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# Characterization of *Arabidopsis* seedlings growth and development under trehalose feeding

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## Abstract

Trehalose is the alpha, alpha-1, 1-linked glucose disaccharide. Its metabolism is found in a wide variety of organisms and is seen as evolutionary old. Trehalose metabolites are, however, present at only very low concentrations and their role in plants are not understood. The physiological effects of 100 mM trehalose on growth and carbon allocation in seedlings are characterized in this paper. Trehalose feeding to *Arabidopsis thaliana* elicits strong responses. On 100 mM trehalose, seedlings germinate and extend cotyledons but fail to develop primary leaves. The primary roots do not grow beyond 2-3 mm and there is not any starch in root tips. In light, growth arrest on 100 mM trehalose can be rescued by exogenous supply of metabolisable sugar. Trehalose feeding results in anthocyanin accumulation and chlorophyll reduction. Trehalose causes cells of the root extension zone to swell and lysis. Trehalase expression analysis showed that WT seedlings grown on trehalose have 10-fold induced *AtTRE1* expression compared to the sorbitol treatment.

***Keywords*:** trehalose, T6P, trehalase, carbon allocation, growth, Arabidopsis

## Introduction

Trehalose is the alpha, alpha-1, 1-linked glucose disaccharide, which is found ubiquitously and is therefore thought to be evolutionary ancient (Elbein et al., 2003). Its metabolism has recently been recognized to play an important role in carbon signaling in plants (Paul et al., 2008; Rolland et al., 2006). Trehalose is a carbon reserve. It has been shown that Trehalose has several biological functions. In fungal spores, trehalose hydrolysis occurs during early germination and presumably provides glucose for energy and biosynthesis (Thevelein, 1984). Trehalose is a stress protectant and protects proteins and membranes from denaturation by replacing water as it makes hydrogen bonds to polar residues (Brumfiel, 2004; Croweet et al., 1998; Wolkers et al., 2003). Plants generally contain only trace amounts of trehalose (Muller et al., 1995; Zentella et al., 1999). Exceptions to this exist and these are plants with extreme drought stress resistance such as *Selaginella lepidophylla* that accumulate quantitative amounts of trehalose. All plants seem to contain genes for trehalose metabolism (Blazquez et al., 1998; Leyman et al., 2001; Shima et al., 2007; Vogel et al., 1998). Synthesis of trehalose in plants is typically via its phosphorylated intermediate, trehalose-6-phosphate (T6P). Trehalose-6-phosphate synthase (TPS) converts UDP-Glucose and Glucose-6-phosphate to T6P. Trehalose phosphate phosphatase (TPP) de-phosphorylates T6P to trehalose. Trehalase cleaves trehalose to two glucose molecules (Elbein et al., 2003). Moreover, it has been shown that *Arabidopsis* encodes a single active TPS and a family of putative TPS-like proteins that have specific regulatory functions in actively growing tissues (Vandesteene et al., 2010).

Minor alterations of T6P steady states in plants yield dramatic and pleiotropic phenotypic changes (Pellny et al., 2004; Pramanik and Imai, 2005; Schluepmann et al., 2003; Schluepmann and Paul, 2009). Additionally, deletion of the T6P synthase (TPS), gene *AtTPS1*, in Arabidopsis is lethal and can be overcome by complementation with active TPS enzyme (Eastmond et al., 2002; Schluepmann et al., 2003). Evidences are thus accumulating that suggest an important regulatory role for T6P in the coordinating of metabolism with development (Paul et al., 2008). It is not understood, however, how

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T6P controls carbon utilization and why changes in the steady state level of T6P yield such strong phenotypic changes.

Attempts to produce trehalose in plants by over-expressing yeast *TPS* in tobacco yielded drought resistant plants (Holmstrom et al., 1996; Romero et al., 1997). Expression of *E.coli TPS-TPP* fusions in rice also yielded drought tolerance and in addition salt tolerance (Garg et al., 2002; Jang et al., 2003). Trehalose metabolism has been implicated in biotic stress resistance as well. Spraying wheat with a trehalose solution confers resistance to *Blumeria graminis* infection. Trehalose appears to activate plant defense responses e.g. papilla deposition, phenylalanine ammonia lyase and peroxidase activities (Reignault et al., 2001). The data suggests that trehalose and/or T6P may be a key component in plant-microorganism interactions (Iturriaga et al., 2009). The underlying mechanism is unclear so far. Isolation and characterization of *Arabidopsis* mutants resistant to exogenous trehalose at 100 mM could be a main achievement in understanding trehalose mechanisms against biotic and abiotic stresses.

In the present work, the Arabidopsis seedlings of growth inhibition due to T6P accumulation on ½ MS medium supplemented with 100 mM trehalose is characterized further. This characterization is necessary since the effects of 100 mM trehalose may be different from the effects of 25 mM trehalose combined with 10 µM Validamycine A that were used previously to describe the effect of trehalose (Fritzius et al., 2002; Roman et al., 2007; Wingler et al., 2000). The characterization of the physiological effects of 100 mM trehalose on seedlings presented in this paper will enable us to isolate and interpret the mutants from the suppressor screen. Results showed that seedlings that had long roots with primary and secondary leaves, high level of T6P and low level of starch after growing on 100 mM trehalos could be used as trehalose resistant mutants.

## Material and Methods

### *Plant material and growth conditions*

In this study, *Arabidopsis thaliana* accession Columbia-0 was used as Wild type (WT). Seeds of WT and TreF 46.2, a line expressing *E.coli* cytosolic trehalase behind the CaMV35S promoter (Schluepmann et al., 2003) were used in this study. Seeds were sterilized 5 minutes with 70% Ethanol followed by 10 minutes in 20 % commercial bleach (4% w/v chlorine) and washed 5 times in sterile milli-Q water. Sterilized seeds were plated on agar solidified half strength MS medium (Murashige and Skoog, 1962) supplemented with 50 mM trehalose with or without 50 mM sugars (sucrose, maltose, glucose, fructose, sorbitol and palatinose) and stratified in darkness at 4ºC for 2 days before the plates were transferred to a growth chamber at 25ºC under a 16-h-light/8-h-dark photoperiod. In this experiment, seedlings were grown vertically for 14 days. After 7 days, pictures were taken and the root length was measured with the Image J program (Wayne Rasband, NIH Maryland, USA).

### *Starch staining and confocal microscopy*

For analysis of starch distribution, all the seedlings were taken and destained in 70% and then 90% v/v ethanol. Staining was done with Lugol solutions and then washed with milli Q water. Pictures were taken using a Normarski microscope (Jena, Germany). For confocal Laser scanning microscopy (Zeiss, Germany), the roots were stained with propidium iodine (1µg/ml).

### *Chlorophyll and anthocyanin measurements*

Chlorophyll a, b and total chlorophyll were measured spectrophotometrically as described by Jeffery and Humphery (1975). In brief, 14 days seedlings were ground in liquid nitrogen and extracted with 80% v/v acetone. Then, the absorbance was measured (at 647, 652 and 664nm) and used to calculate chlorophyll content.

Anthocyanin content of seedlings was determined using the protocol of Mita et al., (1967). Frozen and homogenized seedlings (20 mg) were extracted for 1 day at 4°C in 1 mL of 1% (v/v) hydrochloric acid in methanol. The mixture was centrifuged at 13,000 rpm for 15 min and the absorbance of the supernatant was measured at 530 and 657 nm. Relative anthocyanin concentrations were calculated with the formula [$A_{530}$ – (1/4 x $A_{657}$)]. The relative anthocyanin amount was defined as the product of relative anthocyanin concentration and extraction solution volume. One anthocyanin unit equals one absorbance unit [$A_{530}$ – (1/4 x $A_{657}$)] in 1 mL of extraction solution.

## T6P measurements

Fifty mg fresh weight of 14 days old seedling were snap frozen, then ground using a dismembranator (Braun, Germany) before extraction with 800 µl of chloroform/ acetonitrile/water at a ratio of 5:7:2 for 2 hours at –10°C. After 5 min 6000 g centrifugation (Braun, Germany) at 4°C for 5 min, the acetonitrile/water phase was recovered and the chlorophorm phase

back-extracted with 400 µl water, the water and acetonitrile water phases were combined and dried under vacuum over night. Samples were taken up in 1 ml water prior to solid phase extraction (SPE). After loading, the SPE phase was rinsed with 4 volumes 5 mM NaOH. Then, samples were eluted twice with 0.5 ml 2% v/v formic acid. Eluates were combined and dried under a flow of nitrogen, resuspended in 0.2 ml water, filtered and 10 µl injected onto the AS-11-HC column (250 ×2 mm, Dionex, USA) for HPLC-PAD (Dionex) or HPLC-MS (Ion trap, Agilent). The ion exchange column was eluted with a 5-100 mM gradient of NaOH. Addition of T6P (12.8 nmol) during the extraction and or immediately loading onto the HPLC allowed calculation of T6P recoveries and an approximate evaluation of amounts of T6P in the extracts.

### *RNA isolation, RT-PCR and quantitative PCR (Q-PCR) analysis*

Seeds of *Arabidopsis thaliana* accession Columbia-0 (WT) were grown on ½ MS medium for 10 days. The plant material was snap frozen in liquid nitrogen, pulverized with glass beads for 2 minutes at 2800 rpm in a dismembrator (Braun, Germany). The total RNA was isolated with RNeasy plant mini kit (QIAGEN USA, Valencia, CA). The RNA concentration and purity were determined by measuring at 260 nm. 10 ng RNA was treated with 2 U DNAse I (DNA- free, Ambion, Austin, USA) to remove genomic DNA. Absence of DNA was analyzed by performing a PCR reaction (40 cycles, similar to the real–time PCR program) on the DNaseI- treated RNA using Taq-DNA polymerase. RT-PCR experiments were performed using 1 µg of total RNA extracted and used for first-strand cDNA synthesis with sixty units M-MLV Reverse Transcriptase (promega, Madison, WI), 0.5 µg of odT16v (custom oligo from invitrogen, Carlsbad, CA) and 0.5 µg random hexamer (invitrogen).

Q-PCR was carried out by ABI-prism 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA). Per each reaction, 12.5 µl of CYBR green PCR Master Mix (Applied Biosystems, UK) and 2.5 µl of trehalase specific primer (AtTRE1-F 5'-gctgcaccacgaaccagtaga-3' and AtTRE1-R 5'-ttcttcgttctccacgttgga-3'; Efficiency: 1.98) were used. Relative quantitation of gene expression was based on the comparative Ct method (User Bulletin No. 2: ABI PRISM 7700 Sequence Detection system, 1997) using *AtACTIN2* as a calibrator reference.

## Results

### *100 mM trehalose cause an accumulation of starch in source and depletion in sink tissues*

Supply of the 100 mM trehalose to the *Arabidopsis* seedlings (WT) led to the growth arrest and development arrest in leaves. In WT seedlings, the root length was very short (1.9± 0.6 mm after 14 days) and emergence of primary leaves was entirely inhibited. The trehalase expressing seedlings (TreF, line 46.2) had 12 times longer roots than WT ones after 14 days growth on 100 mM trehalose. TreF seedling root lengths on trehalose were as long as them on the sorbitol osmoticum control (figure 1).

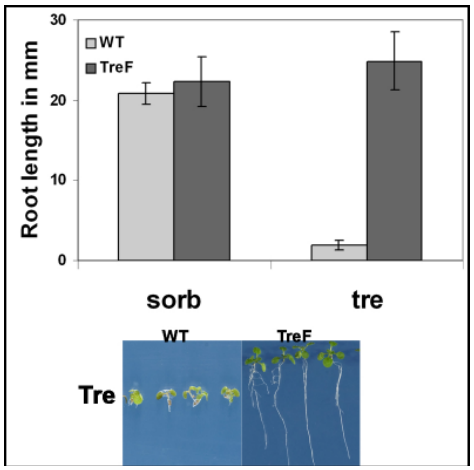


**Figure 1.** The effect of 100 mM trehalose on the root growth of WT seedlings. WT seeds were germinated and grown under long day conditions on ½ MS medium with 100 mM trehalose or sorbitol. Root length was measured after 14 days. Each experiment was repeated three times. Error bars indicate Standard deviation. The abbreviations are WT (Wild type), tre (trehalose), and sorb (sorbiyol).

Trehalose in the medium led to an accumulation of large amounts of starch in the seedling source tissue, cotyledon, and to a depletion of starch in the columnella cells of the root cap, a sink tissue (figure 2a-c). Confocal microscopy of the seedling roots stained with propidium iodine revealed swelling as well as lysis of the cells in the extension zone of roots grown on 100 mM trehalose but not on 100 mM sorbitol (figure 2d-f). In addition to altered starch distribution and reduced root growth, trehalose appeared to alter cell wall elasticity compared with sorbitol.

Distribution of starch in TreF and WT was studied in 14 d seedlings using Lugol staining. Staining revealed that the reaction to trehalose was not fully homogenous when examining a large number of WT seedlings: 72% of the seedlings responded with massive trehalose accumulation in the cotyledons whilst 28% failed to stain. The response to trehalose of seedlings expressing *E.coli* trehalase (TreF line) was homogenous, as cotyledons of these seedlings did not stain with Lugol. Seedlings of the TreF line displayed starch in the columnella cells of the root tips (not shown).

Quantification of starch in the WT and TreF seedlings on trehalose is shown in figure 2 g. WT seedlings contained 11 $mg \cdot g^{-1}$ FW (fresh weight) starch on medium with 100 mM sorbitol. On trehalose, the starch level in WT was increased to 52 $mg \cdot g^{-1}$ FW. TreF seedlings on trehalose contained the same amount of starch as WT on sorbitol.

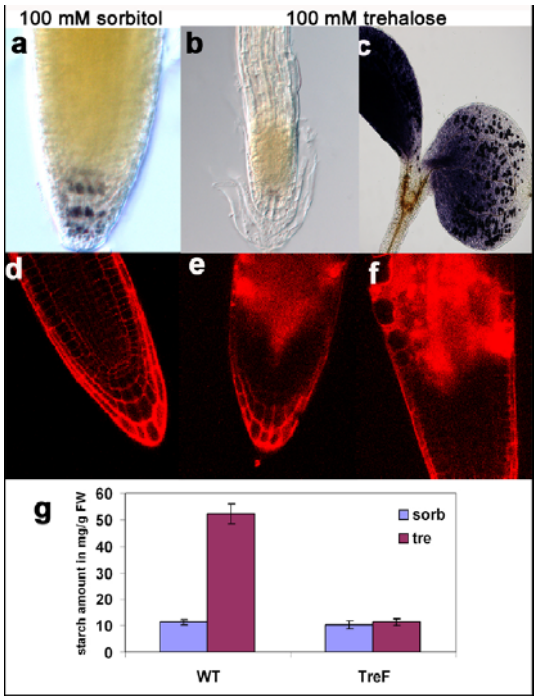


**Figure 2.** Starch staining and quantification. Seedlings were grown 14 d in long day conditions, then stained with $KI/I_2$ and studied using Nomarski microscopy. (a) Starch in the columnella of WT roots grown on 100 mM sorbitol osmoticum control, (b) Starch in the columnella of WT roots grown on 100 mM trehalose, (c) Starch accumulation in cotyledons of WT seedlings grown on 100 mM trehalose. Confocal microscopy of seedling roots with propidium iodine, (d) Typical root on 100 mM sorbitol osmoticum control, (e, f) typical swelling and lyses of cells at the extension zone of seedling roots on 100 mM trehalose, (g) Starch amounts of whole seedlings from WT and TreF on 100 mM sorbitol or trehalose after 14 days. The FW stands for Fresh Weight.

***Trehalose-6-phosphate mediated growth arrest on trehalose is due to the altered carbon allocation***

The seedling response to sugars without added trehalose was analyzed at 50 mM concentrations except for the toxic mannose where 6 mM was used (figure 3 without tre). The growth on 50 mM sorbitol, the osmoticum control equaled that on the half strength MS medium and thus suggests that osmoticum has little effect on growth at these sugar concentrations. Growth was enhanced when seedlings were supplied 50 mM of glucose, fructose, sucrose or maltose compared to the seedlings supplied with sorbitol; seedlings therefore utilized the supplied metabolisable carbon for growth. Interestingly, growth on the 100 mM of either fructose or glucose was not as vigorous as growth on 50 mM of each glucose and fructose or 50 mM of glucose or fructose. This was the case for both WT and trehalase expressing seedlings. Growth on palatinose equaled that on sorbitol

suggesting that this sugar, like sorbitol was not utilized. Trehalose at 50 mM inhibited root growth significantly to 30% of the control levels. Seedlings did not germinate on 50 mM 2-deoxy glucose or 6 mM mannose.

The seedling response to the 50 mM sugar combined with 50 mM trehalose was investigated (figure 3A, B). Sucrose, maltose and a combination of fructose and glucose completely alleviated the growth inhibitory effects of trehalose. Fifty milimolar of fructose or glucose alleviated the trehalose mediated growth inhibition partially. Sorbitol and palatinose were ineffective against the growth inhibition. The T6P accumulation on trehalose did not rescue inhibition of the seedling germination due to 2-deoxyglucose, and so T6P unlikely acted as an inhibitor of HXK2 mediated signaling. This was further supported by the fact that T6P accumulation did not rescue mannose inhibition of germination. After 7 days, growth of the TreF expressing seedlings on trehalose equaled that on sorbitol (figure 3A, C- with trehalose). If grown for longer periods of time, these seedlings thrived on trehalose with growth exceeding that on sorbitol, presumably because glucose from trehalose cleavage was used for the growth. Addition of sucrose, maltose or a combination of glucose and fructose increased growth of trehalase expressors on trehalose, their root lengths being twice as long on medium with trehalose and the metabolisable sugars.

### *Chlorophyll and anthocyanin contents correlate with the growth response*

WT seedlings growing on trehalose had dark red-rimmed cotyledons. Chlorophyll and anthocyanin contents were determined in the WT and TreF seedlings. While total chlorophyll content of WT seedlings were reduced 4 fold after 14 days growth on trehalose compared to the growth on sorbitol

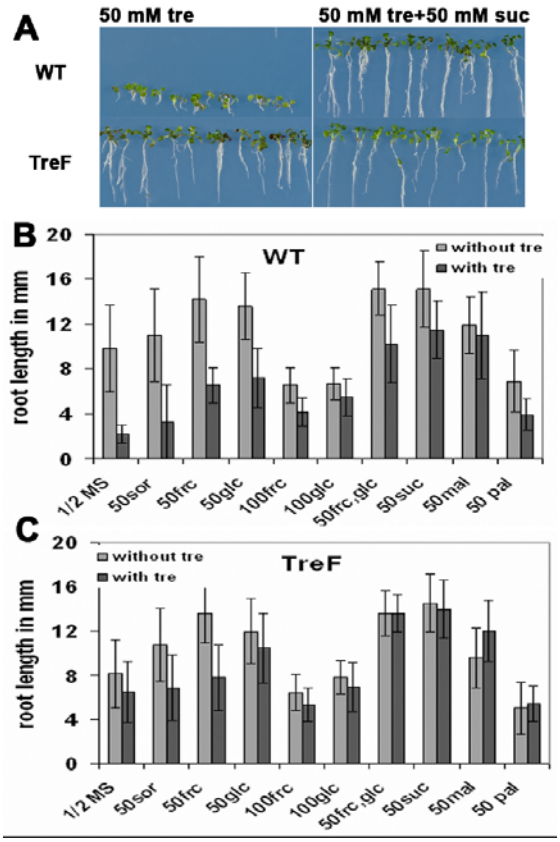


**Figure 3.** Effects of metabolisable sugar on the growth inhibitory effect of trehalose. Seedlings were grown for 14 days in long day conditions on half strength MS medium containing 50 mM trehalose with or without different sugars. Root lengths were determined using Image J. WT, seedlings from WT; TreF, seedlings from the *E.coli* trehalase, *TreF*, expressing line 46.2; (A) WT and TreF seedlings on 50 mM trehalose (50 mM tre) with or without 50 mM sucrose (50 mM tre+50 mM suc). (B) Root lengths of WT on half strength MS medium (1/2MS) with different sugar combinations. Sugars concentration in this experiment was at 50 mM, except for mannose, glucose, fructose and a combination of fructose and glucose, where the concentration were 6mM, 100 mM, 100 mM and 50 mM fructose combined with 50 mM glucose, respectively. (C) Root lengths of TreF in media with different combinations of sugar.

(figure 4a), the Chla/Chlb ratio increased by feeding of 100 mM trehalose (3.75 on trehalose compared to 2.13 on sorbitol).

Anthocyanin was 5 fold induced in the WT seedlings after 14 d growth on trehalose compared to sorbitol (figure 4b). The TreF seedlings did not show any change in chlorophyll and anthocyanin levels by the trehalose feeding (not shown).

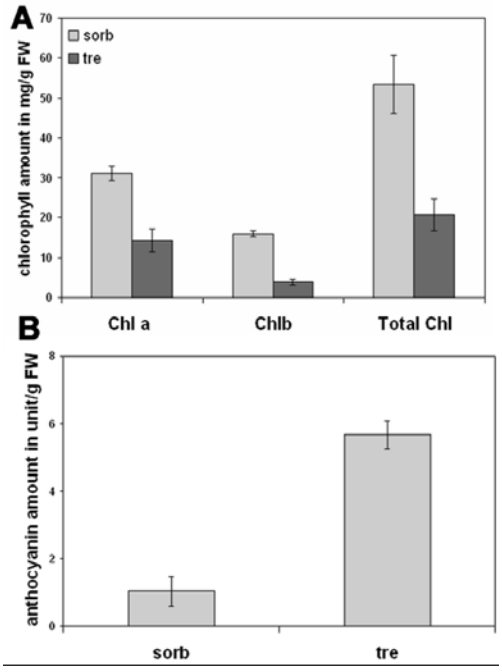


**Figure 4.** The effect of 100 mM trehalose on chlorophyll and anthocyanin content in WT seedlings. Seedlings were grown for 14 days on 100 mM of either sorbitol or trehalose in long day conditions. a) Chlorophyll and b) anthocyanin contents. Each experiment was repeated three times. Error bars indicate Standard deviation. WT: Wild Type, Chla: chlorophyll A, Chlb: chlorophyll B, Total Chl: total chlorophyll, FW: fresh weight.

***T6P measurements***

T6P levels were determined in 50 mg FW tissue using a pre-purification SPE procedure followed by HPLC-PAD and HPLC-MS. Results confirmed an accumulation of T6P in the WT seedlings grown for 14d on trehalose. The T6P level in 14d seedlings was 10.2 ± 2.5 nmol·g$^{-1}$ FW, compared to the 2.78 ±1.3 nmol·g$^{-1}$ FW on sorbitol (figure 5a).

***Trehalase expression***

We analyzed the expression levels of the *AtTRE1*gene, the only trehalase gene in Arabidopsis, to find out the trehalase expression status in WT seedlings growing on 100 mM trehalose. The analysis was performed using mRNA from 10 d old seedlings grown on ½ MS medium with 100 mM sorbitol or trehalose, and Q-PCR technique. Results showed that WT seedlings grown on trehalose had 10-fold induction of the *AtTRE1* expression compared to sorbitol (figure 5b).

## Discussion

Trehalose supplied to the growth medium of seedlings inhibits growth and allocation of carbon to the root and shoot (Fritzius et al., 2001; Schluepmann et al., 2004; Schluepmann and Paul, 2009; Wingler et al., 2000). This growth inhibition was previously studied by combining 25 mM trehalose with 10 μM Validamycine, a trehalase inhibitor. In this research, we used 100 mM trehalose without addition of any trehalase inhibitor.

Supplied trehalose is transported through the plant tissues and enters the cells, since plants expressing trehalase in the cytosol thrive on medium with trehalose (Schluepmann et al., 2003; Schluepmann et al., 2004). In the light conditions, growth arrest on trehalose is due to T6P accumulation (Schluepmann et al., 2004; Schluepmann and Paul, 2009) and chlorophyll reduction in the WT seedlings implies that these typical senescence processes correlate with growth inhibition. T6P measurement carried out in this research revealed that trehalose is absorbed by the seedlings leading to an increase in the steady state of T6P supporting the notion that growth arrest of the seedlings on trehalose is due to T6P accumulation.

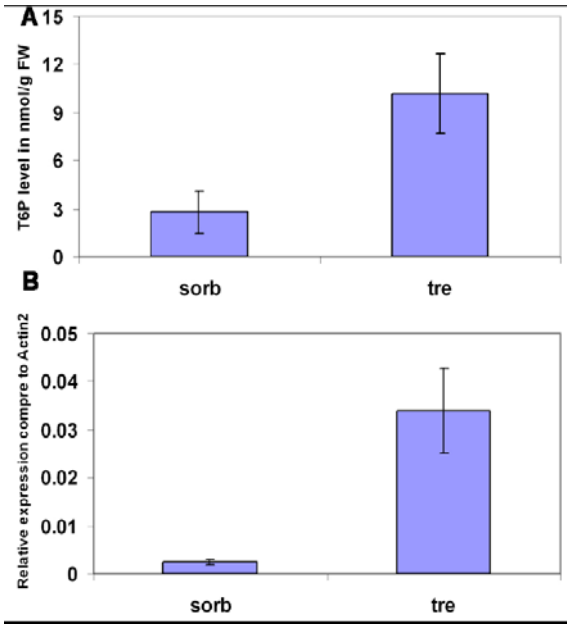


**Figure 5.** T6P quantification and trehalase gene expression. a) T6P level in WT. Seedlings of WT were grown for 14 days on half strength MS supplemented with 100 mM of either sorbitol (sorb) or trehalose (tre). T6P was quantified by HPLC-MS Scanning, b) Trehalase gene expression in WT. Seedlings were grown on agar solidified half strength MS for 10 days before RNA extraction and Q-PCR analysis of gene-expression. Levels of gene expression were determined with reference to *AtACTIN2*. Each experiment was repeated three times. Error bars indicate Standard deviation. WT stands for Wild Type.

***The trehalose pathway regulates carbon partitioning in light***

Accumulating evidences suggest an important regulatory role for T6P in growth and development of the seedlings (Paul et al., 2008; Schluepmann et al., 2003).

It is not understood however, how T6P controls carbon utilization and why changes in the steady state level of T6P yield such strong phenotypic changes.

Growth inhibition by trehalose can be overcome by expression of *E.coli* trehalase, TreF, a soluble enzyme targeted to the cytosol suggesting that trehalose supplied exogenously is imported into the plant cells, then cleaved by expressed trehalase and the released carbon is utilized for growth (Schluepmann et al., 2003). Growth inhibition on trehalose can be overcome by expression of the *E.coli* trehalose phosphate hydrolase, an enzyme that cleaves T6P into Glucose-6-phosphate and glucose suggesting that T6P accumulation is causing growth arrest (Schluepmann et al., 2004). Studying the effect of sugars on T6P mediated growth arrest may therefore reveal interactions between T6P and sugar signaling pathways that control carbon utilization in the source tissues or that control carbon allocation and transport.

The T6P-mediated inhibition of growth is likely due to starvation of the sink tissues important for growth, such as shoot and root apical meristems. It has been shown on 25 mM trehalose that carbon allocation is reversed by high accumulation of starch in cotyledons (Wingler et al., 2000). Interestingly, supply of the metabolisable sugar in addition to 100 mM trehalose relives the growth inhibitory effects of trehalose suggesting that starvation causes growth arrest; it further suggests that trehalose does not affect the ability of sink tissue to metabolise the allocated carbon. Increased elasticity of the cell walls in the extension zone and absence of starch accumulation in columnella cells of the root tip suggest that T6P accumulation throughout the plant tissues likely causes starvation of the sink tissues important for growth, such as shoot and root apical meristems. Sink starvation is not caused by the sink's inability to metabolize carbon since the supplied carbon is utilized and the effects of the T6P accumulation are then overcome. Indeed, starch is no longer formed at the root tip in the columnella when the WT seedlings are grown on 100 mM trehalose (Ramon et al., 2007; Wingler et al., 2000). Since *pgm1* (*pgm1* cannot synthesize starch) seedlings are also growth arrested on trehalose (Fritzius et al., 2001), T6P inhibition of the growth is not due to the carbon partitioning into starch in the cotyledons. Inhibition, therefore, is more likely due to carbon loading/unloading or transport. These effects can be at subcellular level, for example, export from chloroplast to the cytosol. Alternatively, that can be at the plant level i.e. the

interface between mesophyll and vascular bundles or simply involve transport inhibition in the phloem.

This can be exploited in a genetic screen program to identify plants altered in T6P metabolism or target processes of T6P. Mutant seedlings that overcome growth arrest on 100 mM trehalose are either altered in trehalose import, trehalose catabolism, T6P synthesis or in the responses to T6P. Thus, screening for suppressors of growth inhibition on 100 mM trehalose will not only uncover mutants in trehalose metabolism or the control thereof but also mutants that overcome T6P mediated changes in carbon allocation. Characterization of the physiological effects of 100 mM trehalose on Arabidopsis seedlings presented in this paper will help to interpret the mutants obtained from the proposed suppressor screen. Mutants capable of growth on 100 mM trehalose have been obtained which are being used to further extend our understanding of how T6P accumulation arrests the seedling growth.

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# Cost of resistance to herbivory in the annual plant *Arabidopsis thaliana*

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## Abstract

This study examines the assumption that plant resistance to herbivory has fitness costs. To assess costs, a standard method was used for examining the significant negative genetic correlation between the resistance character and damage in the presence of herbivory and with fitness in the absence of herbivory. Seeds of five plants from four genotypes of *Arabidopsis thaliana* were sown under controlled conditions in a growth chamber. Half of the resulting two months-old rosettes were used for glucosinolate analysis and for herbivory assessment. The other half were transplanted into an enclosure in the natural habitat of this plant and their fitness (fruit number) were measured after harvesting the plants. Caterpillars from *Spodoptera exigua* were obtained from lab culture for herbivory assessment. Two second-instar caterpillars from *S. exigua* were placed on each of the rosettes. Larval weight of caterpillars was measured after 5 days. One hundred mg dry mass of leaves from 5 rosettes of each genotypes were used for HPLC analysis. There were genetic variations in types and quantities of glucosinolate between genotypes. The results from herbivory assessment showed that the larval weight of *S. exigua* fed on some genotypes was significantly lower than others, and therefore there was genetic variation in resistance to herbivore for *A. thaliana* genotypes. The statistical analysis showed that the larval weight of *S. exigua* was negatively correlated with the total glucosinolate concentration and with fruit number. Therefore, under the condition of this experiment, glucosinolates reduced damage by *S. exigua* and exhibited significant fitness costs.

***Keywords:*** *Arabidopsis thaliana*, Costs of resistance, Glucosinolates, Herbivore, *Spodoptera exigua*

## Introduction

The idea that a plant must allocate limited resources among growth, reproduction, and defense has been central to the ecological and evolutionary theories (Coley et al., 1985; Frank, 1993; Herms and Mattson, 1992) and underpins recent ideas about life history trade-offs. Stated simply, if a plant allocates a greater proportion of resources to defense, then less should be available for growth and /or reproduction. If this relationship did not exist, then there would be no cost to counteract the benefit of resistance, and all plants should be resistant. The many examples of polymorphisms in the levels of resistance within and among populations suggest strongly that costs are prevalent (e.g., Parker, 1992; Simms, 1992), and for decades the notion of evolutionary trade-offs associated with resistance has been widely accepted. However, failures to detect costs of resistance to herbivores and pathogens (Agren and Schemske, 1993; Brown, 1988; Simms and Rausher, 1987) have raised questions about whether such costs exist in plant populations (Simms, 1992; Simms and Triplett, 1994) and thus, about the appropriateness of theories that postulate such cost. Many researchers have attempted to detect costs of resistance to herbivores but they have not observed significant costs. Early attempts, based on nongenetic approaches, initially suggested that costs might be common. However, most of these approaches have not determined whether there is a negative genetic correlation between resistance and fitness. For example, although some investigators have calculated the cost of resistance in the currency of adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADPH), or carbon (Chew and Rodman, 1979), these prove not to be meaningful in an evolutionary context unless the costs can be expressed in the relevant units of plant fitness.

Others have inferred the existence of costs of resistance from the detection of significant negative phenotypic correlations between fitness and resistance (Baldwin et al., 1990; Coley, 1986). Such studies must be viewed with caution because environmental covariances may cause the phenotypic covariances to differ in both sign and magnitude from the underlying genotypic

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covariances (Rausher, 1992). However, the failure may be a result of reasons other than the actual absence of costs of resistance (Charlesworth, 1990). For example, costs may not be revealed as a pairwise negative correlation between resistance and fitness because these two traits are mutually correlated with other characters (Houle, 1991). Costs may also go undetected because they may be manifested only under certain environmental conditions that differ from those used in experiments (Bergelson, 1994).

*Arabidopsis thaliana* (Brassicaceae) is a predominantly self- fertilizing, annual herb that is native to Europe and now wildly distributed in many parts of north-temperate regions of the world (Baskin and Baskin, 1972; Ratcliffe, 1961). *A. thaliana* is a prime model system of plant molecular genetics, and is currently used to explore the molecular basis of resistance to herbivores (Kroymann et al., 2003). *A. thaliana* produces both secondary compounds (most notably the glucosinolates, a class of secondary compounds characteristic of the Brassicaceae and leaf trichomes that could potentially deter oviposition and insect feeding (Mauricio, 1998).

This paper examines the existence of fitness costs of resistance in populations of the annual plant *Arabidopsis thaliana*. Assessment of costs of resistance requires the presence of genetic variation for the resistance characters. Resistance characters are, by definition, traits that reduce the amount of damage an individual plant experiences. To assess costs, a standard method was used for examining the significant negative genetic correlation between the resistance character and damage in the presence of herbivory and with fitness in the absence of herbivory.

## Materials and methods

Seeds of five plants were collected from four populations (genotypes), 2 from dune and 2 from inland area in the Netherlands. Seeds of each population were sown under controlled conditions in a growth chamber (20º C, 18-h light, 70% humidity). Seeds produced by these plants were germinated and half of the resulting two months-old rosettes were used for glucosinolate analysis and for herbivory assessment. To include all environmental condition, the other half was transplanted into an enclosure in the natural habitat of this plant. Rosettes from each of four genotypes in 5 replicates were transplanted into a randomized complete block design. The rosettes were transplanted into small holes with minimal disturbance of the surrounding vegetation. Fitness (fruit number) was measured after harvesting the plants.

One hundred mg dry mass of leaves of 5 rosettes of each genotype was used for the HPLC analysis. Extraction, purification and glucosinolate measurements were performed following the procedure used by Van Dam et al. (2003) with sinigrin as the external standard. Glucosinolates were extracted with 70% methanol solution, desulphatased with arylsulphatase on a DEAE-Sephadex A25 column and separated on a reversed phase C-18 column on HPLC with an acetonitril-water gradient. The elution program was a linear gradient starting at 0% acetonitrile (ACN) that increases to 35% ACN in water over 30 minutes. Detection was performed with a single wavelength detector set to 229 nm. Glucosinolates that could not be identified were indicated based on their UV absorption spectrum.

To show genetic variation for resistance to the glucosinolstes reduce damage caterpillars from *Spodoptera exigua* were obtained from a lab culture, reared on an artificial diet in a growth chamber at 25°C, 16h/8h L/D photoperiod, 70% RH. Rosettes of 5 plants in 5 replicates from the same rosettes, as used for HPLC analysis, were used for this experiment. Two second-instar (second growth stage) caterpillars from *S. exigua* were placed on each rosette. Larval weight of caterpillars was measured after 5 days. Differences in larval weight show that resistance characters in genotypes are different.

Data were analyzed with SPSS 13. Normality of the data was checked by post-hoc analysis of the residuals using the Kolmogorov-Smirnov test for normality. Differences in larval weight of herbivores and differences in glucosinolate concentration between populations were tested with ANOVA. The correlation between glucosinolates and larval weight of herbivores and with fruit number was analyzed with the Pearson test.

## Results

### *Glucosinolate differences in HPLC analysis*

Results indicated a genetic variation in glucosinolates in the studied plants. Twelve principal glucosinolates were found in the leaves of plants grown in the growth room. They were classified into four structural types according to Fahey et al. (2001): indol glucosinolates (I), aliphatic with straight and branched chains glucosinolates or olefins (D), alcohols side chains glucosinolates (E) and sulfur-containing side chains glucosinolates (A). Individual plants and populations (genotypes) differed in glucosinolate

composition. Epiprogoitrin, 4-hydroxy glucobrassicin and an unknown sulfur-containing glucosinolate were found only in one of dune gynotype and gluconapin was found only in dune plants. 3-OH propylglucosinolate and an unknown alkenyl glucosinolate were found only in one of the inland genotype. The concentration of sinigrin was high in dune genotype compared to the inland genotypes. The plants from dune had a significantly higher concentration of total glucosinolates as compared to the inland plants. The aliphatic glucosinolate were found in high concentration on dune genotypes. Concentration in plants from dune was also higher than in inland plants but this was not significant (table 2).

**Table 1**. Mean (± SE) of larval weight (mg) of *Spodoptera exigua* fed on dune and inland populations.

| Herbivore | ***Dune 1*** | Dune 2 | Inland 1 | Inland 2 |
|---|---|---|---|---|
| Larval weight of *S. exigua* | 20 ± 11.7 a | 17.9 ± 8.79 a | 128.5 ± 73.4 b | 122.2 ± 34 b |

The values in each row, followed by a different character are significantly different (ANOVA, Tukey test, $P<0.05$). $n= 25$.

**Table 2**. Glucosinolate type of leaves for dune and inland plants grown in growth room.

| Glucosinolate type | Dune 1 | Dune 2 | Inland 1 | Inland 2 |
|---|---|---|---|---|
| I | 1.01 ± 0.16 a | 1.26 ± 0.09 a | 1.04 ± 0.19 a | 0.97 ± 0.07 a |
| D | 14.06 ± 2.41 b | 25.80 ± 1.85 a | 2.14 ± 0.39 c | 8.35 ± 0.79 bc |
| E | 0 | 0 | 4.37 ± 0.79 | 0 |
| A | 0.59 ± 0.09 b | 1.31 ± 0.07 a | 1.01 ± 0.17 ab | 1.31 ± 0.07 a |
| Total glucosinolate concentration | 15.66 ± 2.67 b | 28.37 ± 1.99 a | 2.56 ± 1.53 b | 10.63 ± 0.99 b |

Mean concentration (±SE) (µmoles/g dry weight) for each type is given, $n= 5$. I = indol glucosinolates; D = aliphatic glucosinolates with straight and branched chains (olefins); E = glucosinolates with alcohols side chains and A = glucosinolates with sulfur-containing side chains. The values in each row, followed by a different character are significantly different (ANOVA, Tukey test, $P<0.05$).

***Herbivory Assessment***

The results from herbivory assessment showed that the larval weight of *S. exigua* fed on some genotypes was significantly lower than others ($P<0.001$), and indicating genetic variation in resistance to herbivore for *A. thaliana* genotypes (table1).

***Herbivory in relation to glucosinolates and fitness***

The result showed that the larval weight of generalist herbivore *S. exigua* was negatively correlated with total glucosinolate concentration ($r = -0.64$, $P<0.001$) and with the olefin group in the leaves (figure 1). The larval weight of the generalist herbivore *S. exigua* was also negatively correlated with gluconapin, sinigrin and 4 methoxyglucobrassicin ($r = -0.71$, $P<0.001$; $r = -0.65$, $P = 0.002$; $r = -0.59$, $P = 0.006$ respectively).

At the same time there was a significant negative genetic correlation between glucosinolates and fruit numbers ($r = -0.45$, $P = 0.05$) (figure 2).

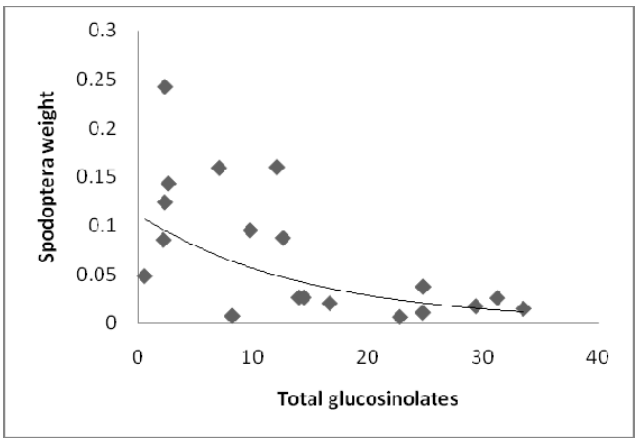


**Figure 1.** Pattern between total glucosinolates and *Spodoptera* weight.

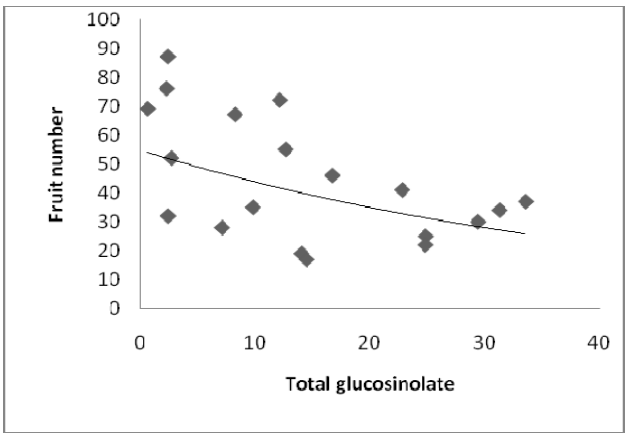


**Figure 2.** Pattern between total glucosinolates and fruit number.

## Discussion

First, this experiment indicated variation in glucosinolate concentration among the studied populations. Significant genetic differences in glucosinolates have already been reported for *Arabidopsis thaliana* (Mosleh Arany, 2009) and for *Brassica oleracea* (Mithen et al., 1995). Second, this experiment demonstrated that the main effect of glucosinolates lies in defense against a generalist herbivore as there was a negative correlation between glucosinolates and *S. exigua* weight. The negative impacts of glucosinolates on generalist herbivore *Trichoplusia ni* also reported by Kliebenstein et al. (2002). In addition to glucosinolate composition, glucosinolate concentration also negatively impacted generalist herbivory for both *S. exigua* and *Trichoplusia ni* (Kroymann et al., 2003). Third, results of this experiment provided evidences that resistance characters to herbivory (glucosinolate here) exhibited fitness costs. Mitchell-Olds et al (1996) demonstrated the cost of resistance to herbivory and disease in *Brassica*. They showed that genetic resistance to the fungal pathogen, *Leptosphaeria maculans* was cost-free, while resistance to *Peronospora parasitica* showed a negative genetic correlation between disease resistance and growth rate. Mauricio (1998) showed cost of resistance to natural enemies in field populations of *Arabidopsis*. The herbivore in his study was not identified, so that it is not clear whether they are specialist or generalist herbivores.

This study shows significant fitness cost for one of two types of resistance characters, glucosinolates. *Arabidopsis* has another resistance character that can reduce damage by herbivores, trichome density. Most plant species possess multiple resistance characters. Based on the assumption that there are costs of resistance, several authors have suggested that there should be a trade-offs among resistance characters (Bjorkman and Anderson, 1990). There are several evidences for such a relationship. Rehr et al. (1973) found that *Acacia* species possess either cyanogenic glycosides or symbiotic ant-based defense but not both. Bjorkman and Anderson (1990) showed that a morph of a South American blackberry lacking glandular trichomes had significantly tougher leaves than a morph with trichomes. By contrast, Steward and Keeler (1988) found no relationship between indol alkaloide and three physical resistance characters in 19 species of the genus *Ipomoea*. This study did not examined the relationship between two resistance characters in *Arabidopsis* but Mauricio (1998) found a significant positive correlation between trichome density and total glucosinolate concentration in *A. thaliana*.

This study demonstrated that under the condition of this experiment, glucosinolstes reduced damaged by *S. exigua* and exhibited significant fitness costs.

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# Investigating protein features contribute to salt stability of halolysin proteins

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## Abstract

The study used various screening techniques, clustering, decision tree and generalized rule induction (association) (GRI) models and molecular phylogenic relationship to search for patterns of halophilicy and to find features contribute to halolysin salt stability. We found that Met was the sole N-terminal amino acid in halolysin proteins, whereas other amino acids found at that position of other proteases and termitase. Eighty-three protein features were shown to be important in feature selection modeling, and just one peer group with an anomaly index of 2.42 declined to 1.87 after being run using only important selected features. The depth of the trees generated by various decision tree models varied from 1 to 5 branches. Compared to datasets without feature selection the number of peer groups in clustering models was reduced significantly ($p<0.05$). In most decision tree models, the frequency of Gly - Gly was the most important feature for decision tree rule sets and this feature was used in antecedent to support the rules in most GRI association rules. Significant differences ($p < 0.001$) found in charged amino acids between halolysin and other proteins with more Asp and Glu in halolysin proteins, while more hydrophobic residues and aliphatic amino acids were found in other proteases.

***Keywords:*** bioinformatics, modeling, protein, halophilic, halolysin

## Introduction

*Halobacteria*, extremely halophilic red-pigmented bacteria, have been intensively studied during the past decades (Sumper, 1987; Oren, 1994; Kamekura, 1998; Mukohata et al., 1999; Joo and Kim, 2005), through which our understandings of various biological processes such as energy metabolism (Gonzalez-Hernandez and Pena, 2002), environmental response (Elevi Bardavid and Oren, 2008), gene regulation (Conover and Doolittle, 1990), and the Archaea l cell cycle (Cui et al., 2006) have been greatly increased. Their extraordinary ability to grow in hypertonic solution (above 300 g of NaCl per liter) and their potential ability to hydrolyze proteins are the main reasons for rapid increase in research in this field (Kristjansson et al., 1986). A microorganism corresponding to the description of *Halobacteria salinarum* was isolated from salted fish more than 80 years ago (Soppa, 2006). Since then, many haloArchaea l species have been isolated, which, after considerable renaming, are currently grouped into 25 genera. Several years ago, it was decided that the species *Halobacterium salinarum*, *Halobacterium halobium*, and *Halobcterium cutirubrum* are so similar that they should be regarded as strains of one species named Halobacterium salinarum. *Halobacterium salinarum* shows very high genetic variability that was attributed to the large number of insertion sequences (Yang et al., 2006).

A small percentage of proteins can tolerate salinity and dryness stress. The enzymes from extremely halophilic bacteria represent a fascinating example of adaptation. . These enzymes function in vivo and in vitro at ranges of 4 to 5 M NaCl and upon exposure to low salt densities they lose their activities very rapidly (Binbuga et al., 2007; Pesenti et al., 2008; Zhu et al., 2008). Recently, genes for a number of halophilic enzymes have been cloned, including dihydrofolate reductase from *Haloferax volcanii* (Fine et al., 2006), glutamate dehydrogenase from *Halobacterium salinarium* (Ingoldsby et al., 2005), and malate dehydrogenase from *Haloarcula marismortui* (Zaccai et al., 1986). The mechanism of halophilicity of these enzymes, however, has not been fully elucidated at the molecular level. It has been shown Glu243Arg, a mutant protein of the malate dehydrogenase, was more halophilic, and

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required significantly higher concentrations of NaC1 or KCI for equivalent stability (Madern et al., 1995). Proteases are key enzymes in many processes important to the cell and are widely used in biotechnology and industry. Many representatives of the *Archaea* domain are extremophiles, thriving in conditions lethal to most cells. Thus, *Archaea* represent an important resource of enzymes, including proteases, in applied research as well as for basic enzymology. For applications requiring low water activity such as high salt or organic solvents, haloArchaea l and their enzymes have great potential to act as biocatalysts (Kamekura et al., 1992; De Castro et al., 2008).

Halolysin, a halophylic alkaline serine protease, has been extracted from *Archeaebacterium* and some other bacteria such as *Natrialba asiatica*, *Haloferax mediterranei*, *Natrialba magadii* and *Halobacterium sp. NRC-1* (Kamekura et al., 1992; Kamekura and Seno, 1993; Kamekura et al., 1996; De Castro et al., 2008). Halolysinfrom Halophilic archaeon is active at NaCl concentration of 4-4.5 M, loses its activity at salt concentration lower than 2M and is a very interesting sample of studying adaptation to harsh conditions (Feng and Yang, 2008; Strahl and Greie, 2008). The purpose of this study was to find the most important features contributing to these enzymes' ability to stand high concentration of salts and find other similar possible enzymes. Here we studied phylogenic relationship, feature selection, screening models, association models and statistical analyses among halolysin and other proteases extracted from few bacteria, fungi and plants in order to investigate features contributing to salt tolerance.

## Material and Methods

Nine halolysin sequences (A42605, AAG20619, AAV66536, BAA01049, BAA10958, CAP14928, NP_281139, P29143 and YP_001690274) were extracted from UniProt Knowledgebase (Swiss-Prot and TrEMBL). To find similar proteases, peptidases and termitase sequences, p29143 halolysin sequence was used to blast with available databases and 37 plant protease, 8 fungal proteases and 6 termitase were found and saved as FASTA format. To draw phylogenic tree, three software (CLCbio, MEGA4 and CLASTAL W) were used with similar parameters (i.e. Neighbor joining algorithm). Similar consensus sequences with 100% restrictions from alignment sequence with lower E value were chosen. Forty hundred and thirty nine protein features such as length, weight, isoelectric point, count and frequency of each element (carbon, nitrogen, sulphur, oxygen and hydrogen), count and frequency of each amino acid, count and frequency of negatively charged, positively charged, hydrophilic and hydrophobic residues, count and frequency of dipeptids, number of α-helix and β-strand and other secondary protein features were extracted.

To investigate protein features contributing to resistance of halolysin proteins to salty conditions and to compare them with other proteases and termitase studied in this paper, we divided dataset proteins into two groups: 1) T/F groups (T = halolysin proteins and F = other proteins; plant, bacterial and fungal proteases and termitase). 2) H/B/F/P/T groups (H = halolysin proteins, B = bacterial proteases, F = fungal proteases, P = plant proteases and T = termitase; comparing halolysin proteins with individual class of other proteins). The Protein name (either T/F or H/B/F/P/T) variable was set as the output variable and others as input variables. All features were classified as continuous variables, except the N-terminal amino acid, which was classified as categorical. A dataset of these protein features was imported into Clementine software (Clementine_NLV-11.1.0.95; Integral Solution, Ltd.).

Various decision tree algorithms were applied to the datasets to identify the most important features and find possible patterns that contribute to protein classes. These models allowed the development of classification systems that automatically included in their rules only the attributes important in making a decision. Attributes that did not contribute to the accuracy of the tree were ignored. This process yielded very useful information about the data and could be used to reduce the data to relevant fields only before training another learning technique, such as a neural network. As various algorithms were available for performing classification and segmentation analysis, and herein we used different decision tree and cluster analysis models. All models were run both with and without feature selection criteria to investigate the effects of the feature selection algorithm on other models behavior. All models run as previously described (Ebrahimi et al., 2009; Bijanzadeh et al., 2010; Ebrahimi and Ebrahimie, 2010).

### *Screening Models*

#### *Anomaly detection model*

This model was used to identify outliers or unusual cases in the data. Unlike other modeling methods that store rules about unusual cases,

anomaly detection models store information on what normal behavior looks like. This makes it possible to identify outliers even if they do not conform to any known pattern. While traditional methods of identifying outliers generally examine one or two variables at a time, anomaly detection can examine large numbers of fields to identify clusters or peer groups into which similar records fall. Each record then can be compared to others in its peer group to identify possible anomalies. The further away a case is from the normal center, the more likely it is to be unusual.

***Feature selection algorithm***

The feature selection algorithm was applied to identify the attributes having a strong correlation with the thermostability of enzymes. The algorithm considers one attribute at a time to determine how well each predictor alone predicts the target variable. The important value for each variable is then calculated as (1–*p*), where *p* is the *p* value of the appropriate test of association between the candidate predictor and the target variable. The association test for the categorized output variables differs from the test for continuous variables. In our study, when the target value was categorical (as in our datasets), *p* values based on the F statistic were used. The idea was to perform a one-way ANOVA F test for each predictor; otherwise, the *p* value was based on the asymptotic t distribution of a transformation of the Pearson correlation coefficient. Other models, such as likelihood-ratio chi-square (also tests for target-predictor independence), Cramer's V (a measure of association based on Pearson's chi-square statistic), and Lambda (a measure of association that reflects the proportional reduction in error when the variable is used to predict the target value) were conducted to check the possible effects of calculation on feature selection criteria. The predictors were then labeled as important, marginal, and unimportant, with values > 0.95, between 0.95 and 0.90, and < 0.90.

***Clustering Models***

***K-Means***

The K-Means model can be used to cluster data into distinct groups when the content of the groups is unknown. Unlike most learning methods in Clementine, K-Means models do not use a target field. This type of learning, with no target field, is called unsupervised learning. Instead of trying to predict an outcome, K-Means tries to uncover patterns in the set of input fields. Records are grouped so that those which are within a group or a cluster tend to be similar to each other, whereas those which are in different groups are dissimilar. K-Means works by defining a set of starting cluster centers derived from the data. It then assigns each record to the cluster to which it is most similar based on the record's input field values. After all cases have been assigned, the cluster centers are updated to reflect the new set of those records assigned to each cluster. The records are then checked again to see whether they should be reassigned to a different cluster, and the record assignment/cluster iteration process continues until either the maximum number of iterations is reached or the change between one iteration and the next fails to exceed a specified threshold.

***Two-Step cluster***

The Two-Step cluster model is a two-step clustering method. The first step makes a single pass through the data, during which it compresses the raw input data into a manageable set of subclusters. The second step uses a hierarchical clustering method to progressively merge the subclusters into larger and larger clusters, without requiring another pass through the data. Hierarchical clustering has the advantage of not requiring the number of clusters to be selected ahead of time. Many hierarchical clustering methods start with individual records as starting clusters and merge them recursively to produce ever-larger clusters.

***Decision Tree Models***

***Classification and regression tree (C & RT)***

This model uses recursive partitioning to split the training records into segments by minimizing the impurity at each step. A node is considered pure if 100% of cases in the node fall into a specific category of the target field.

***CHAID***

This method generates decision trees using chi-square statistics to identify optimal splits. Unlike the C&RT and QUEST models, CHAID can generate non-binary trees that means some splits can have more than two branches.

***Exhaustive CHAID***

This model is a modification of CHAID thatmore thoroughly examins all possible splits, but it takes longer to compute.

***QUEST***

The QUEST model provides a binary

classification method to builde decision trees. It is designed to reduce the processing time required for large C & RT analyses while also reducing the tendency are performed in classification tree methods to favor predictors that allow more splits.

***C5.0***

The C5.0 model builds either a decision tree or a rule set. The model works by splitting the samples based on the field providing the maximum information gained at each level. The target field must be categorical. Multiple splits into more than two subgroups are allowed.

***Association Model***

The generalized rule induction (GRI) model discovers association rules in the data. GRI extracts a set of rules from the existing data, pulling them out the rules with the highest information content. Information content is measured using an index that takes both the generality (support) and accuracy (confidence) of rules into account.

Statistical analyses; general linear model comparisons (pairwise comparisons with Tukey test and confidence level of 95.0% were done by the SPSS software (version 13, Michigan, USA).

## Results

More than 72% (155) of proteins studied here were bacterial proteases while 17.21% (37), 4.19% (9), 3.72% (8) and 2.79% (6) were plant proteases, halolysins, fungal proteases and termitase. The average length, weight, isoelectric point, and aliphatic indices of proteins studied here were 573.27 ± 260.91, 60.95 ± 28.30, 6.68 ± 1.59, and 81.40 ± 7.80 (mean ± SD). The average counts of sulphur, carbon, nitrogen, oxygen, and hydrogen were 12.86, 2695.85, 733.94, 852.92, and 4196.46, respectively, and the average counts of hydrophobic, hydrophilic, and other residues were 292.84 ± 134.88, 169.84 ± 75.40, and 110.58 ± 61.90 (mean ± SD). The frequencies of hydrogen, carbon, oxygen, nitrogen, and sulphur in all enzymes were 0.494 ± 0.005, 0.317 ± 0.003, 0.087 ± 0.003, 0.101 ± 0.005 and 0.002 ± 0.001, and the frequencies of hydrophobic, hydrophilic, other, negatively, and positively residues were 0.509 ± 0.048, 0.303 ± 0.051, 0.188 ± 0.033, 56.55 ± 36.38, and 43.66 ± 25.14, respectively. The frequencies of amino acids ranged from a low amount of 0.0001 ± 0.00001 for Ile, Asp and Gln to a high amount of 0.176 ± 0.024 for Ala.

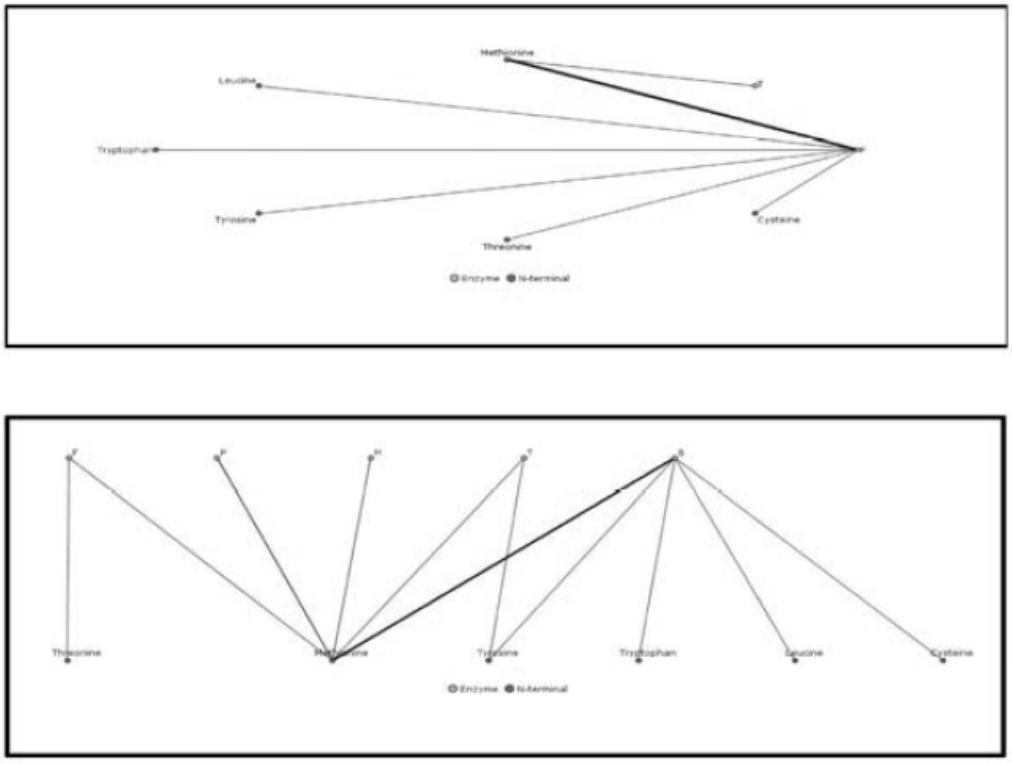


**Figure 1.** Web graph of N-terminal amino acids in a) T (halolysin proteins) and F (bacterial, Fungi and plant proteases and termitase), and b) bacterial proteases (B), fungal proteases (F), halolysin proteins H), plant proteases (p) and termitase (T) groups, thicker lines showing higher incidences of amino acids.

In 97.21% of proteins, the N-terminal amino acid was Met and in 0.93% of proteins the same position was occupied by Tyr. In 0.47%, the last amino acid was Cys, Leu, Thr and Trp. The average non-reduced Cys extinction coefficient at 280 nm was 71367.07 ± 31759, non-reduced Cys absorption was 1.21 ± 0.28, the reduced Cys extinction coefficient was 71109.49 ± 31629.72, and the reduced Cys absorption was 1.20 ± 0.28 (mean ± SD). Figure 1 is a web graph that illustrates the strength of the relationship between N-terminal amino acids and halophilic properties of proteins. Met exhibited a strong relationship with all proteins (a thicker line shows a stronger relationship). Met was the only N-terminal amino acid found in halolysin proteins, whereas Cys, Leu, Tyr, Try and Thr were found at N-terminal position of other proteases and termitase proteins. When halolysin proteins were compared with individual classes of other proteases and termitase, Met exhibits a strong relationship with all proteins and was the only N-terminal amino acid found in halolysin proteins, whereas Tyr and Thr were found at N-terminal position of fungal proteases and termitase proteins and Tyr, Try, Cys and Leu were found at the N-terminal in bacterial proteases.

The results showed that halolysin proteins can be inserted in a separate phylum between eukaryotes (plants and fungi) and bacteria, called Archea (figure 2). Some bacterial proteases such as thermophilic proteases [Q45670 (b118), EDL64549 (b147), ZP-01860436 (b149), YP-002603898(b108) and YP-002603888] showed close relationship with halolysin proteins. Plant proteases from pherphion family with EEF49096 (Tripeptidyle peptidase II, putative) and some bacterial proteases are classified in a separate group. According to figure 2, plants proteins are located at the top of phylogenic tree while fungi proteins with other bacteria proteases such as CAD85094 (b129) and CAD43134 (b50) are put near the top of the tree, confirming their place as eukaryotes. The results of protein blast showed that some parts of the proteins are conserved in all proteins studied here (E value 0). These conserved proteins have been known as putative, pattern formation or hypothetical proteins with a common amino acid sequence of Sec 7; this central region serves as exchange factor.

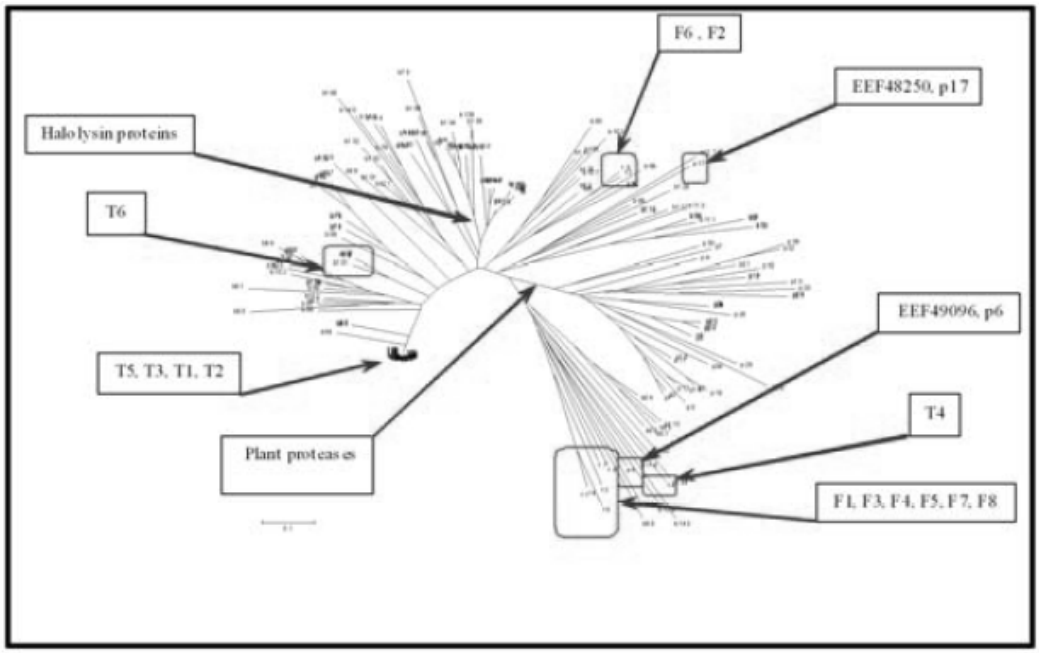


**Figure 2.** Phylogenic tree generated by MEGA4 software, showing halolysin proteins position regarding to other proteases and termitase (f: Fungi protease, T: Termitase, P: Plant protease, H: Halolysine).

When feature selection model applied on dataset of protein features compared halolysin with other proteins (T/F groups), 83 of 215 features were ranked as important ($p > 0.95$) in contribution to halolysin ability to stand harsh conditions (table 1) and 15 features were found to be marginal ($0.90 < p > 0.95$). When the halolysin was compared with each individual protein classes (H/B/F/P/T), 176 out of 215 features were ranked as important and 15 features as marginal. Each time, a node was generated with just important features and was used whenever it was necessary to run all other models on feature selection dataset (as mentioned in Materials and Methods).

**Table 1:** Results of feature selection on important and marginal features contributing to the optimum temperature of proteins

| No | Field | Value | Rank | No | Field | Value | Rank |
|---|---|---|---|---|---|---|---|
| **1** | Freq. of Gly-Gly | 1.0 | Important | **50** | Freq. of Phe-Ala | 0.991 | Important |
| **2** | Freq. of Ala –Asp | 1.0 | Important | **51** | Freq. of Ala - Cys | 0.99 | Important |
| **3** | Freq. of Gly-Asp | 1.0 | Important | **52** | Freq. of His- Asp | 0.989 | Important |
| **4** | Freq. of Asp-Pro | 1.0 | Important | **53** | Mature peptide | 0.989 | Important |
| **5** | Freq. of Glu-Leu | 1.0 | Important | **54** | Freq. of Glu-Val | 0.989 | Important |
| **6** | Freq. of Aspartic Acid | 1.0 | Important | **55** | Freq. of Cys-Ala | 0.988 | Important |
| **7** | Freq. of Cys-Trp | 1.0 | Important | **56** | Freq. of His- Glu | 0.988 | Important |
| **8** | Freq. of Gly-Arg | 1.0 | Important | **57** | Freq. of Glu-Lys | 0.987 | Important |
| **9** | Freq. Negatively Charged | 1.0 | Important | **58** | Freq. of Gly-Thr | 0.987 | Important |
| **10** | Freq. of Gly | 1.0 | Important | **59** | Active site | 0.986 | Important |
| **11** | Freq. of Glu-Tyr | 1.0 | Important | **60** | Freq. of Glu-Ser | 0.985 | Important |
| **12** | Freq. of Asp-Leu | 1.0 | Important | **61** | Freq. of Met | 0.984 | Important |
| **13** | Gene | 1.0 | Important | **62** | Freq. of His- Lys | 0.984 | Important |
| **14** | Freq. of Ile | 1.0 | Important | **63** | Freq. of Phe-His | 0.984 | Important |
| **15** | Freq. of Asp-Asp | 1.0 | Important | **64** | Freq. of lie-Lys | 0.983 | Important |
| **16** | Freq. of Asp-Gly | 1.0 | Important | **65** | Count of Phe | 0.983 | Important |
| **17** | Freq. of Asp-Glu | 1.0 | Important | **66** | Freq. of Cys-Pro | 0.982 | Important |
| **18** | Freq. of Ala -Lys | 1.0 | Important | **67** | Count of Ile | 0.981 | Important |
| **19** | Isoelectric point | 1.0 | Important | **68** | Freq. of Phe-Trp | 0.981 | Important |
| **20** | Freq. Positively Charged | 1.0 | Important | **69** | Freq. of Ala -lle | 0.98 | Important |
| **21** | Freq. of Phe | 1.0 | Important | **70** | Freq. of Glu-Gly | 0.977 | Important |
| **22** | Freq. of lie-Arg | 1.0 | Important | **71** | Freq. of Gly-Phe | 0.977 | Important |
| **23** | Freq. of Lys | 1.0 | Important | **72** | Freq. of Ala -Pro | 0.976 | Important |
| **24** | Freq. of Glu-Gln | 1.0 | Important | **73** | Positively Charged residues | 0.976 | Important |
| **25** | Freq. of Glu-Pro | 1.0 | Important | **74** | Freq. of Ser | 0.975 | Important |
| **26** | Freq. of Glu-lle | 1.0 | Important | **75** | Freq. of Gly-His | 0.975 | Important |
| **27** | Freq. of Asp-Gln | 1.0 | Important | **76** | Freq. of Gly-Ala | 0.973 | Important |
| **28** | Freq. of Ala -Thr | 1.0 | Important | **77** | Freq. of Ala -Met | 0.971 | Important |
| **29** | CDS | 1.0 | Important | **78** | Freq. of His- Cys | 0.965 | Important |
| **30** | Freq. of Cys-Glu | 1.0 | Important | **79** | Freq. of lie-Phe | 0.963 | Important |
| **31** | Freq. of Asp-Arg | 1.0 | Important | **80** | Freq. of Asp-Ala | 0.962 | Important |
| **32** | Freq. of lie-lie | 1.0 | Important | **81** | Freq. of sulphur | 0.958 | Important |
| **33** | Freq. of Ala -Ser | 0.999 | Important | **82** | Freq. of Gly-Pro | 0.957 | Important |
| **34** | Freq. of Asp-His | 0.999 | Important | **83** | Freq. of Gly-Lys | 0.953 | Important |
| **35** | Freq. of Glu | 0.999 | Important | **84** | Count of Asp | 0.944 | Marginal |
| **36** | Freq. of Asp-Lys | 0.999 | Important | **85** | Count of Met | 0.943 | Marginal |
| **37** | Freq. of Asp-Phe | 0.999 | Important | **86** | Freq. of lie-Met | 0.94 | Marginal |
| **38** | Freq. of Phe-Asn | 0.998 | Important | **87** | Count of His | 0.933 | Marginal |
| **39** | Count of Lysine | 0.998 | Important | **88** | Freq. of Ala -Tyr | 0.93 | Marginal |
| **40** | Freq. of Phe-Glu | 0.997 | Important | **89** | Freq. of His- Ser | 0.927 | Marginal |
| **41** | Freq. of Glu-Glu | 0.996 | Important | **90** | Count of Beta-strand | 0.926 | Marginal |
| **42** | Freq. of Glu-Thr | 0.995 | Important | **91** | Freq. of Tryp | 0.924 | Marginal |
| **43** | Freq. of Phe-Phe | 0.995 | Important | **92** | Freq. of lie-Leu | 0.922 | Marginal |
| **44** | Freq. of Asp-Thr | 0.994 | Important | **93** | Freq. of Cys-Ser | 0.92 | Marginal |
| **45** | Freq. of His- Leu | 0.993 | Important | **94** | Freq. of Ala –Glu | 0.919 | Marginal |
| **46** | Freq. of Phe-lie | 0.993 | Important | **95** | Freq. of Glu-Cys | 0.918 | Marginal |
| **47** | Freq. of His | 0.992 | Important | **96** | Freq. of Phe-Lys | 0.912 | Marginal |
| **48** | Freq. of Gly-Asn | 0.992 | Important | **97** | Freq. of Glu-Asp | 0.908 | Marginal |
| **49** | Freq. of Gly-Ser | 0.992 | Important | | | | |

When the anomaly detection model was used on T/F groups, the records were divided into just one peer groups with an anomaly index cutoff of 2.42 and 3 records of this peer group of 215 records were found to be anomalies. When the models were applied using feature selection criteria, one peer groups with an anomaly index cutoff of 1.92 was found. When the model was used on H/B/F/P/T groups, one peer group with three records and anomaly index of 2.41 and 1.87 for dataset with or without feature selection filtering was found, respectively.

When the K-Means model was applied on T/F groups, the records were put into 5 groups or clusters (46, 14, 90, 10 and 55). When the model was applied on dataset with feature selection filtering, again five clusters with 58, 56, 21, 26 and 54 records were generated. When the halolysin was compared with each individual class of proteins, (H/B/F/P/T groups), 47 of the records were put into the first cluster and 14, 89, 10, and 55 records were put into the second, third, fourth, and fifth clusters, respectively. When the K-Means model was applied on the dataset with the feature selection filtering, again five clusters were generated, with 56, 3, 12, 77, and 67 records in each cluster.

Two-Step method clustered records (from T/F groups) into two groups with 52 and 159 records in each cluster, and three clusters (with 109, 52 and 54 records in each cluster) were created for the filtered dataset using feature selection criteria. Two clusters (52 and 195 records and 163 and 52 records) were created with or without the feature selection filtering; when the model applied on H/B/F/P/T groups.

When halolysin proteins (T group) were compared with other proteases and termitase (F group), the C5.0 model generated a decision tree with a depth of 2 and cross-validation of 98.1 ± 0.8. The most important feature used to build the tree was the frequency of oxygen. If the value of this feature was equal to or less than 0.111, the proteins fell into F category (bacterial, fungal and plant proteases and termitase); otherwise they were put into the T category. In this category, if the frequency of Tyr was equal to or less than 0.036, they were placed in the F subgroup; otherwise they were put into the T subgroup (halolysin proteins). When a 10-fold cross-validation was applied to the same dataset, again a tree with a depth of 2 and cross-validation of 97.6.1 ± 1.1 was created. The same protein features and values were used to create tree branches. When the same models were applied to datasets using the feature selection filtering, a tree with the same depth (2) and cross-validation of 96.3 ± 1.1 and 89.1 ± 1.0 were generated for C5.0 and C5.0 with a 10-fold cross-validation, respectively. The frequency of Glu-Leu features were used to create the first branch (value < 0.007 in F mode and > 0.007 T Mode); in T mode if the frequency of Gly was equal to or less than 0.121 they were put in F mode (proteases and termitase); otherwise they were in T mode (halolysin proteins).

When the H/B/F/P/T dataset was used, the C5.0 model generated a decision tree with a depth of 5 and cross-validation of 86.9 ± 1.7. The most important feature used to build the tree was the count of sulphur. If the value of this feature was equal to or less than 18, the proteins fell into the bacterial proteases category; otherwise they were put into the plant proteases category. In the bacterial proteases subgroup, the frequency of Glu-Ser was used to create the next tree branches, with < 0.009 as the bacterial protein mode and > 0.009 as the halolysin protein mode. In the plant proteases subgroup, if the value for the frequency of other residues was equal to or less than 0.164, they were placed in the fungal proteases subgroup; otherwise they were put into the plant proteases subgroup. When a 10-fold cross-validation was applied to the same dataset, again a tree with a depth of 5 and cross-validation of 85.5 ± 1.5 was created. The same protein features and values were used to create tree branches. When the same models were applied to datasets using feature selection filtering, a tree with a depth of 4 and cross-validation of 87.5 ± 2.2 and 86.1 ± 2.5 were generated for C5.0 and C5.0 with 10-fold cross-validation. The same protein features were used to create the first and second subgroups.

In the C&RT node, a tree with a depth of 1 was created, and the most important feature used to build the tree was the frequency of Gly - Gly (value < 0.026 for the F mode and > 0.026 for the T mode (the halolysin protein). The same results were obtained when the feature selection was selected. When the halolysin was compared with each individual class of other proteins (H/B/F/P/T groups), a tree with a depth of 4 was created, and the most important feature used to build the tree was the count of sulphur (value < 18.5 for bacterial and > 18.5 for plant proteases). The frequency of Gly - Gly was used to create the second level for the first subgroups (< 0.026 for bacterial and > 0.026 for halolysin proteins) and the frequency of Glu (< 0.032 for plant and >0.032 for bacterial proteases). The same results were obtained when a feature selection was used.

In the Quest modeling, a tree with a depth of 2 was generated, and the frequency of Gly - Gly (with a value equal to or less than 0.021) was used

to create the first tree branches (the F mode) and the frequency of Ala-Lys was used to generate the next subgroup (< 0 for the halolysin protein and > 0 for other proteases and termitase). The same results occurred when a feature selection filtering was applied. When H/B/F/P/T groups compared, a tree with a depth of 2 was generated, and the count of Cys (with a value equal to or less than 7.654) was used to create the first tree branches (bacterial proteases) and the frequency of Gly - Gly was used to generate the next subgroup (< 0.021 for bacterial proteases and > 0.021 for halolysin proteins). In the plant subgroup, the frequency of Lys (0.113) was used to create fungal and plant proteases. The same results occurred when a feature selection filtering was applied.

A tree with a depth of 2 was generated when the CHAID model was applied to the data with and without feature selection. If the frequency of Lie-Ala was < 0.005, the mode was F; if it was > 0.005 and the frequency of Lie-Ala was equal to or less than 0.006, the mode was T. The same trees with the same features and values were generated when exhaustive CHAID models were applied on datasets with and without the feature selection. When H/B/F/P/T groups were compared and the CHAID model was applied to the data with and without the feature selection, a tree with a depth of 3 was generated. If the count of hydrophobic residues was < 180, the mode was bacterial proteases; if it was > 417, the mode was plant proteases. If the count of hydrophobic residues range from 180 to 196 and the frequency of hydrogen was equal to or less than < 0.492, the mode was bacterial proteases; otherwise it was the termitase. When the counts of hydrophobic residues were > 196 and < 225, it formed the next branch, and three other branches were created when the same feature was between 225 and 268, 268 and 341, 341 and 386, and 386 and 417 (figure 3). The same trees with the same features and values were generated when exhaustive CHAID models were applied on datasets with and without the feature selection. The best percentage of correctness, performance evaluation, and mean correctness in the decision tree models were observed in the C5.0 model, followed by the CR&T, CHAID, and finally the Quest models (table 2).

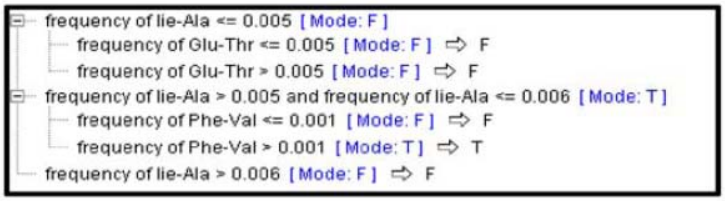


**Figure 3.** A decision tree generated by the CHAID modeling method without feature selection filtering, comparing halolysin proteins with the others (T/F groups).

**Table 2**. Percentage of correctness, wrongness, performance evaluation (T & F), and mean correct and incorrect in various decision tree models, in datasets without feature selection (a) and with feature selection (b), comparing halolysin proteins with others (T/F groups).

| **(a)** | | | | |
|---|---|---|---|---|
| | **% Correct** | **% Wrong** | **Performance evaluation (T)** | **Performance evaluation (F)** |
| **C5.0** | 100 | 0 | 3.173 | 0.043 |
| **C5.0 with 10-fold validation** | 100 | 0 | 3.173 | 0.043 |
| **CR&T** | 98.14 | 1.6 | - | - |
| **QUEST** | 99.53 | 0.47 | - | - |
| **CHAID** | 99.53 | 0.47 | - | - |
| **Exhaustive CHAID** | 99.53 | 0.47 | - | - |
| **(b)** | | | | |
| **C5.0** | 99.53 | 0.47 | 3.173 | 0.038 |
| **C5.0 with 10-fold validation** | 99.53 | 0.47 | 3.173 | 0.038 |
| **CR&T** | 98.14 | 1.84 | 2.933 | 0.033 |
| **QUEST** | 99.53 | 0.47 | 3.173 | 0.038 |
| **CHAID** | 100 | 0 | 3.173 | 0.043 |
| **Exhaustive CHAID** | 100 | 0 | 3.173 | 0.043 |

The GRI node analysis created 100 rules with 215 valid transactions with minimum and maximum support of 3.26% and 8.37%, respectively. Maximum confidence reached 100% and minimum confidence decreased to 50.0%. When the feature selection was used, minimum support, maximum support, maximum confidence, and minimum confidence changed to 0.47%, 9.3%, 100%, and 50.0%. The highest confidence (100%) and support (4.19%) occurred when the frequency of oxygen was > 0.12 and count of hydrogen was < 3652 or N-terminal was Met or both together. When the feature selection filtering was applied the highest confidence and support were 100% and 3.72 when the frequency of Gly - Gly was > 0.022 and the frequency of Ala-Lys < 0.0001 or the frequency of Gly was higher than 0.008. When the halolysin protein was compared with other individual protein classes (H/B/F/P/T groups), a GRI node analysis created 100 rules with 215 valid transactions with minimum and maximum support of 14.88% and 17.21%, respectively. Maximum confidence reached 100% and minimum confidence decreased to 97.22%. When the feature selection was used, minimum support, maximum support, maximum confidence, and minimum confidence changed to 14.88%, 17.67%, 100%, and 97.74%. The highest confidence (100%) and support (16.28%) in both methods (with/without feature selection filtering) occurred when the count of Lys was lower than 28.5, the frequency of Gly-Pro was greater than 0.002, and the frequency of Asp-Leu was less than 0.006 (table 3).

Statistical analyses showed significant differences ($p < 0.01$) in positively and negatively charged amino acids between halolysins and other proteins. Halolysin proteins had higher average of negatively charged amino acids comparing to other proteins. Asp and Glu, two negatively charged amino acids with average of 0.091 and 0.053, showed higher average comparing to other amino acids in halolysin proteins. More than 20% of amino acids in halolysin proteins were negatively charged comparing with just 9% in other proteins; resulting in at least two times more negatively charged amino acids presence in halophilic proteins. The Ratio of negatively charged amino acids to positively charged amino acids in halolysin and other proteins were 3 and 1.3 times.

A significant difference ($p < 0.01$) was found in 21 features of primary protein structure in halolysins and plant proteases. Positively charged amino acids (such as Lys, Arg and His) showed higher frequencies in plant proteases. A highly significant difference ($p < 0.0001$) was found in hydrophobic amino acids (Val, Pro, Phe, Ile, Leu and Met) of plant proteases and halolysin proteins resulting in the same significant differences of hydrophobic compounds in those proteins. Cys and Met, as N-terminal amino acids, were found to be more frequent in plant proteases than halolysins and other proteases studied in this paper forming more di-sulphid bonds in plant proteases. In halolysins, about 50% of Cys were in the N-terminal position while just 20% of the N-terminal amino acid in plant proteases was Cys.

A significant difference ($p < 0.05$) was found in aliphatic index in plant proteases and halolysin proteins, which could be due to aliphatic amino acids (Ile, Val, Pro, Met and Leu). More beta-strand was found in plant proteases which could be due to higher number of Lys, His and Cys. The frequency of Pro in plant proteases was higher than its frequency in halolysin proteins ($14.11 \pm 9.54$ and $13.78 \pm 1.09$, (mean ± SD), respectively). Some dipeptid bonds (such as Met-Met, Met-Cys and Cys-Cys) were more frequent in plant proteases and they could contribute in more beta-strand formation.

## Discussion

Salt dependence and salt tolerance microorganisms are newly discovered microorganisms, classified as new taxa with new names within the microbial taxonomy. Some use the term for all organisms that require some level of salt for growth, including concentrations around 35 g/l as found in seawater. Halobacterium species are obligatory halophilic microorganisms that have been adapted to optimal growth under conditions of extremely high salinity. They contain a correspondingly high concentration of salts internally and exhibit a variety of unusual and unique molecular characteristics. Since their discovery, extreme halophiles have been studied extensively by chemists, biochemists, microbiologists, and molecular biologists to define both molecular diversity and universal features of life. A notable list of early research milestones on halophiles includes the discovery of a cell envelope composed of an S-layer glycoprotein, Archaea l either lipids and purple membrane, and metabolic and biosynthetic processes operating at saturating salinities. These early discoveries established the value of investigations directed at extremophiles and set the stage for pioneering phylogenetic studies leading to the three-domain view of life and

**Table 3:** The association rules found in the data by the generalized rule induction (GRI) method, comparing halolysin proteins with the others (T/F groups)

| Antecedent | Confidence % |
|---|---|
| Freq. of Gly - Gly > 0.022 and Freq. of Ala -Lys < 0.000 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. of Gly > 0.122 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. of Phe < 0.018 and Freq. of Gly > 0.122 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. of Glu > 0.048 and Ile < 18.500 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. of Asp > 0.083 and Ile < 18.500 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Phe < 9.500 and Freq. of Gly > 0.122 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. Positively Charged < 0.048 and Ile < 18.500 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. Negatively Charged > 0.134 and Ile < 18.500 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Positively Charged residues < 25.500 and Freq. of Gly > 0.122 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Isoelectric point < 4.480 and Ile < 18.500 | 100.0 |
| Freq. of Gly > 0.122 and Isoelectric point < 4.480 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Phe < 9.500 and Freq. sulphur < 0.002 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. Positively Charged < 0.048 and Freq. Positively Charged > 0.046 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. Negatively Charged > 0.134 and Isoelectric point > 4.385 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Positively Charged residues < 25.500 and Freq. sulphur < 0.002 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Freq. sulphur < 0.002 and Isoelectric point < 4.480 | 100.0 |
| Freq. of Glu-Leu > 0.008 and Isoelectric point < 4.480 and Isoelectric point > 4.385 | 100.0 |
| Freq. Negatively Charged > 0.198 | 100.0 |
| Isoelectric point < 4.170 and Isoelectric point > 4.040 | 100.0 |
| Freq. of Gly - Gly > 0.022 and Isoelectric point < 4.480 | 88.89 |
| Freq. of Glu-Leu > 0.008 and Ile < 18.500 | 88.89 |
| Freq. of Glu-Leu > 0.008 and Phe < 9.500 and Isoelectric point > 4.385 | 87.5 |
| Freq. of Glu-Leu > 0.008 and Freq. Positively Charged < 0.048 and Isoelectric point > 4.385 | 87.5 |
| Freq. of Glu-Leu > 0.008 and Positively Charged residues < 25.500 and Isoelectric point > 4.385 | 87.5 |
| Freq. of Glu-Leu > 0.008 and Isoelectric point < 4.480 | 72.73 |
| Freq. of Gly - Gly > 0.022 | 66.67 |
| Isoelectric point < 4.480 | 60.0 |
| Freq. of Gly > 0.122 | 57.14 |
| Freq. Negatively Charged > 0.134 and Isoelectric point < 4.480 | 55.56 |
| Freq. of Gly - Gly > 0.016 | 55.0 |
| Freq. of Ala -Lys < 0.000 and Isoelectric point < 5.265 | 55.0 |
| Freq. of Asp > 0.078 and Positively Charged residues < 32.500 | 55.0 |
| Lysine < 9.500 and Isoelectric point < 4.480 | 52.94 |
| Freq. of Asp-Gln > 0.004 and Isoelectric point < 5.025 | 52.63 |
| Freq. of lie-Arg < 0.000 and Isoelectric point < 5.065 | 50.0 |
| Freq. of Asp-Gln > 0.004 and Freq. of Ile < 0.042 | 50.0 |
| Freq. of Asp-Gln > 0.004 and Freq. of Gly > 0.106 and Freq. Positively Charged < 0.062 | 50.0 |
| Freq. of Asp-Gln > 0.004 and Freq. of Glu > 0.042 and Isoelectric point < 5.025 | 50.0 |
| Freq. of Asp-Gln > 0.004 and Phe < 9.500 and Isoelectric point < 5.445 | 50.0 |
| Freq. of Asp-Gln > 0.004 and Positively Charged residues < 32.500 and Freq. of Asp > 0.058 | 50.0 |
| Freq. of Ala -Lys < 0.000 and Freq. Negatively Charged > 0.114 | 50.0 |
| Freq. of Ile < 0.038 and Isoelectric point < 5.555 | 50.0 |
| Freq. of Phe < 0.018 and Isoelectric point < 5.610 | 50.0 |
| Freq. of Asp > 0.078 and Phe < 10.500 | 50.0 |
| Ile < 19.500 and Isoelectric point < 5.405 | 50.0 |
| Phe < 9.500 and Isoelectric point < 5.265 | 50.0 |
| Freq. of Ala -Asp > 0.010 | 50.0 |
| Freq. of Ala - Cys > 0.002 and Isoelectric point < 4.760 | 50.0 |
| Freq. of Lysine < 0.018 and Isoelectric point < 5.155 | 50.0 |
| Freq. of Asp > 0.078 and Isoelectric point > 4.385 | 50.0 |
| Lysine < 9.500 and Freq. of Gly > 0.114 | 50.0 |
| Lysine < 9.500 and Freq. of Phe < 0.018 and Positively Charged residues < 28.500 | 50.0 |
| Lysine < 9.500 and Lysine > 5.500 and Positively Charged residues < 25.500 | 50.0 |
| Lysine < 9.500 and Phe < 9.500 and Freq. Positively Charged < 0.052 | 50.0 |
| Lysine < 9.500 and Positively Charged residues < 25.500 and Isoelectric point < 4.760 | 50.0 |
| Freq. Positively Charged < 0.048 and Isoelectric point < 4.480 | 50.0 |
| Freq. Negatively Charged > 0.134 and Freq. Positively Charged < 0.050 | 50.0 |
| Positively Charged residues < 25.500 and Isoelectric point < 4.760 | 50.0 |
| Isoelectric point < 4.480 and Isoelectric point > 4.285 | 50.0 |
| Isoelectric point < 4.170 | 50.0 |

classification of Halobacterium as a member of the Archaea l domain. It has been shown that some proteins and enzymes are responsible for living organism's tolerance against hypersaline conditions; therefore defining features contribute to this valuable characteristics of proteins paves roads toward engineering new strains of plants growing in harsh salty conditions. To date, some studies have looked at phylogeny, taxonomy and nomenclature of halophilic strains and various models have been employed to determine the most important features that contribute to these organisms' ability to stand hypersalinity media. In this study, we applied different modeling techniques to study more than 70 features of some halophilic proteins and compared them with similar proteases and termitase (found after multiple alignments) in an attempt to understand their ability to withstand salty conditions. We used different screening, clustering, and decision tree modeling on two datasets: one with and the other without feature selection filtering.

The phylogenic tree (figure 1) showed that halophilic organisms can be placed in a separate phylum between eukaryotes and bacteria, *Archea*, which is in line with previous studies (Pruess et al., 2003; Li et al., 2008; Wimmer et al., 2008). Although the results of feature selection modeling showed that 83 features (from 252) had a value greater than 0.95, the frequency of Gly - Gly ranked as the most important feature (table 1), and it was used in some decision tree models to create the main subgroups and branches. The number of peer group (one group) did not change when feature selection filtering was applied but anomaly index cutoff decreased from 2.42 (without feature selection) to 1.92 (with feature selection) showing the positive effects of feature selection filtering on removing outliers. Although the number of records in the clusters changed between the models with and without feature selection, the number of clusters generated by K-Means modeling did not. In the TwoStep model, the number of clusters decreased from three (without feature selection) to just two (with feature selection) groups.

The depth of trees generated by the various decision tree models varied from 1 (in the C&RT model with T/F comparison, with/without the feature selection dataset) to 5 (in the C5.0 model with 10-fold cross-validation on H/B/F/P/T groups) branches. The best cross-validation results were obtained in the C5.0 model when H/B/F/P/T groups compared. The protein features were used by various decision tree models to create trees varied from the count of sulphur (in the C5.0, C5.0 with 10-fold cross-validation and C&RT model on H/B/F/P/T groups) to the frequency of Gly - Gly (in the C&RT T/F and Quest models) and the count of Cys, Leu-Ala and hydrophobic residues in Quest, T/F CHAID and H/BFPT CHAID. In most GRI association rules (100 rules), the frequency of Gly - Gly was used as an antecedent to support the rules. Although previous studies have shown the importance of acidic amino acids (Glu and Asp) residues (Lanyi, 1969; Lanyi, 1974) and Gly (Lai, Hong et al. 2000; Robert, Le Marrec et al. 2000) in halophilic proteins, in this study, for the first time, we looked not only at individual amino acid composition, but also the importance of dipeptid amino acid composition in salt stability of these proteins and found Gly - Gly as the most important feature contributes to halotolerant capacity of these proteins. Performance evaluations in the decision tree models tested were found to be the same in all models. No significant differences in the percent of correctness, performance evaluation, and mean correctness of various decision tree models were found when feature selected datasets were used, but when feature selection datasets were used the number of peer-groups in clustering models reduced significantly.

Charged amino acids prevent charged ions from attaching to proteins and they have a significant role in stabilizing protein against salty conditions, and keep water molecules around these components. Sequence comparisons showed that, in general, the halophilic proteins contain an excess of negatively charged amino acids over positively charged amino acids, and the number of negatively charged amino acid residues is higher than that in their non-halophilic homologs (Kushner and Onishi, 1966; Rao and Argos, 1981; Tokunaga et al., 2008). The additional negative charges are located mostly on the protein surface, presumably helping to stabilize the protein molecule by competing with the salt for hydration (Lanyi, 1974). It has also been proposed that hydrophobic interactions play an important role in the ability of these proteins to cope with the salt stress in a hypersaline environment (Mevarech et al., 2000; Kastritis et al., 2007; Memmi et al., 2008). It has been shown that negatively charged amino acids such as Asp and Glu may contribute to protein ability to resist salty conditions; as shown in a higher percentage of negatively charged amino acid residues (18.5%) in halophilic strains than its non-halophilic counterparts (Pieper et al., 1998). Our finding were in line with the previous studies showing higher average of negatively charged amino acids in halolysin proteins with highly significant difference ($p < 0.001$) comparing to other proteins. It has been shown the cumulative

amount of Lys and Arg amino acids and even the content of Val were remarkably high in salt stability Archaea (Ferrer et al., 1996). Higher hydrophobic amino acids found in plant proteins could be related to their function as inside proteins tending to aggregate as a sphere surrounded by water to increase their stability inside the cells and this may clarify more positively charged amino acid such as Lys, Arg and His found in plant proteases, although it have been mentioned that this feature may also contribute to salt stability in some organisms (White and Jacobs, 1990; Srimathi et al., 2007; Valery et al., 2008). The results showed that Met was the sole N-terminal amino acid in halolysin proteins whereas other amino acids such as Cys, Thr, Tyr, Try and Leu were also found at this position of other proteases and termitase. In similar studies, it have been shown the N-terminal sequence of halophilic species play important role in their resistance to salty conditions (Baker et al., 1992; Wakai et al., 1995; Ferrer et al., 1996; Ihara et al., 1997; Porciero et al., 2005). A significant difference ($p<0.05$) in aliphatic index was found between plant proteases and halolysin proteins which could be due to the presence of more aliphatic amino acids such as Ile, Val, Pro, Met and Leu in plant proteases and this difference or higher number of dipeptid bonds may be responsible for more beta-strands in plant proteases (Hose et al., 2001; Lahav et al., 2002; Mishra and Jha, 2009).

We analyzed the performance of different screening, clustering, and decision tree algorithms for discriminating halophilic and non-halophilic proteins. Our results showed that the amino acid composition can be used to discriminate between protein groups. We found that most of the mentioned algorithms can be used to discriminate between halophilic and non-halophilic proteins with accuracy in the range of 98–100 %. Our analysis detected no significant difference in performance between different methods used in this paper. Interestingly, all decision tree models had a similar accuracy (higher than 98 %), and no differences were observed between analysis with and without feature selection. The best performance and correctness results were obtained with C5.0 and CHAID algorithms. Thus, we suggest that these decision tree models can be used as an effective tool to discriminate halophilic and non-halophilic proteins.

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# The effect of silver thiosulfate (STS) on chlorophyll content and the antioxidant enzymes activity of potato (*Solanum tuberosum* L.)

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## Abstract

Potato (*Solanum tuberosum* L.) auxiliary buds c.v. White Desiree were cultured in MS medium containing 0, 50, 100, 150 and 200 µM concentrations of silver thiosulfate (STS) under *in vitro* condition. After eight weeks, the effect of silver ions ($Ag^+$) in the form of silver thiosulfate complex (STS), as an ethylene action inhibitor, on chlorophyll contents of leaves, ascorbate peroxidase, guaiacol peroxidase and catalase activities of roots and leaves were studied. Application of silver (STS) in culture medium increased chlorophyll content comparing to the control plants significantly. After treatments of potato plants with STS, ascorbate peroxidase and guaiacol peroxidase activities in roots were higher than shoots while catalase activity was higher in leaves than roots. However, increasing of STS concentration in the culture medium resulted in higher activities of antioxidant enzymes with some variations.

***Keywords*:** Antioxidant Enzymes, Chlorophyll Content, Potato, Silver Thiosulfate

## Introduction

Potato is an important tuberous crop plant worldwide (Torabi et al., 2008). Improvement of its growth and culture condition is important under *in vitro* culture for propagation and the increase of yield. Growth and development of potato under *in vitro* culture is sensitive to generation and accumulation of ethylene in closed vessels (Ehsanpour and Jones, 2001; Perl et al., 1988; Sarkar et al., 1999). Ethylene ($C_2H_4$), an unsaturated hydrocarbon, is a simple plant hormone that affects some of the growth and development processes in plants (Gianinetti et al., 2007). It regulates abscission, organ senescence, ripening, and plant defense (Abeles et al., 1992). Accumulation of ethylene is associated with abnormalities in *in vitro* conditions (Chi et al., 1991). The negative effects of ethylene on potato plants can be controlled using silver ions as inhibitors of ethylene biosynthesis and action (Beyer, 1976).

Higher plant cells, as aerobic cells, require oxygen for production of energy (Shcolnick and Keren, 2006). During the $H_2O$ production from $O_2$, reactive oxygen species (ROS) can be formed (Fath et al., 2002; Shao et al., 2008). The most important of these ROSs are superoxide radical ($O^{\cdot}{}_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\cdot}$) (Dalton et al., 1986). Excessive accumulation of ROSs in cells can cause damage to cellular macromolecules such as lipids, nucleic acids and proteins (Hernandez et al., 1993). In response to the ROS generation and accumulation, plant cells can induce their antioxidant defense systems (Larson, 1988; Mizuno et al., 2005). The formation and accumulation of ROSs may be prevented by enzymatic and non-enzymatic antioxidant defense systems. Superoxide dismutase (SOD), proxidases and catalase (CAT) are some of the antioxidant enzymes which can participate in elimination of ROSs. SOD catalyzes the dismutaion of $O^{\cdot}{}_2^-$ to $H_2O_2$ and $O_2$, whereas CAT and non-specific peroxidases destroy the generated $H_2O_2$ in different cell compartments (Moran et al., 1994; Anderson et al., 1995). APX, dehydroascorbate reductase (DHAR) and glutathione reductase can participate in Halliwell-Asada pathway (Ascorbate-glutathion cycle) which removes $H_2O_2$ in cynaobacteria and plant chloroplasts (Dalton et al., 1986; May et al., 1998). Low molecular mass antioxidants as ascorbic acid, glutathione and tocopherols are non-enzymatic defense system against ROSs (Blokhina et al., 2003).

Silver (Ag) with density of 10.5 g $cm^{-3}$ is a heavy metal (Toppi and Gabbrielli, 1999). Silver can be uptake and transport through copper-transport

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systems in many organisms (Lee et al., 2002). Silver and copper are group IB transition metals and have similar physico-chemical traits. As first reported by Beyer (1979) and subsequently noted by others (Rodrigues et al., 1999), ethylene binding site in ethylene receptors contains $Cu^{+}$ as cofactor that is required for high-affinity ethylene binding and silver ions ($Ag^{+}$) can inhibit ethylene action by substituting for $Cu^{+}$ at ethylene receptor (Beyer, 1979). It is known that several genes involved in ethylene perception in higher plants. Silver thiosulfate complex (STS [$Ag(S_2O_3)_2^{3-}$]) is an inhibitor of ethylene action which dissociates in the plant tissues and free silver ions. These ions act efficiently as anti-ethylene agents (Veen and Van De Geijn, 1978).

Previous investigations have demonstrated that heavy metals such as cadmium can induce oxidative stress and change of antioxidant enzymes (Schuzendubell and Polle, 2002), but to our knowledge, so far no report has been published on the effect of silver (Ag), as a heavy metal, in silver thiosulfate complex (STS [$Ag(S_2O_3)_2^{3-}$]) on changes of chlorophyll content and activity of the antioxidant enzymes in potato (*Solanum tuberosum* L.) c.v. White Desiree under *in vitro* condition.

In this study, we aimed to understand the effect of silver thiosulfate (STS) as an ethylene action inhibitor on chlorophyll content and the activity of antioxidant enzymes in potato plant.

## Materials and Methods

### *Plant material and culture conditions*

Potato plants, cultivar White Desiree, were propagated on MS (Murashig and Skoog, 1962) medium supplemented with agar (1% w/v) and sucrose (3% w/v), pH 5.8. Then, auxiliary buds were transferred to MS medium containing concentrations of 0 (control), 50, 100, 150 and 200 µM STS. All cultures were then kept in the culture room with a 16/8-h light/dark photoperiod with 2000 Lux intensity at 25 ± 2 ˚C for eight weeks. STS solutions were prepared by mixing 800 µM $AgNO_3$ and 3200 µM $Na_2S_2O_3.5H_2O$ in 1:1 ratio based on the method of Ehsanpour and Jones (2001).

### *Chlorophyll measurement*

Total chlorophyll content from 0.1 g fresh leaves from eight-week-old potato plants was extracted. According to the method of Arnon (1949) using 80% acetone in darkness and measured at 645 and 663 nm by spectrophotometer.

### *Protein extraction and enzyme assay*

For protein and enzyme extraction 0.1 g of fresh leaf and root from eight-week-old plants were homogenized using a mortar and pestle with 1 ml of 100 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone (PVP). The whole extraction procedure was carried out on ice. The homogenates were then centrifuged for 30 min at 14000 rpm at 4˚C and supernatants were used for protein and enzyme activity measurement.

Ascorbate peroxidase (APX, EC 1.11.1.11) and guaiacol peroxidase (GP, EC 1.11.1.7) activities were determined according to the method of Nakano and Asada (1981). The reaction buffer for APX activity contained 50 mM sodium phosphate buffer (pH 7), 0.5 mM ascorbic acid, 0.1 mM EDTA, 1.25 mM $H_2O_2$ and 0.02 ml enzyme extract in a final volume of 1ml. Ascorbate oxidation was measured at 290 nm at extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$. For GP activity mixture (1 ml) contained 50 mM sodium phosphate buffer (pH 7), 0.1 mM EDTA, 0.1 mM $H_2O_2$ and 0.1 ml enzyme extract. Enzyme activity was assayed by monitoring formation of tetraguaiacol from guaiacol at 470 nm at extinction coefficient of 26.6 $mM^{-1}$ $cm^{-1}$ in the presence $H_2O_2$.

Catalase (CAT, EC 1.11.1.6) activity assay was also carried out according to the method of Aebi (1984). The decrease in $H_2O_2$ was measured at 240 nm and activity was calculated as µM $H_2O_2$ consumed per minute (extinction coefficient 39.4 $mM^{-1}$ $cm^{-1}$).

Total soluble protein was determined using modified Bradford (1976) method described by Olson and Markwell (2007). Bovine serum albumin as the standard protein was used.

### *Statistical analysis*

All experiments were carried out in three replications and mean values ± standard deviation were presented. Data were subjected to ANOVA and the mean differences were compared by Dunkan test at $p < 0.05$.

## Results

Presence of silver thiosulfate (STS) in culture medium increased the total chlorophyll content of plants in comparison with the control. The highest amount of chlorophyll was observed at 100, 150 and 200 µM STS while, the lowest amount of chlorophyll was observed in the medium without STS (figure 1).

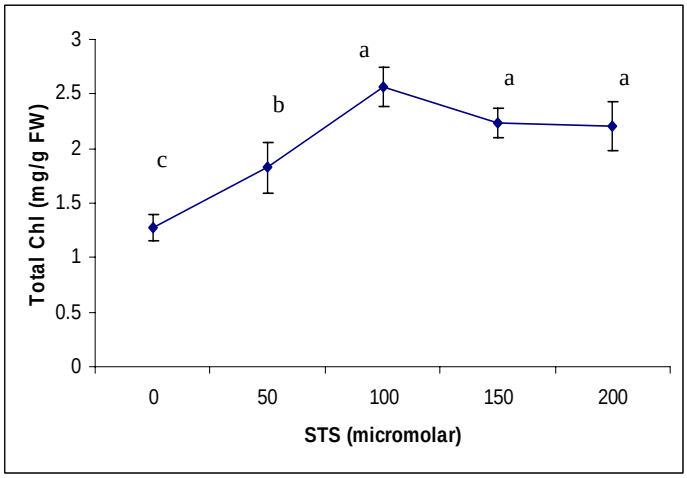


**Figure 1.** Effect of STS on chlorophyll content of potato leaf c.v. White Desiree. Values are means of three replications ± Std. Uncommon letters are significant ($p < 0.05$) based on the Duncan test.

By increasing the STS concentration in the culture media, the activity of the assayed enzyme was also increased in roots. So that the APX activity was increased significantly at 200 µM STS whereas, the activity of GP and CAT increased at 100 µM STS. The activity of APX and GP in roots was much higher than that in leaves. In contrast, CAT activity in leaves was higher than roots and did not show a significant difference in leaves of potato (figure 2).

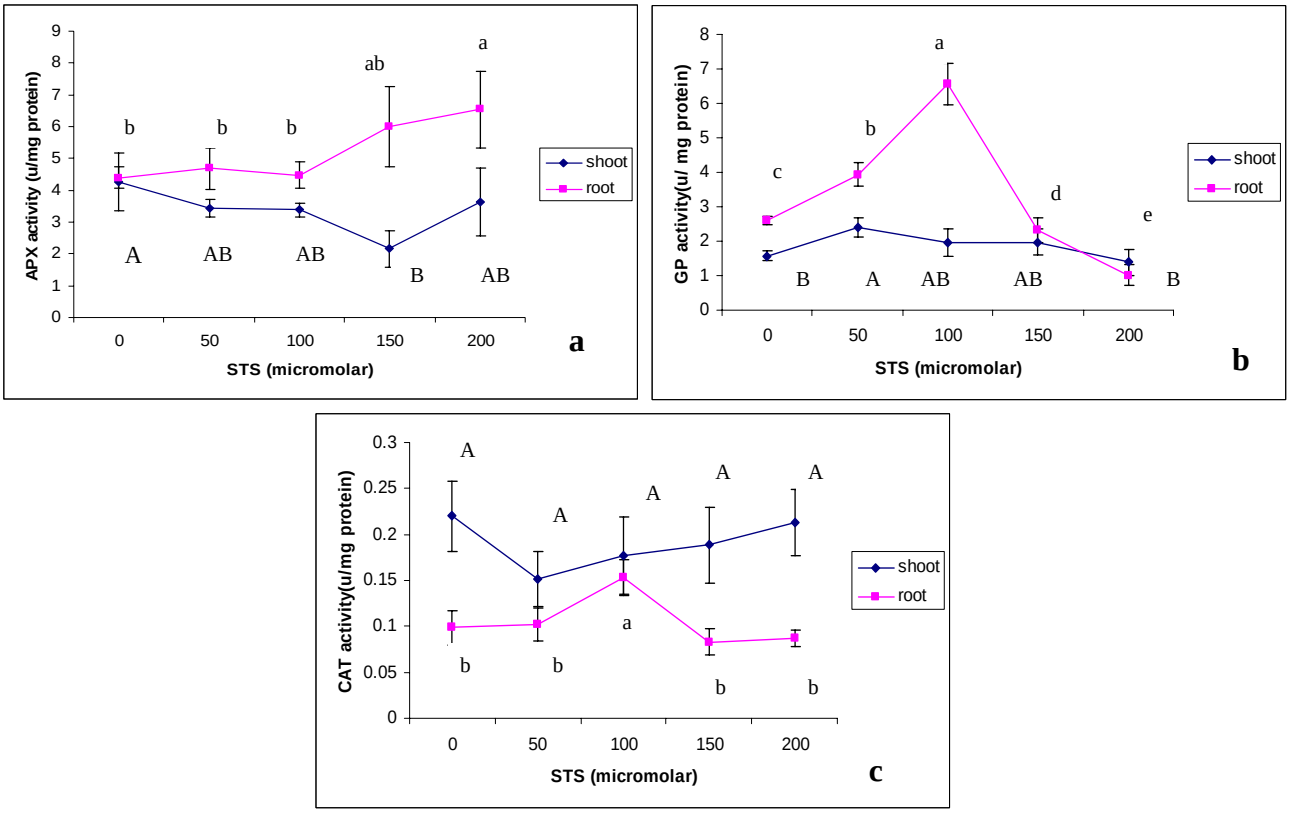


**Figure 2.** The effect of STS on (a) Ascorbate peroxidase (APX), (b) guaiacol peroxidase (GP) and (c) Catalase (CAT) activities in roots and leaves of potato cultivar White Desiree. Values are means of three replications ± Std. Uncommon letters are significant ($p < 0.05$) based on the Duncan test.

## Discussion

Chlorophyll has a unique and essential role in higher plants (Eckhardt et al., 2004). Biosynthesis and breakdown of chlorophyll in plants are complex pathways that are regulated by different factors. It has been documented that ethylene has a negative effect on chlorophyll content of plants (Jona et al., 1997). For example, Jakob-Will et al., (1999) reported that ethylene induced expression of chlorophyllase genes (Chlase) in Citrus fruits, but inhibition of ethylene action by STS increases the chlorophyll content. The STS also increases the leaf area (Ehsanpour and Jones, 2001; Perl et al., 1988) as well as, chlorophyll content, viability and the number of protoplasts in potato cultivar Delaware (Ehsanpour and Jones, 2001). In the present study, we found similar results in chlorophyll content by increaseing the STS concentration. Increasing of the chlorophyll content and leaf area may be due to the inhibition of ethylene action by STS treatment. It has been reported that accumulation of ethylene and depletion of oxygen in thigthly closed vessel is associated with various morphological abnormalities during *in vitro* plant tissue culture (Ehsanpour and Jones, 2001; Perl et al., 1988; Sarkar et al., 1999; Sarkar et al., 2002). Therefore, the potato growth can be improved by suppression of ethylene action using the STS treatment.

Heavy metal-induced changes in CAT, APX and GP activities has already been reported (Gallego et al., 1996; Chaoui et al., 1997; Gallego et al., 1999; Roa & Sresty, 2000). In our study, APX and GP activities were much higher in roots while CAT showed lower activity in roots. The high level of APX and GP activities in roots indicated efficient conversion of $H_2O_2$ to $H_2O$. In contrast, the CAT activity in roots was lower than shoots, it is possibly due to some compensation mechanism between APX and CAT enzymes. However, the details of this hypothesis will need to be studied in the future. CAT activity remained without significant changes in comparison to the control plants. Although catalase may be present in all plant cells, it tends to be restricted largely to peroxisomes. The Catalase has a high *K*m for $H_2O_2$, as substrate, and this enzyme alone can not be sufficient for omitting and degrading all the generated $H_2O_2$ (Halliwell, 1974). Thereby, according to our study, the catalase seems poorly suited scavenger for $H_2O_2$ in root of potato plant c.v. White Desiree under STS treatment and other enzymatic (APX and GP) and non-enzymatic pathways could also cooperate to detoxify ROSs in the root tissues.

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# Identifying thrips (Insecta: Thysanoptera) using DNA Barcodes

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## Abstract

*Thrips tabaci* Lindeman is an extensively distributed pest insect in many areas that affects plants through direct feeding and at the same time, it makes damage as a vector of different viruses. As a basic first step to control pests is authentic identification, but the inability to determine morphological characters of thrips species makes this process very difficult. For creating an identification key for *T. tabaci*, an economically important species present in Iran, four individuals were selected from four different sites of Mashhad and the vicinity, each as a separate population. The method was based on nucleotide sequencing analysis of the mitochondrial cytochrome C oxidase I (*COI*) gene. Phylogenetic analyses conducted by the neighbor-joining method yielded almost identical phylogenetic reconstructions of trees that separated thrips based on the geographic origin. Molecular data indicate that different thrips species are located in distinct groups. These results show that molecular keys can be a useful method to provide much-needed information on thrips identification for pest management officers and quarantine purposes.

***Keywords***: *Thrips tabaci*, mitochondrial DNA, barcoding, phylogenetic tree, molecular identification

## Introduction

Among the 5800 thrips species described worldwide only 1% are known as pest species with about ten species as vectors of plant viruses (Morris and Mound, 2003). Onion thrips, *Thrips tabaci* Lindeman, is the most harmful species of the Thysanoptera order. It is extremely polyphagous, most damages being reported on bulb plants, tobacco, cabbage, and ornamental plants. The damage is caused either by feeding of larvae or adults. Its role has been proved in the transmission of different viruses such as Tomato Spotted Wilt Virus (TSWV), Eggplant Mottled Dwarf Virus (EMDV), and Iris Yellow Spot Virus (IYSV) to several plants (Babaie and Izadpanah, 2003; Boonham et al., 2002; Gera et al., 1998; Zen et al., 2008**)**. For the first time, *T. tabaci* was reported by Afshar (1938) on tobacco, cotton, cucumber, potato, onion and cabbage in Iran. *Thrips tabaci* is wide-spread in Iran and has been reported from most areas (Alavi et al., 2007) and is the major foliage pest in field cultures. This pest can cause considerable damage due to its feeding behaviour and their rapid reproduction. When conditions are hot and dry, a generation can be completed in only 2-3 weeks (Cranshaw et al., 2005). It is estimated that the yield loss caused by thrips in Iran (onion farms) is more than 50% (Alimousavi et al., 2007). *Thrips tabaci* is a very small insect that shows a high degree of similarity in appearance, particularly in preadult stages, (e.g. larval thrips are often mistaken for Collembola, whereas adults are commonly confused with Staphylinidae beetles (Vierbergen, 1995), which can make them extremely difficult to identify at the species level. On the other hand, in plant consignments, rapid identification is important to prevent the introduction of new pests into non-infested areas. Consequently, the rudiment and accurate recognition of thrips species is important in species-specific control programs, especially for thrips that have determined insecticide resistance (Roehrdanz, 1997).

‘‘DNA barcoding’’ is a method based on DNA sequencing of a standard gene region (Herbert et al., 2003b). It can be helpful in species diagnosis because sequence divergences are usually much lower among individuals of a species than between closely related species (Herbert et al., 2003a).

Recent researches show that it is possible to create credible identification systems established on the analysis of sequence diversity in small fragments of DNA (Tautz et al., 2003) and theoretical aspects (De Salle et al., 2005; Savolainen et al., 2005), methods (Blaxter et al., 2005; Steinke et al., 2005), and applied cases (Chase et al., 2005;

Monaghan et al., 2005) of the DNA barcoding

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are today under quite an intense development. Hebert et al. (2003b) focused this discussion by proposing that a DNA barcoding system for animal life could be based upon sequence diversity in the mitochondrial gene cytochrome C oxidase I (*COI*).

Insect mitochondrial genome (mtDNA) analysis is a powerful tool for the study of population genetics and phylogenetics. In the past few years primer sequences for the PCR amplification of various insect mtDNA genes have been published (Kambhampati and Smith, 1995). For example, congeneric species of moths show an average sequence divergence of 6.5% in *COI*) whereas divergences between conspecific individuals average only 0.25%. Similar values were obtained in birds, with intraspecific divergences at *COI* averaging 0.27%, while congener divergences averaged 7.93% (Herbert et al., 2004).

The use of genetic markers, like mtDNA, represents a valuable addition or alternative to classical methods of species identification. The strategy used in the present research is based on nucleotide sequencing analysis of the *COI* gene for the rapid and accurate identification of *T. tabaci*.

## Materials and Methods

### *DNA extraction and COI sequencing*

Four populations of *T. tabaci* were collected during 2007-2008 from Mashhad and the vicinity in Iran. DNA for PCR templates was extracted from an adult using DNeasy blood and tissue qiagen kit following the manufacturer's protocol. A double-stranded *COI* template was generated using the universally conserved mtDNA *COI* primers, LCO1490 and HCO2198 (Folmer et al., 1994). PCR reactions were performed with 10 mM dNTPs, 5 U/µl Amplitaq, 25mM $MgCl_2$, 10X PCR buffer, 20mM sense and antisense primers. The PCR thermal regime was 60 s at 94°C, 30 s at 52°C, and 90 s at 72°C with 35 cycles using a Biomethra thermocycler. PCR-amplified products were purified using Bioneer's PCR purification kit. Samples were sequenced from both directions using an ABI 377 sequencer.

### *Data analysis*

Sequences were edited and aligned using BioEdit 7.0.5.2 (Hall, 1999) (figure 1). The nBLAST program (http://www.ncbi.nlm.nih.gov/blast/) was employed to identify similarities between the sequences obtained in this work and previously published data (*Haplothrips* spp., *Thrips palmi*, *T. vulgatissimus*, *T. tabaci*, *Frankliniella occidentalis*) (table 1). A pair wise sequence divergence (the evolutionary distances) was calculated using the Kimura two-parameter distance model with MEGA4 (Kimura, 1980); Sequences were compared to identify intra- and interspecific nucleotide differences (tables 2 and 3). To visualize these patterns of divergence, the neighbor-joining tree (Saitou and Nei, 1987) and minimum evolution trees were constructed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test by 100,000 replicates (Felsenstein, 1985). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 397 positions in the final alignment. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

## Results

DNA was sequenced from four samples of thrips from Mashhad and the vicinity. DNA sequencing resulted in a fragment of the *COI* gene. All samples were successfully amplified. We selected a 413bp segment of DNA for further analysis. Distance estimates (figure 1) were used to generate a neighbor joining (NJ) tree. This tree (figure 2) shows the thrips sequences in six major clades, corresponding to the *Frankliniella occidentalis*, *Haplothrips* spp., *Thrips palmi*, *T. vulgatissimus and T. tabaci* (two clades) species. *COI* sequences of specimens from Mashhad formed two single clades, whereas the sequences of two other species formed two related sister clades, which together formed a larger group. The grouping of the taxa on the tree corresponded to species designation and geographic region, within each major clade and there was segregation based on their origins.

The trees (maximum parsimony analysis and minimum evolution trees not shown) produced by this analysis showed the same overall topology as the NJ tree.

Our result confirm the previous data reported by Crespi et al. (1996) that supports the deep phylogenetic split between *Terebantia suborder* (i.e. *Thrips* spp. and *Frankliniella occidentalis*) and *Tubulifera suborder* (include *Haplothrips* spp.) and corroborates the sister-taxon relationship of these two probably monophyletic suborders. Distance values (table 2) are correlated with geographic distance between specimen collection sites. For example, Palestine territory is the next region to Iran (in this research) and calculated sequence distances between individuals *T. tabaci* from these locations ranged to 0.019. In contrast, the sequence distance between Iran and the United Kingdom specimens, is much higher (0.043).

**Table 1.** Thrips species used in the study.

| **Species** | **Geographic Region** | **Specimen/Clone Designation** | **GenBank Accession No.** |
|---|---|---|---|
| Genus *Frankliniella* | | | |
| *F. occidentalis* | South Africa | CSL T166 | AM932023 |
| *F. occidentalis* | Italy | CSL T185 | AM932026 |
| *F. occidentalis* | Kenya | ENTOBAR0588 | FN545993 |
| *F. occidentalis* | UK | ENTOBAR0484 | FN545981 |
| Genus *Haplothrips* | | | |
| *H. cenchricola* | Spain | ENTOBAR0545 | FN545925 |
| *H. distinguendus* | UK | ENTOBAR0591 | FN545929 |
| *H. setiger* | Spain | ENTOBAR0695 | FN545939 |
| *H. statices* | UK | ENTOBAR0633 | FN545936 |
| *H. subtilissimus* | UK | ENTOBAR0603 | FN545933 |
| Genus *Thrips* | | | |
| *Th. palmi* | India | ENTOBAR0576 | FN546147 |
| *Th. palmi* | Dominican Republica | CSL T122 | AM932013 |
| *Th. tabaci* | Bosnia and Herzegovina | CSL T97 | AM932006 |
| *Th. tabaci* | UK | ENTOBAR0652 | FN546169 |
| *Th. tabaci* | Palestine | ENTOBAR0583 | FN546148 |
| *Th. tabaci* | Japan | IW | AB277235 |
| *Th. tabaci* | Japan | SM | AB277237 |
| *Th. tabaci* | Bosnia and Herzegovina | CSL T123 | AM932014 |
| *Th. tabaci* | Japan | ON2 | AB277236 |
| *Th. tabaci* | Bosnia and Herzegovina | ENTOBAR0654 | FN546171 |
| *Th. tabaci* | UK | CSL T223 | AM932043 |
| *Th. tabaci* | Bosnia and Herzegovina | ENTOBAR0419 | FN546157 |
| *Th. tabaci* | Palestine | ENTOBAR0585 | FN546150 |
| *Th. tabaci* | Palestine | ENTOBAR0584 | FN546149 |
| *Th. tabaci* | Iran | FUM11 | Current study |
| *Th. tabaci* | Iran | FUM 12 | Current study |
| *Th. tabaci* | Iran | FUM13 | Current study |
| *Th. tabaci* | Iran | FUM14 | Current study |
| *Th .vulgatissimus* | UK | ENTOBAR0629 | FN546059 |
| *Th. Vulgatissimus* | UK | ENTOBAR0363 | FN546068 |

**Table 2.** Pairwise Kimura 2-parameter distances between groups of *T. tabaci* (±SE).

| | **Bosnia** | **UK** | **Palestine** | **Japan** | **Iran** | |
|---|---|---|---|---|---|---|
| **Bosnia** | | 0.006 | 0.009 | 0.003 | 0.009 | |
| **UK** | 0.03 | | 0.007 | 0.007 | 0.008 | |
| **Palestine** | 0.041 | 0.039 | | 0.01 | 0.005 | |
| **Japan** | 0.004 | 0.033 | 0.043 | | 0.009 | |
| **Iran** | 0.04 | 0.043 | 0.019 | 0.04 | | |

**Table 3.** Mean distances between groups based on different species of thrips by Kimura 2-parameter distance (± SE).

| | ***F. occidentalis*** | ***Haplothrips spp.*** | ***T. palmi*** | ***T. vulgatissimus*** | ***T. tabaci*** |
|---|---|---|---|---|---|
| ***F. occidentalis*** | | 0.034 | 0.026 | 0.024 | 0.026 |
| ***Haplothrips* spp.** | 0.369 | | 0.033 | 0.034 | 0.033 |
| ***T. palmi*** | 0.236 | 0.373 | | 0.022 | 0.024 |
| ***T. vulgatissimus*** | 0.206 | 0.379 | 0.198 | | 0.022 |
| ***T. tabaci*** | 0.237 | 0.379 | 0.221 | 0.197 | |

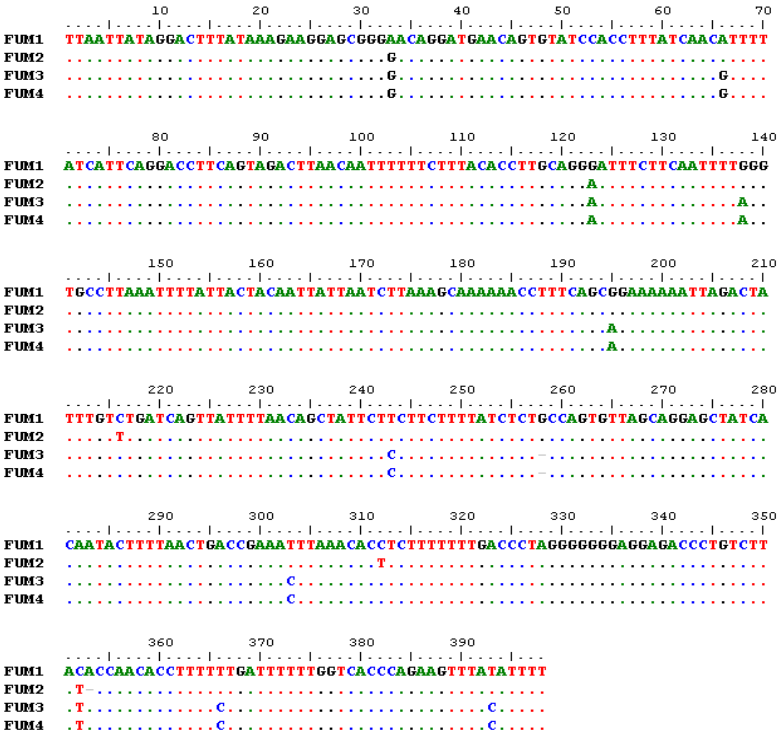


**Figure 1.** Aligned sequences from the mitochondrial cytochrome C oxidase I (*COI*) gene of four populations of *Thrips tabaci* species from Iran. Dots indicate nucleotides that are identical throughout the compared sequences, R indicates G/A nucleotides and dashes indicate insertions/deletions.

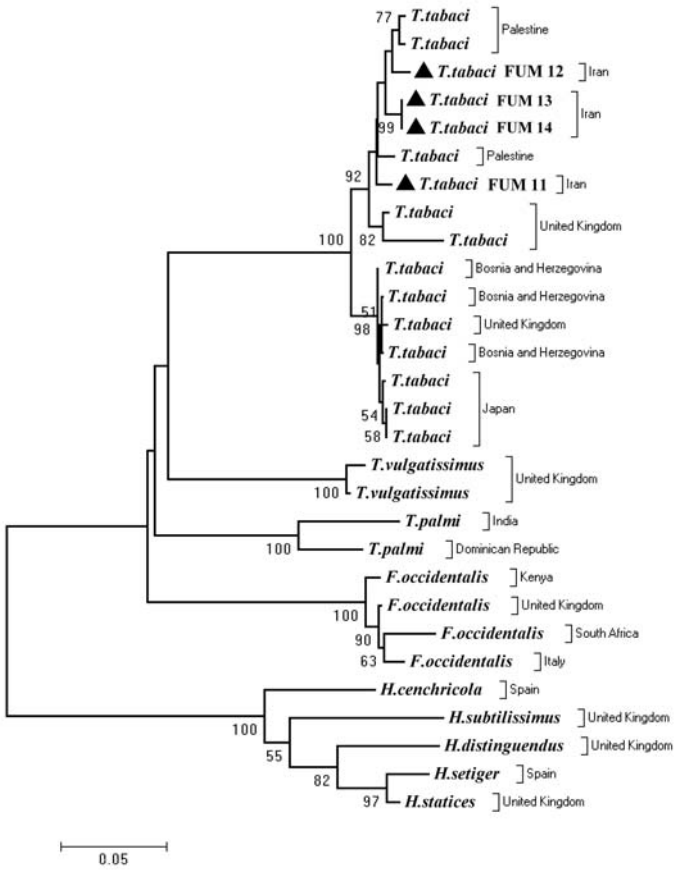


**Figure 2.** Unrooted neighbor-joining tree (with 100,000 replicate) constructed with the Kimura two-parameter distance calculation based on mtDNA *COI* sequence data. Taxa are labeled with the collection site. Bootstrap support ≥ 50% is indicated at branches.

## Discussion

Recently, the *COI* gene has been used for identification purposes in projects known as species barcoding. The idea behind barcoding is to sequence the *COI* gene of as many different species as possible and then use the *COI* sequence to identify unknown specimens by comparing their COI sequence the catalogued or named species (Blaxter, 2004; Hebert et al., 2003a, b; Tautz et al., 2003; Van Driesche et al., 2008). Whereas geographic isolation and genetic drift contribute to pronounced intraspecific phylogeographic structure, gene flow retards the genetic divergence of populations (Avise et al., 1987). The latter may be massive enough to reverse adaptive differentiation, unless the integrity of populations is maintained by reproductive solation (Brunner et al., 2004).

Our analyses clearly indicate that genetic differentiation is significant among populations of *T. tabaci* collected from different locations and then mtDNA sequences could be used in many studies to determine the origin of an invasive species. An example is the study by Havill et al. (2006) to determine the origin of the hemlock woolly adelgide, *Adelges tsugae* Annand (Homoptera: Adelgidae), which has invaded eastern North America. *COIt* has a great ability to help identify the invasive species (Scheffer et al., 2006) and natural enemies (Greenstone et al. 2005). Perdikis et al. (2003) used mitochondrial DNA sequences to distinguish between two closely related predatory hemipterans encountered in field studies.

Different markers are useful for inferring phylogeny of this insect group. For example Inoue and Sakurai (2007) used partial sequences of *COI*, *28S* ribosomal, and *EF-1α* for determining the phylogenetic relationships between the species of thrips and the vector competence of thrips for tospoviruses.

This study investigated the utility of *COI* for identifying thrips species. As demonstrated in this work, there is a relationship between phylogeny and origin evolution of thrips species. This can reveal that climate changes have important effects on diversification of species of thrips. Variation and polymorphism is common between species, nevertheless, it is often ignored by taxonomists. Molecular studies have the potential for detection of genetic polymorphism within species, and such information will be useful in identification of important species, study of population genetic, ecology, vector transmission, insecticide resistance, biological control and quarantine.

In conclusion, *COI* appears to be a good candidate marker to be used in DNA barcoding projects and can be particularly suitable in combination with the sequencing of additional genes or when biological and morphological characteristics are also studied to supplement *COI* data.

## Acknowledgements

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# Cadmium-induced genotoxicity detected by the random amplification of polymorphism DNA in the maize seedling roots

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## Abstract

In recent years, several plant species have been used as bioindicators, and several tests have been developed to evaluate the toxicity of the environmental contaminants. In this study, the root length and DNA band pattern of root tips in maize (*Zea mays* L.) seedlings were exposed at different concentration of cadmium pollutant (40 and 80 mg $L^{-1}$). The results indicated that the root length of maize seedlings reduced with an increasing cadmium concentration. A random amplification of polymorphic DNA (RAPD) analysis from the extracted DNA was carried out using twenty three 10-base pair random primers. Eleven primers produced 72 bands between 221-3044 base pairs in gel electrophoresis. DNA damage became evident as the presence and/or absence of DNA fragments in the treated samples compared to the control groups. The number of disappearing bands in profiles increased from 33 at 40 $mgL^{-1}$ of cadmium concentration to 45 after exposure to 80 mg $L^{-1}$ cadmium concentration compared to total bands in control. Three new bands appeared in 40 $mgL^{-1}$ of cadmium concentration but five in 80 $mgL^{-1}$ of cadmium concentration. The results showed that RAPD analysis could be a useful tool for detection of genotoxic effects of cadmium toxicity on plants.

***Keywords:*** Cadmium, Corn, DNA Damage, RAPD Analysis, Root Growth

## Introduction

The heavy metal cadmium (Cd) is considered as one of the most dangerous environmental pollutants which usually originates from industrial and agricultural activities such as mining waste disposal and application of pesticides or fertilizers (Agar and Taspinar, 2003). The toxic effects of cadmium have been demonstrated in different plant and animal species. Cd oxidative stress can be involved in Cd toxicity, by either oxygen free radical production (Stohs and Bagchi, 1995; Schutzendubel et al., 2001), or by decreasing the enzymatic and nonenzymatic antioxidants (Sandalio et al., 2001; Fornazier et al., 2002; Cho and Seo, 2004; Surjenru et al., 2007). Cd not only inhibits seed germination, root growth and mitotic index of cells, but also induces damage to different cellular components such as membrane, proteins and DNA (Zhang et al., 1994; Liu et al., 1992; Patra and Panda, 1998; Waisberg et al., 2003; Jimi et al., 2003). Recently, advances in molecular biology have led to using the DNA based techniques (RFLP, RAPD, AFLP, SSR and VNTR) for DNA damage analysis in eco-genotoxicity (Savva, 1996, 1998). RAPD is used extensively for species classification and phylogenetic analysis. A novel application of RAPD method is as biomarker assay to detect DNA damage and mutational events, such as rearrangements point mutation, small insert or deletions of DNA and ploidy changes in cells of bacteria, plants, invertebrate and vertebrate animals (Atienzar et al., 2000). The aim of this study was to detect DNA damage induced by Cd using the RAPD technique. Detection of the genotoxic effect involves comparison of RAPD profiles of the root tip DNA generated by control and treated maize seedlings.

## Materials and Methods

### *Plant materials and treatments*

Seeds of *Zea mays* (var KSC.704) were first surface sterilized by using 20-min incubation in 5% (w/v) sodium hypochlorite, followed by three times washing with distilled water. The seeds were then germinated at 24°C and subsequently transferred to pots containing a mixture of sand and perlite (1/1, v/v). The seedlings were grown in a greenhouse under growth conditions of 16 h light and 8 h dark, an average minimum temperature of 18°C, an

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average maximum temperature of 28°C, and the mean humidity of 60%. The heavy metal treatment was performed on 3-days old seedlings exposed to 40 and 80 $mgL^{-1}$ Cd (in the form of $CdN_2O_6.9H_2O_2$) for 7 days.

***DNA extraction and RAPD experiment***

Approximately 1.5 cm of the seedling root tips of control and treated samples were collected and ground in liquid nitrogen. Total genomic DNA was extracted using modified CTAB assay (Oard and Dronavalli, 1992). The PCR amplification was carried out with twenty three 10-base pair random primers (Eurofins MWG Operon-company) and genomic DNA as the template. PCRs were performed in a reaction mixture of 20 µl containing approximately 80 ng of the genomic DNA dissolving in sterile distilled water, 10X PCR buffer (2µl), 1.5 mM MgCl , 0.25 mM of each dNTP, 2 µl of 10 µM primer and 1 U *Taq* DNA polymerase. The RAPD protocol consisted of an initial denaturing step of 5 min at 94°C, followed by 35 cycles at 94°C for 1min (denaturation), 35°C for 1 min (annealing), and 72°C for 2 min (extension) with an additional extension period of 10 min at 72°C. The PCR amplification products were separated on 1% agarose gel using Tris-Borate-EDTA (TBE) buffer and GeneRuler 100bp DNA ladder (Fermentas, Germany). All the PCR examinations were carried out by Bioer XP thermal.

***Statistical analysis and computations***

The root growth experiment was conducted as randomized complete block designed with three replicates. Raw data were imported to Microsoft Excel program for calculation and graphic analysis. The SPSS (version 17.0) program was used for analysis of variance and comparison of the means was performed by Duncan's method at $P < 0.05$.

## Results

***Effect of Cd on root growth***

The result (Figure 1) shows the inhibitory effect of Cd treatment on the root length of maize seedlings at two concentrations (40 and 80 $mgL^{-1}$). The root length decreased 12.42% and 28.32% (significant at $P<0.05$) compared to untreated seedlings.

***Effect of Cd on RAPD bands pattern***

Out of the 23 random primers tested, only 11 primers gave stable bands (tables 1 and 2). The RAPD fingerprints showed substantial differences between unexposed and exposed seedlings to Cd, with apparent changes in the number and size of the amplified DNA fragments (figures 2 to 5). The number of disappearing bands in 80 $mgL^{-1}$ Cd concentrations was greater than 40 $mgL^{-1}$.

Disappearing of RAPD bands at 40 $mgL^{-1}$ Cd concentration was occurred with primers OPA-2, OPA-8, OPA-9, OPB-7, OPD-5, OPF-14, OPN-2, OPN-4 and at 80 $mgL^{-1}$ Cd concentration with primers OPA-2, OPA-9, OPD-2, OPD-3, OPD-5, OPF-14, OPN-2, OPN-4. At 40 $mgL^{-1}$ Cd concentration, two new bands (550 and 350bp) appeared with OPD-03 and one new band (472bp) appeared with OPB-07 primer. New bands at 80 $mgL^{-1}$ Cd concentration appeared with OPB-07 primer (one new band; 1073bp), OPN-04 primer (one new band; 679 bp), OPF-14 primer (one new band; 1051bp), OPA-2 primer (one new band; 900 bp) and OPD-5 primer (one new band; 472bp). Eleven primers produced 81 bands between 221-3044 base pairs in gel electrophoresis. The negative control (lane 2 of figure 2) showed that the PCR did not have external contamination.

## Discussion

In this study, the root length was severely inhibited by Cd. Changes in the root length of maize seedlings exhibited an inverse relationship with Cd concentration. As Figure 1 shows, exposure of maize seedlings to 80 $mg\,L^{-1}$of Cd concentration has more inhibitory effect on the root length than 40 $mgL^{-1}$ of Cd concentration. These results confirmed that Cd is a toxic agent for plant growth as described by Suzuki (2005). Out of the 23 decamer oligonucleotide primers tested, only 11 primers gave specific and stable results and ten primers of these 11 primers indicated changes in the RAPD profiles following cadmium treatment (Table 1 and Figures 2, 3, 4 and 5). Meanwhile, these primers gave a total of 72 bands ranging from 221-3044 base pairs in gel electrophoresis (Table 2). In this study, DNA damage was shown by RAPD profiles via disappearance or appearance of bands. The number of disappearing RAPD bands in profiles increased from 33 at 40 $mg\,L^{-1}$ of Cd concentration to 45 after exposure to 80 $mg\,L^{-1}$ of Cd concentration, compared to total bands in control. Disappearing bands are likely to be due to changes in oligonucleotide priming sites, originated from rearrangements and less likely from point mutations and DNA damage in the primer binding sites (Nelson et al., 1996; Liu et al., 2005; Enan, 2006; Liu et al., 2009). Three and five new bands were appeared in 40 and 80 $mg\,L^{-1}$ of Cd concentration.

**Table 1.** Nucleotide sequences of the twenty-three 10-mer primers* used for the random amplification of polymorphism DNA.

| Primer | Nucleotide sequence (5′-3′) |
|---|---|
| OPA-01 | CAGGCCCTTC |
| OPA-02** | TGCCGAGCTG |
| OPA-03 | AGTCAGCCAC |
| OPA-04 | AATCGGGCTG |
| OPA-07 | GAAACCGGTG |
| OPA-08** | GTGACGTAGG |
| OPA-09** | GGGTAACGCC |
| OPA-10** | GTGATCGCAC |
| OPA-17 | GACCGCTTGT |
| OPA-18 | AGGTGACCGT |
| OPA-20 | GTTGCGATCC |
| OPB-07** | GGTGACGCAG |
| OPB-10 | CTGCTGGGAC |
| OPC-02 | GTGAGGCGTC |
| OPC-05 | GATGACCGCC |
| OPC-14 | TGCGTGCTTG |
| OPD-02** | GGACCCAACC |
| OPD-03** | GTCGCCGTCA |
| OPD-05** | TGAGCGGACA |
| OPF-14** | GGTGCGCACT |
| OPN-02** | ACCAGGGGCA |
| OPN-04** | GACCGACCCA |
| OPS-09 | TCCTGGTCCC |

*All primers were provided by Eurofins MWG Operon-company (Ebersberg, Germany).
**Primers which gave optimum bands in the experiment.

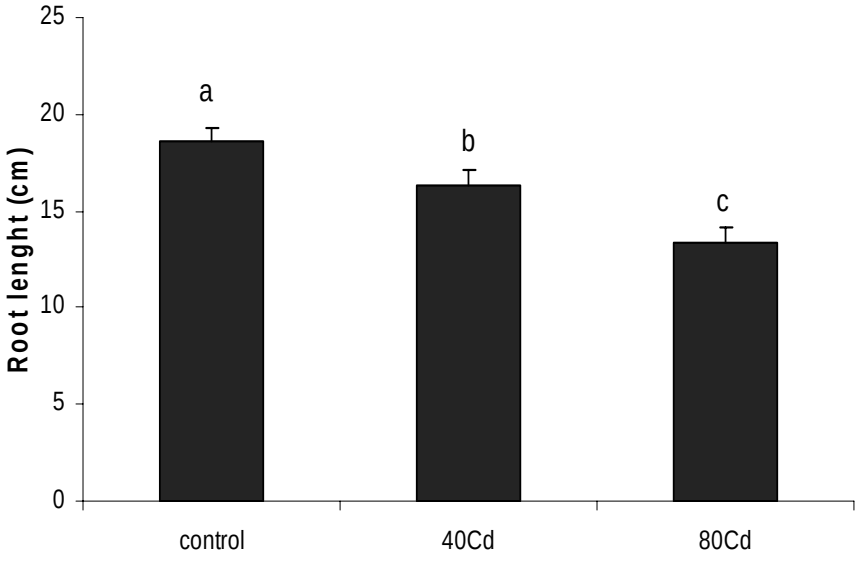


**Figure 1.** Effects of two Cd concentrations (40 and 80 mg $L^{-1}$) on root length of the maize seedlings

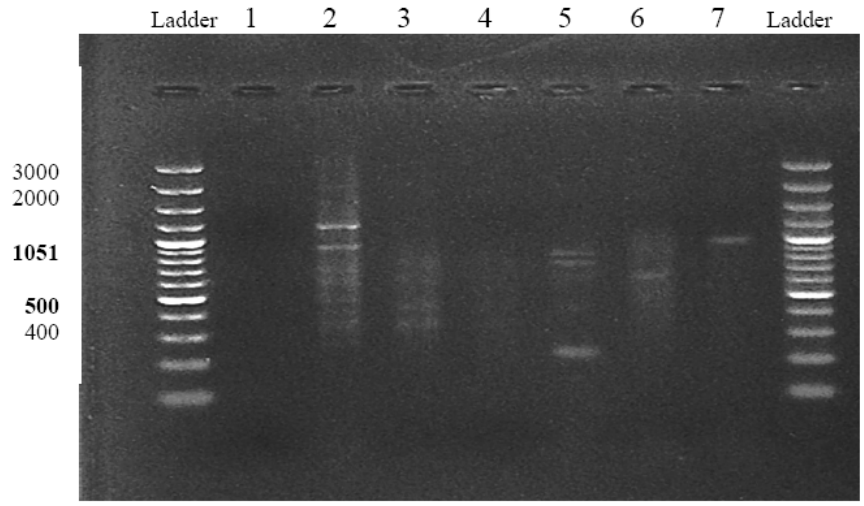


**Figure 2.** RAPD profiles of genomic DNA from root tips of maize seedlings unexposed and exposed to two Cd concentrations (40 and 80 mg $L^{-1}$) respectively, using primers OPD-5 (lanes 2, 3, and 4) and OPF-14 (lanes 5, 6, and 7). Lane 1 is negative control. Ladder: 100 bp DNA ladder (100-3000).

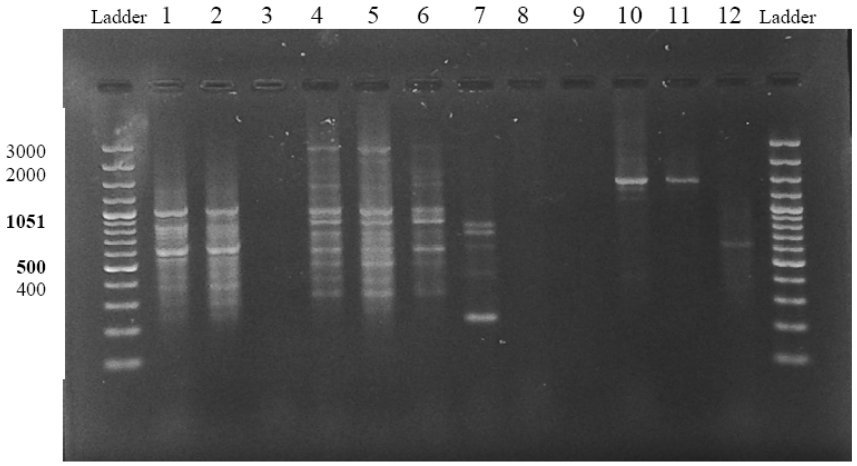


**Figure 3.** RAPD profiles of genomic DNA from root tips of maize seedlings unexposed and exposed to two Cd concentrations (40 and 80 mg $L^{-1}$) respectively, using primers OPD-2 (lane 1, 2, and 3), OPD-3 (lane 4, 5, and 6), OPN-2 (lane 7, 8, and 9) and OPN-4 (lane 10, 11, and 12). ladder: 100 bp DNA ladder (100-3000).

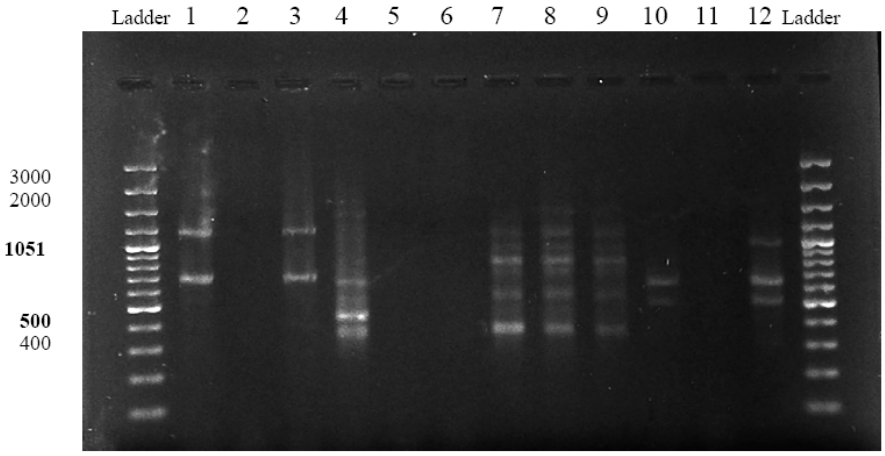


**Figure 4.** RAPD profiles of genomic DNA from root tips of maize seedlings unexposed and exposed to two Cd concentrations (40 and 80 mg $L^{-1}$) respectively, using primers OPA-8 (lane 1, 2, and 3), OPA-9 (lanes 4, 5, and 6), OPA-10 (lanes 7, 8, and 9) and OPB-7 (lanes 10, 11, and 12). ladder: 100 bp DNA ladder (100-3000).

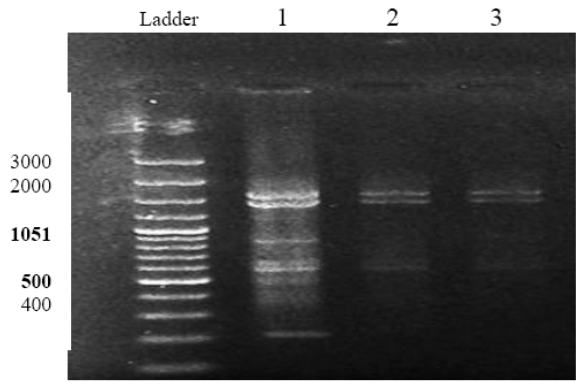


**Figure 5.** RAPD profiles of genomic DNA from root tips of maize seedlings unexposed and exposed to two Cd concentrations (40 and 80 mg $L^{-1}$) respectively, using primers OPA-2 (lanes 1, 2, and 3). ladder: 100 bp DNA ladder (100-3000).

**Table 2.** Molecular sizes (bp) of appeared and disappeared bands by random primers using PhotoCap software

| | **Treatments** | | | | |
|---|---|---|---|---|---|
| Primers names | Total bands in control | 40 ($mgL^{-1}$) Cd concentration | | 80 ($mgL^{-1}$) Cd concentration | |
| | | Appearance of new bands | Disappearance of control bands | Appearance of new bands | Disappearance of control bands |
| OPA-2 | 1679, 1500, 1205, 874, 657, 603, 513, 221 | | 1205, 874, 513, 221 | 900 | 1205, 874, 513, 221 |
| OPA-8 | 1197, 717 | | 1197, 717 | | No band disappeared |
| OPA-9 | 1282,1200,873, 690, 582, 470, 382 | | 1282,1200,873, 690, 582, 470, 382 | | 1282,1200, 873, 690, 582, 470, 382 |
| OPA-10 | 1631, 1194, 1060, 868, 786, 600, 505, 396 | | No band disappeared | | No band disappeared |
| OPB-7 | 690, 547 | | 690, 547 | 1073 | No band disappeared |
| OPD-2 | 1366, 1078, 960, 844, 626, 568, 463, 400, 316, 262 | | No band disappeared | | 1366, 1078, 960, 844, 626, 568, 463, 400, 316, 262 |
| OPD-3 | 3044, 2458, 1773, 1500, 1249, 1112, 936, 788, 647, 548, 453, 357 | 550, 350 | No band disappeared | | 2458, 453 |
| OPD-5 | 2234, 1243, 1000, 805, 729, 643, 521, 432, 373 | 472 | 2234, 1243, 1000, 729, 521, 432 | 472 | 2234, 1243, 1000, 805, 729, 643, 521, 373 |
| OPF-14 | 908, 790, 667, 462, 243 | | 908, 790, 462, 243 | 1051 | 908, 790, 667, 462, 243 |
| OPN-2 | 891, 809, 679, 476, 252 | | 891, 809, 679, 476, 252 | | 891, 809, 679,476, 252 |
| OPN-4 | 1500, 1446, 1249, 460 | | 1446, 1249, 460 | 679 | 1500, 1446, 1249, 460 |

Structural changes or some changes in DNA sequence due to mutation and/or large deletions (bringing two pre-existing annealing sites closer) create in new priming sites. Previous studies had shown that changes in DNA fingerprint offer a useful biomarker assay in ecotoxicology (Savva, 1996; Savva, 1998). Cd could induce DNA damage such as single- and double-strand breaks, modified bases, abasic sites, DNA-protein crosslink, oxidized bases and even bulky adducts in organisms (Hamada et al., 1997; Aust and Eveleigh, 1999; Bisova et al., 2003; Waisberg et al., 2003; Atesi et al., 2004; Hsiao and Stapleton, 2004; Jimi et al., 2004; Becher et al., 2004; Liu et al., 2005, 2009 and Cencki et al., 2009). Our finding support this claim that DNA polymorphisms detected by RAPD can be considered as a powerful biomarker assay for detection of the genotoxic effects of environmental pollutants like heavy metals. As a tool in risk assessment, the RAPD assay can be used in characterization of Cd hazard in soil. The RAPD-PCR based assay is fast, reliable and easy to conduct in any laboratory for assessment of environmental hazardous metals on plants.

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# Chromosome number and meiotic behaviour of two populations of *Onobrychis chorassanica* Bunge (*O.* sect. *Hymenobrychis*) in Iran

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## Abstract

The genus *Onobrychis* belongs to family Fabaceae and has about 130 species throughout the world. *Onobrychis* sect. *Hymenobrychis* with nearly 14 species in Iran is one of the important sections of the genus. The chromosome number and meiotic behaviour were studied in two populations of *Onobrychis chorassanica* belonging to this section native to Iran. This report is the first cytogenetic analysis of this taxon. Both populations are diploid and possess $2n = 2x = 14$ chromosome number, consistent with the proposed base number of $x = 7$. Although these taxa displayed regular bivalent pairing and chromosome segregation at meiosis, some meiotic abnormalities were observed. The meiotic abnormalities included varied degrees of chromosome stickiness including B-chromosome, cytomixis, asynchronous nucleus, fragmented chromosomes, occurrence of laggard chromosomes, chromosome bridges, desynapsis, micronucleous and tripolar cells.

***Keywords*:** chromosome number, Fabaceae, meiotic behaviour, *Onobrychis chorassanica*, *O.* sect. *Hymenobrychis*.

## Introduction

The genus *Onobrychis* with nearly 130 species is mainly distributed in the north temperate regions, but centers of diversity are in the eastern Mediterranean area and western Asia. *Onobrychis* includes annual or perennial, mostly caulescent herbs (rarely spiny shrubs), which have an indumentum with simple hairs or rarely are glabrous. A few taxa of the genus such as *O. viciifolia* are cultivated as fodder or for ornamental value (Lock and Simpson, 1991; Mabberley, 1997; Yakovlev et al., 1996). *O.* sect. *Hymenobrychis* with nearly 14 species in Iran is one of the important sections of the genus. The taxonomy of the genus continues to be the subject of much confusion, mainly because of the different approaches to species delimitation, resulting in varying numbers of recognized species (Aktoklu, 2001; Ball, 1978; Boissier, 1872; Duman and Vural, 1990; Hedge, 1970; Sirjaev, 1925). Recently some new taxa of the genus have been described from Iran (Ranjbar et al., 2004, 2006, 2007a, 2007b, 2009a, 2009b, 2010c and 2010d).

Most of the cytological studies in the genus have concentrated on the chromosome count (Baltisberger, 1991; Karshibaev, 1992; Slavivk et al., 1993), with little work focused on detailed karyological criteria for taxonomic purposes (e.g. Khatoon et al., 1991; Mesicek and Sojak, 1992). From these and other reports (e.g. Abou-el-Enain, 2002; Diaz-Lifante et. al., 1996; Fedorov, 1969; Goldblatt, 1981a, 1984, 1985, 1988; Goldblatt and Johnson, 1991; Romano et. al., 1987), it is evident that the chromosome count is known for just over a quarter of the species. Two chromosome numbers, $x = 7$ and $x = 8$, and three ploidy levels, $2n = 2x = 14$, $2n = 4x = 28$, $2n = 8x = 56$ and $2n = 2x = 16$, $2n = 4x = 32$, are present in the genus.

Studies on the impact of karyotypic characters on the interspecific and phylogenetic relationships and also on meiotic behaviour in the genus are still limited (Ranjbar et al., 2009b).

The evolutionary trend in *Onobrychis* has been discussed briefly based on the chromosome number. Goldblatt (1981) suggested that $x = 8$ is ancestral in the genus and those species with $x = 7$ are derived through aneuploid loss. However, Falistocco (1991) and Gomurgen (1996) argued that evolution within the genus has occurred by increasing the basic number from $x = 7$ to $x = 8$. Phylogenetic studies based on other lines of evidence have indicated that the primary centre of genetic diversity of *Onobrychis* is in the Mediterranean region and that the ecological

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separation of this region into western and eastern sectors represents a main event in the evolution of the genus (Ashurmetov and Normatov, 1998).

The present work aimed at increasing the knowledge about chromosome numbers and meiotic behaviour of the two Iranian populations of *O. chorassanica* belonging to the *O.* sect. *Hymenobrychis*. Such findings would help researchers to promote the understandings of the relationships between the chromosomal criteria and taxonomic delimitations.

## Materials and methods

Chromosome number and meiotic behaviour were analyzed in two populations of *O. chorassanica*. These populations were collected from different regions of Khorasan Province; located between Quchan and Sabzevar (13636), and Mashhad and Chenaran, Ferazy village (13639). For cytogenetic study, 15 young flower buds from at least 5 plants at an appropriate stage of development were fixed in 96% ethanol, chloroform and propionic acid (6:3:2) for 24 h at room temperature and then stored in 70% alcohol at 4°C until used. Anthers were squashed and stained with 2% acetocarmine. All slides were made permanent by the Venetian turpentine. Photographs of chromosomes were taken on an Olympus BX-41 photomicroscope at initial magnification of X 1000. Chromosome counts were made from well-spread metaphases in intact cells, by direct observation and from photomicrographs. Voucher specimens are kept at BASU, Hamedan, Iran.

## Results

All the studied samples from both populations were diploid and possessed $2n = 2x = 14$ chromosome number, consistent with the proposed base number of $x = 7$. Two populations of *O. chorassanica* were also investigated here for meiotic behaviour of their pollen mother cells (PMCs). Data with regard to the meiotic stage as well as abnormalities that observed in each stage is presented in table 1. A total of 1476 diakinesis/metaphase I (D/MI), 680 anaphase I/telophase I (AI/TI), 277 metaphase II (MII) and 470 anaphase II/telophase II (AII/MII) cells were analyzed. The meiotic irregularities observed in the studied taxa included: chromosome stickiness, B-chromosomes, precocious division of centromeres, chromosome bridges resulting from stickiness, the occurrence of laggard chromosomes, formation of micronuclei in tetrad cells, formation of tripolar cells, desynapsis and cytomixis which have been discussed bellow (figure 1).

## Discussion

Results showed that both populations were diploid and possessed $2n = 2x = 14$ chromosome number, consistent with the proposed base number of $x = 7$. Goldblatt (1981) suggested $x = 14$ as the basic number for the subfamily Faboideae, $x = 8$ for the tribe Hedysareae and $x = 8$ or 7 for the genus *Onobrychis*. He assumed that $x = 8$ is ancestral in the genus and that species with $x = 7$ are derived through aneuploid loss. The occurrence of the aneuploid series of $2n = 22$, 27, 28 and 29 in *O. viciifolia*, i.e. $2n = 3x + 1$, $4x - 27$, 28 and 29 in *O. viciifolia*, i.e. $2n = 3x + 1$, $4x - 1$, $4x$ and $4x + 1$, respectively (Corti 1930; Sacristan 1966) demonstrates the role of aneuploid alteration from the higher chromosome numbers based on multiples of $x = 7$ in the evolution of the species. Then, we confirmed that the chromosome number of $x = 7$ in *O.* sect. *Hymenobrychis* has been derived from other numbers through aneuploid changes of the diploid or polyploid numbers.

Stebbins (1974) reported that the presence of more polyploid species than diploids in a genus is the evidence that these species have a geographical distribution different from those of their diploid ancestors. Ashurmetov and Normatov (1998) assumed that the primary centre of genetic diversity of the genus *Onobrychis* is the Mediterranean countries, while Yildiz et al., (1999) and Ranjbar et al., (2009b) argued that it is in north and-south western Asia. The observations of the present study as well as the available data on chromosome number of *Onobrychis* indicate that, among the approximately 50 species with known chromosome counts, the diploid species, either annual or perennial, represent 40% of the whole, while the polyploids represent 60%. Polyploids are encountered mainly in north-western Asia, especially in Anatolia, Turkey, Turkmenistan and Uzbekistan. Diploids are distributed throughout temperate Asia, Mediterranean countries and south-western Asia especially Iran.

Meiosis is an event of high evolutionary stability which culminates in a reduction of chromosome number. The normal and harmonious course of meiosis ensures gamete viability. The cytological events of gametogenesis are controlled by a large number of genes that act from premeiotic to postmeiotic mitosis. Mutations in these genes cause anomalies that may impair fertility. Furthermore, many abnormalities affecting plant fertility or

causing total male sterility have been detected during the evaluation of meiotic behaviour in some species.

***Laggard, fragmented and sticky chromosomes***

Laggards, for being unable to orient at the metaphase plate were observed during metaphase I. The laggards at this phase of division might have been degenerated or may have resulted in the formation of polyads particularly at the resting phase (Basi et al., 2006). According to Nicklas and Ward (1994), non-oriented bivalents may be related to impaired attachment of kinetochores to the spindle fibers. Pagliarini (1990) reported that

**Table 1.** Number of pollen mother cells (PMCs) analyzed and percentage of PMCs meiotic behaviour in two population of *Onobrchis chorassanica*.

| **Populations**<br>**Meiotic characters** | **cho39 (Mashhad)** | **cho36 (Quchan)** |
|---|---|---|
| Total cell number | 776 | 700 |
| D/MI | 51 | 86 |
| % D/MI | 6.57 | 12.2 |
| % Cytoplasmic connection | 11.76 | 0 |
| % Sticky & laggard chromosome | 5.88 | 19.76 |
| % B-chromosome | 0 | 2.32 |
| % Precocious segregation | 0 | 4.32 |
| AI/TI | 358 | 320 |
| % AI/TI | 46.13 | 45.5 |
| % Fragmented chromosome | 0.27 | 0 |
| % Laggard chromosome | 0.27 | 0 |
| % Bridge | 0.27 | 1.4 |
| MII | 157 | 120 |
| % MII | 20.23 | 17.4 |
| % Fragmented chromosome | 5.73 | 0 |
| % Desynapsis | 3.18 | 0 |
| % Micronucleus | 3.18 | 46.6 |
| % Cytomixis | 1.27 | 0 |
| % Asynchronous nucleus | 0 | 20 |
| AII/TII | 210 | 260 |
| % AII/TII | 27.06 | 14.45 |
| % Micronucleus | 0.47 | 0 |
| % Tripolar cell | 0.47 | 0 |
| % Cytomixis | 0 | 0.47 |
| *N* | 7 | 7 |

Abbreviations: D/MI = Diakinesis/Metaphase I; AI/TI = Anaphase I/Telophase I; MII = Metaphase II; AII/TII = Anaphase II/Telophase II; *n* = Chromosome number.

laggards may result from late chiasma terminalization. Ascending chromosomes are the result of precocious migration. According to Utsunomiya et al., (2002), they generally consist of univalent chromosomes formed during late prophase stages by precocious chiasma terminalization in early metaphase I. They may even result from low chiasma frequency or from the presence of asynaptic or desynaptic genes (Pagliarini, 2000). Laggards may produce micronuclei, if they fail to reach the poles in time to be included in the main telophase nucleus (Koduru and Rao, 1981; Utsunomiya et al., 2002), leading to the formation of micro-pollen and probably to gametes with unbalanced chromosome numbers (Mansuelli et al., 1995), such as aneuploids (Defani-Scoarize et al., 1995). Laggards were observed in both populations.

***B-chromosomes***

B-chromosomes or accessory chromosomes that occur in addition to the standard or A-chromosomes in some plants, are smaller than other chromosomes and do not form any association with them. B-

chromosomes, when present in high numbers affect negatively the growth and vigor of the plants, while in low numbers may benefit the plant possessing them (Jones and Houben, 2003). B-chromosomes were observed only in Quchan population (figure 1H).

***Precocious division of centromeres***

In Quchan population, the number of cells with univalents presenting precocious migration to the poles during metaphase I was high (figure 1I), while this abnormality was not observed in Mashhad population. Because univalents usually do not suffer regular segregation in the first division, the frequency of univalents in diakinesis/metaphase I, has been used as a standard measure of meiotic disturbances in other species (Scholes and Kaltsikes, 1974).

***Cytomixis***

The phenomenon of cytomixis consists in the migration of chromosomes between meiocytes

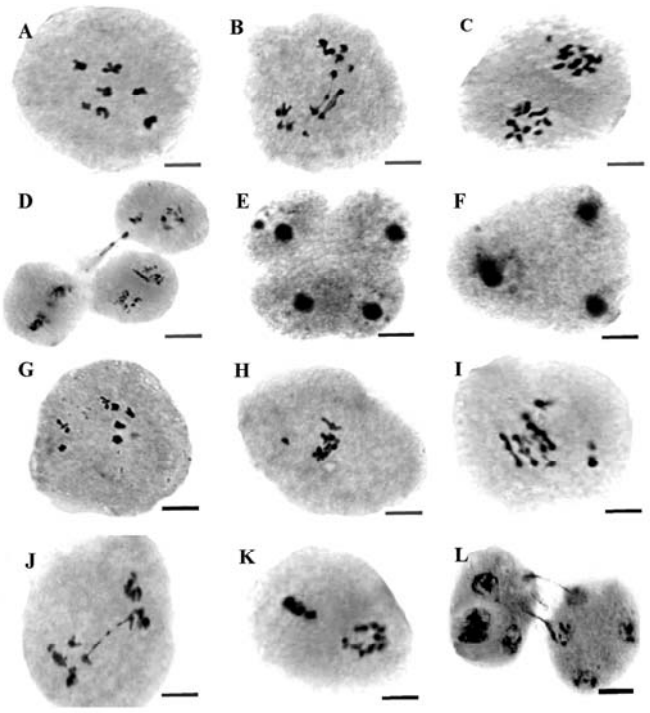


**Figure 1.** (A – F) Representative meiotic cells in Mashhad population of *O. chorassanica*: (A) Diakinesis. (B) Bridge. (C) Desynapsis. (D) Cytomixis. (E) Micronucleus. (F) Tripolar cell. (G – L) Representative meiotic cells in Quchan population of *O. chorassanica*: (G) Diakinesis. (H) B-chromosome. (I) Metaphase I with univalent in precocious ascension. (J) Bridge. (K) Asynchronous nucleus. (L) Cytomixis (Scale bar = 3 μm).

through cytoplasmic connection. Since cytomixis creates variation in the chromosome number of the gametes, it could be considered as a mechanism of evolutionary significance (Ghaffari, 2006). This phenomenon was occurred in both populations in metaphase II and telophase II cells (figures 1D and L).

***Chromosome bridges***

Chromosome bridges resulting from stickiness were observed in two populations in anaphase I cells (figures 1B and J). The thickness of bridges observed and the number of chromosomes involved in their formation varied among different meiocytes. Genetic as well as environmental factors have been considered as the reasons for chromosome stickiness in different plant species (Nirmala and Rao, 1996).

***Micronucleus***

Micronucleus is another abnormality that was found in both populations (figure 1E). Chromosomes that produced micronuclei during meiosis were eliminated from microspores as microcytes. The micronucleus reached the microspore wall and formed a kind of bud, separated from the microspore. The eliminated microcytes gave origin to small and sterile pollen grains (Baptists-Giacomoelli et al., 2000).

***Tripolar cells***

Failure of chromosome movement occurred in

one of the poles of anaphase cells, leading to the formation of tripolar cells. Such cells produce normal reduced and unreduced daughter cells. This phenomenon was found in Mashhad population (figure 1F). Such unreduced meiocytes may lead to the information of 2*n* pollen grains (Sheidai et al., 2007).

In conclusion, both diploid populations of *O. chorassanica* with the chromosome basic number of $2n = 2x = 14$ showed different meiotic abnormalities. These abnormalities were observed in Mashhad population in higher frequencies than those in Quchan. They are genetically controlled and have been reported in populations of different legume species like *Oxytropis* and *Astragalus* (Ranjbar et al., 2010a, b). Varied ranges of meiotic abnormalities in populations with the same chromosome number is considered as a means for generating different kinds of recombinants, influencing the variability within natural populations in a possibly adaptive manner (Rees and Dale, 1974). Like other meiotic abnormalities, cytomixix occurred in Mashhad population higher than in Quchan. Cytomixis may lead to production of aneuploid plants or result in the production of unreduced gametes, as reported in several species. Unreduced gamete formation is of evolutionary importance as it can lead to the production of plants with higher ploidy level (Falistocco et al., 1995).

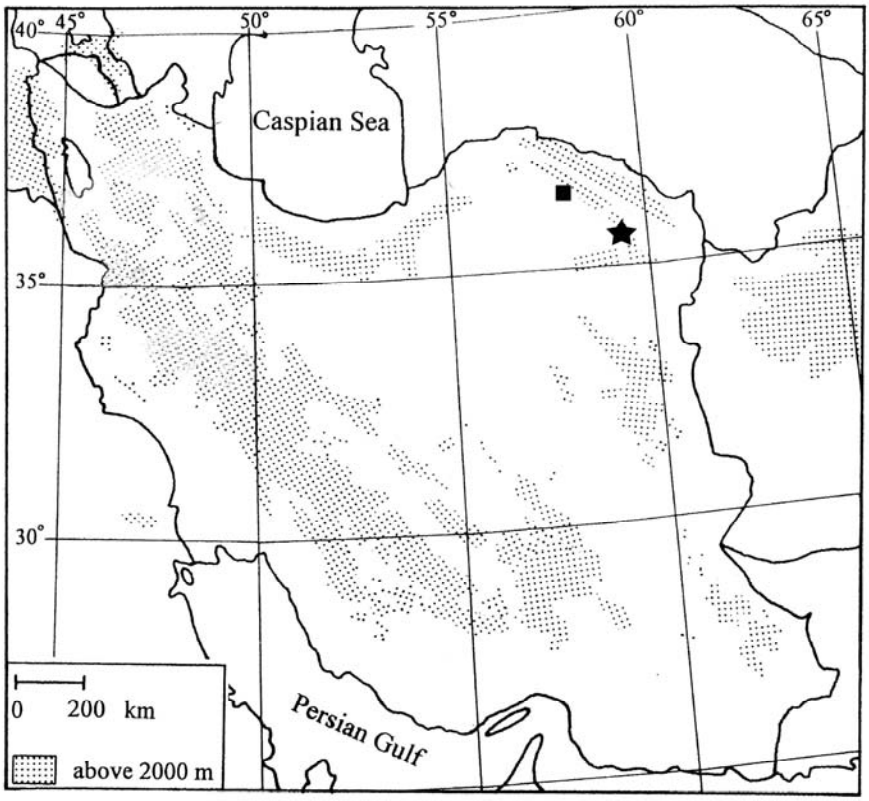


**Figure 2.** Distribution of Mashhad (★) and Quchan (■) populations of *Onobrchis chorassanica* in Khorassan Province.

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