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Effect of Salinity Stress on *Capsicum annuum* Callus Growth, Regeneration and Callus Content of Capsaicin, Phenylalanine, Proline and Ascorbic Acid

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Abstract: The present research was conducted to study salinity effect on callus growth and regeneration from the local Chilli pepper cultivar as well as calli content of capsaicin, phenylalanine, proline and ascorbic acid. The results showed that the Pericarp gave the highest fresh and dry weight of 511.6 mg and 56.95 mg respectively at 9 dSm^{-1} compared with other interactions. Moreover the lowest fresh and dry weight was recorded for the root calli grown at 12 dSm^{-1}. The highest regeneration percentage was 87.20% at 3 dSm^{-1} and the lowest was 6.70% at 9 dSm^{-1}. For explant effect on regeneration, the highest percentage was 71.1% for shoot tips and the lowest was 23.30 % from the pericarp. However no plants were regenerated at 12 dSm^{-1} from all explants and at 9 dSm^{-1} from calli induced from roots, placenta and pericarps. Calli induced from Pericarp contain significantly higher Proline amount at 12 dSm^{-1} which was 34.65 µg/g and the lowest was 2.57 µg/g at 3 dSm^{-1}. Moreover Phenylalanine ranged from 28.23 µg/g at 3 dSm^{-1} and 41.50 µg/g at 12 dSm^{-1}. While a wide range between the explants in the Ascorbic acid amount was recorded. The highest was 47.21 µg/g from the Placenta calli and the lowest was 0.98 µg/g from the Shoot tip calli. On the other hand calli produced from Placenta gave the highest amount of Capsaicin 53.11 µg/g at 9 dSm^{-1} which was not significantly different than the placenta and the pericarp at 12 dSm^{-1} and the shoot tips, placenta and the pericarp at 9 µg/g. In conclusion Chili pepper callus tolerated salinity via the accumulation of Ascorbic acid, Proline, Phenylalanine and Capsaicin. Moreover Chili Pepper grown *In vitro* under salt stress contained high amount of Capsaicin the important pharmaceutical compound. Finally pepper plants were regenerated from salt stressed calli might be salt tolerant under field conditions.

Key words: Chili pepper, *In vitro* proline, phenylalanine, ascorbic acid and capsaicin.

1. Introduction

Chili pepper is an important source for vitamin A, C and E1 as well as secondary products such as flavonoids, phenolic acids and carotenoids [1, 2]. Moreover, it contains capsaicin which gives the fruits a punchy test. This alkaloid has wide medicinal and pharmaceutical applications. It has been used as pane killer and to treat arthritis [3] and to reduce cholesterol. Capsaicin has also been used as fungicide [4].

Tissue culture technique provides suitable environment for studying the secondary metabolites and the factors that affect their production with a systematic approach to improve their production [5]. Plant secondary metabolites production is influenced by biotic and abiotic stress signals [2]. Salinity is a major constrain which poison plant cells due to free radicals formation. To cope with the stress, plants developed several mechanisms including morphological and structural changes and increasing the secondary products [6]. Salt stress effect on plant cells metabolism and the production of carbohydrates, proteins [7, 8], and enzymes [9] have been investigated *in vitro* in some plants. The main

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objective of current research was to study the effect of salt stress on chili pepper callus growth and it's contained of capsaicin and some amino acids as well as plant regeneration from salt stressed calli.

2. Materials and Methods

The present research was conducted at The Ministry of Science and Technology/Directorate of Agricultural Research, Genetic Engineering Department during the years 2012-2014. Local chili pepper cultivar was used in the study.

The seeds and the fruits were surface sterilized for one min with 70% Ethanol. Then they were submerged in 4% sodium hypochlorite (NaOCI) for 20 min with continuous stirring and washed with sterile distilled water trice 5 min each.

The seeds were grown on MS medium [10] supplemented with 2 mg/L Glycine, 2 mg/L GA_3, 0.5 mg/L Nicotinic acid, 0.5 mg/L Pyridoxine,0.1 mg/L Thiamine, 100 mg/L Inositol, 3000 mg/L Sucrose and 6000 mg/L Agar. Fourteen days old seedlings were used as explants source. Calli were induced from shoot tips (1 cm), cotyledon leaves, hypocotyls and roots by culturing them on the same medium with out GA_3 and with the addition of 2 mg/L of Kint and IAA. While the placente and the pericarp were grown on the same medium but with addition of 2 mg/L 2,4-D.

Salt stress was achived by the addition of sodium chloride to MS medium to give electric conductivity of 6, 9 and 12 dSm^{-1} media. The control was 3 dSm^{-1}, which was achived by using half strength MS medium. Constan weight (100 mg) of the induced calli from all the explants were cultured on the corisponding medium with ten replications for each salt level and after 6 weeks fresh and dry weights were masured.

Capsaicin was extracted from all calli and estimating according to the procedure of AlOthman et al. [11] with slight modifications. Amino acids were estimated using the method of Jones and Gilli [12] and Ascorbic acid was estimated according to Mitic et al. [13] with slight modifications calli from all stress media were transferred to horrmoe free MS medium for regeneration. All the experiments were conducted in C.R.D (Completely Randomized Design) and the results were analysed by GenStat soft ware program [14].

3. Results

3.1 Sodium Chloride Effect on Callus Fresh and Dry Weights

The results (Tables 1 and 2) showed significant increase in the fresh and dry weights of the calli as the salinity level increased and decreased at 12 dSm^{-1}. The highest fresh and the dry weights were 432.72 mg and 34.96 mg respectively at 9 dSm^{-1} salinity level compared with 283.57 mg and 25.44 mg at 3 dSm^{-1}. Moreover the pericarp gave significantly higher calli fresh and dry weights than the other explants which were 398.59 mg and 42.17 mg respectively. However the roots gave the lowest calli fresh and dry weights of 275.38 mg and 17.28 mg respectively. Analysis of the interaction between the salt levels and the explants showed that the Pericarp gave the highest fresh and dry weights of 511.6 mg and 56.95 mg respectively at 9 dSm^{-1} compared with other interactions. The lowest fresh and dry weights were for the root calli grown at 12 dSm^{-1}.

The results (Table 3) showed that the percentage of plant regeneration is decreased as the salinity level increased. The highest percentage was 87.20% at 3 dSm^{-1} and the lowest was 6.70% at 9 dSm^{-1}. For explant effect on regeneration, there was no significant difference between calli induced from shoot tips and Cotyledons but both were significantly higher than the other explants. The highest percentage of plant regeneration was 71.1% for shoot tips followed by 66% Cotyledons. The lowest percentage was 23.30 % which was recorded from calli induced from the pericarp. The interaction analysis showed that at 3 dSm^{1} 100% regeneration was obtained from the calli induced from shoot tips, cotyledons, hypocotyls and roots. However no plants were regenerated at 9 dSm^{-1}

Table 1 Effect of salinity stress on callus fresh weight (mg) from different chili pepper explants (100 mg initial weight) after 6 weeks.

Explants	Salt levels dSm^{-1}				Mean
	3	6	9	12	
Shoot tips	269.95	377.42	415.61	328.46	347.86
Cotyledon	319.81	350.14	436.84	332.43	359.8
Hypocotyls	315.07	390.76	422.71	319.87	362.1
Roots	220.05	243.55	354.16	283.75	275.38
Placenta	286.44	436.11	455.42	321.03	374.75
Pericarp	290.08	456.99	511.60	335.68	398.59
Mean	283.57	375.83	432.72	320.20	

L.S.D $_{(0.05)}$ salt levels 14.45 interaction 22.46 explants 17.23.

Table 2 Effect of salinity stress on callus dry weight (mg) from different chili pepper explants (100 mg initial weight) after 6 weeks.

Explants	Salt levels dSm^{-1}				Mean
	3	6	9	12	
Shoot tips	17.60	31.3	34.55	27.42	27.72
Cotyledon	21.51	28.99	31.83	25.63	26.99
Hypocotyls	23.14	36.64	36.25	22.51	29.63
Roots	17.98	23.49	14.12	13.54	17.28
Placenta	37.13	42.49	36.06	23.85	34.88
Pericarp	35.29	44.46	56.95	31.98	42.17
Mean	25.44	34.56	34.96	24.16	

L.S.D $_{(0.05)}$ salt levels 3.64 interaction 8.91 explants 4.46.

Table 3 Plant regeneration % from salt stressed callus induced from different chili pepper explants.

Explants	Salt levels dSm^{-1}			Mean
	3	6	9	
Shoot tips	100	90.0	23.3	71.1
Cotyledon	100	86.7	11.3	66.0
Hypocotyls	100	56.7	5.7	54.1
Roots	100	80.0	0	60.0
Placenta	66.7	73.3	0	46.7
Pericarp	56.7	13.3	0	23.3
Mean	87.2	66.7	6.70	

L.S.D $_{(0.05)}$ salt levels 5.32 interaction 13.02 explants 7.52.

from calli induced from roots, placenta and pericarps. The results indicated that although fruit explants gave calli with higher fresh and dry weights than the other explants (Tables 1 and 2); they gave lower percentage of plant regeneration under the conditions of the current experiment. Moreover, despite that, calli color changed to dark brown under the effect of salinity but it did not lose its regeneration ability (Fig. 1) except at 12 dSm^{-1} no plants were regenerated from all calli grown in this salinity level.

Fig. 1 Plant regeneration from stressed dark callus .

The results (Table 4) showed that as salinity level increased the calli proline content increased. The highest amount was 18.62 µg/g at 12 dSm^{-1} which was significantly higher than the other treatments. Calli induced from pericarp and placenta calli contain 16.35 and 16.12 µg/g proline respectively which were significantly higher than other explants. Interaction analysis showed that calli induced from pericarp contain significantly higher proline amount at 12 dSm^{-1} which was 34.65 µg/g and the lowest 2.57 µg/g at 3 dSm^{-1} compared with the other treatments.

The results (Table 5) showed that calli induced from the placenta and the pericarp gave 43.23 and 40.34 µg/g phenylalanine respectively and both are significantly different than the other explants. Calli induced from the Shoot tips contained the lowest amount of phenylalanine 25.30 µg/g. In addition as the salinity increased the amount of phenylalanine was increased as well. It was ranged from 28.23 µg/g at 3 dSm^{-1} and 41.50 µg/g at 12 dSm^{-1}. Calli induced from placenta and pericarp grown on media supplemented with 6, 9, or 12 dSm^{-1} contain significantly higher amount of phenylalanine compared with the other combinations except the root calli grown on 12 dSm^{-1}.

The results (Table 6) showed that salinity had adverse effect on calli content of ascorbic acid. The lowest amount was 11.51 µg/g at 3 dSm^{-1} and increased to 20.10 µg/g at 6 dSm^{-1} which was significantly different than the other salinity levels and reduced gradually to 17.58 µg/g at 12 dSm^{-1}. Wide range between the explants in the ascorbic acid amount was recorded. The highest was (47.21 µg/g) from the placenta calli and the lowest was (0.98 µg/g) from the shoot tip calli. The interaction analysis showed no constant relationship between the salinity level and the ascorbic acid amount among the explants. For the placenta and the pericarp the maximum amounts were (62.91 µg/g and 48.62µg/g respectively) at 6 dSm^{-1} and reduced as the salinity level increased. While for the shoot tip, cotyledons and the hypocotyls the maximum amount was at 9 dSm^{-1}. However for the roots, ascorbic acid amount continued to increase as the salinity level increased.

Table 4 Effect of salinity Stress on proline content (µg/g) of callus induced from different explants of chili pepper.

Explants	Salt levels dSm^{-1}				Mean
	3	6	9	12	
Shoot tips	6.65	4.01	10.5	12.56	8.43
Cotyledon	6.24	3.14	9.23	14.76	8.34
Hypocotyls	7.34	5.56	13.98	12.03	9.73
Roots	6.03	6.79	7.18	14.07	8.52
Placenta	8.22	10.36	22.62	23.64	16.21
Pericarp	2.57	10.22	17.97	34.65	16.35
Mean	6.18	6.68	13.58	18.62	

L.S.D $_{(0.05)}$ salt levels 3.22 interaction 9.05 explants 5.83.

Table 5 Effect of salinity stress on phenylalanine content (µg/g) of callus induced from different explants of chili pepper.

Explants	Salt levels dSm^{-1}				Mean
	3	6	9	12	
Shoot tips	18.09	16.15	33.16	33.78	25.30
Cotyledons	27.34	31.7	37.17	43.41	34.91
Hypocotyls	29.36	35.76	38.53	35.11	34.69
Roots	24.15	29.01	33.52	43.45	32.53
Placenta	38.14	40.48	46.23	48.06	43.23
Pericarp	32.27	39.88	43.98	45.21	40.34
Mean	28.23	32.16	38.77	41.50	

L.S.D $_{(0.05)}$ salt levels 2.07 interaction 9.03 explants 5.12.

Table 6 Effect of salinity stress on Ascorbic acid content (µg/g) of callus induced from different explants of chili pepper.

Explants	Salt levels dSm^{-1}				Mean
	3	6	9	12	
Shoot tips	0.98	1.25	1.36	0.33	0.98
Cotyledon	2.13	5.12	7.20	7.15	5.40
Hypocotyls	1.70	3.95	11.53	9.30	6.62
Roots	1.32	4.10	5.02	12.24	5.67
Placenta	34.79	62.91	46.83	44.29	47.21
Pericarp	28.16	48.62	43.35	32.17	38.08
Mean	11.51	20.10	19.22	17.58	

L.S.D $_{(0.05)}$ salt levels 0.95 interaction 6.13 explants 4.38.

Table 7 Effect of salinity stress on Capsaicin content (µg/g) of callus induced from different explants of chili pepper.

Explants	Salt levels dSm^{-1}				Mean
	3	6	9	12	
Shoot tips	21.87	32.84	41.87	30.54	31.78
Cotyledon	16.08	17.22	29.67	33.26	24.06
Hypocotyls	11.43	14.1	26.35	22.56	18.61
Roots	6.09	6.48	18.78	10.32	10.42
Placenta	37.94	44.55	53.11	51.62	46.81
Pericarp	32.17	35.11	49.14	48.12	41.14
Mean	20.93	25.05	36.49	32.74	

L.S.D $_{(0.05)}$ salt levels 1.11 interaction 6.22 explants 4.07.

The results (Table 7) of capsaicin content analysis showed that as the salinity increased the capsaicin increased and the highest amount was (36.49 µg/g) recorded at 9 dSm^{-1}. However at 12 dSm^{-1} Capsaicin reduced to 32.74 µg/g. For explant effect calli induced from Placenta gave the highest amount (46.81 µg/g) compared with the other explants. Moreover the interaction analysis showed that calli produced from placenta gave the highest amount of capsaicin (53.11 µg/g) at 9 dSm^{-1} which was not significantly different than the placenta and the pericarp at 12 dSm^{-1} and the shoot tips, placenta and the pericarp at 9 µg/g. The lowest amount (6.09 µg/g) was recorded from the root calli at 3 dSm^{-1}.

4. Discussions

Callus fresh and dry weights reduction at 3 dSm^{-1} might be related to nutrients deficiency in this medium since it contained half the amount of salts. Full strength MS is required for plant cell division and growth. On the other hand the callus growth reduction at the highest salt level is due to the osmotic stress in this medium which inhibit mineral nutrients uptake and affect the ions availability to the cells [15]. In contrast other researchers found that high salinity level increase the plant growth hormones such as ABA and jasmonates which have an important role in salt tolerance [16] In addition the gradual increase in the fresh and dry weights from 6 to 9 dSm^{-1} may be due to cell adaptation to the high salinity by the regulation of the osmotic pressure in the cells.

High salinity level prevented plant regeneration from calli produced from all the explants. Accumulation of Na^+ and Cl^- ions in the cells inhibit the growth regulators syntheses which are required for cell differentiation. High ions concentration dehydrates and poisons the cells. Salinity results in the dehydration of the cells and reduces the availability of nutrients which is reflected in the inhibition of their growth [17]. No plants regenerated from placenta and pericarp calli, this might be because of the phenolic compounds produced by the fruit tissue. Several

researchers reported high phenolic compounds in pepper fruits [18, 19].

When plants expose to salt stress it accumulate secondary products [20] and amino acids [21, 22]. Proline and phenylalanine are among the amino acids which are produced by some plants under salt stress. From the current experiment the proline amount increased as the salinity increased. Proline is accumulated in the cytoplasm to survive the osmotic stress caused by high salt concentration. Moreover it has been reported that proline provides osmoprotection [23, 24]. The high amount of proline in the cell will increase the phenol compounds through the biosynthesis pathway of proline–linked pentose phosphate which will enhance shikimate and phenyl propanoid synthesis [25] which may suppress callus growth these results are disagree with Abbas et al. [26]. They found significant increment in callus fresh weight in the presence of proline in callus culture media.

On the other hand accumulation of Ascorbic acid in the cells is an adaptive mechanism to tolerate salinity stress [27]. Ascorbic acid is an antioxidant which alleviate the harmful effects of NaCl salinity [28, 29]. Transforming potato with GalUR gene which enhanced its ascorbic acid content, reduced the damage occurred by salt stress. Thus Upadhyaya et al. [30] concluded that engineering of ascorbate pathway enzymes is a major step in the development of salt tolerant crop plants. Pretreatment of lentil seeds with ascorbic acid reduced the effects of salt stress and improved its grain yield [31].

Accumulation of alkaloids in plants growing under biotic and abiotic stress has been reported by many researchers [8]. Particularly capsaicin production is increased in pepper grown under salt stress [32]. The same result was reported by Ali et al. [33] as salinity increased the capsaicin and vitamin C was increased.

In conclusion Chili pepper callus tolerated salinity via the accumulation of ascorbic acid, proline, phenylalanine and capsaicin. Moreover chili pepper grown *in vitro* under salt stress contained high amount of capsaicin the important pharmaceutical compound. Finally pepper plants regenerated from salt stressed calli might be salt tolerant under field conditions with high content of capsaicin.

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