

SALT-INDUCED CHANGES IN GENE EXPRESSION OF TOMATO PLANTS

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ABSTRACT

Salinity is considered a hindrance for growth and productivity of several economic crops worldwide. In this paper we investigate the effects of different concentrations of NaCl (0, 25, 50 and 100 mM) on growth and gene expression of two tomato cultivars differing in salt tolerance, a salt sensitive variety (GS12) and a salt tolerant cultivar (Adora). Salt stress caused GS12 plants to be affected negatively with significant decrease in all growth parameters; meanwhile Adora plants grew in a comparable pattern to control untreated plants. When differential display polymerase chain reaction was applied to test the difference in gene expression in both cultivars, GS12 plants showed down regulation of some genes while, Adora plants showed up regulation of other genes. We managed to identify a zinc finger protein gene and a nitrite reductase gene from Adora tomato plants which appeared to contribute to the ability of these plants to withstand salt stress and grow in a much similar manner like control untreated plants. These two genes could contribute in further studies of tolerance to other stresses when introduced to other crop plants or other tomato cultivars.

Keywords: salt tolerance, nitrite reductase gene, zinc finger protein, tomato

1. INTRODUCTION

Salt stress is a widespread abiotic stress which limits the production of crops and alters the distribution of plants per region. Researchers try to understand plant response to salt stress by identifying new model plants. Different genes are believed to be involved in salt tolerance of which are those genes which control ion compartments in the cells, ion exit, selectivity of ions and the scavenging of reactive oxygen species (ROS) [1, 2].

Plants respond to salinity in a complex manner which involves changes in the morphology as well as changes in the biochemistry and physiology of cells [3].

Reduction of growth is the most pronounced morphological symptom of salt stress [4], which is the result of altered changes in ion balance, water state, mineral composition, photosynthetic machinery and membrane instability [5].

Reduction in the yield of pepper and melon was reported as a result of salt stress [6].

Fruit size, total yield and photosynthesis of salt stressed tomato were reduced after excessive salt exposure [7].

Tomato plants are moderately sensitive to salt stress. The presence of accessions of many salt-tolerant wild tomato plants has proved to make tomato a model crop to compare strategies of salt tolerance. Recent studies focused on the ability to characterize genetic and physiological characteristics of wild tomato species as compared with with tomato cultivars [8].

Researchers proposed a lot of mechanisms to explain the salt tolerance of wild tomato species, such as antioxidative mechanisms, ion exit and distribution, and osmotic regulation. Tal and Shannonn [9] proved that *Solanum cheesmanii* acts as a halophyte with the power to accumulate Na⁺ in the aerial parts. But, in another study, the ability of *S. cheesmanii* to overcome salt stress was attributed to Na⁺ exclusion from leaves [10].

It is of high importance to identify different responses of plants to abiotic stresses and to analyze genes which improve tolerance to different stresses [11]. Many procedures have been used to analyze the expressed gene which is differentially regulated in response to many abiotic stresses in higher plants. These procedures include differential display screening using differential display polymerase chain reaction (DD-PCR) [12, 13]. The simplicity of DD-PCR and the need to have a relatively small amount of mRNA proved the method to be productive. By using DD-PCR, several stress-responsive genes have been identified [14].

The DD-PCR technique gives a flexible and sensitive way to analyze and identify differentially expressed genes. It is widely used to identify several cDNAs which correspond to genes regulated by heat, salt, drought, sucrose and during developmental stages of plant growth [15].

In this study differential display was used to characterize changes in mRNA that occur in two salt-treated tomato cultivars differing in salt tolerance, one being salt sensitive (GS12) and the other being salt tolerant (Adora), and to identify novel salt-responsive genes.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

This paper includes the results of two large scale experiments which were carried out during the two successive late summer seasons of 2013 and 2014 in the botanical garden of the faculty of Science at Mansoura University, Egypt to investigate the response of two tomato cultivars differing in salt tolerance, a salt sensitive variety "GS12" and a moderately salt-tolerant cultivar "Adora", to different concentrations of NaCl (0, 25, 50 and 100 mM). GS12 seeds were purchased from Syngenta seeds supplier in Egypt while Adora seeds were purchased from Gaara seeds supplier in Egypt. Adora seeds are a hybrid known to be salt tolerant to 20 ppm soil salinity.

Seed sowing was done on August 4th, in 84 cells, foam trays filled with growing media of peat and vermiculite (1:1, v/v) and transplanted into pots containing 6 kg of clay-sandy soil (1:1, w/w) on 8th September on both growing seasons.

Half strength Hoagland solution [16] was used to water seedlings regularly in two days intervals until transplantation. Salinization took place after one week from transplantation; Hoagland nutrient solution containing 25 mM, 50 or 100 mM NaCl were used to water the four groups of plants while control plants were watered as before. Each treatment at each experiment was arranged in a completely randomized design with four replicates; each replicate included five plants (20 plants per treatment). Salinization took place every two weeks until the end of the experiment. Plants were watered with distilled water if needed in between salt treatments. Harvesting and sampling of tomato plants took place after 49 d of salt treatment initiation. Leaves were harvested, immediately frozen in liquid nitrogen and stored at -80°C until use.

2.2. Measurement of growth parameters

Growth parameters (root length, shoot length, fresh weight, dry weight and water content) were determined throughout the entire period of the experiment to evaluate the sequence of growth characters of the different treated tomato plants.

2.3. RNA extraction

In liquid nitrogen, frozen leaves were ground to a fine powder. Total RNA for DD-PCR was extracted using the GeneJET Plant RNA Purification Mini Kit (Thermo Scientific) following the manufacturer's instructions. The quantity of total RNA was evaluated by measuring its A260. By using denaturing agarose electrophoresis, the quality of the RNAs was checked.

2.4. DD-PCR

DD-PCR [12] was performed using total RNA extracted from the 8th fully expanded leaflets of GS12 and Adora tomatoes collected after 49 d of salt treatment initiation. RNA was extracted as above. Removal of potential chromosomal DNA contamination was done by DNase I (Thermo Scientific) treatment. Reverse transcription reactions were done using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

3 µg total RNA was used to synthesize first-strand cDNA by using RevertAid reverse transcriptase (Thermo Scientific) and anchored dT18VN (TVN, V= A, C, or G; N= A, C, T, or G) as a primer in 20 µl reaction according to instructions of the manufacturer. The reaction mixture contained 4 µl of RevertAid 5X Reaction Buffer with MgCl₂, 2 µl of 10 mM dNTPs, 1 µl of 10 pmol anchored (dT18VN) primer, 3 µg RNA in 5 µl RNase-free water, 1 µl RevertAid reverse transcriptase, 1 µl RiboLock Ribonuclease Inhibitor (Thermo Scientific) and 6 µl RNase-free water. The reactions were incubated at 42 °C for 60 min. The reverse transcriptase was inhibited by incubation at 72 °C for 5 min and stored at -20 °C till use as template for PCR.

Before starting the RT-PCR a routine PCR with Taq polymerase was run with 1 µl of template RNA and primers. The negative results indicated that the RNA samples were totally free from DNA and amplified PCR product will be produced from cDNA templates.

The reaction mixture for PCR contained 12.5 µl of PCR master mix (ThermoScientific), 1 µl of anchored (dT18VN) primer (10 pmol) and 1 µl of one of each of the three arbitrary 10-mers, DD1 (5'-AAGCCGAAGC-3'), DD2 (5'-TCAGCACGGA-3'), and DD3 (5'-TGGATTGGTC-3'), 3 µl cDNA and 1 µl Taq DNA polymerase (5 units/ µl) (Fermentas, Lithuania) and made up to a total volume of 25 µl using nuclease-free water.

Forty cycles of PCR were performed at 95 °C for 30 s, 42°C for 1 min, and 72°C for 1 min. A 5 min denaturation at 95 °C (one cycle) at the beginning of the reaction and a 5 min extension at 72 °C at the end were performed. Plant 18s rRNA primers (forward, 5'-CCAGGTCCAGACATAGTAAG-3'; reverse, 5'-GTACAAAGGGCAGGGACGTA-3' [17] were used as an internal control for normalization.

On 1.5% (w/v) agarose gels, products of the reaction were separated and then stained with ethidium bromide. Bands differently induced in the RNA samples were excised, directly purified after DNA isolation using the GenJET Gel Extraction Kit (Thermo Scientific).

2.5. DNA Sequencing.

The selected purified DNA products were then further processed for DNA sequencing in Animal Research Health institute, Giza, Egypt.

2.6. Alignments and phylogenetic analysis

Nucleotide sequences were analysed in BLAST 1.0 or 2.0 [18] (<http://www.ncbi.nlm.nih.gov/blastn>) and aligned in ClustalW [19] (<http://www.ebi.ac.uk/clustalW>). The nucleotide sequences were checked through Blastx (www.ncbi.nlm.nih.gov:80?BLAST/) to detect the correct frame for the amino acid translation. The amino acid sequences were aligned in ClustalW.

2.7. Statistical analysis

Growth parameters data were subjected to one-way analysis of variance (ANOVA) with Post Hoc L.S.D. (least significant difference) test. * p value < 0.05 was accepted statistically significant. Statistical analysis was performed with statistical package for social science for windows (SPSS, version 13.0, 2004, Chicago, IL, USA).

3. RESULTS

3.1. Changes in growth parameters

Treatment of GS12 and Adora tomato plants with different concentrations of NaCl showed significant decrease in all growth parameters determined, being highly pronounced in the salt sensitive cultivar, GS12.

The values of the different growth parameters appeared to be lower in GS12 plants than in Adora treated plants. The following sequence of treatments for GS12 and Adora (Control > 25 mM NaCl > 50 mM NaCl > 100 mM NaCl) was displayed throughout the entire period of the experiment. From Table 1, it is obvious that GS12 growth was greatly reduced by salt stress at 50 and 100 mM NaCl. Adora plants appeared to tolerate salt stress as compared with control plants with appreciable decrease at 100 mM NaCl.

3.2. Identification of salt responsive genes by DD-PCR

In the present work, the focus was on qualitative differences (i.e., presence or absence of cDNA fragments) rather than quantitative variation in band intensity among samples revealed by DD. Differential display PCR (DD-PCR) was used to compare overall differences in gene expression between GS12 and Adora tomato plants in response to salt stress.

Differential display (DD) was performed to detect changes in tomato leaves under salt-stress conditions. Figure 1 shows amplification products obtained by this approach. On a 1.5% agarose gel, products from the sample total RNA were separated and detector signals were converted into virtual images.

Three arbitrary primers were used for DD-PCR amplification. Figure 1 represents the scanning of the three arbitrary primers (DD1, DD2 and DD3). In case of GS12 salt sensitive tomato, with a total of 3 arbitrary primers, of the 11 differential bands displayed on the gel, 6 gene fragments were found to be induced in response to salt stress. Meanwhile, Adora salt tolerant tomato, with a total of 3 arbitrary primers, of the 20 differential bands displayed on the gel, 9 gene fragments were found to be induced in response to salt stress.

For GS12 leaves, selected three arbitrary primers generated 11 bands and the size of amplification products ranged from 300 bp to 1300 bp. The number of bands generated per primer ranged from 2 to 5. Out of 11 bands generated, 4 (36.36%) were polymorphic and 7 (63.63%) were monomorphic, indicating different gene expression (Table 2). For Adora leaves, selected three arbitrary primers generated 20 bands and the size of amplification products ranged from 100 bp to 3000 bp. The number of bands generated per primer ranged from 4 to 9. Out of 20 bands generated, 13 (65%) were polymorphic and 7 (35%) were monomorphic, indicating different gene expression (Table 2). The details of amplification products are given in Table 2. The largest number of differential bands was detected for primer DD2 (13 bands), while the lowest was scored for primer DD3 (6 bands).

In all cases, DD-PCR patterns generated by the salt stressed leaves were clearly different from those obtained using control cDNA. The results obtained from three primers are presented in Figure 1 and Table 2. The principal events observed following the salt stress were presence and absence of cDNA fragments compared with the normal control plants. Also, it was observed the different gene expression in Adora plants compared with GS12 plants in response to salt stress as compared with control plants. The magnitude of response being highly observed in Adora plants treated with 50 mM NaCl.

3.3. Sequencing and identification of cDNA fragments

Two DD-derived cDNA clones from Adora plants treated with 50 mM NaCl were characterized as a partial sequence of the tomato zinc finger protein and nitrite reductase genes by sequence analysis and homology search of GenBank databases.

One up-regulated fragment (Figure 1b) was further characterized. Sequence analysis of this fragment was 483 bp in length and was similar with four Genbank sequences (99% identity with accession no. HG975513.1, 99% identity with NM_001279257.1, 99% identity with AK246932.1 and 99% identity with BT013336.1). The BLAST searches of GenBank databases revealed that it encodes zinc finger protein (ZF2). Therefore, we named the gene zinc finger protein (ZF2) gene.

The second up regulated fragment was 399 bp in length and was similar with three Genbank sequences (99% identity with accession no. XM_004248688.2, 99% identity with AC244757.7 and 99% identity with BT014587.1). The BLAST searches of GenBank databases revealed that this encodes plant nitrite reductase gene (NIR). Therefore, we named the gene nitrite reductase gene (NIR).

Blast search revealed that the obtained protein sequence for ZF2 protein (AKP17131) from Adora tomato treated with 50 mM NaCl shares sequence homology with zinc finger proteins from different plants. It shows high sequence similarity to zinc finger protein isolated from *Solanum lycopersicum* (NP_001266186) (98%) and zinc finger protein isolated from *Solanum chacoense* (AAU12056) (73%).

Blast search revealed that the obtained protein sequence for NIR protein (AKP17132) from Adora tomato treated with 50 mM NaCl shares sequence homology with nitrite reductase proteins from different plants. It shows high sequence similarity to NIR protein isolated from *Solanum lycopersicum* (XP_004248736.1) (98%), nitrite reductase protein isolated from *Solanum tuberosum* (XP_006349625.1) (98%) and nitrite reductase protein isolated from *Nicotiana sylvestris* (XP_009783559.1) (92%).

4. DISCUSSION

Salt stress is one of the most important limiting factors when growth and development of plants are considered. Worldwide, salinity is found to be a hindrance to the development and productivity of several crops among them tomato plants. Tomato, being moderately sensitive to salt stress, is considered a very important vegetable crop in the world. During the past few years, researchers used to investigate the possible mechanisms of salt tolerance in wild tomato species and the ability of new tomato hybrids to tolerate different salt concentrations in the soil [20].

From the above results, treatment of two tomato cultivars differing in salt tolerance, GS12 (salt sensitive) and Adora (salt tolerant), with different concentrations of NaCl (25, 50 and 100 mM) resulted in significant decrease in all growth parameters determined. The magnitude of response being greatly pronounced in GS12 plants especially under high salt concentrations 50 and 100 mM. Adora plants appeared to tolerate salt stress at 25 and 50 mM but at 100 mM a significant decrease was observed in all growth parameters as compared with control untreated plants.

Similar results were also reported for salt sensitive tomato [21,22] and strawberry [23] which were grown in saline soil. On the other hand, several authors reported that fresh weight and dry mass significantly increased under saline conditions in some horticultural crop species including salt tolerant tomato [24] and cucumber [22].

Negative effects of salinity on seed germination and vegetative growth of tomato have been reported. More specifically, root biomass and shoot growth were reduced. Similar reports on salinity stress responses in tomato showed reduced stem and leaf dry weights, leaf area and stem thickness [24].

The ability of Adora tomato plants to tolerate salt stress was further investigated using DD-PCR. While GS12 tomato showed down regulation of several genes, Adora tomato showed up regulation of two genes, namely zinc finger protein (KT236084) and nitrite reductase (KT236085).

Sakamoto et al. [25] showed the early induction of a subset of Arabidopsis Cys2/His2-type zinc-finger transcription factors after drought, salinity, or ABA treatment. Mukhopadhyay et al. [26] analyzed OSISAP1 which is a stress inducible gene proposed to encode a zinc-finger protein in rice. In transgenic tobacco, overexpression of this gene showed to increase as a result of stress tolerance, as shown by salinity-, chilling- and drought-tolerance analyses. The retention of green color, fresh weight and leaf development as well as percentage germination were improved in transgenic lines, as compared with wild type tobacco, under stress and recovery conditions.

As shown in our results, the expression of zinc finger protein gene in 50 mM NaCl treated Adora plants, could attribute to the power of the plants to withstand such stress and grow in a comparable manner to that of control untreated plants.

The zinc finger protein has homology to other zinc finger proteins in other plants as mentioned above. Inhibition of apoptosis is accelerated by the zinc-finger domain which is required for dimerization to inhibit the tumor necrosis factor. High-salinity stress leads to inhibition of cell division and acceleration of cell death [27]. Overexpression of zinc finger protein gene can help to avoid stress associated injuries such as chlorophyll loss and apoptosis in transgenic plants and better recovery from stress.

Debouba et al. [28] treated salt-sensitive tomato plants with different concentrations of NaCl (0, 25, 50, and 100 mM). They showed that when salinity increased, a decrease in dry weight production and protein contents in the leaves and roots was pronounced. Under salinity, nitrite reductase activity was decreased in both the leaves and roots. These results are in accordance with our results. In GS12 plants there was down regulation of nitrite reductase gene whereas Adora plants showed up regulation of nitrite reductase gene at 50 mM NaCl. These results show that nitrite reductase plays an important role in salt tolerance.

Yuan et al. [20] showed that over expression of nitrite reductase gene in creeping bentgrass improved salt stress resistance and is associated with increased cell membrane integrity, water

retention, capacity for maintaining potassium homeostasis, chlorophyll content, and total N accumulation.

In conclusion, this study has characterized a zinc-finger protein gene and a nitrite reductase gene from salt tolerant tomato cultivar (Adora) which may be used to engineer stress tolerance in other crop plants or other tomato cultivars. It also has not escaped our notice that these two proteins may have functions in other stresses that have been shown to induce their expression.

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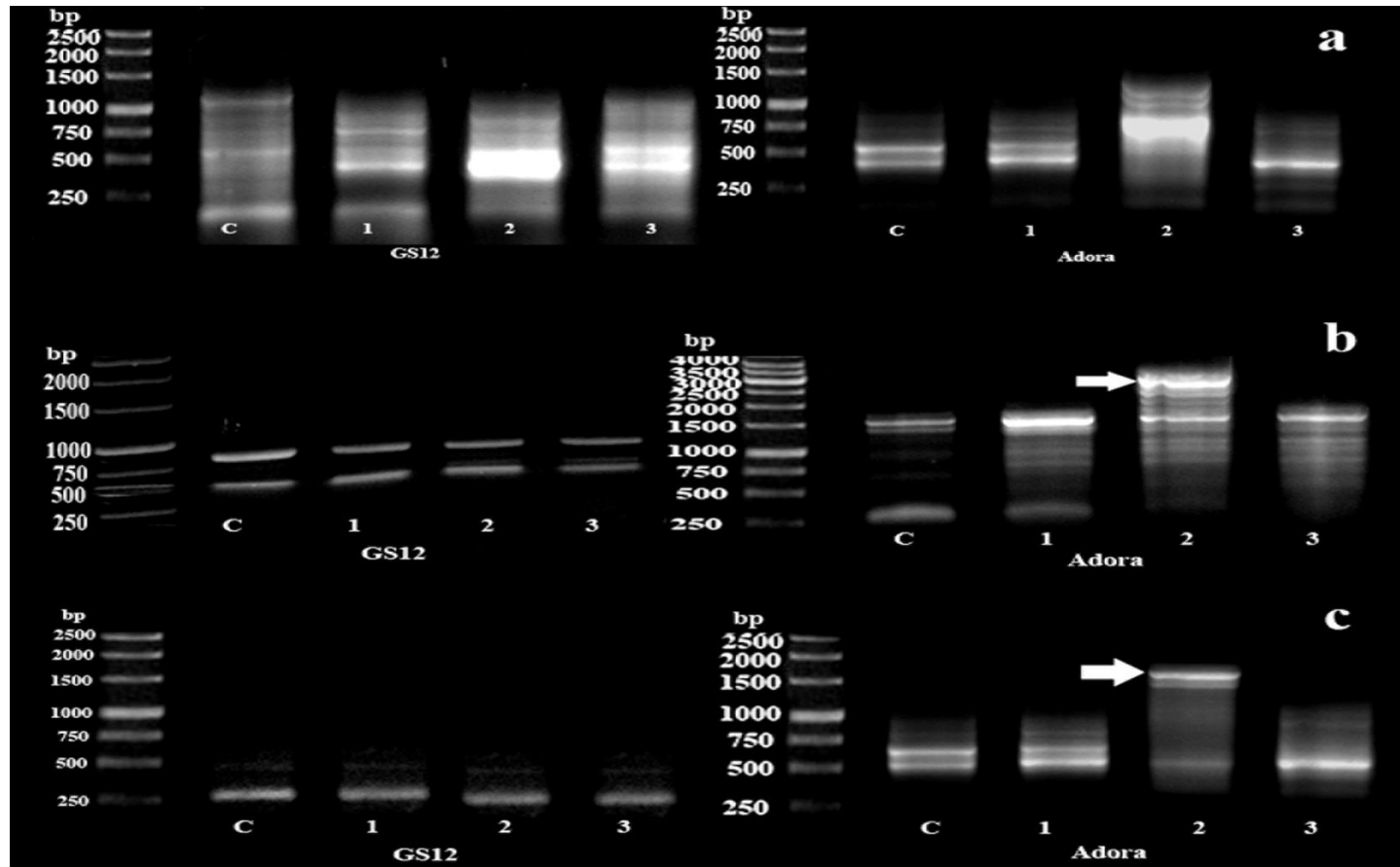


Figure 1: Representative 1.5% agarose gels of differential display patterns generated from GS12 and Adora samples. a: differential display using primer DD1, b: differential display using primer DD2, c: differential display using primer DD3. Lane numbers: C=control, 1=25 mM NaCl, 2=50 mM NaCl and 3= 100 mM NaCl. White arrows indicate the bands which were excised from gel and sequenced.

Table 1: The effect of different concentrations of NaCl on growth parameters of salt sensitive GS12 and salt tolerant Adora tomato plants grown on clay-sandy soil. * Mean values are significantly different from control at $p \leq 0.05$.

parameters Treatments		Shoot length (cm)	Root length (cm)	Shoot diameter (cm)	Shoot fresh wt.(g)	Root fresh wt.(g)	Whole plant fresh wt. (g)	Shoot dry wt.(g)	Root dry wt.(g)	Whole plant dry wt. (g)	Whole plant water content (g)
		GS12	Control	62.75	50.50	0.719	66.25	37.50	103.75	20.59	20.09
25 mM NaCl	53.80*		43.20*	0.695*	63.00*	30.00*	93.00*	15.26*	16.32*	31.58*	61.42*
50 mM NaCl	50.00*		42.00*	0.683*	60.33*	23.33*	83.66*	14.95*	14.90*	29.85*	53.81*
100 mM NaCl	47.80*		40.55*	0.668*	56.55*	21.67*	78.22*	9.82*	10.54*	20.36*	57.86*
Adora	Control	63.35	52.80	0.785	72.50	38.10	110.60	28.69	21.68	50.37	60.23
	25 mM NaCl	62.50*	52.00*	0.785	70.00*	38.05*	108.05*	27.95*	20.78*	48.73*	59.32*
	50 mM NaCl	62.10*	50.30*	0.729*	69.05*	36.94*	105.99*	24.91*	18.59*	43.5*	62.49*
	100 mM NaCl	60.56*	49.70*	0.701*	66.04*	33.55*	99.59*	20.26*	14.04*	34.3*	65.29*

Table 2: Total number of differential bands and number of polymorphic bands generated by differential display using three arbitrary primers

	Primer		GS12					Adora				
			Number of cDNA fragments (bands)			Polymorphism (%)	Mol. Wt range (bp)	Number of cDNA fragments (bands)			Polymorphism (%)	Mol. Wt range (bp)
	Name	Sequence (5'-3')	Total	monomorphic	polymorphic			Total	monomorphic	polymorphic		
1	DD1	AAGCCGA AGC	5	3	2	40	130 0- 100	7	2	5	71.43	130 0- 100
2	DD2	TCAGCACG GA	4	2	2	50	900- 500	9	4	5	55.56	300 0- 300
3	DD3	TGGATTGG TC	2	2	-	0	500- 300	4	1	3	75	130 0- 500
Total			11	7	4	36.36		20	7	13	65	