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Standardization of Tissue Culture Protocols for Callus Induction and Plant Regeneration from Mature Embryo of Sorghum [*Sorghum bicolor* L. Moench]

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Abstract: Three local genotypes of grain sorghum were studied for thire ability to induse callus from mature embryos using different hormonal treatments. MS medium were supplemented with different concentrations of plant regulators in order to get the best rate of callus induction, frequency of regeneration, and rooting rate of plantlets. The obtained plants were acclimatized to geta plant capable of growing under greenhouse conditions. All experiments were laid according to Complete Randomized Design (CRD) with three replications.
For the surface sterilization of the grains 5% NaOCl for 20 minutes was the best concentration. It was observed that the addition of 2 mg/L of 2,4-D (2,4- dichlorophenoxyacetic acid) to MS medium could increase in the frequency of callus induction and embryogenic callusformatioin comparing with other treatments. Adding 1.5 mg/L TDZ(Thidiazuron), 1.5 mg/L BAP(6-benzyl adenine) and 1 mg/L IAA(Indole-3-acetic acid) to the growth medium had the best effect on regeneration and number of plantlets, while the addition of 1 mg/L NAA(1-naphthylacetic acid) was found to be the best for enhancing both number and length of the roots.
Key words: *Sorghum bicolor*, mature embryo, callus, 2,4-D, regeneration, TDZ.

Introduction

Sorghum bicolor (L.) Moench is an important cereal crop plant which has a worldwide annual production about 60 million tones. It ranks as the sixth most planted crop in the world, after wheat, rice, maize, soybean and barley. Sorghum is being a staple for both human and livestock, and a potential source of biofuel, that it can be used as food, feed, fodder, fiber and fuel, beside the rising demand for Sorghum towards the production of alcohol, beer and other Sorghum based derivatives such as syrup and jaggery[1,2]. Sorghum originates from tropical Africa, which makes it highly tolerant to drought and well adapted to arid countries in northeast Africa, where it is mainly grown, as well as to dry areas in the United States and India[3]. In the developing world, improving Sorghum through genetic transformation is the latest in a long series of technologies that have been applied to this crop. Cereal crop improvement through genetic transformation requires establishment efficient plant regeneration is a prerequisite for a complete genetic transformation protocol[4]. On the other hand Sorghum is considered to be one of the most recalcitrant species among the cereals for in vitro response and genetic transformation[2,5,6]. Also, the regeneration of differentiated cereal plant cells from callus remains a major limiting step in obtaining high numbers of cereal clones or independent transgenic cereal lines[7]. Elhag and Butler (1992)[8] have studied the effect of genotype, explant age and medium composition on callus induction and plant regeneration from mature and immature embryos of Sorghum. Vasil and Vasil (1986)[9] suggested that the differential response of the genotypes may be due to differential expression which in turn depends on the spatial and temporal distribution, beside physiological and developmental stages. Previous studies on sorghum tissue culture revealed that callus has been also induced in Sorghum from both immature and mature embryo[2,10]. Mature seeds are the most preferred explants for in vitro protocols as they can

be stored, available round the year and can be easily handled[2]. Studies in cereals showed that, the use of 2, 4-Dichlorophenoxyacetic acid (2,4-D) in callus induction from mature embryos was a critical factor. In general, auxins 2,4-D in the range of 1–3 mg/L, is essential for the formation of embryogenic callus from cereal embryos[11]. It was reported that cereals require low level of 2, 4-D to initiate the callus cultures and its higher concentrations have been found to be less effective in the formation of embryogenic callus[12,13]. Kresovich *et al*., (1987)[14] reported that the frequency of regeneration ranged from 26 percent to 69 percent across the genotypes in Sorghum. Previous work on Sorghum tissue culture using immature inflorescence, increasing the level of kinetin to a concentration of 0.5 mg /L in the MS medium increased the frequency of embryogenic callus ormation[15].

It was observed also that, auxin and cytokine combination could improve the regeneration[16,17]. In order to overcome the genotypic limitations of plant regeneration in Sorghum, callus induction medium must be supplemented with strong cytokine like Kinetin with 2,4-D[6]. In monocotyledons species, several authors reported that TDZ induces multiple shoot formation[18].It was confirmed that TDZ is capable of promoting callus regeneration and it has potential for enhancing the regeneration of cereal and grass species[18].

Multiple shoots were observed using 10μM TDZ in the study of[19], in another study[17] it was reported that the use of a combination of 1.5 mg/L of BAP, TDZ and 1.0 mg/L IAA could enhance multiple shoot production.

Several factors could affect the rate of rooting like plant cultivar, plant regulator used and growth medium[20]. Choosing the type of auxin and its concentration are the most important factors in the ability of rooting in plants[20]. Adding 1mg/l NAA resulted in the best number and thickness of roots[20]. Studies showed that using (50, 30%) diluted MS medium may increase plant rooting cultured in vitro[21].

According to the importance of callus induction and plant regeneration from mature embryos of Sorghum, we will focus in this work on standardization of tissue culture protocols for different genotypes and explants with different hormonal treatments in order to get a well-established regeneration system, which is further useful for the use in developing transgenic crop.

Materials and methods

Plant material

Seeds used in this study were belong to three local genotypes of grain sorghum Ezraa3, Ezraa7 and Rezenia cultivars and were obtained from General commission of agriculture research in Damascus- Syria.

Seeds sterilization

Seeds were surface sterilized in 70% ethanol for 3 min, rinsed twice with sterilized distilled water, incubated further in 6% sodium hypochlorite solution for 15 min, and rinsed for 4 times in sterilized distilled water. The surface sterilized seeds were incubated at 25 °C for 2 h in sterilized distilled water for imbibing.

Treatments of plant materials

Callus induction: The mature embryos were removed from the imbibed seeds and placed on MS medium[22] supplemented with 30 g/L sucrose 7 g/L agar and different concentration of 2,4-D as shown in Table(1). They were incubated at 25±2 °C in darkness for 6 weeks, after which callus were obtained.

Table (1) Plant regulators (mg/L) used for each growth stage

Stage	2,4-D	Kinetin	TDZ	BAP	IAA	NAA
Callus induction	0	0	-	-	-	-
	0	0.5	-	-	-	-
	0.5	0	-	-	-	-
	0.5	0.5	-	-	-	-
	1	0	-	-	-	-
	1	0.5	-	-	-	-
	1.5	0	-	-	-	-
	1.5	0.5	-	-	-	-

	2	0	-	-	-	-
	2	0.5	-	-	-	-
	2.5	0	-	-	-	-
	2.5	0.5	-	-	-	-
Plant regeneration	-	-	-	-	-	-
	-	0.5	-	-	-	0
	-	1	-	-	-	0
	-	0.5	-	-	-	0.5
	-	0.5	-	-	-	1
	-	-	0.5	0.5	0.5	-
	-	-	0.5	1	1	-
	-	-	1.5	1.5	1.5	-
	-	-	0.5	0.5	1	-
	-	-	1	1	1	-
	-	-	1.5	1.5	1.5	-
	-	-	-	-	-	-
Rooting	-	-	-	-	-	0
	-	-	-	-	-	0.5
	-	-	-	-	-	1
	-	-	-	-	-	1.5
	-	-	-	-	-	2

Shoots regeneration: The obtained callus was transferred to shoot regeneration medium (Table1), incubated at 25 ± 2 ℃ in the presence of light (2000 lux) for 16h then 8 h of darkness and subcultured every 4 weeks.

Roots formation: Plantlets were cultured on rooting medium (MS salts 0.5X concentration), incubated at 25 ± 2 ℃ in the presence of light (2000 lux) for 16h then 8 h of darkness, (Table1).

Well-established root system were transferred to the vermiculate soil, covered for 5 days with plastic covers to prevent desiccation, grown in tissue culture room and transferred to greenhouse 15 days later.

Statistical analysis

For each genotype a completely randomized design with 3 replicates (25 embryos per replicate) was constructed to collect data on the frequency of callus induction, embryogenic callus formation, frequency of regeneration and the rate of obtained shoots and roots numbers were documented. The experimental data were analyzed statistically using Xls-Stat software in order to determine the variation among treatments, explants and genotypes.

Result and discussion

Callus Induction

Callus began to appear after 6 to 8 days of embryos cultivation at the induction medium, callus was initially a mushy texture and quantity of a few, but after sub culturing properties of callus improved and became more compact. Embryogenic callus was formed after 45 days of culturing, where the callus induction and embryogenic callus formation frequency were documented.

The effect of different levels of 2,4-D, and Kinetin in the development of callus

Results showed significant differences in the frequency callus induction among used levels of growth regulator 2,4-D, studied cultivars and interaction among them, while no significant differences appeared in both presence and absence of Kinetin, the same results were obtained by[15]. As shown in Table (2), the frequency of callus induction using (1.5, 2, 2.5 mg/L) 2,4-D were (85.56, 85.00, 77.78 %, respectively) and were the highest significantly, while no callus induction appeared in the control treatment (2,4-D free), which confirm that callus induction frequency mainly determined according to the presence of 2,4-D in the medium, these are in agreement with Mangula *et al.,* (2000)[13]who mentioned that using (1-3 mg/L) 2,4-D had the best effect on callus induction. The interaction between all cultivars and different levels of 2,4-D showed that the frequency of

callus induction were the highest using (1.5, 2, 2.5 mg/L) 2,4-D with all studied cultivars (Table2). These results insure that auxin 2,4-D has a fundamental role in callus induction, which agrees with reports of[23,24], and this role which auxin 2,4-D plays is a result of encouragement of mitotic cell division, where auxin is working to increase the synthesis of nucleic acids RNA, beside its activation action of enzymes that promote chemical reactions necessary to provide required materials for cell division, such as the revitalization of the enzyme RNA Polymerase[25].

The effect of different levels of 2,4-D, and Kinetin in callus induction

Results showed that there were significant differences in the frequency of embryogenic callus formation among the used levels of 2,4-D and Kinetin, studied cultivars, and the interactions among them. Table (2) shows that the frequency of embryogenic callus formation was significantly the highest (50, 43.33 %,) using (1.5, 2 mg/L2,4-D respectively), followed by (30.00, 22.78 %) using (1, 2.5 mg/L2,4-D respectively), while no embryogenic callus formation obtained in the control treatment (2,4-D free). These results refer to the importance of 2,4-D presence in the medium of callus induction, in levels ranging from (1 – 2.5 mg/L), on the other hand both high levels (more than 2.5 mg/L) and low levels (less than 1 mg/L) could negatively affect the frequency of callus induction.

No significant differences in the frequency of embryogenic callus formation in the presence of Kinetin (0.5 mg/L) compared to the control (Kinetin free). These results confirm that the frequency of embryogenic callus formation mainly determined by the presence of 2,4-D in the medium of induction.

Cultivars Rezenia and Ezraa3 had the highest significantly frequency of embryogenic callus formation (34.72, 33.61 %, respectively), while the lowest significantly was Ezraa7 (12.22 %). As for the interaction between species, 2,4-D and Kinetin, cultivar Rezenia had the highest frequency of embryogenic callus formation (53.33, 73.33 %) using (1 , 2 mg/L respectively) 2,4-D in the absence of Kinetin, and (53.33, 70.33, respectively) using 1.5 mg/L 2,4-D in the presence and absence of Kinetin (Table 2). These results are in agreement with[26,27,28] who confirmed that high concentrations of KIN negatively affect callus induction and the frequency of embryogenic callus formation.

Table (2) Effect of Kinetin and 2,4-D in callus induction and embryogenic callus formation.

Callus Induction (%)				Embryogenic Callus (%)				Treatments	
Average for each treatment	**Ezraa3**	**Ezraa7**	**Rezenia**	**Average Varity**	**Ezraa3**	**Ezraa7**	**Rezenia**	**Kinetin mg/l**	**2,4-D mg/l**
0.00^{E}	0.00^{H}	0.00^{H}	0.00^{H}	0.00^{E}	0.00^{M}	0.00^{M}	0.00^{M}	0	0
0.00^{E}	0.00^{H}	0.00^{H}	0.00^{H}	0.00^{E}	0.00^{M}	0.00^{M}	0.00^{M}	0.5	0
46.67^{D}	50.00^{EFG}	50.00^{EFG}	40.00^{G}	13.33^{DE}	16.66^{JKLM}	10.00^{KLM}	13.33^{JKLM}	0	0.5
48.89^{D}	43.33^{FG}	40.00^{G}	63.33^{DEF}	16.67^{D}	23.33^{HIJKL}	0.00^{M}	26.66^{GHIJK}	0.5	0.5
74.44^{BC}	76.66^{ABCD}	73.33^{ABCD}	73.33^{ABCD}	40.00^{B}	33.33^{EFGHIJ}	33.33^{EFGHIJ}	53.33^{ABCDE}	0	1
68.89^{C}	66.66^{CDE}	70.00^{BCDE}	70.00^{BCDE}	20.00^{D}	26.66^{GHIJK}	3.33^{LM}	30.00^{GHIJ}	0.5	1
76.67^{ABC}	73.33^{ABCD}	80.00^{ABCD}	76.66^{ABCD}	42.22^{B}	40.00^{EFGHI}	33.33^{EFGHIJ}	53.33^{ABCDE}	0	1.5
78.89^{ABC}	83.33^{ABCD}	76.66^{ABCD}	76.66^{ABCD}	44.44^{B}	43.33^{DEFGH}	20.00^{IJKLM}	70.33^{AB}	0.5	1.5
91.11^{A}	90.00^{AB}	90.00^{AB}	93.33^{A}	61.11^{A}	66.66^{ABC}	43.33^{DEFGH}	73.33^{A}	0	2
80.00^{ABC}	86.66^{ABC}	66.66^{CDE}	86.66^{ABC}	38.89^{BC}	63.33^{A}	3.33^{LM}	50.00^{BCDEF}	0.5	2
85.56^{AB}	83.33^{ABCD}	80.00^{ABCD}	93.33^{A}	22.22^{D}	46.66^{CDEFG}	0.00^{M}	20.00^{IJKLM}	0	2.5
84.44^{ABC}	80.00^{ABCD}	86.66^{ABC}	86.66^{ABC}	23.33^{CD}	43.33^{M}	0.00^{M}	26.66^{M}	0.5	2.5
-	61.11^{A}	59.44^{A}	63.33^{A}	-	33.61^{A}	12.22^{B}	34.72^{A}	**Average for each cultivar**	
62.41^{A}	62.22^{A}	62.22^{A}	62.78^{A}	28.81^{A}	33.89^{A}	35.56^{A}		0	Average KIN
60.19^{A}	56.67^{A}	60.00^{A}	63.89^{A}	23.89^{A}	33.33^{AB}	33.89^{A}		0.5	
0.00^{D}	0.00^{D}	0.00^{D}	0.00^{D}	0.00^{D}	0.00^{E}	0.00^{E}		0	Average 2,4-D
47.78^{C}	45.00^{C}	46.67^{C}	51.67^{C}	15.00^{D}	20.00^{DE}	20.00^{DE}		0.5	
71.67^{B}	71.67^{B}	71.67^{B}	71.67^{B}	30.00^{B}	30.00^{BCD}	41.67^{BC}		1	
77.78^{AB}	78.33^{AB}	78.33^{AB}	76.67^{B}	43.33^{A}	41.67^{BC}	61.67^{A}		1.5	
85.56^{A}	78.33^{AB}	88.33^{A}	90.00^{A}	50.00^{A}	65.00^{A}	61.67^{A}		2	
85.00^{A}	83.33^{AB}	81.76^{AB}	90.00^{A}	22.78^{BC}	45.00^{B}	23.33^{D}		2.5	

Factor	LSD (%1) Embryogenic Callus (%)	LSD (%1) Callus Induction (%)
2,4-D Concentration	11.78	11.05
Kinetin Concentration	16.74	15.70
Cultivar	20.51	19.23
Inter action between 2,4-D and Kinetin concentration	16.66	15.63
Inter action between Kinetin and cultivar	29.00	27.20
Inter action between 2,4-D and cultivar	16.04	15.04
Inter action between Kinetin 2,4-D cultivar	29.69	21.27
CV%	32.65	13.41

Different letters on top refer to significant differences ($P < 0.01$).

Regeneration

The effect of hormonal mixtures in the frequency of plant regeneration (%) of callus

Regeneration was significantly affected by the use of different hormonal mixtures. As shown in Table (3) the highest frequency of regeneration (25%) was obtained using (1.5 mg/L TDZ, 1.5 mg/L BAP, and 1 mg/L IAA), followed by (12.5%) using (1.5 mg/L TDZ, 1.5 mg/L BAP, and 0.5 mg/L IAA), then (12.5 %) using 0.5 mg/L NAA and 1 mg/L Kinetin, while the callus did not show any response to regeneration in the rest of treatments and in control treatment. Cultivar $Ezraa_7$ showed the highest frequency of regeneration (9.545 %) comparing with Ezraa3 (2.273%). Frequency of regeneration in the interaction between cultivars and different hormonal mixtures was significantly the highest (32.5%) in cultivar Ezraa7 using (1.5 mg/L TDZ, 1.5 mg/L BAP and 1 mg/L IAA), (25%) using (1.5 mg/L TDZ, 1.5 mg/L BAP, and 0.5 mg/L IAA), and (20%) using (1 mg/L Kinetin , and 0.5 mg/L NAA), followed by cultivar Ezraa3 (20%) using (1.5 mg/L TDZ, and 1.5 mg/L BAP, and 1 mg/L IAA). Frequency of regenerating did not go above 10 % for Ezraa7 using (1 mg/L TDZ, 1 mg/L BAP, and 1 mg/L IAA), 5% using(1 mg/L TDZ, 0.5 mg/L BAP, and 1 mg/L IAA), and 2.5% using (0.5 mg/L Kinetin, and 0.5 mg/L NAA), while in cultivar Ezraa3 frequency of regeneration didn't exceed 5% using (1 mg/L Kinetin , and 0.5 mg/L NAA), which means that using lower levels of auxin and higher levels of cytokine could increase the frequency of regeneration.

The effect of hormonal mixtures in the number of roots on callus

It was observed that the number of formed shoots could be significantly affected according to the use of different hormonal mixtures, studied cultivars and interaction between them. As shown in table (4) the highest rate of shoots numbers was (18.25) and it was obtained using (1.5 mg/L TDZ, 1.5 mg/L BAP, and 1 mg/L IAA), followed by (8.12) using (1.5 mg/L TDZ, 1.5 mg/L BAP, 0.5 mg/L IAA, 1 mg/L Kinetin, and 0.5 mg/L NAA), while no shoots were formed in other treatments. The rate of shoots numbers formed on callus was the highest in cultivar Ezraa7 (5.63) comparing with Ezraa3 (1.34). As for the interaction of cultivars with different hormonal mixtures the rate of shoots numbers was significantly the highest in the cultivar Ezraa3 (24) using (1.5/L TDZ, 1.5 mg/L BAP, and 1 mg/L IAA), followed by Ezraa7 (16.25) using (1.5 mg/L TDZ, 1.5 mg/L BAP, and 0.5 mg/L IAA), while the number of shoots was the lowest in Ezraa7 using (0.5 mg/L Kinetin, and 0.5 mg /L NAA), and no shoots were formed in other treatments.

Results showed that the best growth medium for plant regenerating (frequency of regenerating and number of formed shoots) is the one containing (1.5 mg/L TDZ, 1.5 mg/L BAP, and 1 mg/L IAA), corresponding to results of [17]. TDZ has the ability to increase the frequency of regenerating[18], that it belongs to synthetic cytokines which stimulate differentiation of buds from callus[29]. It was noticed that the rate of formed shoots could be affected according to different hormonal mixtures used, that the treatment including 1mg/L of KIN, and 0.5 mg/L of NAA has given the best frequency of regenerating (20%) and number of shoots (12) in the cultivar Ezraa7, these results are in agreement with[15,30], so in order to control plant regenerating, growth medium should be supplemented with suitable amounts of auxins and cytokines, the same was mentioned by[29]. The highest frequency of regenerating in cultivars Ezraa7 and Ezraa3 were relatively low (32, 20 %), this may be due to the source of the explants, which was used for callus induction, where mature embryos were used, this result was also observed by[31] who mentioned that the frequency of regenerating from mature embryos was lower comparing with immature embryos, but the ease of access to mature embryos and their availability throughout the year is the reason behind their use as a source of callus.

The effect of hormonal mixtures in the number of roots formed in the regeneration stage

As shown in Table (3) There were significant differences in the rate of roots numbers among different hormonal mixtures, studied cultivars, and the interactions between them. The highest rate of roots numbers was (19.38 roots) using both treatments (1mg/L NAA), and (1 mg. L -1TDZ , 0.5 mg/L BAP, and 1 mg/L IAA), and it was (16.75 roots) using (1mg/L NAA, 1 mg/L TDZ, 1 mg/L BAP, and 1 mg/L IAA), while it was (15.25 roots) using (0.5 mg/L TDZ, 0.5 mg/L BAP, and 0.5 mg/L IAA), and (13.88 roots) using (0.5 mg/L NAA, and 1 mg/L Kinetin). There were no significant differences among the studied cultivars. As for the interaction between studied cultivars and different hormonal mixtures, cultivar Ezraa3 had the highest rate of roots numbers (24.75 root) using (1 mg/L KIN and 0.5 mg/L NAA), then both cultivars Ezraa3 and Ezraa7 (19.50, 19.25 roots, respectively) using (1 mg/L TDZ, 0.5 mg/L BAP, and 1 mg/L IAA) , and in Ezraa7 (18.50 roots) using (0.5 mg/L TDZ, 0.5 mg/L BAP, and 0.5 mg/L IAA), and (17.25 root) using (1 mg/L TDZ, 1 mg/L BAP, and 1 mg/L IAA), while the lowest rate was Ezraa7 (2.5 roots) using (1.5 mg/L TDZ, 1.5 mg/L BAP, and 0.5 mg/L IAA). These results indicate that using higher auxin concentrations comparing with cytokine could affect roots numbers positively, which is an expected result according to the well-known role of auxins in the formation of roots[32]. In this study we could not get any shoot plant from Rezenia cultivar with all used treatments, which may be due to the difference in Genetic makeup with the rest of the studied cultivars, and thus the difference in the response to the applied hormonal treatments[9].

Table (3): The effect of plant growth regulator in the frequency of regeneration, number of shoots and number of roots

Regeneration%			Number of shoot			Number of root			Treatment Factor				
Average of cultivar	Ezraa3	Ezraa7	Average of cultivar	Ezraa3	Ezraa7	Average of cultivar	Ezraa7	Ezraa3	IAA Mg/l	BAP Mg/l	TDZ Mg/l	NAA Mg/l	KIN Mg/l
0.00^{C}	0.00^{D}	0.00^{D}	0.00^{C}	0.00^{C}	0.00^{C}	6.87^{DE}	6.00^{EFG}	7.75^{DEFG}	-	-	-	0	0
0.00^{C}	0.00^{D}	0.00^{D}	0.00^{C}	0.00^{C}	0.00^{C}	2.62^{E}	2.75^{FGH}	2.50^{GH}	-	-	-	0.5	0
0.00^{C}	0.00^{D}	0.00^{D}	0.00^{C}	0.00^{C}	0.00^{C}	19.38^{A}	26.25^{A}	12.50^{CDE}	-	-	-	1	0
1.25^{C}	0.00^{D}	2.50^{CD}	0.37^{C}	0.00^{C}	0.75^{cC}	11.75^{BCD}	23.50^{aAB}	0.00^{H}	-	-	-	0.5	0.5
12.50^{B}	5.00^{CD}	20.00^{bAB}	7.12^{B}	2.25^{C}	$1200.^{B}$	13.88^{ABC}	3.00^{FGH}	24.75^{AB}	-	-	-	0.5	1
0.00^{C}	0.00^{D}	0.00^{D}	0.00^{C}	0.00^{C}	0.00^{C}	15.25^{ABC}	18.50^{ABC}	12.00^{CDEF}	0.5	0.5	0.5	-	-
6.25^{BC}	0.00^{D}	12.50^{BC}	2.12^{C}	0.00^{C}	4.25^{C}	9.25^{CDE}	11.75^{CDEF}	6.75^{EFGH}	0.5	1	1	-	-
12.50^{B}	0.00^{D}	25.00^{A}	8.12^{B}	0.00^{C}	16.25^{B}	4.62^{E}	2.25^{H}	7.00^{DEFGH}	0.5	1.5	1.5	-	-
2.50^{C}	0.00^{D}	5.00^{CD}	0.75^{C}	0.00^{C}	1.50^{C}	19.38^{A}	19.25^{ABC}	19.50^{ABC}	1	0.5	1	-	-
5.00^{BC}	0.00^{D}	10.00^{BCD}	1.62^{C}	0.00^{C}	3.25^{C}	16.75^{AB}	17.25^{ABC}	16.25^{BCD}	1	1	1	-	-
25.00^{A}	20.00^{AB}	32.50^{A}	18.25^{A}	12.50^{B}	24.00^{A}	2.62^{E}	2.75^{FGH}	2.50^{GH}	1	1.5	1.5	-	-
-	2.27^{A}	9.54^{A}	-	1.34^{A}	5.63^{A}	-	10.14^{A}	12.11^{A}	Average treatment				

Different letters on top refer to significant differences ($P < 0.01$).

Rooting

The effect of different concentrations of NAA in the length of roots

Results showed that the rate of roots length could significantly affected according to the use of different levels of auxin NAA, studied cultivars, and the interaction between them. The rate of roots length was significantly the highest (8.30, 6.90, 6.70 cm) using (1, 0.5, 1.5 mg/L NAA respectively), while the lowest length appeared in both control (NAA free), and using 2 mg/L NAA (3.25, 3.15 cm, respectively) with no significant differences between them. No significant differences in the rate of the roots length between the two cultivars studied $Ezraa_7$ and $Ezraa_3$ (5.93, 5.28 cm, respectively). As for the interaction between the different cultivars and NAA levels, the highest root lengths were in $Ezraa_7$ using (1, 0.5 mg/L NAA), and $Ezraa_3$ using 1.5 mg/L NAA (9.86, 8.40cm , respectively), while the rate of roots length was significantly the lowest in $Ezraa_7$ and $Ezraa_3$ using 2 mg/L NAA, and with control treatment (NAA free), (2.00, 3.20, 3.30cm, respectively). It was observed that auxin NAA can greatly affect the root length when used in the ½ MS medium, that root lengths were rabidly increased using NAA concentrations ranging from 1 to 1.5 mg/L, but it was noticed that root length began decreasing with increasing NAA concentration over this limit, indicating the importance of controlling the concentration of NAA in the medium of rooting for the best rate length of the roots length, that this character has big importance in increasing the chances of plant survival, growth and

development , according to the importance of roots in water and minerals nutrients absorbance, the same results obtained by [17,32,33].

The effect of different concentrations of NAA in the number of roots

Results showed that there were significant differences in the rate of roots numbers according to different levels of auxin NAA, cultivars, and the interaction between them. Table (4) shows that the rate of formed roots numbers was significantly the highest (5.10 roots) using 1 mg/L NAA, while the lowest was in the control (2.00 roots) (NAA free), while no significant differences appeared between the two studied cultivars. As for the interaction between cultivars and different levels of NAA, the results showed that the average number of roots was significantly the highest in cultivars Ezraa7 and Ezraa3 (5.60, 4.60 roots) without significant differences between them using 1 mg/L NAA, while it was significantly the lowest in the same two cultivars in the control treatment (NAA free) (1.80, 2.20 roots, respectively).

Resulting that the presence of NAA has an important role in increasing the numbers of formed roots, and that the best medium for roots formation was the one containing 1 mg/L NAA, in the two cultivars studied, This corresponds to the results of[2,20], while the use of higher levels of NAA resulted in decreasing numbers of formed roots, and this agrees with results of[34].

Table (4): Effect of NAA concentration on length and number of roots

Root length (cm)			Number of root			Treatment
Average cultivar	**Ezraa3**	**Ezraa7**	**Average cultivar**	**Ezraa3**	**Ezraa7**	NAA Mg/l
3.25^{B}	3.20^{DE}	3.30^{DE}	2.00^{B}	2.20^{CD}	1.80^{D}	0
6.90^{A}	5.40^{BCD}	8.40^{A}	3.40^{AB}	3.00^{BCD}	3.80^{ABC}	0.5
8.30^{A}	6.20^{BCD}	9.86^{A}	5.10^{A}	4.60^{AB}	5.60^{A}	1
6.70^{A}	7.30^{ABC}	6.10^{BCD}	3.80^{AB}	3.20^{BCD}	4.20^{AB}	1.5
3.150^{B}	4.30^{CDE}	2.00^{E}	3.60^{AB}	3.20^{BCD}	4.00^{ABC}	2
	5.28^{A}	5.93^{A}		3.24^{A}	3.92^{A}	**Average treatment**

Factor	**LSD (1%) Number of root**	**LSD (1%) Root length(cm)**
Treatment	1.83	3.27
Cultivar	1.75	3.15
Inter action between treatment and cultivar	1.81	3.26
CV%	17.44	20.03

Different letters on top refer to significant differences ($P < 0.01$).

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