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Pisum sativum L. a biotic stress tolerance, Genomic and *in vitro* approach.

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Abstract

Pisum sativum L sterilized embryos were cut from mature seeds and cultured on Murashige and Skoog(MS) medium complemented with vitamins and growth regulators. Results indicate that 2,4-D at (2.5) mg/l give good production of callus. *Pisum* callus treated with (0.75) % of ethyl methane sulphonate (EMS) for (30) min were cultured on MS medium containing (0 – 200) mM NaCl. Polymerase chain reaction (PCR) screening shows the presence of 4 and 6 PCR fragments corresponding to putative pea DHN and CAD genomic DNA respectively. These DNA fragments were diverse in length from about 50 to 900 base pair bp. for DHN gene and from about 50 to 750 bp. for CAD gene. PCR results for callus grown on increased salinity levels plus treatment with EMS shows the presence of one sharp band for both genes for about 50 bp. leangth in compared with control.

Keywords: *Pisum*, genomic, in vitro, salt, tolerance

INTRODUCTION

Though (***Pisum sativum*** L.) is one of the mainly ancient crops in the New World and is presently the most important grain legume for personal human consumption in the globe. Demands for salt tolerance cells is one of the main goals for many researchers Basu *et al*., 1997 and Al – Oubaidi ,2006. Ecological elements such as salinity, drought, and extreme temperature could influence the production and development of important crops and this could be convert into reduced yield Cushman and Bohnert 2000. Salinity stress tolerance has developed genetically through acclimatization or physiologically by exposure of plant cells to abiotic stress gradual increases concentration Farooq *et al*., 2009. Looking for alternative forage and grain legumes could be extremely useful because of their important characteristics traits such as salinity tolerance, soil improvement through fixing atmospheric nitrogen and producing grain with high protein content Rubio *et al*., 2002(5). Ttissue culture has been used in the selection of salt and drought tolerant cell lines. These lines have been used to regenerate plants resistant to harsh environmental conditions in several crops such as potato, tomato and wheat Miki *et al*., 2001(6); Queiros, 2007(7).

Requesting of applicable approaches to improve crop productivity under harmful environmental situation requires a well understanding of the mechanisms involved during crops response to abiotic stresses Roorkiwal and Sharma 2012(8), yet this a complex event, information about genes involved in the system is essential. Tracing the competitor genes responsible for stress tolerance through sequence similarity and functional studies are important for marker-assisted breeding Schena *et al*., 1995(9). Such genes present a useful resource for comparative genomics and can be used as molecular markers in genetic transformation to develop desired cultivars.

Cinnamyl alcohol dehydrogenase (CAD) gene is expected to play a key role in plant defense against many abiotic and biotic stresses Raes et al 2003(10). Dehydrins (DHNs) represent a family of hydrophilic proteins, which accumulate during seed development. Due to their hydrophilic property, DHNs are believed to protect plant tissues against various stresses causing cellular dehydration such as salinity, drought and cold Vaseva et al 2011(11), although some DHNs have a role to serve as antioxidants scavenging free radicals Sun and Lin 2010(12).

Distinguishing and cloning genes in plant that have tolerance for abiotic stress is an important application in modern plant research, producing mutants with modified phenotypes and physiological responses is one of the powerful approach for determining the biological functions of genes in an organism Gharsallah *et al*., 2016(13).Ethyl Methane Sulphonate (EMS) is considered the most common chemical mutagen used in plants. EMS alkylate guanine bases and leads to mispairing-alkylated G pairs with T instead of C, resulting in primarily G/C- to- A/T transitions Talebi *et al*., 2012(14).

MATERIALS AND METHODS

Sterilized Explants (embryos) of ***Pisum sativum*** **L**. were excised and cultured in universal tubes containing MS medium Murashige and Skoog,1962(15) with concentration of the auxin 2,4-D (2.5mg/l, which incubated in dark at a temperature 23 ± 1 °C .

Induction of mutation through chemical mutagens (EMS)

The protocol of callus treatment with (EMS) reported in Al – Oubaidi, 2006(2) for the development of mutant calli in Glycine max L. was employed ,The callus were treated with varying concentrations of (EMS). The solutions of the (EMS) at the concentrations (0.5, 0.75, 1.0,) %were prepared and filter sterilized through a 0.45 µm Millipore filter and placed in sterile petri dishes inside the air flow cabinet where the callus were soaked in (EMS) for (15, 30, 60,) minutes. Constant weight of callus by 250 mg were took and placed on same fresh callusing media as used for normal callus and by 10 replications for each treatment and for each period of time. Then they were incubated under the previous conditions, to determine the appropriate concentration of the (EMS) and the appropriate soaking duration for the callus, the fresh and dry weight of callus were recorded after four weeks. EMS solution were prepared at a concentration of 0.75% and immersion callus in it for 30 minutes , The constant weight of callus (250 mg) were took and cultured on culture media containing salt levels (0, 50,100,150or200)mM NaCl and by 10 replications for each level of salt. The fresh and dry weight of callus formation was recorded after 30days Ramawat, 2008(16).

DNA extraction:

Pisum sativum callus induced in four different salinity levels joined with EMS on MS medium plus control treatment were used for the identification of abiotic stress responsive competitor genes. Total genomic NA was extracted from callus using CTAB method Doyle and Doyle 1987(17).

PCR primers:

DHN F 5, ATG TCT CAG TAT CAA AAC C 3,
DHN R 5, CTA GTG TCC AGT ACA TCC TCC 3,
CAD F 5, GAA GTG TGC CGA CGA TAA GC 3,
CAD R 5, GGC TTC AGA AAC CAA AGT CAC C 3,

PCR conditions:

PCR amplification for the two genes was carried out in Eppendorf gradient thermal cycler in 25 µl reaction kit (Bioneer PCR premix reaction kit) containing 20 ng template DNA. The amplification profile included: initial denaturation for 5 min at 94 ºC followed by 35 cycles of denaturation for 1 min at 94 ºC, annealing at temperature specific for each target gene (48, 55 ºC

for DHN and CAD genes respectively) for 1 min and extension at 72 ºC for 1 min, final extension was allowed for 5 min at 72 ºC. amplified PCR products were resolved on 2% agarose gel and electrophorese time were 45, 60 min for DHN and CAD genes respectively.

Experimental design and statistical analysis:

A Randomized completely block design (CRD) was used. Least significant differences (LSD) were calculated. The difference between means were compared according to the least significant differences (LSD) at the probability of 5% Steel and Torrie, 1980(18) .

RESULTS AND DISCUSSION

Effect of 2, 4-D on callus fresh and dry weight (mg):

Adding 2,4-D significantly effect on the fresh weight of callus .The result in table (1) shows that the concentration of 2.5 mg/l 2,4–D gave the highest fresh weight of callus which reached to (182.75) mg while the lowest fresh weight of callus was found in control (0.0)mg . For dry weight the result in the same table shows that the concentration of 2.5 mg/l of 2, 4–D gave the highest dry weight of callus which reached to (21) mg and this treatment significantly different from other treatments except the treatment of 3.5 mg/l which reached to (19.25) mg while the lowest dry weight of callus was found in control (0.0) mg.

These results are supported by the result of Ebony *et al.,* 2010(19) who observed that callus growth was faster than the callus produced at lower concentration of 2,4-D. (20), reported that, callus fresh weight for four cultivars of wheat had a positive correlation with the increasing the concentrations of 2,4-D. The differences between increased or decreased the callus weight may be due to differences in cell's sensitivity to plant growth regulators, their capacity to dedifferentiate and/or the endogenous concentrations of plant growth regulators within these explants Mitchell, *et al.,* 2009(21).

Callus induction requires a balanced ratio of auxin and cytokinin as stated by Skoog and Miller 1957(22).In number of plant species, callus induction favors higher auxins and cytokinins, but (15) stated that seed and newly branched in apical meristems are rich source of auxins so that a little amount of 2,4-D was required for callus initiation (16). Also (23) found there are decreasing in response of callus which induced when increasing2, 4-D concentration which inhibit growth of the cells.

Table 1: Effect of different concentrations of 2, 4-D on callus fresh and dry weight (mg) after four weeks of culture on MS medium.

Concentrations of 2, 4-D(mg\l)	Cont	1.5	2.5	3.5	LSD (0.05)
Fresh weight - mean (mg)	0.0	110.75	182.75	146.25	33,76
Dry weight-mean (mg)	0.0	10.00	21.00	19.25	6.37

Effect of different treatments of EMS on callus fresh and dry weight:

The effect of EMS treatment on callus proliferation showed in Table(2).The highest average of callus fresh weight found (305) mg which callus was soaked in 0.75% of EMS, this treatment is not significantly different from the treatment of 0.5 and 1.0% which reached to(259,293) mg respectively , for the time treatments the highest dry weight was for the treatment of (30) min which reached to (337) mg that mean this treatment significantly different from the treatment (60) min which reached to (188) mg,but not significantly different from the treatment (15) min. which reached to (332) mg.

For the callus dry weight there were no significant different between the treatments of (0.5, 0.75 and 1.0) % showed in table (2), but for the time treatments, the highest dry weight was for the treatment of (30) min which reached to (29.5) mg that mean this treatment significantly different from the (60) min .which reached to (18.4) mg ,but no significantly different from the (15) min which reached to (28.6) mg .

It is likely that only higher doses and along time resulted in the restriction of cell growth, which did not affect lower doses and a short time after interaction of the mutagens with plant growth regulators Khawar and Özcan. 2006 (24). Ethyl Methane Sulphonate (EMS) an alkylating chemical mutagen and causes a high frequency of gene mutation and a low frequency of chromosome aberration Van-Harten 1998(25), Bahera *et al*., 2012(26).

The most important parameters for inducing mutation by EMS are concentration, duration of treatment and solution temperature Alcantara *et al*., 1996(27).

Table 2: Effect of different treatments of EMS on callus fresh and dry weight (mg) respectively after four weeks of culture on medium .

Concentrations of EMS %	Time(min)			Mean
	15	30	60	
0.5	328	357	93	259
0.75	362	359	193	305
1.0	305	295	279	293
Mean	332	337	188	
LSD (0.05)	Concentrations =N.S. Time = 88.3 Concentration × Time =152.9			
Concentrationsof EMS%	**Time(min)**			**Mean**
	15	**30**	**60**	
0.5	27.2	31.3	7.8	22.1
0.75	31.8	27.7	17.7	25.7
1.0	26.8	29.5	29.9	28.7
Mean	28.6	29.5	18.4	
LSD (0.05)	Concentrations = N.S. Time = 7.99 Concentration × Time =13.76			

The effect of EMS on callus salt tolerance:

After the re-cultivation on salt levels 0-200 mM of NaCl salt. The results were recorded in table (3) showed that EMS helped with increasing salt tolerance in callus *Pisum sativum* cells. It was obvious that the treatment of 50 mM gave highest average of callus fresh weight reached to (409) mg this treatment significantly different than all other treatments. The least average of callus fresh weight recorded with the treatment 200 mM which gave (242.6) mg. For the callus dry weight the treatment of 50 mM gave highest average of callus fresh weight reached to (49.4) mg this treatment significantly different than all other treatments.

The callus formation was affected by the salinity of the medium. Although callus growth was observed in all the salt concentrations, there was a gradual decrease in callus growth as the salinity increased Thiagarajan, 2013(28). Callus viability was significantly reduced for each increase in NaCl concentration. Similar observations were reported by (29) on tobacco callus. In general, increasing NaCl concentration in the medium, resulted in a significant reduction (more negative) in callus cell sap osmotic potential.

This was described by Handa *et al*., 1983(30) as a reason behind cell adjustment of osmotic potential under stress conditions. (31) Reported more negative osmotic potential of *Brassica campestris* callus exposed to salinity; this was closely correlated with water stress and was accompanied by an increase in Proline content. Proline accumulated significantly for each increase in NaCl concentration. Protein content decreased significantly with each increase in NaCl concentration .This confirms the results obtained by (32) on salt stressed rice callus. And (33).

Table 3: The effect of 0.75 % EMS for 30 min. on callus salt tolerance for Both fresh and dry weight after four weeks of culture on medium .

Salt Concentrations (mM)	cont	50	100	150	200
Fresh weight-mean (mg)	385.4	409.0	243.0	328.8	242.6
Dry weight-mean (mg)	37.8	49.4	32.0	40.4	34.6
LSD (0.05)	For fresh weight =40.57			For dry weight =5.006	

Table 4: DNA concentration and purity extracted from *Pisum sativum* callus.

Sample	DNA concentration ng/µl	260/280	260/230
Control	33.4	1.78	1.51
50mM NaCl	116.3	1.64	1.09
100mM NaCl	59.9	1.57	0.78
150mM NaCl	75.3	1.54	0.73
200mM NaCl	54.9	1.47	0.73

Table (4) demonstrate the concentration and purity of DNA extraction using CTAB method. PCR amplification for DHN gene Fig. 1 shows the highest number of DNA

Fragments was found in control treatment, which shows at least four bands range in length from 50 to 900 bp. The high number of DNA fragments indicated a dehydrin multigene family in *Pisum sativum.* This is corresponds with (34).Moreover (8) also reported the presence of many amplicons for DHN gene in Chickpea plants. Accumulation of dehydrins in plants is a common response for drought (34), according to (35) the accumulation of dehydrins is associated with a tolerance mechanism governing to the maintenance of cellular turgor. (36) suggested that amino acid composition of dehydrins with high content of charged and polar residues may promote their special protective functions under cell dehydration conditions.

Fig. 2 demonstrate the DNA fragments amplified in PCR for CAD gene, the results shows that the highest number of amplicon was in control treatment with length varied from about 50 to 750 bp., in comparison with the different increasing salinity levels which shows almost one clear sharp band about 50 bp. length. CAD gene play a key role in plant defense against divers abiotic and biotic stresses (10).

Induced stresses using rising salinity incorporated with EMS mutagenesis generates randomly distributed mutations throughout the genome. As a result, chemical mutagenesis can be used not only to understand the role of specific amino acid residues in protein function but also to search for loss- or gain- of –function mutants, also EMS can be used for generating breeding lines (37, 38). Present study was operated with an objective to recognize abiotic stress responsive genes in *Pisum*. Two abiotic stress responsive competitor genes previously validated for their significance in stress response in various model crops and other legumes were amplified which provides basic information that can be exploited in overcoming various abiotic stress related problems limiting *pisum* production and subsequently in breeding superior varieties producing more yield under abiotic stresses conditions by presence of favorable alleles for stress response in single variety using modern molecular breeding approaches.

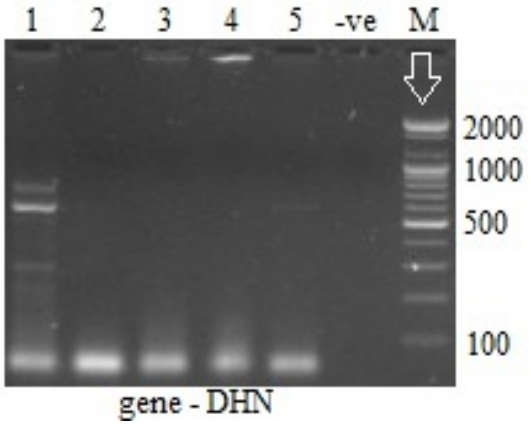

Fig. 1: Amplification of DHN gene extracted from DNA *Pisum* callus; 1= control, 2= 50 mM NaCl, 3=100 mM NaCl, 4=150 mM NaCl, 5= 200 mM NaCl,-ve= negative control, M= 100bp ladder DNA.

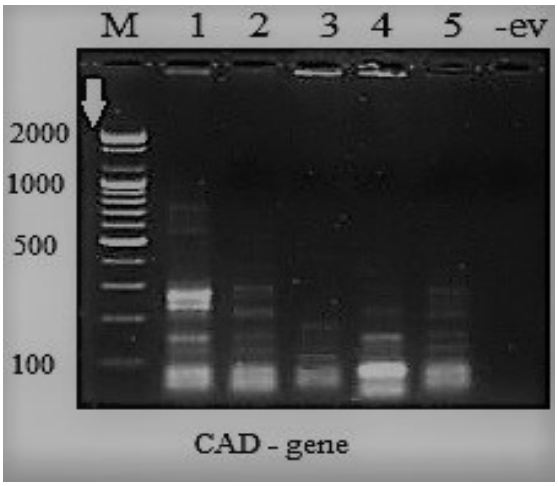

Fig. 2: Amplification of CAD gene extracted from DNA *Pisum* callus; M= 100 bp ladder DNA, 1= control, 2= 50 mM NaCl, 3=100 mM NaCl, 4=150 mM NaCl, 5= 200 mM NaCl,-ve= negative control.

CONCLUSION

Induced stresses using rising salinity incorporated with EMS mutagenesis generates randomly distributed mutations throughout the genome. As a result, chemical mutagenesis can be used not only to understand the role of specific amino acid residues in protein function but also to search for loss- or gain- of –function mutants, also EMS can be used for generating breeding lines. Present study was operated with an objective to recognize abiotic stress responsive genes in *Pisum*. Two abiotic stress responsive competitor genes previously validated for their significance in stress response in various model crops and other legumes were amplified which provides basic information that can be exploited in overcoming various abiotic stress related problems limiting *pisum* production and subsequently in breeding superior varieties producing more yield under abiotic stresses conditions by presence of favorable alleles for stress response in single variety using modern molecular breeding approaches.

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