an indispensable component of photosystem II (PSII) by stabilizing the deoxygenation/oxygenation system (8, 10), in addition to regulating the activity of some enzymes such as asparagine, ATPase, and Amylase (10, 33). All of these roles show that NaCl under a concentration of 50 mM increases the biomass. As for the high levels of salinity, which usually correspond to an increase in the level of NaCl, the cellular concentration of Na^+ and Cl^- ions increases.

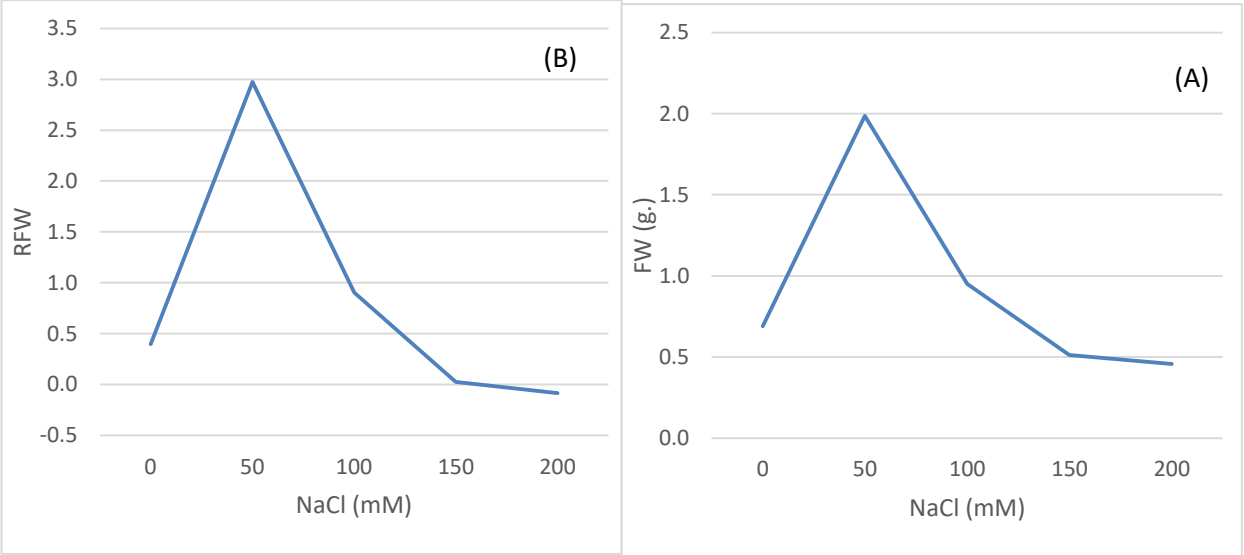

Figure 1. Effect of treating bean calli cultures growing in MS medium for 35 days of cultivation with different concentrations of NaCl on FW (A) RFW (B).

This leads to the occurrence of osmotic stress and results in many toxic effects on the plant cell, including a decrease in the level of other cations, especially K^+, an increase in the production of Reactive Oxygen Species (ROS), and inhibition of cell membranes, protein synthesis, cellular metabolism, and nutrients absorption (36). In light of this, the concentration of 150 mM can be considered the critical salt limit at which the FW of *P. vulgaris* L. callus cultures decreases due to the sharp decrease in the FW after 35 days of cultivation in the nutrient medium.

Growth characteristics

Some basic growth parameters of bean callus cultures were studied. Treatments with GSH and GA_3 significantly improved growth parameters under normal and salt stress conditions. Media not treated with NaCl with high concentrations of GSH and GA_3 recorded the highest average of FW, DW, and RFW of callus cultures, reaching 973.7 mg, 52.80 mg, and 0.947, respectively. At the same time, an improvement in growth parameters for the same concentrations was observed under conditions of salt stress compared to callus

cultures exposed to NaCl and not treated with GSH and GA_3. As for the BI, a noticeable increase was recorded in callus cultures treated with NaCl, while treatment with GSH and GA_3 protected the callus tissue from damage by oxidation of phenols (Table 1). Exposure of plant tissue to abiotic stress is usually accompanied by a decrease in the growth of some sensitive plant species, which represents the first response. This results in a decrease in morphological indicators of plant tissue such as FW, DW, and RFW, accompanied by an increase in the BI (Table 1). The reason for this can be attributed to the lack of plant cell absorption of water and nutrients from the nutrient medium due to the increase in salt content inside the cell, causing a decrease in the osmotic capacity of the cell. The result can also be attributed to reasons related to hormonal disorders resulting from the effect of salinity. The current results are consistent with the findings of Ahmad et al. (1) on the susceptibility of *Solanum tuberosum* L. plants growing in vitro to salinity stress, and the findings of Khalid and Aftab (18) that indicated the negative effect on the morphological indicators of plants. Solanum tuberosum grown outside the living body by treatment with a concentration of 80 mM of NaCl, and the results of Seki et al. (34) on the treatment of microflora plants of *Fragaria ananassa* Duch with different concentrations of NaCl. GSH represents is an antioxidant characterized by its ability to withstand various stresses which the plant cell suffers from, including salt stress. It has a number of physiological roles such as reducing the flow of Na^+ which has a negative effect, reducing oxidative stress, preventing lipid peroxidation, and thus maintaining the stability of the permeability of biological membranes, which positively reflects on plant cell growth (13). GA_3 is a phytohormone which enhances cell expansion and elongation, the development of vascular tissue, maintenance of apical dominance, regulation of the photosynthetic and gravitropic behavior, and thus it ultimately enhances the biomass of NaCl stressed and non-stressed plants (11). In this study, exogenous addition of GA_3 improved callus growth and, thus, it successfully counteracted the harmful effects of salt stress. These results are consistent with what was indicated by Khalid and Aftab (18), who described the positive role of GA_3 in enhancing the tolerance of plant tissues to salinity stress.

Table 1. Effect of NaCl, GSH and GA_3 on some growth parameters of bean calli cultures induced from hypocotyl after 35 days of cultivation

NaCl (mM)	GSH (mM)	GA_3 (mg L^{-1})	FW (mg)	DW (mg)	RFW	BI
		0	693.7	29.69	0.397	3.6
	0	1.0	809.7	36.93	0.619	2.3
		2.0	798.0	33.23	0.596	3.3
		0	650.0	26.57	0.300	2.0
0	0.025	1.0	709.7	35.48	0.419	3.3
		2.0	830.0	38.28	0.660	1.2
		0	715.0	28.44	0.430	3.6
	0.05	1.0	906.7	43.66	0.813	3.5
		2.0	973.7	52.80	0.947	3.1
		0	402.3	13.93	-0.195	2.7
	0	1.0	450.0	19.29	-0.100	2.4
		2.0	500.7	20.78	0.001	3.7
		0	528.7	24.25	0.057	3.2
150	0.025	1.0	523.7	23.70	0.047	4.1
		2.0	556.3	25.81	0.113	4.3
		0	527.0	27.61	0.054	3.8
	0.05	1.0	627.7	28.83	0.255	3.7
		2.0	545.7	30.76	0.091	3.8
	L.S.D < 0.05		69.88	5.948	0.1391	0.7096

Physiological characteristics

Studying some physiological characteristics is a priority in order to prepare a report that explains the extent of their influence on growth characteristics and the relationship among growth parameters. The results showed that the content of Na^+ and K^+ and the ratio of Na^+/K^+ were affected significantly, and that treatment with NaCl recorded an increase in the accumulation of Na^+ and a decrease in the content of K^+ and thus an increase in the ratio of Na^+/K^+. On the other hand, treatments with

GSH and GA_3 resulted in a decrease in Na^+ accumulation and an increase in K^+ content and thus a decrease in the Na^+/K^+ ratio (Table 2). Physiological factors negatively affected the growth indicators of callus cultures grown under conditions of salt stress. This is true because salinity causes insufficient absorption of water and nutrients by the plant cell due to decreased osmotic capacity, which is a common indicator of salt stress (12). To avoid the harmful effect of osmotic stress due to salinity stress, the plant cell has developed certain mechanisms which enable it to protect itself from various stresses, including the salinity stress. One of these mechanisms can be provided by treatment with GSH, which contributes to increasing the levels of proline, free amino acids, and total soluble sugars of cells exposed to salt. It is known that amino acids accumulate in high concentrations, causing osmotic adjustment in plants under salt stress. Proline plays a vital role in osmotic adjustment and stability to protect enzymes, proteins and membranes against the harmful effects of osmotic stress of salinity (6). Our results are in agreement with results in previous studies, including Nahar (23) on *Vigna radiata* L. and Sadak et al. (28) on *Cicer arietinum* L. The direct effect of salt stress is the damage to cell membranes at high concentrations of NaCl. This deterioration can be overcome by exogenous addition of some plant growth regulators, including GA_3, which maintains the stability or permeability of biomembranes through its role in synthesizing proteins important in plant biological processes. Previous studies, including Misratia et al. (21), explained the physiological role played by GA_3 in tolerating the harmful effects of salinity through its role in increasing the accumulation of free proline, which maintains membrane permeability and increases the absorption of macro- and micronutrients which plants suffer greatly from its deficiency under high salinity.

Table 2. Effect of NaCl, GSH, and GA_3 on some physiological parameters of bean calli cultures induced from hypocotyl after 35 days of cultivation

NaCl (mM)	GSH (mM)	GA_3 (mg L^{-1})	Na^+ (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	Na^+/K^+
0	0	0	4.67	44.00	0.106
		1.0	4.33	47.20	0.082
		2.0	3.59	46.23	0.077
	0.025	0	2.59	49.27	0.053
		1.0	2.45	51.03	0.048
		2.0	1.04	51.07	0.020
	0.05	0	1.39	49.80	0.028
		1.0	1.18	52.13	0.023
		2.0	0.62	52.77	0.012
150	0	0	60.87	20.50	2.970
		1.0	57.20	24.40	2.347
		2.0	51.33	22.17	2.320
	0.025	0	54.27	24.43	2.225
		1.0	47.67	26.83	1.778
		2.0	33.73	23.33	1.447
	0.05	0	34.47	22.73	1.516
		1.0	27.87	20.20	1.385
		2.0	23.47	24.27	0.993
L.S.D < 0.05			2.422	2.551	0.015

Biochemical characteristics

Studying the biochemical characteristics is important in determining the extent of changes occurring to them as a result of various treatments and their reflection on the physiological characteristics. The results showed that the accumulation of H_2O_2, MDA, and CAT activity increased significantly as a result of treatment with NaCl. At the same time, treatment with GSH and GA_3 contributed to reducing the negative effect of H_2O_2 and MDA accumulation by increasing the activity of CAT (Table 3). Salt stress stimulates the production of ROS and causes oxidative stress (32), as ROS react with biomolecules and cause pigment oxidation, lipid peroxidation, protein denaturation, and mutation at the molecular level, ending in damage to plant cell biomembranes (22). The results in the current study are consistent with the findings reported in previous studies (19, 20, 26, 41). Authors of these studies suggested that increased

antioxidant activity of enzymes gives plant tissues greater resistance to stress-induced damage. GSH is part of the AsA-GSH cycle that scavenges H_2O_2 inside the plant cell, as it reacts with O^-_2 and OH. It is an effective electron donor during the process of ROS detoxification and oxidation. It not only detoxifies ROS but also regulates antioxidant enzymes. It is used to convert toxic xenobiotics into less toxic complexes that are transported into the vacuole (13, 17). The results of our study are similar to the results in previous studies, including Nahar (2015) on *Vigna radiata* L. and Sadak et al. (32) on *Cicer arietinum* L. Plant hormones, including GA_3, act as a signal to stimulate gene expression of many genes that contribute to plant cell tolerance to salinity stress (30). In this study, exogenous application of GA_3 reduced oxidative damage through increasing CAT activity in stressed and unstressed callus cultures. Wen et al. (35) attributed this to the ability of GA_3 to stimulate the gene express-Ion responsible for the synthesis of proteins that enhance plant cell tolerance to salt. The results of the current study are consistent with the results of the study by Khalid and Aftab (18) who suggested an effective role for the growth regulators GA3 in enhancing the tolerance of plant tissues to salt stress by influencing biotechnology parameters.

Table 3. Effect of NaCl, GSH, and GA_3 on some biochemical parameters of bean calli cultures induced from hypocotyl after 35 days of cultivation.

NaCl (mM)	GSH (mM)	GA_3 (mg L^{-1})	H_2O_2 (µM g^{-1} FW)	MDA (µM g^{-1} FW)	CAT (IU mg^{-1} protein)
		0	51.98	3.281	7.857
	0	1.0	52.82	3.369	10.405
		2.0	54.41	3.613	10.000
		0	48.47	2.793	10.095
0	0.025	1.0	52.88	3.502	9.310
		2.0	46.95	2.616	10.595
		0	44.49	2.305	10.976
	0.05	1.0	35.98	1.951	13.119
		2.0	36.28	1.951	12.976
		0	81.85	4.678	8.690
	0	1.0	61.27	4.123	8.833
		2.0	61.42	4.123	8.405
		0	57.51	3.946	9.048
150	0.025	1.0	55.72	3.369	9.143
		2.0	54.34	3.281	9.143
		0	55.72	3.591	9.143
	0.05	1.0	52.18	3.192	8.857
		2.0	48.58	3.680	9.714
L.S.D < 0.05			7.181	0.4324	0.8775

CONCLUSION

The present study demonstrated that salinity significantly affects the growth, physiological, and biochemical performance of *Phaseolus vulgaris* L. callus cultures grown in vitro. The critical salt concentration was determined at 150 mM NaCl, beyond which callus growth declined markedly. Exogenous application of Glutathione (GSH) and Gibberellic acid (GA_3) effectively mitigated the adverse effects of salt stress by improving fresh and dry weights, maintaining favorable Na^+/K^+ balance, and enhancing antioxidant activity, particularly catalase (CAT). The combination of 0.5 mM GSH with 2.0 mg L^{-1} GA_3 resulted in the highest improvement in growth and biochemical indicators, while reducing oxidative stress markers such as H_2O_2 and MDA. These findings confirm that GSH and GA_3 act synergistically to enhance salt tolerance in bean callus cultures, suggesting their potential role in improving stress resilience in leguminous crops under saline conditions.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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