Effect of Acridine Orange in Promoting Growth and Physiological Characteristics of *Fragaria Ananassa* Duch Under Salinity Stress in Vitro

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Effect of Acridine Orange in Promoting Growth and Physiological Characteristics of *Fragaria Ananassa* Duch Under Salinity Stress in Vitro.

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Abstract. This experimental were conducted out at plant tissue culture lab., Center of Desert studies to test the effect of four concentrations of Acridine Orange viz., 0 , 10 , 50 and 100mM L^{-1} under four levels of salinity viz., 0 , 30 , 60 and 90 mM NaCl L^{-1} on some phenological traits including: leaves number, branches number, plant length and fresh weight. Membrane stability index and electrolytic leakage also were in vitro measured. The experiment was factorial and arranged in a complete randomized design (CRD) with five replicates. Results revealed that 50 mML^{-1} of Acridine orange gave highest leaves number of 14.67 leaves $explant^{-1}$, branches number of 3.75 branches $explants^{-1}$, plant length of 1.48cm and fresh weight of 0.71g. whereas, 10 mM L^{-1} membrane stability index of 76.63. Furthermore, 30 mML^{-1} possesses highest leaves number of 11.75 leaves $explants^{-1}$, branches number of 2.83 branch $explant^{-1}$, plant length of 1.29cm and fresh weight of 0.58g. The highest level of salinity (90 mM) gave highest of electrolyte of 69.07.It could concluded that 50 m mol l^{-1} of acridine orange was the best concentration to promote growth of strawberry in vitro. Moreover, Plantlets were more tolerant to 30 m mol L^{-1} of Nacl in vitro.

Keyword: Acridine orange, strawbwrry, In vitro, Chemical mutagem, Saline stress.

1. Introduction

Strawberry (*Fragaria ananassa* Duch) belongs to Rosaceae. This species is perennial fruit crop. that adapts to a wide range of environments. Its originated in northern America [1]. This plant categorized as small fruits ranked in the fourth consumptive fruit after apple, orange and banana [2]. Highest world consumption of strawberry fruit lead to release highest quantities of waste as Well as special taste, antioxidant, anti-inflamatory and antimicrobial properties [3]. Strawberry considered as a good source of natural antioxidant such as carotenes, vitamins, phenols, flavonoids and glutathiones. Therefore, it possesses highly potential as antioxidants against ROS [4, 5]. Stunting growth that affect memory [6]. Salinity matter is regarded a big problem that faces agricultural sector in terms of twenty percent of cultivated lands in dry and semi dry region especially Iraq were negatively affected traditional irrigation methods, highly-salinity water consumption and surplus chemical fertilizers contributed on gradually accumulation of salts around rhizosphere and soil surface. Soils became non arable due to the negative effects of salinity represented by osmosis, nutrient unbalance, toxicity and chemical and physical properties. some factors were studied that

increase the plant tolerance to salinity stress. Such these factors, genetic divergence of strawberry.

Genetic variance strawberry cv. Albion could be occurred by chemical mutagens. Its behavior was compared under different levels of saline stress in vitro using biochemical and physiological mechanisms. Furthermore, genetic variance was determined in using molecular markers. In vitro mutations induction represented as interesting methods to induce genetic divergence. Therefore, selection of cells successfully was applied on many fruits crops using explants cells and tissues invitroing. Therefore, mutation induction became effective tool for developing the novel genetic material in plants especially over stress exposure [7]. N-7 of guanine is main effect site that be as target for chemical mutagens. Accordingly, the prior concept was recently observed in nitrogenous bases. There bases could be replaced by Acridine and insert in DNA strip or G:C transferred into A:T. Thus, it could be suggested that uncoupling on N-7 of guanine caused mutation induction [8] . Kaya et al [9] referred that Strawberry exposed to 50 mM of saline stress reduced the activity of SOD and CAT enzymes. while POD was increased. However, this effect was changed by addition of 0.l sodium nitroprusside resulted in minimum of negative effects of saline stress on Strawberry. Dziadezyk et al [10] obtained 18 tolerant clones from Strawberry grown on media contained 200 mM of NaCl. Thereafter, these clones were subcultured on mid saline medium contained 75 mm of NaCl hen selected the most tolerant clone pau /27/08 . Hussein et al [11] found that treated cultural medium of strawberry by 500, 750 and 1000 mg NaCl L^{-1} reduced chlorophyll of 10.13 mg g^{-1}, shoot length of 2.10cm, shoot dry weight of 1.22mg, root length of 2.13cm and root dry weight of 0.96mg. these results could assist to select the tolerant individuals of strawberry using in vitro. Akbar-Mozafari et al [12] pointed that in vitro exposed strawberry to 0 , 50 and 100 mM of NaCl caused negatively effects on growth parameters, pigments content, relative water content, membrane stability index and unbalance ions in mature plants. Zahedi et al [13] found that strawberry grew under 0, 40 and 80 m M NaCl possessed low yield, reduced qualitative fruit characteristics and increased oxidative stress. Jamalian et al [14] stated that strawberry plants exposed to saline stress for five weeks caused increased of ferulic, caffeic and coumaric acid. These saline tolerance indices considered as defense mechanisms for antioxidant compounds. Therefore, this study was conducted out to assess the effect of acridine orange as chemical mutation agent on some phenological traits of strawberry grown on different levels of sodium chloride as saline agent using in vitro culture.

2. Materials and Methods:

Lab trial was conducted out at tissue culture labs, center of desert studies, University of Anbar during 2019-2020. Transplants of strawberry cv. Albion were brought from Hit-Anbar 135 km north west Baghdad, this variety possesses special properties. Runners directly separated from field then transferred into lab. Consequently, runners tips were cut at 1cm [15], carefully cleaned with liquid soap. Meanwhile, they were washed under tap water for 1 hour. Cleaned separated tips were translocated into laminar air flow cabinet for surface sterilization.

2.1.Explants and tools sterilization

Metallic and glass work supplies such as surgical blade holders, forceps, petri dishes, beakers and flasks then were cleaned by cleaners and water. Meanwhile, these glasses were washed by distilled water and put in an oven on 200°c for 2 hours [16]. Furthermore, distilled water was sterilized in autoclave on 121°c, pressure of 1.04 Kg cm^{-2} for 15 min. Trivially, explants were sterilized by soaked into Clorox (sodium hypochlorite 6% w v^{-1}) then diluted upto 3% from sterilization solution. Drops of surfactant (tweens20) was added to reduce surface tension then soaked in 70% ethanol for 1 min. Consequently, explants were washed with distilled water three times for 5 min then transferred to petri dishes to take of the runners tips with length of 0.5 cm separated using sterilized blades and forceps.

2.2.MS Medium

Standard MS medium used as medium of explant culture [17]. This MS was supplied by

company Caisson, thirty grams per liter form it was weighed. Thereafter, plant growth regulators were applied as required the fortified by PPM (plant preservative mixture) to inhibit microbial contamination [18]. Additionally, activated charcoal was applied with 4.0 g l^{-1}, H potential was adjusted from 5.7 upto 5.8 using NaOH/HCl. Seven grams of agar per liter were added to culture medium the volume were completed upto 1 liter. Cultural medium was dissolved using heating on magnetic hot plate stirrer till boiling. Medium was distributed in vials of 10 ml for each vial and closed. Vials were autoclaved on 121°c,atm of 1.04 Kg cm^{-2} for 20 min.

2.3.Regeneration and proliferation

In generation stage, growth tips of Albion variety were sown on MS solidified by 0.5 mg BA L^{-1} and 0.1 mg IBA L^{-1}, then explants were incubated at 24°c±1 and light intensity of 1000 lux for 16/8 h (light/dark) for 4 weeks. Whereas, in proliferation plantlets that produced from generation were transferred onto MS fortified with 1.0 mg BA and 0.1 mg IAA L^{-1} as in prior stage.

2.4.Chemical mutagen preparation

Three concentrations of acridine orange were prepared. So, drops of dimethylsulfoxide (DMSO) 3-5 drops were added to solution of acridine orange to be entirely dissolved then 10, 50 and 100 Mm acridine per liter were prepared. Hydrogen potential of concentrations' solutions was adjusted to 7.2 based on its effects on gene mutation proportional to chromosomal one [19]. Consequently, solution of each concentration was put in sterilized jars. Thereafter, solutions were autoclaved on atm of 1.04 Kg cm^{-2}, temperature of 121°c for 20 min. After a while, solutions were cooled then plantlets were completely soaked in each concentration of solution for 1 hour in laminar. Thereafter, plantlets were taken and washed with distilled water for 10 min with shaking 3-5 times. Meanwhile, plantlets were in vitro planted on MS as explained in generation stage for 1 month.

2.5.Saline stress exposure

MS was prepared as in plantlets generation stage then fortified by 4 concentrations of NaCl viz., 0, 30, 60 and 90 Mm. Thereafter, plantlets were planted on saline-stressed MS the left for 6 weeks.

2.6.Measured indices

In vitro trial was conducted out using factorial experiment system with 5 replicates. Each vegetative growth was considered as experimental unit. Growth data were recorded on number of leaves per plantlet, number of branches per plantlet, plantlet length and fresh weight. Whereas, membrane stability index (MSI) and electrolyte leakage (EL) were measured according to Prochazkova et al. [20] and Karray-Bouraoui et al. [21], respectively.

2.7.Statistical analysis

Factorial experiment was applied using CRD with five replicates except MSI and EL these were displayed using histogram [22]. Data were analyzed via downloading them on GENSTAT 12.

3. Results and Discussion:

3.1.Leaves number (leaves plantlet^{-1})

Table 1 illustrates that the acridine mutagen levels differed significantly among treatments, level 50 mmol L^{-1} proportionally appeared the highest concentration of leaves number per plantlet ranked after control, which reached 14.67 leaves $plantlet^{-1}$, then the level 10 $mmolL^{-1}$ (10.67 leaves $plantlet^{-1}$), so the lowest level was 100 mmol L^{-1} (6.67 leaves $plantlet^{-1}$), while the comparison treatment showed 14.92. leaves $plantlet^{-1}$. However, it noticed from the table that there were significant differences between salinity levels in the number of leaves, the level 0 mmol $liter^{-1}$ that revealed the highest number of leaves at 13.67 leaves $plantlet^{-1}$, then the level 30 mmol $liter^{-1}$ (11.75 leaves $plantlet^{-1}$), followed by the level 90mmol l^{-1} (11.58 leaves $plantlet^{-1}$), while the 60 mmol levels showed the lowest leaves number of 9.92 leaves $plantlet^{-1}$.

Table (1) Effect of chemical mutagens acridine on average leaves number of strawberries under saline stresses in vitro.

Acridine (mmol L^{-1})	NaCl (mmol L^{-1})				Mean	L.S.D
	0	30	60	90		
0	17.00	15.00	13.33	14.33	14.92	
10	13.00	9.33	9.67	10.67	10.67	
50	14.67	14.67	13.33	16.00	14.67	1.84
100	10.00	8.00	3.33	5.33	6.67	
L.S.D			NS			
Mean	13.67	11.75	9.92	11.58		1.84

Table 1 showed there were non-significant differences between the two interactions in the number of leaves. However, control appeared the highest number of 17.00 leaves $plantlet^{-1}$, followed by an overlap between 50mmol L^{-1} of acridine x 90 mmol L^{-1} of NaCl and 16.00 leaves $plantlet^{-1}$. While the interaction presented 100 mmol L^{-1} of the mutagen X 60 mmol L^{-1} of $NaCl^{-1}$ of sodium chloride, the lowest concentration was 3.33 leaves $plantlet^{-1}$.

3.2.Branches number (branches $plantlet^{-1}$)

Table 2 illustrates that the mutagen levels differed significantly across treatments, the level 50 mmol L^{-1} was the highest number of branches, which was 3.75 branches $plantlet^{-1}$, then the level 10 mmol L^{-1} and control (2.92 branches $plantlet^{-1}$) for both, while the level 100 mmol L^{-1} was (1.83 branches $plantlet^{-1}$) of branches number. It noted from the results that there were significant differences between salinity levels on the number of branches. The control showed the highest concentration of branches number of 03.42 branches $plantlet^{-1}$, then the level 30 mmol liter $^{-1}$ (2.83 branches $plantlet^{-1}$), for level 90 mmol L^{-1} (2.75 branches $plant^{-1}$), while the 60 mmol level revealed the lowest number of branches, 2.42 branches $plantlet^{-1}$.

Table (2) Effect of chemical mutagens acridine on average branch number of strawberries under saline stresses in vitro.

Acridine (mmol L^{-1})	NaCl (mmol L^{-1})				Mean	L.S.D
	0	30	60	90		
0	3.67	2.00	2.67	3.33	2.92	
10	3.33	2.67	2.67	3.00	2.92	0.55
50	4.00	4.33	3.67	3.00	3.75	
100	2.67	2.33	0.67	1.67	1.83	
L.S.D			NS			
Mean	3.42	2.83	2.42	2.75		0.55

Table 2, it was evident that there were non-significant differences between the interactions of the two factors on the number of branches. However, numerically an interaction of 100 mmol L^{-1} of the mutagen x 30 mmol liters $^{-1}$ of sodium chloride presented the highest number of 4.33 branches $plantlet^{-1}$, followed by an overlap between 50 mmol L^{-1} X 0 of NaCl (4.00 branches $plantlet^{-1}$). While the interaction of 100 mutagen X 60, the lowest concentration was 0.67 branches plantlet $^{-1}$.

3.3.Plantlets length (cm)

Table 3 shows that the mutagen levels differed significantly among treatments, the control showed the highest plantlet length (cm), which was 1.73 cm, then the level 50 mmol L^{-1} (1.48 cm), the level 10 mmol L^{-1} (1.05) while, level 100 mmol L^{-1} was the lowest of 0.92 of plantlets length. For salinity stress, there was non-significant among levels. However, control gave highest plantlet length was 1.37 cm, then level 30 mmol liter $^{-1}$ (1.29 cm), so the level 60 mmol $liter^{-1}$ was the lowest (1.24 cm).

Table (3) Effect of chemical mutagens acridine on average strawberry plantlets length under

saline stress in vitro.

Acridine (mmol L^{-1})	NaCl (mmol L^{-1})				Mean	L.S.D
	0	30	60	90		
0	1.87	1.40	1.80	1.83	1.73	0.22
10	0.97	1.13	0.80	1.30	1.05	
50	1.60	1.53	1.63	1.13	1.48	
100	1.03	1.10	0.73	0.80	0.92	
L.S.D			NS			
Mean	1.37	1.29	1.24	1.27		NS

Moreover, table 3 shows non-significant differences among treatments of interaction but numerically control gave highest plantlet length of 1.87 cm, followed by an interaction 0 X 90 mmol liter^{-1} of 1.83 cm. whereas, interaction of 10 mmol liter^{-1} X 60 mmol liter^{-1} gave only 0.80 cm.

3.4.Fresh weight (mg)

Table 4 presents that the mutagen levels showed significant differences, the control indicated the highest fresh weight, 0.87 mg, then the level 50 mmol liter^{-1} (0.71mg), the level 100 mmol liter^{-1} (0.31 mg), the level 10 mmol liter^{-1} presented lowest weight 0.26 mg of fresh weight. It was noticed from the table that there were significant differences between salinity levels on the fresh weight, as the level of 30 mmol $^{-1}$ showed the highest fresh weight of strawberry plantlets, which was 0.58 mg, then the level 60 mmol L^{-1} (0.54 mg), as the comparison was (0.52 mg), while the 90 mmol levels gave the lowest fresh weight of strawberry of 0.51 mg. Table 4, it obvious that there were significant differences between the interactions of the two factors on the fresh weight of strawberry plantlets. An interaction of 0 of the mutagen X 90 mmol L $^{-1}$ of sodium chloride showed the highest fresh weight of 0.99 mg, followed by an interaction between 50 mmol L^{-1} X 60 mmol liter $^{-1}$ Sodium Chloride (0.94 mg). While the interaction 100 of the mutagen X 90 mmol L^{-1} of NaCl, the lowest fresh weight was 0.20 mg.

Table (4) Effect of chemical mutagens acridine on average fresh weight (mg) for strawberry under saline stress in vitro.

Acridine (mmol L^{-1})	NaCl (mmol L^{-1})				Mean	L.S.D
	0	30	60	90		
0	0.84	0.94	0.70	0.99	0.87	0.03
10	0.31	0.36	0.21	0.18	0.26	
50	0.56	0.67	0.94	0.68	0.71	
100	0.38	0.33	0.30	0.20	0.31	
L.S.D			0.06			
Mean	0.52	0.58	0.54	0.51		0.03

3.5.Cell membrane stability

Figure 1 shows that the treatment of strawberry plants with a chemical mutagen at a concentration of 50 mmol L^{-1} under level of saline tension at 60 mmol L^{-1} led to an increase in the cell membrane stability reaching 90.1, then the control treatment showed the cell membrane stability was 90. However, the treatment of the level of 100 mmol L^{-1} of the mutagen without the saline tension showed the lowest evidence of stability was -5.7.

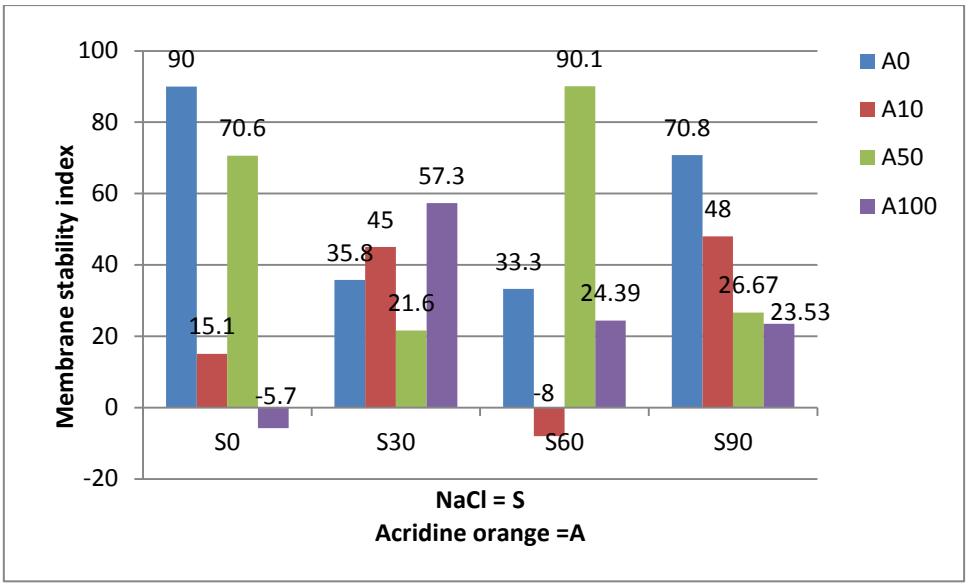

Figure (1) Effect of the chemical mutagen of Acridine orange and NaCl concentration on evidence of membrane stability of strawberry plants in vitro study.

3.6.Electrolytic perfusion

The effect of the chemical mutagen concentrations of acridine orange and different concentrations of sodium chloride in the electrolytic perfusion (Figure 2). the chemical mutagen was exceeded 10 mmol liter^{-1} in increasing the electrolytic perfusion under salt tension at a concentration of 30 mmol liter $^{-1}$ was 86.5. Regarding it was followed by the level of 100 mmol liter^{-1} of the chemical mutagens (78.13), while the concentration of 50 mmol liter $^{-1}$ of the mutagen was the lowest electrolyte perfusion without the saline tension as it reached 25.

The results showed that the level of 50 mmol L^{-1} of mutagens improved the vegetative growth characteristics of strawberry plants, as it increased the number of leaves and branches (table 2), the length of the plant (table 3) and relative leaves weight (table 4). It also increased the cell membrane stability (figure 1) by reducing the electrolyte perfusion (figure 2). This may be attributed to the role of Acridine orange in inducing the substitution of nitrogenous bases, particularly its effect on the nitrogen atom founded in the Guanine base. The substitution of G-T is in the DNA tape and this is called chemical mutagenesis.

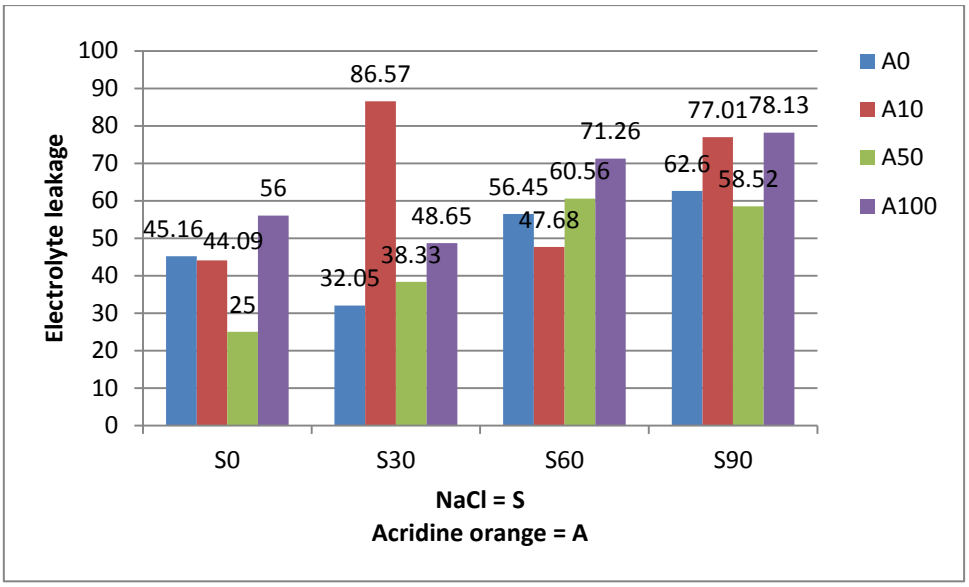

Figure (2) Effect of the chemical mutagen of Acridine orange and NaCl concentration in ex vivo electrolytic perfusion of strawberry plants.

Chemical mutagens interact with the oxygen atom, nitrogen and carbon, which affects the nitrogenous bases and thus affects the genes encoding protein production and thus affects the growth characteristics [8]. In addition, the reason could be due to the fact that the plant develops physiological and biochemical mechanisms with many pathways for ion stability, the manufacture of osmotic substances, free radical scavenges, and hormonal balance. The molecular level of plant adopt itself by activating genes and manufacturing defensive metabolites [23]. The results indicate that chemical mutagenesis have a positive effect on the development of traits, especially the concentration of 50 mmol L^{-1} in the vegetative characteristics and both cell membrane stability and electrolytic perfusion [24]. The effect of Acridine orange is could be attributed to increase the activity of the enzyme Oxidase NADPH as a result of stimulating the production of reactive oxygen species within the vegetable part [25]. Acridine orange has biological importance it binds with sulfur compounds inside the body of the organism, thus it is considered a cationic compound [26]. The binding of Acridine with seventh nitrogen atom in the nitrogenous base Guanine, and this explains Acridine orange's intrusion into the DNA strand and is the site of its main effect [4]. It may be a cause of the synthesis of luciferase and Aldehyde enzymes, and it may also lead to morphological changes in DNA that result in unspecified cloning of a number of genes [27]. The mechanism of binding of the acridine orange dye in the DNA strand, either the binding process is strong, one molecule of them may bind with 4 nitrogen bases, or the binding mechanism may be weak as it occurs at high levels of the dye in the DNA. Therefore, one molecule of them binds to a single nitrogen base and Acridine orange causes chromosome breakage in the legumes. Acridine orange causes chromosomal abnormalities as well as causes mutations in chlorophyll as well as phenotypic mutations [28]. The increased tolerance of plants to NaCl may be attributed to the improvement of some phenotypic characteristics such as cell membrane stability and increased electrolyte perfusion according to Figures 1 and 2 [9]. A transformation in the cell wall [29], which affects the transport of substances to and from the cell, which leads to the entry of the stain Acridine orange into the cell and thus its access into the nucleus and DNA stimulation, which is reflected in the phenotypic characteristics [30]. Correspondingly, the decrease in plant length is attributed to the increased salinity concentration due to an increase in the alkalinity of the medium, which affects the absorption of the elements necessary for growth [31]. The significant decrease in the growth

characteristics of high levels of sodium chloride due to salt tension that may be attributed to the osmotic effect and the negative ionic affected by salinity through increasing the concentration of sodium chloride as it affects in expansion and growth of cells and the decrease in the amount of water entering the cells, which causes a decrease in the water stress of the cultural medium as a result of the increase in salt concentration, which reduces the swelling pressure of the cell, thus it impedes the biochemical processes inside the cell necessary for cell division and thus affects the characteristics of growth phenotypes [32]. The increased salt concentrations led to a decrease in the activity of enzymes, the rate of photosynthesis, and the breakdown of pigments which was reflected in the phenotypic characteristics of strawberry plants [12]. This effect can reduce salinity and increase its tolerance by adding some chemicals. Among the characteristics affected by the salinity, such as the number of leaves, the number of branches, the length of the plant and the wet weight, it occurred as a result of the increase salt concentrations that affect the increase in the osmotic pressure of the cell thus not its expansion, and this is what was observed [11].

4. Conclusions

In vitro culture of strawberry Albion variety runners was induced on MS media solidified by 0.5 mg BA l^{-1} and 0.1 mg IBA L^{-1}, then explants were incubated at 24°c±1 and light intensity of 1000 lux for 16/8 h (light/dark) for 4 weeks. Plantlets were proliferated on MS fortified with 1.0 mg BA and 0.1 mg IAA L^{-1}. Plantlets that produced were subjected to two stress types, chemical mutagen (0, 10, 50 and 100 mmol acridine per liter) and salinity stress using NaCl (0, 30, 60 and 90 mmol per liter. Proportionally, acridine orange at 50 mmol $liter^{-1}$ improved morphological indices thereby improvement leaves number, branches number, plantlet length and fresh weight. For saline-stressed plantlets, these plantlets were proportionally survived under salinity stress via NaCl solution. So, plantlets under were proportionally tolerated 30 mmol $liter^{-1}$ of NaCl in terms of studies morphological indices. Finally, it could be extracted that 50 mmol per liter from acridine orange and 30 mmol per liter from NaCl are preferred to evaluate tolerance of strawberry plantlets to given stresses. Consequently, we could recommend that 50 mmol per liter from acridine orange could be used as chemical mutagen in strawberry in vitro. Moreover, plantlets of strawberry produced in vitro more tolerant to 30 mmol per liter from NaCl. Thus, saline tolerant strawberry could be produced via application acridine orange in vitro.

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