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Effect of NaCl and sodium azide on physiological and enzyme traits of strawberry *In vitro*

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ABSTRACT

The experiment examined the effects of NaCl (0, 2.5, 5 and 10 g l^{-1}) and sodium azide (0, 0.25 and 0.5 mM) on several traits of strawberry plants in vitro. The results showed that increasing NaCl on average increased the proline and carbohydrate accumulation and the activity of peroxidase and catalase enzymes. However, chlorophyll decreased with salt increasing levels. As for the effect of sodium azide an average, it is found that increasing sodium azide concentration at 0.25 and 0.5 mM led to an increase in carbohydrate accumulation while decreasing in proline and chlorophyll accumulation and the activity of catalase enzyme compared with 0 mM sodium azide. Peroxidase activity increased compared with non- mutagenesis treatment (0 mM sodium azide). The interaction between NaCl and Sodium azide, was significant for all studied traits.

Keywords: ***In vitro*, Sodium azide, Sodium chloride, Strawberry, Physiological traits.**

Introduction

Strawberry is one of the most economically important fruit crops worldwide. It has significant nutritional value and various applications in agriculture and industry. However, strawberry cultivation is particularly sensitive to environmental stress, especially salinity (Ferreira et al., 2019). Salinity stress presents a substantial challenge to sustainable strawberry production, as it disrupts physiological processes through ionic toxicity, nutrient imbalances, and osmotic stress, all of which negatively impact yield and also on development, and growth (Attia et al., 2020; Sun et al., 2015). The in vitro propagation technique is a valuable tool that provides controlled environments to study how plants respond to stress (Dziadczyk et al., 2003), allowing for the rapid multiplication of high-quality planting materials. This method is particularly advantageous for investigating the physiological and biochemical mechanisms underlying plant stress tolerance. Al-Shorafa et al. (2014) evaluated the response of two strawberry varieties (Camarosa and Albino) to salinity stress by applying varying NaCl concentrations, the results indicated that both varieties exhibited similar responses to salinity, which led to a significantly increasing proline accumulation in the leaves as increasing in the salinity levels. However, the chlorophyll content of both varieties significantly decreased when exposed to 25 mM of NaCl. Additionally, Mozafari et al. (2018) assessed the impact of 100 mM NaCl on growth of the strawberry variety Queen Elisa. The studies revealed that salinity negatively affected chlorophyll levels, relative water content (RWC), membrane permeability, and disrupted ion exchange in the plants. Salinity is a stress inhibits the physiological and biological processes of plants, alters metabolism and the activity of many enzymes, and leads to partial damage to important metabolic pathways of plants by producing large amounts of ROS (Duman and M., 2018; Zhu, 2001) which are the main source of damage that occurs in the cell when a plant is subjected to abiotic stress, and antioxidant defense systems can remove the toxic effect of ROS, which include enzymatic systems e.g catalase, superoxide dismutase and peroxidase enzymes (Saddique et al.,

2018) which convert H_2O_2 into water. Furthermore, the application of mutagenesis techniques, such as chemical or physical mutagens, holds great potential for inducing genetic variation and allows these differences to multiply over a short time. Combining salinity stress with mutagenesis in an in vitro setting presents an approach to providing suitable conditions for the plant part in controlled places and under sterile conditions (Li et al., 2019; Waugh et al., 2006). Al-Salihy et al. (2018) induced variations in Albion strawberry variety using EMS concentration of 0.1% for 1.5 hours and then planted that variation *In vitro* in MS medium containing varying of sodium chloride concentrations, and they noticed a decrease in the plant content of chlorophyll and an increase in the enzyme peroxidase with increasing NaCl levels.

To induce variation and understand plant responses to salinity, the aim was to investigate the interactions between salinity and mutagenesis in influencing in vegetation growth and physiological traits.

Materials and Methods

The experiment was conducted in the Agricultural Research Center/ Scientific Research Commission. The runners of strawberries (cv. Albion) were sterilized using mercury chloride according to Juameer et al. (2022).

Initiation and multiplication: The explants were planted in initiation media which contain MS salt (Murashige and Skoog, 1962) with 0.5 and 1.5 mg l^{-1} of benzyl adenine (BA) and kinetin respectively (Zobayer et al., 2011). The effect of the interaction of BA with naphthalene acetic acid (NAA) on its ability to shoot propagation from the initiation stage was studied, as it was added in the following concentrations; 0.5, 1 and 2 mg l^{-1} of BA with 0.1, 0.2 and 0.5 mg l^{-1} of NAA.

The average length of the shoot: Shoot formed was calculated by taking the average length of all the shoots that were calculated.

NaCl and sodium azide concentration: Homogeneous plantlets of the Albion variety were treated with the mutagenic sodium azide at a concentration of 0.25 and 0.5 mM for 30 minutes. After the treatment, the plantlets were planted on multiplication medium with 0.5 and 0.2 mg l^{-1} of BA and NAA respectively (according to the multiplication experiment results) containing 0, 2.5, 5 and 10 g NaCl l^{-1}.

The cultures were placed in the growth room for 16 hours with 1000 lux of light and 8 hours at a temperature of 25± 2 °C.

Proline determination: A slightly modified of the Bates et al. (1973) method was used to determine proline, 100 mg fresh weight of shoot ground in 0.8 ml of sulphosalicylic acid (3%), then centrifuged for five minutes at 13500 rpm min^{-1}. 0.5 ml of solution combined with 0.5 ml of glacial acetic acid and acid ninhydrin. After 30 min of boiling, the samples were left to cool. The red layer was separated by adding 2 ml of the toluene, the toluene layer was measured at 520 nm.

Measurement of carbohydrate: With some modifications, the Masuko et al. (2005) method was used to determine the amount of carbohydrates, as the weight of 20 mg of shoot dry weight mashed with 1 ml distilled water then centrifuged for five minutes at 13500 rpm min^{-1}, the intensity of the color was determined using the spectrophotometer microplate reader at 488 nm, as 30 µl of each sample or standard treatments added in the well of 96-well microplate plates, then 100 µl of sulfuric acid were added to it, then 20 µl of phenolic (5%).

Determination of chlorophyll: Chlorophyll was estimated in shoots according to the Arnon (1949) method with some modification, where 1 ml of acetone (80%) was added to 100 mg of shoot fresh weight and then left for 24 hours at a temperature of 10 °C. The spectrophotometer microplate reader was used to measure the color's intensity at 663 and 645 nm.

Estimating the activity of peroxidase and catalase enzymes: 100 mg of shoot fresh weight ground with 500 µl of phosphate buffer (0.05 M, pH 6) then centrifuged at 13500 rpm m^{-1} for 5 min.

Peroxidase activity: With slight modifications, the activity was calculated using the Kim et al. (1988) method, 250 µl each of Gauiacol dye (0.5%), hydrogen peroxide (0.3%), and phosphate buffer (0.05 M, pH 6) were added to each sample (20 µl). The color intensity was determined using the spectrophotometer microplate reader at 470 nm.

Catalase activity: With slight modifications, the Aebi (1984) approach was used to measure the catalase enzymes activity, where 500 µl of hydrogen peroxidase (30 mM) and 1 ml of phosphate buffer (0.05 M, pH 6) were added to 100 µl of each sample. A spectrophotometer set to 240 nm was used to measure the color intensity. The calculation of the enzyme activity followed Lateef et al. (2021).

Statistical analysis: Factorial experiments were applied using the Completely Randomizing Design (CRD) with two factors BA and NAA levels for the propagation experiment, salt and sodium azide concentration for another experiment. Four replicates per treatment and two plants/replication were used. The data were analyzed statistically

according to the Dunkin' test at a probability level of 5%.

Results and Discussion

Effect of BA and NAA on shoot number: According to the results (Table 1), the treatment of 0.5 mg BA l^{-1} produced the greatest number of shoots (9.17 shoots $plant^{-1}$) and 1 and 2 mg BA l^{-1} did not differ from each other significantly. The concentrations of NAA as average did not differ significantly among themselves. As for the interaction between BA and NAA, 0.5 mg BA l^{-1} + 0.2 mg NAA l^{-1} gave 10.25 shoots $plant^{-1}$, while the treatment of 2 mg BA l^{-1} with 0.5 mg NAA l^{-1} gave the lowest number of shoots which reached 0.25 shoots $plant^{-1}$.

Table (1): Effect of different concentrations of BA and NAA on shoot number of the Strawberry cv. Albion.

BA (mg l^{-1})	NAA (mg l^{-1})			
	0.1	**0.2**	**0.5**	**mean**
0.5	9.75a	10.25a	7.50a	**9.17A**
1.0	2.75ab	3.00bc	2.50bc	**2.75B**
2.0	0.25c	1.00c	0.25c	**0.50B**
mean	4.25A	4.75A	3.42A	

Effect of BA and NAA on shoot length: According to the data, the highest average shoot length (3.16 cm) was obtained with 0.5 BA mg l^{-1} (Table 2). The treatments 0.2 and 0.5 mg NAA l^{-1} did not differ significantly from each other, with an average of shoot length which reached 1.71 and 1.81 cm, respectively, and the treatment 0.1 mg NAA l^{-1} gave the lowest average length of the shoot, which was 0.53 cm.

These results are in line with those of Hasan et al. (2010) who observed that a low level of NAA and BA leads to increasing the number of shoots, as well as those of Ara et al. (2012) and Moradi et al. (2011) who found that a low level of cytokinin may stimulate a larger number of branches while a higher concentration causes a reduction in multiplied shoots. Ashrafuzzaman et al. (2013) in their study of the impact of BA on strawberry shoot multiplication of confirmed that a low concentration (0.5 mg of BA l^{-1}) was more efficient for multiplication of shoot than 1 - 3 mg BA l^{-1}. The effect of BA in stimulating shoot multiplication may be due to the ability of BA to stimulate endogenous hormones within plant tissues (Kajla et al., 2018).

Effect of salt and sodium azide on physiological traits:

Proline accumulation: Salinity had a considerable impact on proline accumulation, according to the results (Table 3), 10 g NaCl l^{-1} gave the highest average for proline which reached 2.42 µM proline g^{-1} shoot fresh weight, while the lowest accumulation of proline was 0.77 and 0.76 µM proline g^{-1} shoot fresh weight in 0 and 2.5 g NaCl l^{-1} respectively.

Table (2): Effect of different concentrations of BA and NAA on shoot length (cm) of the Strawberry cv. Albion.

BA (mg l^{-1})	NAA (mg l^{-1})			
	0.1	**0.2**	**0.5**	**mean**
0.5	0.21d	4.20b	5.08a	3.16A
1.0	0.36cd	0.36cd	0.16d	0.29B
2.0	1.03c	0.56cd	0.21d	0.60B
mean	0.53B	1.71A	1.81A	

As for the sodium azide (Table 3), the control treatment (0 mM NaN_3) had the highest accumulation of proline (1.94 µM proline g^{-1} shoot fresh weight), whereas the lowest proline concentration (0.89 µM proline g^{-1} shoot fresh weight) was obtained with 0.25 mM of sodium azide.

Table (3): Effect of salt and sodium azide on proline accumulation (µM g^{-1} shoot fresh weight) in shoot of strawberry cv. Albion.

NaCl (mg l^{-1})	NaN_3 (mM)			
	0	**0.25**	**0.5**	**mean**
0	1.14cde	0.51e	0.66de	0.77C
2.5	1.08cde	0.51e	0.71de	0.76C
5	1.89bc	1.77cd	0.97cde	1.54B
10	3.36a	0.76cde	2.87ab	2.42A
mean	1.94A	0.89B	1.30B	

SA is known to be a common mutant in many animals and plants (Grant and Salamone, 1994) and in fact mutations in living systems are stimulated by the biosynthesis of azide (Owais and Kleinhofs, 1988) and the synthesis of B-azidoalanine, a chemical molecule (Dubey et al., 2017) which cause a DNA point mutation once it's inside the nucleus (Gichner and Veleminsky, 1977), that means the variations are at the genetic level through chromosomal aberration (Ali et al., 2007). Point mutations impair DNA replication, protein inhibition, and growth and metabolic activity (Ragunathan and Panneerselvam, 2007) and the intensity of these effects depends on the concentration of NaN3 (Van Harten, 2002) which changes the balance between growth promoters and growth inhibitors (Gruszka et al., 2012).

The interaction between the salinity and the NaN_3 had significantly affect proline accumulation (Table 3), although the increase in salt levels led to an increase in the accumulation of proline in all levels of

NaN3, on the other hand, there was an accumulation of proline in the control treatment (0 mM NaN_3) higher than the level of 0.25 and 0.5 mM NaN3 at all the salt levels studied. The treatment 10 g NaCl l^{-1} with 0 mM NaN_3 gave the highest accumulation of proline (µM proline g^{-1} shoot fresh weight).

It has been found that proline is important in plant tolerance to salinity because of its function as an enzyme protector (Grant and Salamone, 1994) and reduces the negative effect of sodium chloride on the cell membrane by giving the membrane stability (Mansour, 1998; Yıldız et al., 2010). In most plants, the proline content of the shoot typically increases as the salt level does (Heuer, 2003). Several studies have suggested that proline causes osmotic regulation which makes plants more adapted to grow under saline conditions (Saruhan et al., 2006; Watanabe et al., 2000).

Carbohydrates accumulation: The results (Table 4) showed the salt level 5 g NaCl l^{-1} gave the highest accumulation of carbohydrates which reached 377.4 µg glucose g^{-1} shoot dry weight, while 10 g NaCl l^{-1} gave the lowest accumulation of carbohydrates (263.8 µg glucose g^{-1} shoot dry weight), and the salt levels 0 and 2.5 g NaCl l^{-1} did not differ significantly from each other. Regarding effect of NaN3 in the accumulation of carbohydrates, the treatment of 0.5 mM NaN3 gave the highest accumulation of carbohydrates (398.3 µg glucose g^{-1} shoot dry weight) compared with control and 0.25 mM NaN_3 treatments, which did not differ significantly from each other (Table 4). The interaction was significant between the NaN3 and salt levels, the increase in salt levels led to a decrease in the accumulation of carbohydrates in the control treatment, while the increasing salt levels resulted in an increase in carbohydrates in 0.25 and 0.5 mM NaN_3 (Table 4).

Table (4): Effect of salt and sodium azide on carbohydrate accumulation (µg glucose g^{-1} shoot dry weight) in shoot of strawberry cv. Albion.

NaCl (mg l^{-1})	NaN3 (mM)			
	0	0.25	0.5	mean
0	369.4 abcd	129.7 f	334.1 bcde	277.7 AB
2.5	171.9 ef	224.6 cdef	482.0 ab	292.8 AB
5	212.9 edef	392.4 abc	526.9 a	377.4 A
10	188.2 def	325.8 abcde	250.3cdef	263.8 B
mean	235.6 B	274.9 B	398.3 A	

An increase in carbohydrate accumulation is associated with increasing salt levels, and this may be due to the accumulation of soluble carbohydrates in plants as a response to plant exposure to salinity in some cases, despite the significant decrease in the rate of absorption of CO_2 (Murakeozy et al., 2003). Carbohydrates such as starch and sugars (fructose, glucose, and sucrose) accumulate under salt stress and play a role in osmotic protection, osmotic pressure modification, and carbon storage, as well as removing excess free radicals.

Chlorophyll content: Salinity led to a decrease in chlorophyll, control treatment (0 g NaCl l^{-1}) significantly exceeded the other salt levels in giving the highest average of chlorophyll (33.52 mg chlorophyll g^{-1} shoot fresh weight) while the salt levels 5 and 10 g NaCl l^{-1} gave the lowest chlorophyll rate of 20.79 and 15.04 mg chlorophyll g^{-1} shoot fresh weight respectively (Table 5). Concerning the effect of the sodium azide as an average, the treatment of 0.5 mM NaN3 gave the highest average of chlorophyll (29.94 mg chlorophyll g^{-1} shoot fresh weight) and did not differ significantly from 0 mM NaN3 (Table 5). As for the interaction between the salt levels and the sodium azide, it was significant, the increase in salt levels led to a decrease in the chlorophyll content of the shoot in all NaN_3 concentrations (Table 5).

Table (5): Effect of salt and sodium azide on chlorophyll content (mg g^{-1} shoot fresh weight) in shoot of strawberry cv. Albion.

NaCl (mg l^{1})	NaN_3 (mM)			
	0	0.25	0.5	mean
0	49.27a	26.26cd	35.04ab	33.52A
2.5	36.95ab	43.21ab	26.12bcd	32.43A
5	14.61cd	15.24cd	32.51abc	20.79B
10	10.46d	8.57b	26.10bcd	15.04B
mean	27.82A	18.57B	29.94A	

The decrease in chlorophyll by increasing salt levels is due to osmotic stress, which leads to stopping the nutrient ions' absorption, which causes nutritional imbalance, and reducing transpiration(Sharma et al., 2013). Also, salt stress leads to damage chloroplast membranes (Ashraf and Bhatti, 2000). A decrease in chlorophyll synthesis under stress may be due to inhibition of the activity of enzymes involved in its synthesis (Turan and Tripathy, 2015) e.g, a decrease in the absorption of Mg ions that are involved in the synthesis of chlorophyll (Khan and Frakland, 1983).

It is noted that sodium azide caused a mutation in chlorophyll and the decrease in chlorophyll at 0.25 mM NaN3, these data are consistent with the results

of Srivastava et al. (2019). Owais and Kleinhofs (1988) reported that sodium azide, creates an organic metabolic product that penetrates the nucleus, interacts with DNA, and results in point mutations in the genetic material of the plant.

Effect of salt and sodium azide on enzyme activity

Peroxidase activity (POD): The results (Table 6) showed that increasing salt levels caused an increase of POD enzyme and reached 167.53 unit g^{-1} fresh weight at 10 g NaCl l^{-1}, which significantly outperformed the other levels of NaCl treatments. NaN_3 significantly increased POD activity, as the 0.5 mM NaN_3 gave the highest activity which reached 103.83 unit g^{-1} fresh weight (Table 6). About the interaction between salt levels and the NaN3, It was observed that the increase in salt levels led to an increase in the activity of the POD enzyme at 0 and 0.25 mM NaN3, while for 0.5 mM NaN3 treatment, it was found that 2.5 and 5 g NaCl l^{-1} led to a reduction in the activity of the POD enzyme compared to control treatment (0 g NaCl l^{-1}), while POD enzyme activity increased at 10 g NaCl l^{-1}, where the activity was 127.6, 43.33, 52.17 and 192.83 unit g^{-1} fresh weight in 0, 2.5, 5 and 10 g NaCl l^{-1} respectively (Table 6).

Table (6): Effect of salt and sodium azide on peroxidase activity (unit g^{-1} fresh weight) in shoot of strawberry cv. Albion.

NaCl (mg l^{-1})	NaN_3 (mM)			
	0	0.25	0.5	mean
0	14.08h	1.08l	127.6c	47.58B
2.5	25.67g	6.33i	43.33f	25.11D
5	29.17g	23.83g	52.17e	35.06C
10	246.33a	63.42d	192.83b	167.53A
mean	78.81B	23.67C	103.83B	

The increasing activity of POD enzyme with increasing salt levels at all sodium azide treatments (0, 0.25, and 0.5 mM) might result from an increase in chloride and sodium ions, which increases the activity of some enzymes, including POD (Alhasnawi et al., 2014). This increase in the activity of enzyme could be a reflection of the defense response to cellular damage caused by sodium chloride in medium (Erturk et al., 2007) as it is considered an antioxidant enzyme capturing the roots of hydrogen peroxidase and oxide (O^-), where it works to convert O^- to O_2 with H_2O_2 to finally turn into a water, the higher enzymatic activity, the greater the plants ability to tolerate stress conditions (Quiles and López, 2004).

Catalase activity (CAT): Salt levels of 2.5 and 5 g NaCl l^{-1} increased the activity of the CAT enzyme compared with the control treatment (Table 7), while it reached the lowest activity at 10 g NaCl l^{-1} (1.29 unit min^{-1} g^{-1} fresh weight) as well as sodium azide led to a decrease in CAT activity (Table 7), as the highest activity was 2.07 unit min^{-1} g^{-1} fresh weight at 0 mM NaN3. Concerning the interaction between NaCl and NaN3 (Table 7), the result showed that the highest activity of the CAT enzyme was 2.79 unit min^{-1} g^{-1} fresh weight at the control treatment (0 NaCl, 0 NaN_3), while 0.5 mM NaN_3 without added salt gave the lowest activity (0.82 unit min^{-1} g^{-1} fresh weight). On the other hand, CAT activity increased with increasing salt levels in 0.25 and 0.5 mM NaN_3.

Table (7): Effect of salt and sodium azide on catalase activity (unit min^{-1} g^{-1} fresh weight) in shoot of strawberry cv. Albion.

NaCl (mg l^{-1})	NaN_3 (mM)			
	0	0.25	0.5	mean
0	2.79a	1.75c	0.82g	1.78B
2.5	1.61cd	2.71a	1.60ed	1.97A
5	2.62a	2.00b	1.10f	1.91AB
10	1.26ef	1.11f	1.49de	1.29C
mean	2.07A	1.89B	1.25C	

Increasing CAT activity with salinity is agreed with other researchers (AL Aboudi et al., 2023; Singh et al., 2022). In general, it is noted that the highest CAT activity was in non-mutated plants at most salt levels compared to the activity in mutated plants (0.25 and 5 mM NaN3). Plant growth, metabolism, development, protein synthesis, and DNA replication can all be negatively impacted by sodium azide (Gomez et al., 2019; Szarejko and Maluszynski, 1999), these effects can also alter the balance between growth regulators and growth inhibitors.

Conclusion

The changes that occur as a result of these mutations are indirect, and there is a growing need to identify and understand these effects resulting from mutation so that they can be used in the plant breeding and improvement program.

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