In Vitro Selection of *linum* callus Tolerant to High Temperatures and Plant Regeneration Using Tissue Culture Technique

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Abstract: This study aimed to micropropagate *linum* hypocotyls (7- day old seedlings) using tissue culture technique. Hypocotyls were cultured on agar solidified media. The media were supplemented with various concentrations and combinations of auxins and cytokinins. The study was extended to investigate possible means to increase plant tolerance to heat stress using salicylic acid.In addition, some physiological and biochemical responses were studied with comparing it with plants that have been directly grown from the grains and treated with salicylic acid. Moreover, the variations of protein pattern was studied and separated using gel electrophoresis in the presence of sodium dodecyl polyacrylamide. The study showed the presence of a marked change in the construction of some of the many peptides in studied plant indicate that hardening treatments may modulate the expression of gene or genes that encoding some adaptive or defensive proteins and thus raise the plant's resistance against heat stress.
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Key words: *linum* hypocotyls, heat stress,salicylic acid

1.Introduction:-

High heat stress is one of a biotic factors that has an obvious impact on growth and development of many plants, especially in the last period of the spring season and during summer in most of the world regions. The severe high heat causes several continuous stresses such as water deficient and dryness stress that leads to several physiological and biochemical adverse responses in plants. The impact resulting from the high thermal stress can be prevented to some extent through some compounds such as salicylic acid (Qaiser *et al*, 2010).

Salicylic acid (a common phenolic compound in vascular plants) has a diversified physiological activity and can participate in plant response to environmental stresses.

Salicylic Acid is included within the internal regulators resistant to heat and sicknesses. Some studies has concluded that Salicylic Acid plays an effective role in several physiological and biochemical processes that indicates to consider it within the influencing botanical hormones on the plants growth(Gabrielle *et al*,2011; Mariana *et al*, 2011; Neslihan *et al*, 2012). It was believed that through this improvement, the hardness in plants enables it to resist the drought indirectly when the plants are grown under high thermal strain which causes increase in transportation rate and water loss with reduction in the gap connection and decline of the final outcome of light building in unhardened plants that adversely affects the plants growth (Griffin *et al*., 2004; Patel *et al*,2012).

Biotechnology is one of the proposed alternatives through which they can speed up deriving of new breeds and high productivity that has the ability to resist the different environment stresses.Through these techniques change in the genetic basic building units in the cells and tissue cultures; construct of units or retrieving new plants that compulsory genetically pushed and biochemical & physiology to resist the strains through the laboratory extraction can be created.

The basic target of the present research is the utilization of the laboratory selection methods for the purpose of getting strains of *Linum* which characterized by its ability to endure high temperatures then propagation and retrieve complete plants from such selected cells.

2.Materials and Methods:

2.1.Materials:

***2.1.Sample**:*

Linum (sakha 1) seeds which has an economic importance in industry was used in this study. Also it is used in human food and as natural fiber (Ankit *et al*., 2014)

2.2. The Culture medium:

The utilized medium used was that of (Murashige & Skoog, 1962) (MS) that contains inorganic salts, vitamins and sugar as carbon source (30g/L), various concentrations of growth regulators either individual or through interference with each other were added. pH of the media were controlled at 5.8 by suing either hydrochloric or sodium hydroxide 0.1 standard. After that agar was added (10 g/liter) in

case of hard medium. The nutritional medium was distributed to 9cm Petri dishes (30m/dish) then sterilized in a steam sterilizer on 121°C and under pressure 15 pounds/inch^2 for 15 minutes; It is worth mentioning that all of the chemicals used in the nutrition medium are at high purity. All treatments were incubated in growth chamber at 25°C and light period/dark16/8 hours, using fluorescent bank of cool white under light intensity that reaches to 1000 lax. The nutrition media had been changed every 3 weeks to ensure continuous supply of the necessary nutritionals and the growth regulators used for treatment.

2.2.Methods:

2.2.1. Preparation and Sterilization of Plant Sample

Seeds were sterilized before use through putting them in 70% Ethanol for one minute then Mercuric chloride (0.1%) for 10 minutes and then thoroughly washed with sterilized and distilled water (for 5-7 times) in air laminar flow cabinet (ALFC). The plant parts were planted in the nutrition media and a part of the hypocotyls with length not exceeding10mm was taken.

2.2.2.Acclimatization and transfer the plant to the Soil:

After enhancing shoots and roots formation, the seedlings were transferred cautiously after washing the roots thoroughly with distilled and sterilized water to remove what sticks out of agar taking into account the extreme precision not to destroy or tear the roots to pots containing previously sterilized soil.

Vermiculite was used and put in bag subjects to sterilization.The sterilization continued for ½ hour in the steam sterilization on 121°C and pressure of 15 pounds/inch^2.The plants were surrounded by plastic bags after sprayed with water to maintain high percent of humidity around the plants for a period not less than 3 weeks with noting to raise the cover for one hour gradually in the growth room under conditions of light and dark (16 hours light/8 hours dark) and strong light about 4000 lax at 25°C (Caroline et al, 2012). The plants were irrigated every 3 days with Hoagland solution 1/5 conc. (Hoagland & Arnon, 1950), The average of the required period for adaptation of the *linum* is not less than 30 days.

***The hardening in this experiment included the following factors*:**

- Seeds were soaked in water for 3 hours before planting the separate plant to induce the callus (A) (control).
- Seeds were soaked in 4 molecular conc. salicylic acid for 3 hours (treatment B) before planting the separate plant to induce the callus.
- Hardening the calluses resulting from the separate plant by adding salicylic acid with concentration of 50 molecular to environment of calluses induction (treatment C).
- In addition to a group of plants grown out of seeds directly (without using tissue culture).

2.2.3.Measurements:

The most important measurements that were performed during stimulations of forming calluses from the used plant parts and also during stimulations of stems from callus are: callus shape, growth strength, texture, color, percentage of the formed callus, fresh and dry weight, number of root and green stems which are longer than 1cm and some other measures.

3.Results and Discussion:

3.1. Formation and display of calluses from hypocotyls for *linum* sakha1:

Table (1) indicated the obvious influence of hormone as was determined at concentration 0.25 mg/L the rate of calluses formation from hypocotyls was raised to 92% i.e. equivalent to double of concentration (A) and accompanied with increase in the callus size. At the highest concentration (0.5 mg/L), the percentage deceased to the number equivalent to 17% on concentration (0,25 mg/L) although the volume of the calluses was not affected *(plate1- A)*. The growth regulator of NAA was more uncourageous to form calluses in comparison with the BAP. This may be due to the fact that this regulator of auxian compounds which are much used in the cultures of plant tissues to form the calluses and encourage formation of roots (Pierik,1989). This is contrary to what was seen when using BAP *(plate1-B)* which induced the branches and forming the leaves. This regulator is considered within a group of cytokinins which are in the origin derivatives of Adenine playing an effective and active cell division as well modulating the apical meristems and formation of side buds (Skoog & Tsui,1951) even in the presence of auxin (Indole-3 acetic acid) which is considered one of inhibitors to form buds (Ying et al, 2014).

3.2.The combination influence of NAA and BAB on induction of calluses from hypocotyl for *linum* sakha1.

Table(2) and *(Plate2)* clarified that growth enhancement and callus development from parts of seedlings grown in MS Media plus concentrations of BAP ranging from zero to 0.5 mg/ liter and mutually interfere with NAA with concentrations respectively zero, 0.05, 0.5, 1.0, 2.0, 3.0 mg/ L. In the presence of NAA the number and volume of the arising calluses was increased with increase of regulator concentration.

This pattern was also recorded in concentration of (0.25 mg/L) and the superiority of the auxinic regulator in its influence on the volume of calluses

(+++) and on the percentage for number (75%) at the same concentration (0.05mg /L). This was in consistent wirh the findings of (Dobos *et al*,1994; Soh *et al*,1998; Aghaei, *et al* 2013).

Table (1): Influence of the different concentrations of Cytokinin-6- Benzylamino-purine (BAP) or Auxian Alpha-3- Naphthalene acetic acid (NAA) on of formation and growth of Callus form *linum* hypocotyl sakha 1

Hormone	BAP		NAA	
	Growth rate	Number%	Growth rate	Number%
A	++	47%	++	47%
B	++	52%	+++	95%
C	++	51%	+++	99%
D	+++	92%	+++	99%
E	+++	75%	+++	99%
H			+++	99%

Treatment: nutritional solution MS plus the regulator to give the concentrations (mg/L): **A:** zero **B:** 0.05; **C:** 0.1; **D**: 0.25; **E**: 0.5, from BAP Or **A:** zero **B**: 0.05; **C**: 0. 5; **D**: 1.00; **E**: 2.00; **F**:3.00, from NAA
Values: Are the average of 10 repetitions of each repeated 10 parts of the plant sample in one dish.
%: The number of callus calculated on the basis of growth grade attributed to the total number in the dish.
Growth rate: ++ (medium growth), +++ (big growth)

3.3. Interference between the intermediate concentrations from BAP, NAA and its influence on Calluses hypocotyl of *linum* sakha1

The results in Table(3*)* and *(plate3*) explained the inference between the various concentrations of growth regulator NAA/ and BAP added to the nutritional medium "Murashige & Skoog" that they affect significantly the callus resulting from the planted part of hypocotyls for *linum.*It was found that these environments have a clear influence on production of green growths of callus and these growths responded and gave green leaves specially in the nutritional medium containing (0.05 or 0.1 mg/L) of NAA interfered with (0.5 mg/l) of BAP which is the best in activation of shoot induction of parts of the hypocotyl for *linum*. These findings agreed with those of (Barna&Wakhlu,1994;Pischke et al.,2006; Amoo et al, 2012; Pawar et al,2013).

Table (2): The combination influence of NAA and BAB on induction of calluses from hypocotyl for *linum* sakha1.

Concentration mg / l		**hypocotyl**	
BAP	NAA	Growth rate	Number %
Zero	zero	++	35%
	0.05	+++	75%
	0.5	+++	94%
	1.0	+++	100%
	2.0	+++	100%
	3.0	+++	100%
0.05	zero	++	58%
	0.05	+++	100%
	0.5	+++	100%
	1.0	+++	97%
	2.0	+++	100%
	3.0	+++	95%
0.1	zero	++	47%
	0.05	+++	100%
	0.5	+++	88%
	1.0	+++	95%
	2.0	+++	95%
	3.0	+++	100%
0.25	zero	+++	91%
	0.05	+++	100%
	0.5	+++	98%
	1.0	+++	99%
	2.0	+++	99%
	3.0	+++	98%
0.5	zero	+++	77%
	0.05	+++	97%
	0.5	+++	99%
	1.0	+++	99%
	2.0	+++	99%
	3.0	+++	89%

Table (3): Interference between the intermediate concentrations from BAP, NAA and its influence on Calluses hypocotyl of *linum* sakha1

N0.	Concentration Mg / L		number		Weight(gm)	
	BAP	NAA	shoots	Roots	Fresh	Dry
1	1.00	0.05	15.00	0.50	14.52	0.78
2	0.50	0.10	19.00	4.00	11.28	0.65
3	0.50	0.25	13.00	6.30	11.69	0.66
4	0.50	2.00	9.00	3.00	10.18	0.58
5	0.50	3.00	6.58	4.68	12.31	1.17
6	0.25	2.00	5.79	21.44	11.96	0.724
7	0.25	3.00	5.86	10.63	10.09	0.825

Each reading is a mean value of 10 replicates (each replicate contains 5 callus in one dish).

3.3.Rooting:

The best environment inducing the roots are the environment MS *(Plate 4)* as it creates the highest number of roots (ranges between 5-12 root) within 10 days approximately.

3.4.Hardening by using salicylic acid

From the results represented in Table (4*)* and *(plate 5)* with following up the callus attributes in different treatments, no change was noticed in the rate of its growth or the percentage of the formed number,but the difference was recorded in the general shape of calluses. This difference is due to the excellence of the hardened calluses with salicylic acid concentration 50 molecular to numerous of green growths characterized by standing stems of 115 stems with increase of 5.2 % of the shoots number composed of calluses of control experiment or by increase of 38% of the composed number of induced calluses of the hardened seeds by 4 molecular salicylic acid.This increase led to arise in each of the fresh and dry weight for the calluses of such transaction(c).

Table (4): Attributes of the hardened callus resulting from hypocotyls *for linum* seedlings

Treatments	Enhancement rate and callus growth		Callus Features			Differentiation and weight of callus			
						Weight (g)		Number	
	Growth rate	No.%	color	texture	shape	dry	fresh	shoots	roots
A	+++	100%	green	Rigid-Wet - coherent	It has few protrusion and roots,The green growth abundant and leave range between small to medium length.	0.723	8.42	109.33	0.528
B	+++	100%	green	Wet-	It has snowy protrusions,buds and green shoots medium number and does not have roots	0.418*	6.73*	83.33	zero
C	+++	100%	green to light green at edges	Rigid-friable at edges -wet somewhat	It has protrusions –big size and the green growth is abundant and has small buds and aerobic shoots	1.80*	27.84*	115.00	0.67

- The result relating the display and weight of callus are

average of 10 replicates for each repeated 10 parts of the sample in one dish.

* Significant at 5%; ** Significant at 1%

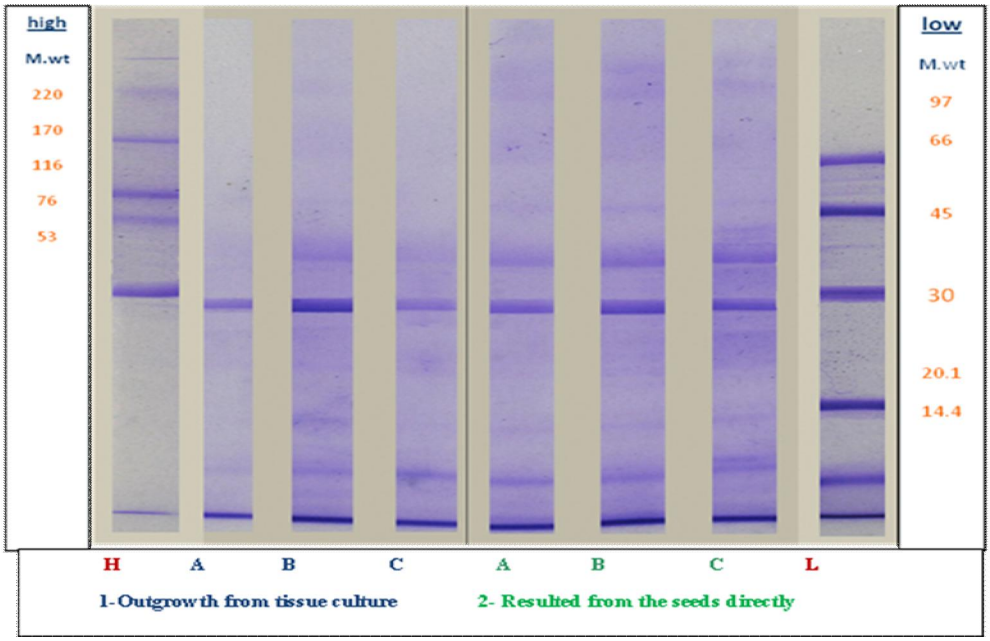
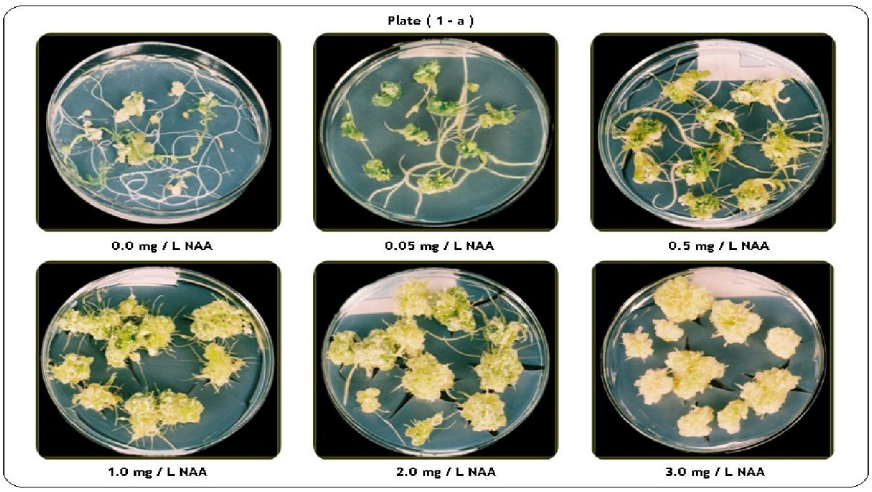


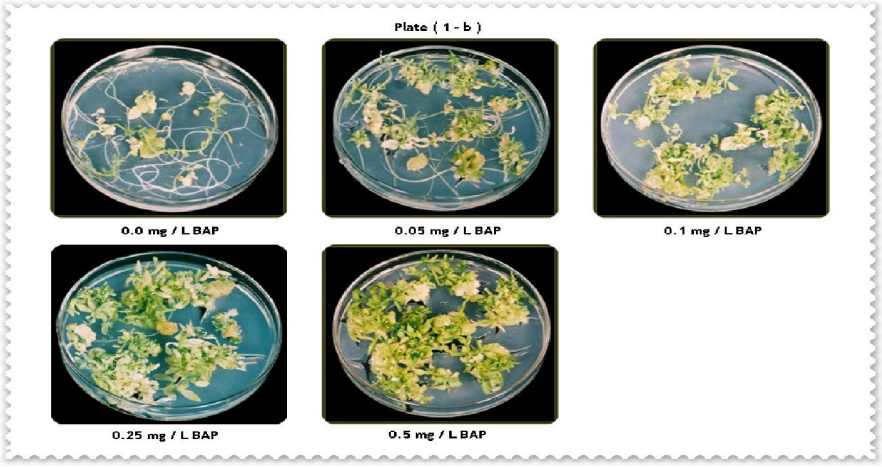
Plate (6): Protein bands (SDS-PAGE) for *linum* plants (Sakha 1)

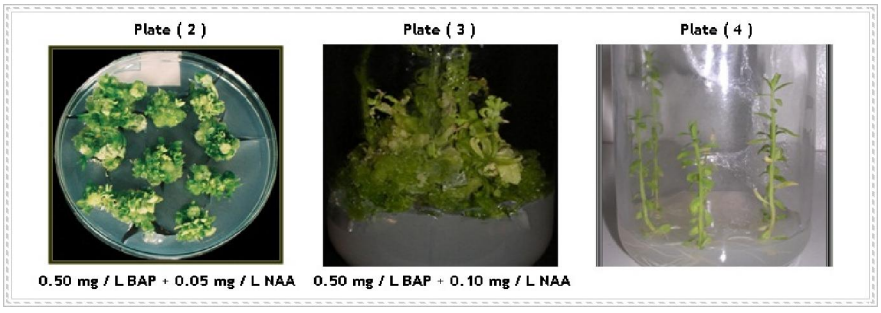
Table (5): Analysis of individual proteins(SDS-PAGE) extracted from *linum* plants (Sakha 1) by using specialized computer software (Packs molecular weights)

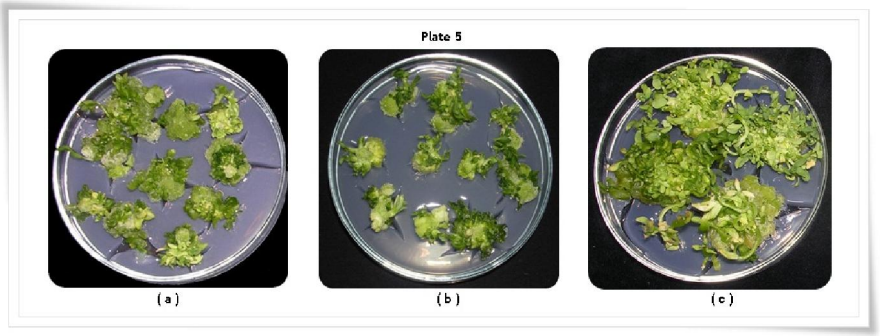
class	Packs molecular weights			Packs molecular weights		
	1-plants recovered from tissues culture			*2-plants grown directly from seeds*		
	C	**B**	**A**	**C**	**B**	**A**
1				358.26	358.26	358.26
2		306.23		306.23	306.23	
3		260.28				
4		228.8		228.8	230.09	
5		179.8	179.8	179.8	179.8	179.8
6	169.26	165.63	165.63	165.63	165.63	165.63
7		155.86	155.86	155.86	155.86	155.86
8		144.76		144.76	144.76	144.76
9		123.81			129.3	128.19
10		109.55	109.55	109.55	109.55	109.55
11		103.08		103.08	103.08	103.08
12			100.89	100.89	100.89	100.89
13				94.348	95.4	96.464
14		92.278		89.259	89.755	89.755
15		86.818		84.444	83.977	84.913
16	79.447	79.447	79.447	79.447	79.447	79.447
17	65.117	66	66	65.242		66
18	63.021	63.021	63.021	63.021	63.021	63.021
19	60.293	60.293	60.293	60.293	60.293	60.293
20	56.475	56.475	56.475	56.475	56.475	56.475
21	54.762	54.762	54.762	54.762	54.762	54.762
22	50.876	51.255	51.831	51.065	49.938	50.876
23	45	46.187	46.359	46.015	45.336	45.505
24	43.521	44.009	43.715	43.911	43.715	43.521
25	42.279	42.467	42.373	42.185	42.185	42.373
26	41.072	41.072	40.889	40.889	40.889	40.889
27	38.933	39.457	39.545	39.37	39.107	39.282
28		37.738	38.076	38.076	37.906	38.076
29				37.071	36.742	36.989
30	35.535	35.456	35.693	35.456	35.456	35.614
31	34.138	34.062	34.062	34.062	34.062	34.062
32		33.163	33.163	33.163	33.163	33.163
33						32.145
34	31.437	31.86	31.718	31.648	31.367	31.089
35	30.134	30.134	30.404	30.336	30.269	30.134
36	27.85	28.01	27.85	28.01	27.85	27.85
37	22.153	22.153	22.153	22.153	22.153	22.153
38	20.448	20.448	20.448	20.448	20.448	20.448

Transaction includes	**Transaction includes**
a)Seeds soaked for 3 hours in water before planting the vegetal separate to induce the callus (control experiment). **b)**Hardening the seeds by soaking in salicylic acid concentration 4 molecular for 3 hours before planting the explant to induce the callus. **c)**Hardening the callus resulting from the enhancement of the explant by salicylic acid concentration 50 micro molecular.	**a)**Dry seeds (control experiment). **b)**Seed soaked in water for 3 hours before planted (control experiment) **c)**Hardened seeds by salicylic acid concentration 3 molecular for 3 hours before planted.









pack appearance

3.5.Protein Bands:

Table (5) and (*plate 6*) showed the presence of significant change in building of some numerous peptides,they depended on the type of agriculture and the used method for hardening in the treated plants.The protein types were characterized by the presence of numerous regions different from each other in the immigration speed and bands density.In addition, it has been noticed the disappearance of some proteins packs while building other packs different from their analogues in the plants. Based on this, it can be said that building of disappearance such peptides relating the study processes are resulted from the possibility of the effect of hardening processes on change or adjustment of gene or genes in order to encode some proteins of adaptation or defense. We may conclude that the thermal shock may lead to the preservation of cells interactions or members, while the acute thermal strain work to cellular damages or death (Martin et al,2013).The thermal doses under the killing level motivate some responses as cells and members protection against acute damage, regain the natural case to the cellular and physiological activity, realization of the high level of thermal resistance (Schoffl *et al*,1998; Meng –Yi *et al*, 2014) and often this is done through motivation and induction of building new chains of the numerous peptides which are known by name of thermal shock proteins and they help in maintaining the cellular entity (Luo et al., 2004).

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