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(54) Title: BIOACTIVE COMPOUNDS OF ASCOPHYLLUM NODOSUM AND THEIR USE FOR ALLEVIATING SALT-INDUCED STRESS IN PLANTS

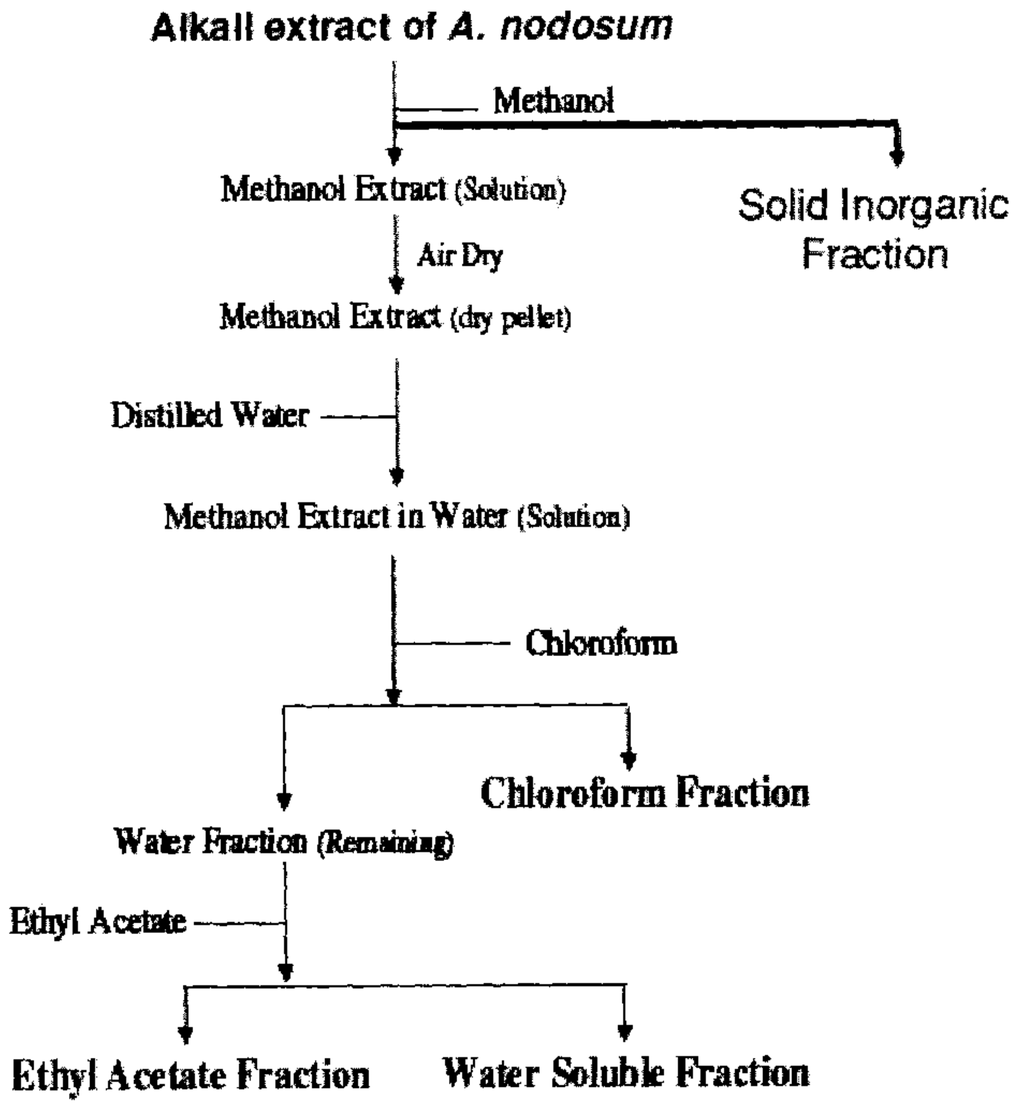

Figure 1

(57) Abrégé/Abstract:
A process for extraction of bioactive organic compounds from alkali extracts of Ascophyllum nodosum. Organic solvent extracts of A. nodosum namely; methanol, chloroform and ethylacetate, were found to alleviate salt induced stress in plants. This effect was due to alteration in the expression of a specific subset of genes induced by compounds present in A. nodosum extracts.

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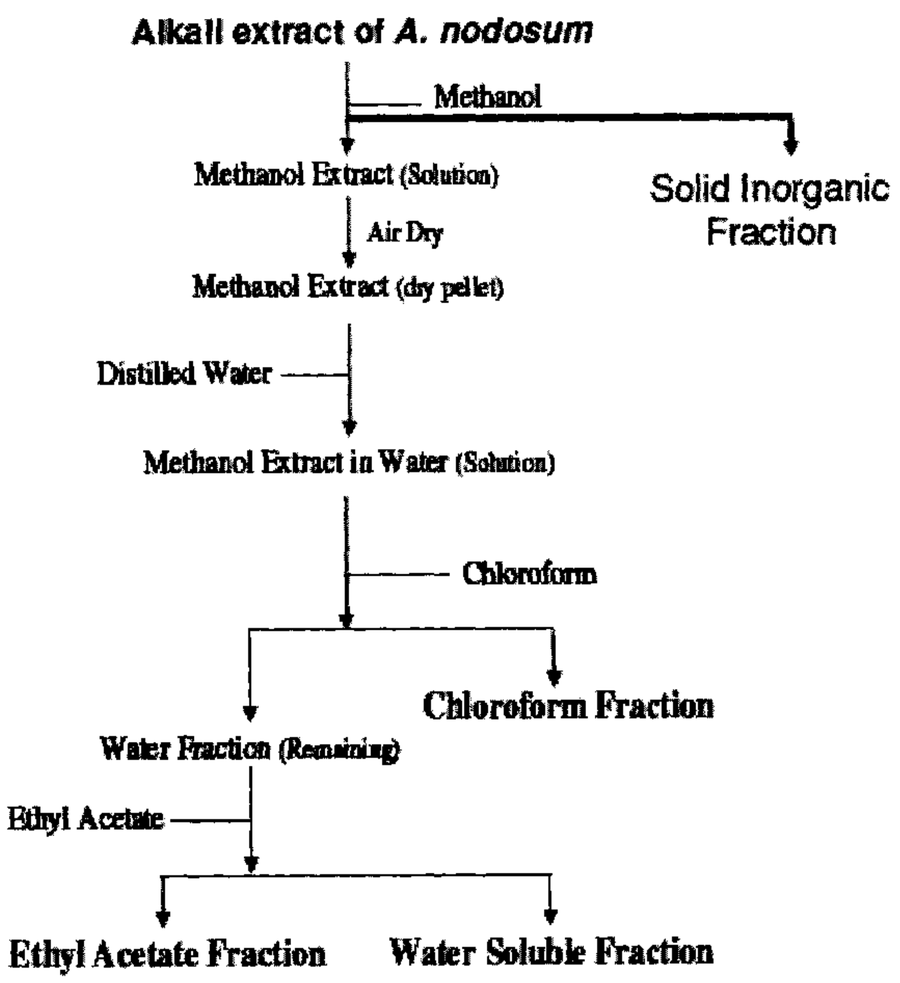

Figure 1

(57) Abstract: A process for extraction of bioactive organic compounds from alkali extracts of *Ascophyllum nodosum*. Organic solvent extracts of *A. nodosum* namely; methanol, chloroform and ethylacetate, were found to alleviate salt induced stress in plants. This effect was due to alteration in the expression of a specific subset of genes induced by compounds present in *A. nodosum* extracts.

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Bioactive Compounds of *Ascophyllum nodosum* and Their Use for Alleviating Salt-Induced Stress in Plants

FIELD OF THE INVENTION

The invention relates to compounds and methods for alleviating salt-induced stress in plants. More specifically, the invention relates to compounds and extracts derived from *Ascophyllum nodosum*, methods of their production, and their use for the alleviating salt-induced stress in plants.

BACKGROUND OF THE INVENTION

Plant growth and productivity is severely affected by various abiotic stresses, like salinity, temperature extremes and drought. Of these, soil salinization is a major constraint in food production on otherwise potentially arable lands, and is thus of particular concern.

Efforts have been made to overcome the problems associated with high soil salinity through the use of plants with a higher level of tolerance to salt stress, by modifying genes encoding different proteins developed through biotechnological approaches. This approach has not yet translated into marketable crop varieties despite several years of research. Further, there has been heightened consumer sensitivity to genetically modified (GM) foods. Accordingly, there continues to be the need for a way to address the problems associated with high soil salinity.

Ascophyllum nodosum (rockweed), a brown algae that grows along the Canadian Atlantic coast, has acquired special mechanisms of salt tolerance, possibly by synthesis of bioactive compounds. Accordingly, the present inventors have sought to develop an alternative approach for alleviating negative effects of salt stress on salt sensitive plants through the isolation of such bioactive compounds.

SUMMARY OF THE INVENTION

Accordingly, an object of the present invention is to provide a means for alleviating salt-induced stress in plants.

According to an aspect of the present invention, there is provided a process for preparing an organic extract useful as a treatment for inducing salinity tolerance in plants, said process comprising the steps of: (a) suspending dried *A. nodosum* in methanol, (b) mixing the suspension, and (c) separating any remaining solid material from the resulting methanol extract,

wherein the methanol extract is useful as a treatment for inducing salinity tolerance in plants.

In an embodiment, the process may further comprise the steps of: (d) removing the solvent from said methanol extract to form a dried residual, (e) resuspending the dried residual from said methanol extract in water, (f) adding chloroform to the resuspended residual from the methanol extract, (g) mixing and allowing the phases to separate into water and chloroform extracts, (h) collecting the chloroform extract, and (i) removing the solvent from said chloroform extract to form a dried residual, wherein the dried residual of said chloroform extract is useful as a treatment for inducing salinity tolerance in plants. In a further embodiment, the process may additionally comprise the steps: (j) resuspending the dried residual from said chloroform extract in water, (k) adding ethyl acetate to the resuspended residual from the chloroform extract, (l) mixing and allowing the phases to separate into water and ethyl acetate extracts, (m) collecting the ethyl acetate extract, and (n) removing the solvent from said ethyl acetate extract to form a dried residual, wherein the dried residual of said ethyl acetate extract is useful as a treatment for inducing salinity tolerance in plants.

In further embodiments, the *A. nodosum* is suspended in the methanol in a ratio of *A. nodosum* to methanol from about 1:1 to about 1:50 volume/volume. In a preferred embodiment, the *A. nodosum* is suspended in the methanol in a ratio of *A. nodosum* to methanol of 1:3 volume/volume.

In yet further embodiments, it may be preferred that steps (f)-(h) be repeated up to 3 times. Similarly, it may be preferred that steps (k)-(m) be repeated up to 3 times.

As a further aspect of the invention, there is provided a method of inducing salinity tolerance in plants, comprising obtaining an organic extract as defined in the above process, and administering the organic extract to a plant under salt stress in an amount effective to ameliorate the salt stress in said plant.

Also provided, as an aspect, is the use of an organic extract as defined in the above process for inducing salinity tolerance in plants.

As another aspect of the invention, there is provided a composition for inducing salinity tolerance in plants, comprising as active agent at least one phytosterol, fungal sterol, terpenoid or fatty acid, or combinations thereof, derived from *Ascophyllum nodosum*.

In the above composition, the fungal sterol may be derived from *Mycosphaerella ascophylli.*

In an embodiment of the above composition, the phytosterol is fucosterol.

As a further aspect, there is provided a method of inducing salinity tolerance in plants, comprising administering a composition as defined above to a plant under salt stress in an amount effective to ameliorate said salt stress in said plant.

Also provided is the use of a composition as defined above for inducing salinity tolerance in plants.

As an additional aspect, there is provided a method of inducing salinity tolerance in plants, comprising extracting at least one phytosterol, fungal sterol, terpenoid or fatty acid, or combinations thereof from *Ascophyllum nodosum*, and administering said organic extract to a plant under salt stress in an amount effective to ameliorate said salt stress in said plant.

In a further aspect of the invention, there is provided a composition of matter comprising at least one phytosterol, fungal sterol, terpenoid, fatty acid, or combinations thereof, from *Ascophyllum nodosum*, for alleviating salinity stress in plants. In an embodiment of the composition of matter, the phytosterol, fungal sterol, terpenoid, fatty acid, or combinations thereof elicit coordinated expression of multiple genes in the plant to induce salinity tolerance.

The invention also provides a composition useful as a treatment for inducing salinity tolerance in plants, obtained according to a process including (a) suspending dried *A. nodosum* or extracts of *A. nodosum* in methanol, (b) mixing the suspension, and (c) separating any remaining solid material from the resulting methanol extract, wherein the methanol extract is provided as a composition useful as a treatment for inducing salinity tolerance in plants.

In an embodiment, the methanol extracts described above may have the solvent at least partially removed such that the extracted organic material, e.g. including one or more of phytosterols, fungal sterols, terpenoids, fatty acids, and combinations thereof, can be used in a plant or seed treatment.

In other embodiments, the methanol extract may be further processed by (d) removing the solvent from the methanol extract to form a dried residual, (e) resuspending the dried residual from the methanol extract in water, (f) adding chloroform to the resuspended residual from the methanol extract, (g) mixing and allowing the phases to separate into water and chloroform extracts, (h) collecting the chloroform extract, and (i) removing the solvent from said chloroform extract to form a dried residual, wherein the dried residual of the chloroform extract is provided as a composition useful for inducing salinity tolerance in plants. Further processing of the dried

residual of the chloroform extract can also be undertaken by (j) resuspending the dried residual from the chloroform extract in water, (k) adding ethyl acetate to the resuspended residual from the chloroform extract, (l) mixing and allowing the phases to separate into water and ethyl acetate extracts, (m) collecting the ethyl acetate extract, and (n) removing the solvent from the ethyl acetate extract to form a dried residual, wherein the dried residual of the ethyl acetate extract is provided as a composition useful for inducing salinity tolerance in plants.

Compositions as described herein can be formulated for use, for instance, as a liquid for spray or root irrigation, or as a solid for seed treatment. Solid and liquid carriers useful in preparing such formulations will be known to those skilled in the art, and can accordingly be used in formulations of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects and advantages of the present invention will become better understood with regard to the following description and accompanying drawings wherein:

FIG. 1 is a schematic representation of the extraction of organic compounds of *A. nodosum*;

FIG. 2 shows the results of NMR analysis of the organic extracts of *A. nodosum*;

FIG. 3 shows a graphical representation of the results of testing organic extracts of *A. nodosum*. As shown, organic extracts of *A. nodosum* alleviate the negative effect of salt on fresh weight in *A. thaliana*;

FIG. 4 shows a graphical representation of the results of testing organic extracts of *A. nodosum*. As shown, organic extracts of *A. nodosum* alleviate the negative effect of salt on number of leaves in *A. thaliana*;

FIG. 5 shows a graphical representation of the results of testing organic extracts of *A. nodosum*. As shown, organic extracts of *A. nodosum* alleviate the negative effect of salt on leaf area in *A. thaliana*;

FIG. 6 shows a graphical representation of the results of testing organic extracts of *A. nodosum*. As shown, organic extracts of *A. nodosum* alleviate the negative effect of salt on plant height in *A. thaliana*;

FIG. 7 shows pictorial results of testing organic extracts of *A. nodosum*. As shown in the photographs, organic extracts of *A. nodosum* alleviates salt-induced stress in *A. thaliana*;

FIG. 8 depicts the results of testing the effect of organic extracts of *A. nodosum* on the expression of stress induced genes in *A. thaliana*, up arrows indicate increased expression, down arrows indicate lowered expression, and horizontal arrows indicate little or no change in expression;

FIG. 9 shows the results of testing the effect of organic compounds of *A. nodosum* on the Na^+ uptake in *A. thaliana*;

FIG. 10 shows volcano plots of gene expression on Day 1 and Day 5 with organic extracts of *A. nodosum*, showing that the organic extracts affect gene expression and make the plant resistant to salinity stress;

FIG. 11 depicts venn diagrams illustrating that organic compounds of *A. nodosum* elicit specific stress tolerance response by up or down regulating specific sets of genes;

FIG. 12 shows the results of analysing expression levels by RT-PCR upon treatment with ethyl acetate extract fractions on Day 1 and Day 5. As observed, organic compounds of *A. nodosum* elicit specific stress tolerance responses by up or down regulating specific sets of genes;

FIG. 13 shows the results of testing catalase activity in lettuce after 24 h under 100 mM NaCl and 150 mM NaCl conditions;

FIG. 14 shows the results of testing catalase activity in lettuce after 48 h under 100 mM NaCl and 150 mM NaCl conditions;

FIG. 15 shows the results of measuring percentage leaf area as affected by salt stress in Lettuce under 100 mM NaCl and 150 mM NaCl conditions;

FIG. 16 shows the results of measuring chlorophyll content in Lettuce after 48 h under 100 mM NaCl and 150 mM NaCl conditions;

FIG. 17 shows the results of measuring chlorophyll content in sugarbeet after 48 h under 100 mM NaCl and 150 mM NaCl conditions;

FIG. 18 shows the results of testing fucosterol and different organic extracts of *A. nodosum* on root length. The code numbers in the figure refers to different organic extracts. As observed, plants treated with fucosterol and extract RS5-45C have a longer root length than untreated plants under salt stress (compared to +Na); and

FIG. 19 shows the reduced root Na^+ uptake under hydrophonics system (ion-exclusion).

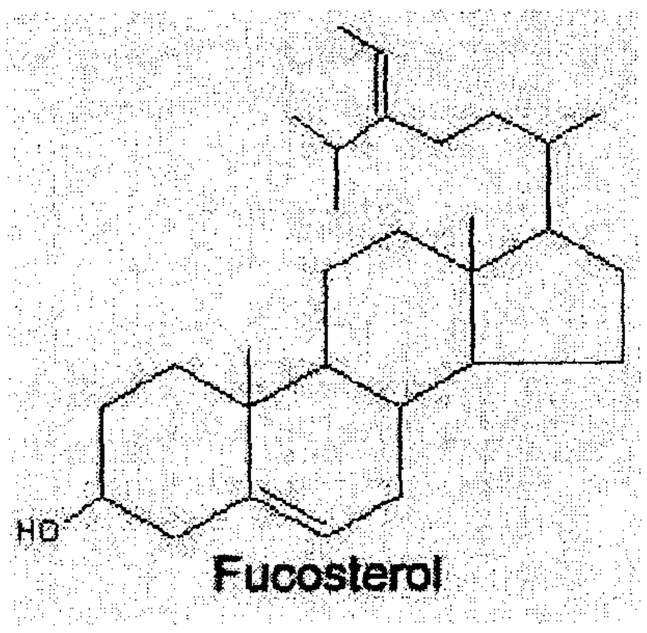
DETAILED DESCRIPTION

The present inventors have identified organic compounds and extracts that alleviate salt stress in plants. This finding has significance for a variety of economically important crops encompassing major plant groups, including cereals (including but not limited to barley, wheat, rice, corn, oats) legumes (including but not limited to pea, mungbean, soybean), brassicas (including but not limited to cauliflower, broccoli, canola, mustard, rapeseed), tubers (including but not limited to beets, potatoes, carrots), and vegetables (including but not limited to tomato, cucumber, lettuce, pepper).

The compounds and extracts are derived from the brown intertidal alga *Ascophyllum nodosum*. This alga is known to have a systemic association with at least one fungus (in particular *Mycosphaerella ascophylli*) which grows in and amongst the internal tissues of the seaweed and is therefore inseparable. Accordingly, compounds and extracts of *A. nodosum* as described herein may also include compounds of fungal origin by virtue of the natural fungal associations with *A. nodosum*.

The fraction of the extract which is beneficial in providing salinity tolerance in land plants has been found to contain predominantly seaweed phytosterols, terpenoids and fatty acids. The extracts may also comprise fungal sterols. In an embodiment the phytosterol is a fucosterol.

Fucosterol (24-ethylidene cholesterol), has the following chemical structure:



Fucosterol

The present invention is particularly advantageous over the prior art since abiotic stress tolerance, including salinity stress tolerance, is imparted by multiple genes (oligogenic). Currently there are limitations to the number of genes that can be efficiently introgressed by genetic modification (transgenic approach) of plants and therefore inducing tolerance to abiotic stresses via the GM approach is limited. Further, chemicals in *A. nodosum* extracts also repress the expression of a number of genes that ultimately leads to salinity tolerance. Taken together, the organic compounds and extracts of *A. nodosum* disclosed herein can be used as an effective alternative to the GMO approach. It is also anticipated that this approach will have more consumer acceptance, and reduce possible ecological damage to the environment by GMOs.

In an embodiment of the invention, the extraction is carried out as follows: dry *A. nodosum* extract powder or dry *A. nodosum* powder is suspended in 100% methanol in a ratio of from about 1:1 to 1:50 volume/volume of powder to methanol, more preferably in a ratio of 1:3 powder to methanol, and mixed (e.g. by vortexing for 10 minutes at room temperature). The extract is then suspended in water (approximately 1:1 to 1:50 of the volume of the original *A. nodosum* powder, more preferably in a ratio of 1:3) and phase partitioned with chloroform

(approximately 1:1 to 1:50 of the volume of the original *A. nodosum* powder, more preferably in a ratio of 1:3), preferably more than once and more preferably three times. The chloroform fractions are combined to provide the chloroform fraction (the analysis of which is discussed in further detail below). The remaining aqueous fraction is phase partitioned with ethyl acetate (approximately 1:1 to 1:50 of the volume of the original *A. nodosum* powder, more preferably in a ratio of 1:3), preferably more than once and more preferably three times. The ethyl acetate fractions are combined, the solvent evaporated and the residual solid formed the ethyl acetate fraction (the analysis of which is discussed in further detail below). Each of the fractions used in the experiments below was dissolved in methanol.

EXPERIMENTS:

Extraction of bioactive compounds from alkali extracts of *A. nodosum*

The schematic representation of the extraction protocol is presented as Figure 1. Briefly, the alkali extract of *A. nodosum* was extracted in three volumes of HPLC grade methanol. The extract was centrifuged and the supernatant was dried to a pellet. The pellet was suspended in distilled water and sequentially fractionated with Chloroform and then Ethyl acetate. The resulting extracts were dried and suspended in pure methanol and used.

Nuclear magnetic resonance (NMR) Analysis of the organic extracts of *A. nodosum*

An NMR analysis revealed the presence of organic compounds, and predominantly fatty acids and sterols in the extracts (Figure 2).

Organic extracts of *A. nodosum* alleviate salt induced stress in plants

Arabidopsis thaliana plants were grown in the green house at 22±2°C under long day photo period (16h light/8h dark). Two-week-old plants were treated with 150 mM NaCl by flooding the roots (at the rate of 25 ml/plant). Twenty four hours after the salt treatment, plants were treated with organic extracts (methanol, chloroform and ethyl acetate) of *A. nodosum* at the rate of 25ml/plant of a solution containing about 1mg/liter of organic compounds. The extract treatment was repeated once after seven days. Observations on plant height, number of leaves, leaf area and fresh weight were recorded after one month of the salt treatment. The changes in the expression of stress inducible genes were studied at five days after treatment.

An analysis of plant fresh weight showed that under unstressed condition the extracts (water soluble, ethyl acetate and chloroform) did not affect the growth of *A. thaliana* plant. However, at

150 mM NaCl stress, ethyl acetate extract of *A. nodosum* showed 52% more fresh weight than plants treated with NaCl alone. Similarly, chloroform extract and water soluble fraction showed 36% and 13% more fresh weight over the untreated controls, respectively (Figure 3). There was a significant increase in the number of leaves in the plants treated with different organic extracts of the algae. Ethyl acetate extract treated plants had 45% more leaves over the untreated control, while chloroform and water soluble also showed 15% and 11% increase (Figure 4). Significant increase in leaf area was also observed in extract treated plants as compared to non treated plants, a 62% increase was observed in ethyl acetate extract treated plants (Figure 5). Similar result was observed in regards to plant height (Figure 6). Figure 7 shows the *A. thaliana* plants following testing with the organic extracts of *A. nodosum,* clearly illustrating in pictorial view the salinity stress response seen in the graphical results of Figures 3-6.

Organic compounds of *A. nodosum* affect gene expression of plant resulting in enhanced tolerance to salt-induced stress

The expression of the following genes was studied by RT-PCR: P5CS1, P5CS2, PDH, Cor 15A, RD29B, DREB2A, NHX1, CHX21, NADK2. The overall pattern of *A. nodosum* induced gene expression is depicted in Figure 8.

Salt stress (150 mM) induced proline synthetase genes P5CS1 and P5CS2, and addition of organic extracts of *A. nodosum* caused a reduction in P5CS1 and P5CS2 transcripts supporting our earlier observation of reduced proline content in the treated plants. No changes were observed in proline degrading gene, PDH due to extract treatment. Transcription factor Cor 15A was induced by extracts while it down regulated RD 29B expression.

Chemical components of *A. nodosum* reduces Na+ uptake in *Arabidopsis*

Plants exposed to high concentration of NaCl accumulate high concentration of Na+ in the tissue that leads to disruption of ionic balance and ultimately cellular function. We conducted an experiment to test if the methanol and ethyl acetate subfraction of *A. nodosum* affect the concentration of Na+ in the leaf tissue of *Arabidopsis* plants. Plant was treated with organic subfractions after 24 h exposure to 150mM NaCl. As expected, sodium content in leaves of NaCl treated plants (150 mM) increased by 84% in comparison to control plants that were not root irrigated with NaCl. Interestingly, treatment of plants with ethyl acetate and methanol fractions caused a decreased accumulation of Na+ in the leaves. Ethyl acetate subfraction was the most active, it reduced the concentration of Na+ in the leaf tissue by 53% while methanol subfraction caused a reduction of 25%. Moreover, ethyl acetate and methanol fraction treatments also

decreased potassium content by 56% and 26% respectively. On the other hand nitrogen and phosphorous content differed only slightly between untreated and treated controls (Figure 9).

Organic compounds of *A. nodosum* elicit global transcriptome changes in *Arabidopsis* leaves

To study the molecular mechanisms of *A. nodosum*-elicited salt tolerance in *Arabidopsis*, we performed global gene expression profiling on the ATH1 GeneChip platform. The ATH1 GeneChip consists of over 22500-probe sets representing nearly 90% of the *Arabidopsis* genome, thus providing a means to ascertain global transcriptional changes elicited by organic sub-fractions of *A. nodosum*. *Arabidopsis* was exposed to 150 NaCl for 24 hours, after which treatment consisted of methanol or ethyl acetate sub-fraction of *A. nodosum*.

Global Expression profile: The change in global transcriptome elicited by organic components in *A. nodosum* extract is depicted in Figure 10. Organic sub-fraction of *A. nodosum* caused significant changes in the transcription of a small subset of the genome, although the majority of transcripts remain unchanged by the treatment. There was little difference in the gene expression profile between the replicates within a treatment and a low *p* value and therefore, we used 1.5 fold level change as the cut off in our analysis. In the ethyl acetate sub-fraction treatment, 184 and 257 genes were up-regulated in day 1 and day 5, respectively. Only six genes were common in day 1 and 5. On the other hand, 91 and 262 genes were down-regulated on day 1 and day 5 by this treatment as illustrated in Venn diagrams (Figure 11). Annotations were done based on MIPS Functional category classifications and listed (Table 1 -4).

CATEGORY 1: Up-regulated genes in ethyl actetate subfraction treatment on day 1

Table 1 lists the genes that were up-regulated on day 1 of ethyl acetate sub-fraction treatment under 150 mM NaCl stress. Of the 184 genes that showed changes, the largest groups were annotated as involved in metabolism (27%), 16% were predicted to be involved in regulating gene expression i.e., transcription factors, 2.2% functions in abiotic stress response and 7.2 in cellular defense. Among all of gene responses, the transcript for late embryogenesis abundant 3 family protein / LEA3 family protein (AT1G02820) and myb-related transcription factor (CCA1; AT2G46830) was observed as the most strongly induced (2.731992 for LEA3; and 3.5 for CCA1). In the abiotic stress group, the genes that showed differential expression included: late embryogenesis abundant protein LEA group 1 (AT5g06760) and LEA 3 family (AT1G02820); drought-responsive protein (AT4g15910) and HVA 22d genes (AT4g24960). The synthesis of hydrophilic proteins is a major response to water-deficit conditions like salinity and drought. LEA proteins, first characterized in cotton during the late stages of seed embryogenesis are a

group of hydrophilic proteins and the encoding genes are ABA inducible. Previous studies report that LEA group 1 (AT5g06760) is regulated by ABA (Zalejski et al., 2006). LEA proteins play a protective role in the dry state and contribute to desiccation tolerance (reviewed by Oliver and Bewley, 1997; Kermode, 1997), although details of their mode of action are not yet clear. However, recent in vitro studies suggest that group I and group III LEA help prevent protein aggregation (Goyal et al., 2005). Also included in the abiotic stress category are other ABA inducible genes: HVA22d (AT4g24960) and Di21 (AT4g15910).

Table 1: Microarray data for selected genes induced by salinity stress in *Arabidopsis thaliana by* ethyl acetate extract treatment after Day 1 of treatment

Array Element	Locus Identifier	Fold (log_2)	Annotation
Abiotic stress			
262113_at	AT1G02820	**2.731992**	late embryogenesis abundant 3 family protein / LEA3 family protein
245523_at	AT4G15910	**1.664332**	drought-responsive protein / drought-induced protein (Di21)
250648_at	AT5G06760	**1.560875**	late embryogenesis abundant group 1 domain-containing protein / LEA group 1 domain-containing protein
254085_at	AT4G24960	**1.542183**	ABA-responsive protein (HVA22d)
Cellular organization and biogenesis			
252607_at	AT3G44990	**2.938349**	xyloglucan:xyloglucosyl transferase, putative / xyloglucan endotransglycosylase, putative / endo-xyloglucan transferase, putative
245465_at	AT4G16590	**2.862751**	glucosyltransferase-related
259736_at	AT1G64390	**1.546902**	endo-1,4-beta-glucanase, putative / cellulase, putative
258675_at	AT3G08770	**2.547661**	lipid transfer protein 6 (LTP6)
253815_at	AT4G28250	**1.841339**	beta-expansin, putative (EXPB3)
256924_at	AT3G29590	**1.739235**	transferase family protein AT5MAT; O-malonyltransferase/ transferase
254185_at	AT4G23990	**1.709051**	cellulose synthase family protein ATCSLG3 (Cellulose synthase-like G3); transferase/ transferase, transferring glycosyl groups
254773_at	AT4G13410	**1.653896**	glycosyl transferase family 2 protein
255524_at	AT4G02330	**1.583472**	pectinesterase family protein
267115_s_at	AT2G32540 AT2G32530	**1.516453**	cellulose synthase family protein /// cellulose synthase family protein [AT2G32540, ATCSLB04 (Cellulose synthase-like B4); transferase/ transferase, transferring glycosyl groups];[AT2G32530, ATCSLB03

			(Cellulose synthase-like B3); transferase/ transferase, transferring glycosyl groups]
256243_at	AT3G12500	**1.529772**	basic endochitinase ATHCHIB (BASIC CHITINASE); chitinase
Unknown genes			
265892_at	AT2G15020	**2.609756**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G64190.1); similar to hypothetical protein [Oryza sativa (japonica cultivar-group)] (GB:AAP46203.1)
256266_at	AT3G12320	**2.335075**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G06980.1); similar to ACI112 [Lycopersicon esculentum] (GB:AAY97870.1)
250292_at	AT5G13220	**2.280981**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G20900.1); similar to P0482D04.10 [Oryza sativa (japonica cultivar-group)] (GB:BAB89663.1); similar to Os04g0395800 [Oryza sativa (japonica cultivar-group)] (GB:NP_001052661.1); similar to Os10g0392400 [Oryza sativa (japonica cultivar-group)] (GB:NP_001064513.1); contains InterPro domain ZIM; (InterPro:IPR010399)
250665_at	AT5G06980	**1.977186**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G12320.1)
251727_at	AT3G56290	**1.897128**	expressed protein similar to Os01g0823600 [Oryza sativa (japonica cultivar-group)] (GB:NP_001044661.1); similar to unnamed protein product [Ostreococcus tauri] (GB:CAL58546.1)
247814_at	AT5G58310	**1.818424**	hydrolase, alpha/beta fold family protein hydrolase, alpha/beta fold family protein
251869_at	AT3G54500	**1.806031**	expressed protein similar to dentin sialophosphoprotein-related [Arabidopsis thaliana] (TAIR:AT5G64170.2); similar to conserved hypothetical protein [Medicago truncatula] (GB:ABD28297.1)
256096_at	AT1G13650	**1.766908**	expressed protein similar to 18S pre-ribosomal assembly protein gar2-related [Arabidopsis thaliana] (TAIR:AT2G03810.3); similar to hypothetical protein [Trypanosoma cruzi strain CL Brener] (GB:XP_813437.1)
262452_at	AT1G11210	**1.727922**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G11220.1); similar to fiber expressed protein [Gossypium hirsutum] (GB:AAY85179.1); similar to cotton fiber expressed protein 1 [Gossypium hirsutum] (GB:AAC33276.1); contains InterPro domain Protein of unknown function DUF761, plant; (InterPro:IPR008480)
248205_at	AT5G54300	**1.712183**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G61260.1); similar to Protein of unknown function DUF761, plant [Medicago truncatula] (GB:ABE84235.1);

			contains InterPro domain Protein of unknown function DUF761, plant; (InterPro:IPR008480)
247030_at	AT5G67210	**1.707944**	expressed protein nucleic acid binding / pancreatic ribonuclease
247463_at	AT5G62210	**1.707315**	embryo-specific protein-related
247214_at	AT5G64850	**1.698314**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G09960.1); similar to 80C09_15 [Brassica rapa subsp. pekinensis] (GB:AAZ41826.1)
249932_at	AT5G22390	**1.689324**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G19260.1); similar to hypothetical protein [Oryza sativa (japonica cultivar-group)] (GB:BAD28434.1); similar to Os12g0155100 [Oryza sativa (japonica cultivar-group)] (GB:NP_001066192.1)
264636_at	AT1G65490	**1.683121**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G65500.1)
252661_at	AT3G44450	**1.670119**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G52740.1); similar to H0717B12.8 [Oryza sativa (indica cultivar-group)] (GB:CAH67161.1)
250158_at	AT5G15190	**1.617973**	expressed protein unknown protein
266800_at	AT2G22880	**1.60535**	VQ motif-containing protein
261033_at	AT1G17380	**1.60292**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G72450.1); similar to PnFL-2 [Ipomoea nil] (GB:AAG49896.1); similar to Os07g0615200 [Oryza sativa (japonica cultivar-group)] (GB:NP_001060268.1); similar to Os03g0402800 [Oryza sativa (japonica cultivar-group)] (GB:NP_001050322.1); contains InterPro domain ZIM; (InterPro:IPR010399)
261247_at	AT1G20070	**1.602362**	expressed protein
260804_at	AT1G78410	**1.589149**	VQ motif-containing protein
247933_at	AT5G56980	**1.569768**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT4G26130.1); similar to cDNA-5-encoded protein (GB:AAA50235.1)
253165_at	AT4G35320	**1.569475**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT2G17300.1); similar to Os02g0715300 [Oryza sativa (japonica cultivar-group)] (GB:NP_001047925.1); similar to Os08g0511400 [Oryza sativa (japonica cultivar-group)] (GB:NP_001062213.1); contains domain N-terminal domain of cbl (N-cbl) (SSF47668)
255763_at	AT1G16730	**1.562988**	expressed protein
263796_at	AT2G24540 AT2G24545	**1.561418**	kelch repeat-containing F-box family protein
246125_at	AT5G19875	**1.558457**	expressed protein

			similar to oxidoreductase/ transition metal ion binding [Arabidopsis thaliana] (TAIR:AT2G31940.1); similar to conserved hypothetical protein [Medicago truncatula] (GB:ABE77569.1)
253943_at	AT4G27030	**1.555481**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G62190.1); similar to Os08g0187900 [Oryza sativa (japonica cultivar-group)] (GB:NP_001061153.1); similar to Ubiquitin-conjugating enzyme (ISS) [Ostreococcus tauri] (GB:CAL55480.1); contains domain UBIQUITIN-CONJUGATING ENZYME VARIANT 1 (PTHR11621:SF1); contains domain UBIQUITIN-CONJUGATING ENZYME E2 (PTHR11621)
249191_at	AT5G42760	**1.542773**	O-methyltransferase N-terminus domain-containing protein similar to hypothetical protein [Oryza sativa (japonica cultivar-group)] (GB:AAN65019.1); contains InterPro domain O-methyltransferase, N-terminal; (InterPro:IPR003455); contains InterPro domain Protein of unknown function Mtu_121; (InterPro:IPR011610)
257710_at	AT3G27350	**1.522115**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G40700.1); similar to Targeting for Xklp2 [Medicago truncatula] (GB:ABE84619.1)
249918_at	AT5G19240	**1.520507**	expressed protein Identical to Putative GPI-anchored protein At5g19240 precursor [Arabidopsis Thaliana] (GB:Q84VZ5;GB:Q8H7A4); similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G19230.1); similar to Os07g0645000 [Oryza sativa (japonica cultivar-group)] (GB:NP_001060451.1); similar to unknown protein [Oryza sativa (japonica cultivar-group)] (GB:BAC07018.1); contains domain PR-1-like (SSF55797)
249134_at	AT5G43150	**1.517847**	expressed protein similar to hypothetical protein MtrDRAFT_AC141109g4v1 [Medicago truncatula] (GB:ABE79896.1)
249011_at	AT5G44670	**1.514902**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT4G20170.1); similar to Os06g0328800 [Oryza sativa (japonica cultivar-group)] (GB:NP_001057533.1); similar to Os02g0712500 [Oryza sativa (japonica cultivar-group)] (GB:NP_001047907.1); similar to unknown protein [Oryza sativa (japonica cultivar-group)] (GB:BAD72474.1); contains InterPro domain Protein of unknown function DUF23; (InterPro:IPR008166)
254508_at	AT4G20170	**1.512831**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G44670.1); similar to Os06g0328800 [Oryza sativa (japonica cultivar-group)] (GB:NP_001057533.1); similar to Os02g0712500 [Oryza sativa (japonica cultivar-

			group)] (GB:NP_001047907.1); similar to unknown protein [Oryza sativa (japonica cultivar-group)] (GB:BAD72474.1); contains InterPro domain Protein of unknown function DUF23; (InterPro:IPR008166)
253425_at	AT4G32190	**1.512381**	centromeric protein-related
259927_at	AT1G75100	**1.506455**	expressed protein JAC1 (J-DOMAIN PROTEIN REQUIRED FOR CHLOROPLAST ACCUMULATION RESPONSE 1); heat shock protein binding
253891_at		**1.500037**	expressed protein similar to unknown protein [Arabidopsis thaliana] (TAIR:AT1G64650.1); similar to unknown protein [Arabidopsis thaliana] (TAIR:AT3G49310.1); similar to expressed protein [Oryza sativa (japonica cultivar-group)] (GB:ABF93637.1); similar to Os10g0519600 [Oryza sativa (japonica cultivar-group)] (GB:NP_001065080.1); similar to Major Facilitator Superfamily protein, expressed [Oryza sativa (japonica cultivar-group)] (GB:ABB47893.2); contains InterPro domain Protein of unknown function DUF791; (InterPro:IPR008509)
Metabolism			
264511_at	AT1G09350	**2.690889**	galactinol synthase, putative ATGOLS3 (ARABIDOPSIS THALIANA GALACTINOL SYNTHASE 3); transferase, transferring glycosyl groups / transferase, transferring hexosyl groups
261191_at	AT1G32900	**2.313856**	starch synthase, putative
266532_at	AT2G16890	**2.057935**	UDP-glucoronosyl/UDP-glucosyl transferase family protein
260727_at	AT1G48100	**2.017334**	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein
252011_	AT3G52720.1	**2.033333**	carbonic anhydrase family protein
249411_at	AT5G40390	**1.945845**	raffinose synthase family protein
251984_at	AT3G53260	**1.789189**	phenylalanine ammonia-lyase 2 (PAL2)
263845_at	AT2G37040	**1.55923**	phenylalanine ammonia-lyase 1 (PAL1)
258589_at	AT3G04290	**1.752056**	GDSL-motif lipase/hydrolase family protein ATLTL1/LTL1 (LI-TOLERANT LIPASE 1); carboxylic ester hydrolase
263841_at	AT2G36870	**1.725838**	xyloglucan:xyloglucosyl transferase, putative / xyloglucan endotransglycosylase, putative / endo-xyloglucan transferase, putative
247360_at	AT5G63450	**2.286131**	cytochrome P450, putative CYP94B1 (cytochrome P450, family 94, subfamily B, polypeptide 1); oxygen binding
252911_at	AT4G39510	**1.944384**	cytochrome P450 family protein CYP96A12 (cytochrome P450, family 96, subfamily A, polypeptide 12); oxygen binding
266246_at	AT2G27690	**1.821355**	cytochrome P450, putative CYP94C1 (cytochrome P450, family 94, subfamily C, polypeptide 1); oxygen binding
249215_at	AT5G42800	**2.829937**	dihydroflavonol 4-reductase (dihydrokaempferol

			4-reductase) (DFR)
245624_at	AT4G14090	**1.7226**	UDP-glucoronosyl/UDP-glucosyl transferase family protein
260955_at	AT1G06000	**1.712522**	UDP-glucoronosyl/UDP-glucosyl transferase family protein
250794_at	AT5G05270	**2.499514**	chalcone-flavanone isomerase family protein
250207_at	AT5G13930	**2.229782**	chalcone synthase / naringenin-chalcone synthase
252123_at	AT3G51240	**2.095258**	naringenin 3-dioxygenase / flavanone 3-hydroxylase (F3H) F3H (TRANSPARENT TESTA 6); naringenin 3-dioxygenase
250533_at	AT5G08640	**1.989595**	flavonol synthase 1 (FLS1)
249774_at	AT5G24150	**1.676203**	squalene monooxygenase 1,1 / squalene epoxidase 1,1 (SQP1,1)
266778_at	AT2G29090	**1.651743**	cytochrome P450 family protein
259681_at	AT1G77760	**1.566168**	nitrate reductase 1 (NR1)
251827_at	AT3G55120	**1.616842**	chalcone-flavanone isomerase / chalcone isomerase (CHI) TT5 (TRANSPARENT TESTA 5); chalcone isomerase
258613_at	AT3G02870	**1.581021**	inositol-1(or 4)-monophosphatase, putative / inositol monophosphatase, putative / IMPase, putative VTC4; 3'(2'),5'-bisphosphate nucleotidase/ inositol or phosphatidylinositol phosphatase
254662_at	AT4G18270	**1.615176**	glycosyl transferase family 4 protein ATTRANS11 (Arabidopsis thaliana translocase 11); catalytic
252363_at	AT3G48460	**1.61335**	GDSL-motif lipase/hydrolase family protein
245275_at	AT4G15210	**1.609439**	beta-amylase (BMY1) / 1,4-alpha-D-glucan maltohydrolase
248311_at	AT5G52570	**1.607389**	beta-carotene hydroxylase, putative BETA-OHASE 2 (BETA-CAROTENE HYDROXYLASE 2); beta-carotene hydroxylase
245734_at	AT1G73480	**1.59682**	hydrolase, alpha/beta fold family protein
250832_at	AT5G04950	**1.587492**	nicotianamine synthase, putative
250344_at	AT5G11930	**1.583021**	glutaredoxin family protein
264931_at	AT1G60590	**1.572077**	polygalacturonase, putative / pectinase, putative
256057_at	AT1G07180	**1.570594**	pyridine nucleotide-disulphide oxidoreductase family protein NDA1 (ALTERNATIVE NAD(P)H DEHYDROGENASE 1); NADH dehydrogenase
263433_at	AT2G22240	**1.553779**	inositol-3-phosphate synthase isozyme 2 / myo-inositol-1-phosphate synthase 2 / MI-1-P synthase 2 / IPS 2
259445_at	AT1G02400	**1.538629**	gibberellin 2-oxidase, putative / GA2-oxidase, putative ATGA2OX6/DTA1 (GIBBERELLIN 2-OXIDASE 6); gibberellin 2-beta-dioxygenase
246700_at	AT5G28030	**1.538054**	cysteine synthase, putative / O-acetylserine (thiol)-lyase, putative / O-acetylserine

			sulfhydrylase, putative
262039_at	AT1G80050	**1.537622**	adenine phosphoribosyltransferase 2 (APT2)
261907_at	AT1G65060	**1.535029**	4-coumarate--CoA ligase 3 / 4-coumaroyl-CoA synthase 3 (4CL3)
267429_at	AT2G34850	**1.533589**	NAD-dependent epimerase/dehydratase family protein MEE25 (maternal effect embryo arrest 25); catalytic
266391_at	AT2G41290	**1.532358**	strictosidine synthase family protein
249540_at	AT5G38120	**1.530133**	4-coumarate--CoA ligase family protein / 4-coumaroyl-CoA synthase family protein
245627_at	AT1G56600	**1.518096**	galactinol synthase, putative ATGOLS2 (ARABIDOPSIS THALIANA GALACTINOL SYNTHASE 2); transferase, transferring glycosyl groups / transferase, transferring hexosyl groups
247175_at	AT5G65280	**1.516934**	lanthionine synthetase C-like family protein
261914_at	AT1G65870	**1.514853**	disease resistance-responsive family protein
261046_at	AT1G01390	**1.513808**	UDP-glucoronosyl/UDP-glucosyl transferase family protein
246490_at	**AT5G15950.1**	**2.141977**	adenosylmethionine decarboxylase family protein
246468_at	AT5G17050	**1.902498**	UDP-glucoronosyl/UDP-glucosyl transferase family protein
Other genes			
256020_at	AT1G58290	**1.528062**	glutamyl-tRNA reductase 1 / GluTR (HEMA1)
264758_at	AT1G61340	**2.169349**	F-box family protein
265962_at	AT2G37460	**1.815725**	nodulin MtN21 family protein
248467_at	AT5G50800	**1.737688**	nodulin MtN3 family protein
264217_at	AT1G60190	**1.70939**	armadillo/beta-catenin repeat family protein / U-box domain-containing protein
249798_at	AT5G23730	**1.556476**	transducin family protein / WD-40 repeat family protein nucleotide binding
248961_at	AT5G45650	**1.535671**	subtilase family protein
266993_at	AT2G39210	**1.53321**	nodulin family protein
248676_at	AT5G48850	**1.517959**	male sterility MS5 family protein
253696_at	AT4G29740	**1.824141**	FAD-binding domain-containing protein / cytokinin oxidase family protein
256894_at	AT3G21870	**1.574575**	cyclin family protein CYCP2;1 (cyclin p2;1); cyclin-dependent protein kinase
Signal transduction			
264767_at	AT1G61380	**1.782612**	S-locus protein kinase, putative
248910_at	AT5G45820	**1.737011**	CBL-interacting protein kinase 20 (CIPK20)
248607_at	AT5G49480	**1.691496**	sodium-inducible calcium-binding protein (ACP1) /

			sodium-responsive calcium-binding protein (ACP1)
260774_at	AT1G78290	**1.66375**	serine/threonine protein kinase, putative
251054_at	AT5G01540	**1.603513**	lectin protein kinase, putative
248191_at	AT5G54130	**1.545963**	calcium-binding EF hand family protein
265030_at	AT1G61610	**1.533198**	S-locus lectin protein kinase family protein
261089_at	AT1G07570	**1.506941**	protein kinase (APK1a)
267546_at	AT2G32680	**1.502409**	disease resistance family protein
258119_at	AT3G14720	**1.500632**	mitogen-activated protein kinase, putative / MAPK, putative (MPK19)
TRANSCRIPTION FACTORS			
266719_at	AT2G46830	**3.590629**	myb-related transcription factor (CCA1)
261569_at	AT1G01060	**2.253258**	myb family transcription factor
263739_at	AT2G21320	**2.252043**	zinc finger (B-box type) family protein
266720_s_at	AT2G46670	**2.214092**	pseudo-response regulator, putative / timing of CAB expression 1-like protein, putative /// pseudo-response regulator, putative / timing of CAB expression 1-like protein, putative
257262_at	AT3G21890	**2.077132**	zinc finger (B-box type) family protein
253799_at	AT4G28140	**2.064143**	AP2 domain-containing transcription factor, putative
263252_at	AT2G31380	**1.991303**	zinc finger (B-box type) family protein / salt tolerance-like protein (STH)
254066_at	AT4G25480	**1.963332**	DRE-binding protein (DREB1A) / CRT/DRE-binding factor 3 (CBF3)
250099_at	AT5G17300	**1.902815**	myb family transcription factor
249769_at	AT5G24120	**1.860477**	RNA polymerase sigma subunit SigE (sigE) / sigma-like factor (SIG5)
254075_at	AT4G25470	**1.845192**	DRE-binding protein (DREB1C) / CRT/DRE-binding factor 2 (CBF2)
259466_at	AT1G19050	**1.814394**	two-component responsive regulator / response regulator 7 (ARR7)
258497_at	AT3G02380	**1.772366**	zinc finger protein CONSTANS-LIKE 2 (COL2)
266820_at	AT2G44940	**1.718757**	AP2 domain-containing transcription factor TINY, putative
256185_at	AT1G51700	**1.676723**	Dof-type zinc finger domain-containing protein (ADOF1)
251665_at	AT3G57040	**1.663682**	two-component responsive regulator / response reactor 4 (RR4)
265418_at	AT2G20880	**1.625219**	AP2 domain-containing transcription factor, putative
255016_at	AT4G10120	**1.624597**	sucrose-phosphate synthase, putative
252917_at	AT4G38960	**1.610185**	zinc finger (B-box type) family protein
245078_at	AT2G23340	**1.593763**	AP2 domain-containing transcription factor,

			putative
263664_at	AT1G04250	**1.591111**	auxin-responsive protein / indoleacetic acid-induced protein 17 (IAA17)
264057_at	AT2G28550	**1.589255**	AP2 domain-containing transcription factor RAP2.7 (RAP2.7)
250598_at	AT5G07690	**1.584711**	myb family transcription factor (MYB29)
246523_at	AT5G15850	**1.582355**	zinc finger protein CONSTANS-LIKE 1 (COL1)
248246_at	AT5G53200	**1.573483**	myb family transcription factor (TRIPTYCHON)
261375_at	AT1G53160	**1.570211**	squamosa promoter-binding protein-like 4 (SPL4)
252214_at	AT3G50260	**1.558081**	AP2 domain-containing transcription factor, putative
264692_at	AT1G70000	**1.543164**	DNA-binding family protein
253411_at	AT4G32980	**1.535375**	homeobox protein (ATH1)
CELLULAR RESCUE AND DEFENSE			
261037_at	AT1G17420	**2.157759**	lipoxygenase, putative
263384_at	AT2G40130	**1.938878**	heat shock protein-related
250083_at	AT5G17220	**1.909543**	glutathione S-transferase, putative
260399_at	AT1G72520	**1.737015**	lipoxygenase, putative
266992_at	AT2G39200	**1.694233**	seven transmembrane MLO family protein / MLO-like protein 12 (MLO12)
253104_at	AT4G36010	**1.655617**	pathogenesis-related thaumatin family protein
258791_at	AT3G04720	**1.63341**	hevein-like protein (HEL)
252921_at	AT4G39030	**1.623038**	enhanced disease susceptibility 5 (EDS5) / salicylic acid induction deficient 1 (SID1)
261150_at	AT1G19640	**1.606685**	S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT)
257644_at	AT3G25780	**1.58864**	allene oxide cyclase, putative / early-responsive to dehydration protein, putative / ERD protein, putative
253496_at	AT4G31870	**1.583689**	glutathione peroxidase, putative
260831_at	AT1G06830	**1.511424**	glutaredoxin family protein
261958_at	AT1G64500	**1.846779**	glutaredoxin family protein
TRANSPORTERS			
261881_at	AT1G80760	**2.267071**	major intrinsic family protein / MIP family protein
264400_at	AT1G61800	**2.264749**	glucose-6-phosphate/phosphate translocator, putative
256751_at	AT3G27170	**2.069786**	chloride channel protein (CLC-b)
262883_at	AT1G64780	**2.039022**	ammonium transporter 1, member 2 (AMT1.2)
252414_at	AT3G47420	**2.003536**	glycerol-3-phosphate transporter, putative / glycerol 3-phosphate permease, putative
263918_at	AT2G36590	**1.808529**	proline transporter, putative

260676_at	AT1G19450	**1.772287**	integral membrane protein, putative / sugar transporter family protein
255877_at	AT2G40460	**1.667473**	proton-dependent oligopeptide transport (POT) family protein
249765_at	AT5G24030	**1.659875**	C4-dicarboxylate transporter/malic acid transport family protein
259185_at	AT3G01550	**1.640112**	triose phosphate/phosphate translocator, putative
262526_at	AT1G17050	**1.632548**	geranyl diphosphate synthase, putative / GPPS, putative / dimethylallyltransferase, putative / prenyl transferase, putative
256336_at	AT1G72030	**1.627288**	GCN5-related N-acetyltransferase (GNAT) family protein
246310_at	AT3G51895	**1.621192**	sulfate transporter (ST1)
250926_at	AT5G03555	**1.589828**	permease, cytosine/purines, uracil, thiamine, allantoin family protein
258293_at	AT3G23430	**1.535768**	phosphate transporter, putative (PHO1)
Energy			
265722_at	AT2G40100	**1.716622**	chlorophyll A-B binding protein (LHCB4.3)
245242_at	AT1G44446	**1.630978**	chlorophyll a oxygenase (CAO) / chlorophyll b synthase
258321_at	AT3G22840	**2.604578**	chlorophyll A-B binding family protein / early light-induced protein (ELIP)

Genes involved in cellular organization and biogenesis like lipid transfer family protein LTP6 (AT3g08770); endo-1,4-beta-glucanase, putative / cellulases (AT1g64390) were induced by this treatment. In the Metabolism group, ethyl acetate sub-fraction treatment activated increased levels of myo-inositol-1-phosphate synthase 2 (AT2g22240) transcripts. Galactinol sythetase genes ATGOLS3 (AT1g09350); ATGOLS2 (AT1g56600) were also up-regulated. Thus, two families of genes that function in the biosynthesis of raffinose oligosaccharide (Myoinositol and Galactinol synthetases) were upregulated by *A. nodosum* extract. Furthermore, raffinose synthase (AT5g40390) was also up-regulated.

Interestingly, several genes in phenylpropanoid pathway, especially flavonoid synthesis were up-regulated. This included Phenylalanine ammonia lyase 1 (PAL1) and PAL2 (that catalyzes the conversion of phenylalanine to cinnamate); chalcone synthase (CHS), (required for the condensation of 4-coumaroyl-CoA and malonyl-CoA to yield naringenin chalcone); chalcone isomerase (CHI); flavonoid 3'-hydroxylase (F3'H), and dihydroflavonol 4-reductase (DFR). Flavonoids are a diverse group of secondary metabolites with a wide array of biological functions like pigmentation, facilitators of plant-microbe interaction, and reproduction. Flavonoids have also been linked to defense responses against biotic and abiotic stresses, such as

pathogens, wounding, and UV light damage. The exact role of flavonoids in salinity stress tolerance is unclear. However, it is possible that flavonoid secondary metabolites will alleviate oxidative stress imposed under high salt concentration.

The transcription factors up-regulated in this category were mainly: zinc finger, myb transcription factors, AP2 domain containing transcription factor. Transcription factors DRE-binding protein (DREB1A) / CRT/DRE-binding factor 3 (CBF3) and DRE-binding protein (DREB1C) / CRT/DRE-binding factor 2 (CBF2) were significantly induced by ethyl acetate sub fraction. The DREB/CBF pathway has been established to be the converging point of NaCl, drought and freezing stress signaling. In the cellular rescue and defense group, glutathione S-transferase (AT5g172200) was shown to be up-regulated.

CATEGORY 2: Up-regulated genes in ethyl actetate sub-fraction treatment on day 5

Category 2 included 257 genes (Table 2) that were up-regulated on day 5 of ethyl acetate sub-fraction treatment. Interestingly, on day 5 of the treatment, the proportion of abiotic stress regulated genes increased to 6.0%. On the other hand, the percentage of genes under transcription factors group decreased on day 5 of the treatment (6.5%) in comparison to day 1 of the treatment. Genes that were up-regulated on day 5 are listed in Table 2. Several group 1 LEA and Group 2 LEA proteins (dehydrins). An ABA induced stress regulation gene, AtHVA22b (AT5g62490), was induced by ethyl acetate sub fraction of *A. nodosum*. Similarly, Di21 (AT4g15910) and ABA responsive protein-related was also up-regulated. Overall, these genes are involved in abiotic stress and ABA dependent.

Table 2: Microarray data for selected genes induced by salinity stress in *Arabidopsis thaliana* by ethyl acetate extract treatment after Day 5 of treatment

Array Element	Locus Identifier	Fold (log_2)	Annotation
ABIOTIC STRESS			
258347_at	At3g17520	15.01491	late embryogenesis abundant domain-containing protein / LEA domain-containing protein
248352_at	AT5G52300	6.511906	low-temperature-responsive 65 kD protein (LTI65) / desiccation-responsive protein 29B (RD29B)
262128_at	AT1G52690	6.839651	late embryogenesis abundant protein, putative / LEA protein, putative
250648_at	AT5G06760	6.689409	late embryogenesis abundant group 1 domain-containing protein / LEA group 1 domain-containing protein
266544_at	AT2G35300	3.490015	late embryogenesis abundant group 1 domain-containing protein / LEA group 1 domain-containing protein

245523_at	AT4G15910	4.048004	drought-responsive protein / drought-induced protein (Di21)
264953_at	AT1G77120	2.547603	alcohol dehydrogenase (ADH)
247437_at	AT5G62490	2.470057	ABA-responsive protein (HVA22b)
258224_at	AT3G15670	1.982609	late embryogenesis abundant protein, putative / LEA protein, putative
263157_at	AT1G54100	1.925271	aldehyde dehydrogenase, putative / antiquitin, putative
256464_at	AT1G32560	1.640385	late embryogenesis abundant group 1 domain-containing protein / LEA group 1 domain-containing protein
263492_at	AT2G42560	1.765456	late embryogenesis abundant domain-containing protein / LEA domain-containing protein
252137_at	AT3G50980.1	4.197867	dehydrin, putative
247095_at	AT5G66400	3.928469	dehydrin (RAB18)
258498_at	AT3G02480	7.061576	ABA-responsive protein-related
253120_at	AT4G35790	1.80686	phospholipase D delta / PLD delta (PLDDELTA)
Cellular Organization and Biogenesis			
253344_at	AT4G33550	4.672816	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
266098_at	AT4G15910	3.967902	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
247718_at	AT5G59310.1	3.581127	lipid transfer protein 4 (LTP4)
265111_at	AT1G62510.1	2.871597	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
257066_at	AT3G18280	1.718598	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
247717_at	AT5G59320	1.545822	lipid transfer protein 3 (LTP3)
254189_at	AT4G24000.1	3.760111	cellulose synthase family protein
264514_at	AT1G09500	2.724271	cinnamyl-alcohol dehydrogenase family / CAD family
255787_at	AT2G33590	2.152222	cinnamoyl-CoA reductase family
260568_at	AT2G43570.1	2.559195	chitinase, putative
260561_at	AT2G43580	2.334174	chitinase, putative
247866_at	AT5G57550	2.133806	xyloglucan:xyloglucosyl transferase / xyloglucan endotransglycosylase / endo-xyloglucan transferase (XTR3)
260560_at	AT2G43590	1.66276	chitinase, putative
249767_at	AT5G24090.1	2.495187	acidic endochitinase (CHIB1)
ENERGY			
248236_at	AT5G53870.1	3.484386	plastocyanin-like domain-containing protein
262347_at	AT1G64110	2.870212	AAA-type ATPase family protein
Metabolism			
253373_at	AT4G33150.1	4.983652	lysine-ketoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme
256746_at	AT3G29320	1.508099	glucan phosphorylase, putative
254806_at	AT4G12430	1.828979	trehalose-6-phosphate phosphatase, putative

251137_at	AT5G01300.1	3.880385	phosphatidylethanolamine-binding family protein
250891_at	AT5G04530.1	3.798344	beta-ketoacyl-CoA synthase family protein
266690_at	AT2G19900	3.346125	malate oxidoreductase, putative
252983_at	AT4G37980	1.598255	mannitol dehydrogenase, putative (ELI3-1)
251100_at	AT5G01670	1.696478	aldose reductase, putative
257881_at	AT3G17180	2.430443	serine carboxypeptidase S10 family protein
266278_at	AT2G29300	2.380858	tropinone reductase, putative / tropine dehydrogenase, putative
251191_at	AT3G62590	2.129764	lipase class 3 family protein
253224_at	AT4G34860.1	2.051706	beta-fructofuranosidase, putative / invertase, putative / saccharase, putative / beta-fructosidase, putative
251642_at	AT3G57520	2.128627	alkaline alpha galactosidase, putative
264524_at	AT1G10070	1.955038	branched-chain amino acid aminotransferase 2 / branched-chain amino acid transaminase 2 (BCAT2)
265199_s_at	AT2G36780	1.738282	UDP-glucoronosyl/UDP-glucosyl transferase family protein /// UDP-glucoronosyl/UDP-glucosyl transferase family protein
263986_at	AT2G42790	1.596132	citrate synthase, glyoxysomal, putative
251668_at	AT3G57010	5.23744	strictosidine synthase family protein
252068_at	AT3G51440.1	2.574864	strictosidine synthase family protein
250859_at	AT5G04660	1.711302	cytochrome P450, putative
266778_at	AT2G29090	1.639061	cytochrome P450 family protein
259058_at	AT3G03470	1.632459	cytochrome P450, putative
257129_at	AT3G20100	1.557462	cytochrome P450 family protein
255521_at	AT4G02280	3.744142	sucrose synthase, putative / sucrose-UDP glucosyltransferase, putative
262047_at	AT1G80160.1	3.467434	lactoylglutathione lyase family protein / glyoxalase I family protein
259054_at	AT3G03480	2.614822	transferase family protein
258374_at	AT3G14360	2.48336	lipase class 3 family protein
263127_at	AT1G78610	2.40754	Mechano sensitive ion channel domain-containing protein / MS ion channel domain-containing protein
267496_at	AT2G30550	1.663507	lipase class 3 family protein
262122_at	AT1G02790	1.63906	exopolygalacturonase / galacturan 1,4-alpha-galacturonidase (PGA3) / pectinase
SIGNAL Transduction			
252872_at	AT4G40010.1	4.47483	serine/threonine protein kinase, putative
249771_at	AT5G24080.1	3.590328	protein kinase family protein

246922_at	AT5G25110.1	2.592862	CBL-interacting protein kinase 25 (CIPK25)
258652_at	AT3G09910	2.277888	Ras-related GTP-binding protein, putative
264783_at	AT1G08650	1.830171	phosphoenolpyruvate carboxylase kinase
255984_at	AT1G34120	1.515543	inositol polyphosphate 5-phosphatase I (IP5PI)
TRANSCRIPTION FACTORS			
249117_at	AT5G43840.1	4.47483	heat shock transcription factor family protein
260097_at	AT1G73220.1	2.520324	sugar transporter family protein
259618_at	AT1G48000	2.411674	myb family transcription factor
263158_at	AT1G54160.1	2.242688	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein
263128_at	AT1G78600	2.14162	zinc finger (B-box type) family protein
250287_at	AT5G13330	2.109079	AP2 domain-containing transcription factor family protein
264510_at	AT1G09530	2.021174	phytochrome interacting factor 3 (PIF3)
258044_at	AT3G21270	2.009734	Dof-type zinc finger domain-containing protein (ADOF2)
264957_at	AT1G77000	1.851241	F-box family protein
260784_at	AT1G06180	1.806539	myb family transcription factor
255585_at	AT4G01550	1.704045	no apical meristem (NAM) family protein
251084_at	AT5G01520	1.557462	zinc finger (C3HC4-type RING finger) family protein
252408_at	AT3G47600	1.541716	myb family transcription factor (MYB94)
256806_at	AT3G20910	1.523143	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein
267010_at	AT2G39250	1.512621	AP2 domain-containing transcription factor, putative
CELLULAR RESCUE and DEFENSE			
247435_at	AT5G62480.1	3.861172	glutathione S-transferase, putative
263374_at	AT2G20560	2.257785	DNAJ heat shock family protein
261285_at	AT1G35720	2.116412	annexin 1 (ANN1)
258158_at	AT3G17790	1.92037	acid phosphatase type 5 (ACP5)
266294_at	AT2G29500	1.583569	17.6 kDa class I small heat shock protein (HSP17.6B-CI)
249575_at	AT5G37670	1.529129	15.7 kDa class I-related small heat shock protein-like (HSP15.7-CI)
249850_at	AT5G23240	1.566699	DNAJ heat shock N-terminal domain-containing protein
265480_at	AT2G15970	1.665163	cold-acclimation protein, putative (FL3-5A3)
TRANSPORTERS			
267080_at	AT2G41190.1	2.617936	amino acid transporter family protein
250506_at	AT5G09930	2.152955	ABC transporter family protein

256757_at	AT3G25620	1.538323	ABC transporter family protein
260163_at	AT1G79900	2.061735	mitochondrial substrate carrier family protein
250161_at	AT5G15240	2.047027	amino acid transporter family protein
245769_at	AT1G30220	1.954904	sugar transporter family protein
264338_at	AT1G70300	1.964731	potassium transporter, putative
256022_at	AT1G58360	1.512825	amino acid permease I (AAP1)
OTHER GENES			
255048_at	AT4G09600	4.802326	gibberellin-regulated protein 3 (GASA3) / gibberellin-responsive protein 3
245982_at	AT5G13170	4.543002	nodulin MtN3 family protein
256789_at	AT3G13672	2.546247	seven in absentia (SINA) family protein
257271_at	AT3G28007	2.408079	nodulin MtN3 family protein
245703_at	AT5G04380	1.898557	S-adenosyl-L-methionine:carboxyl methyltransferase family protein
248676_at	AT5G48850	1.749158	male sterility MS5 family protein
255575_at	AT4G01430	1.73882	nodulin MtN21 family protein
257893_at	AT3G17000	1.732926	ubiquitin-conjugating enzyme, putative
249979_s_at	AT5G18860	1.733286	inosine-uridine preferring nucleoside hydrolase family protein /// inosine-uridine preferring nucleoside hydrolase family protein
255860_at	AT5G34940	1.710485	glycosyl hydrolase family 79 N-terminal domain-containing protein
264729_at	AT1G22990.1	3.242062	heavy-metal-associated domain-containing protein / copper chaperone (CCH)-related
249917_at	AT5G22460.1	3.21146	esterase/lipase/thioesterase family protein
266462_at	AT2G47770.1	3.070209	benzodiazepine receptor-related
247128_at	AT5G66110	2.74753	heavy-metal-associated domain-containing protein
245076_at	AT2G23170	2.689231	auxin-responsive GH3 family protein
250293_s_at	AT5G13370	1.555959	auxin-responsive GH3 family protein /// auxin-responsive GH3 family protein
261768_at	AT1G15550	2.017634	gibberellin 3-beta-dioxygenase / gibberellin 3 beta-hydroxylase (GA4)
256601_s_at	AT3G28290.1	2.251977	integrin-related protein 14a /// integrin-related protein 14a
267429_at	AT2G34850	1.973366	NAD-dependent epimerase/dehydratase family protein
253172_at	AT4G35060	1.936296	heavy-metal-associated domain-containing protein / copper chaperone (CCH)-related
265796_at	AT2G35730	1.705875	heavy-metal-associated domain-containing protein
262644_at	AT1G62710	1.595388	vacuolar processing enzyme beta / beta-VPE
246861_at	AT5G25890	1.513648	auxin-responsive protein / indoleacetic acid-induced protein 28 (IAA28)
Unknown genes			
247061_at	AT5G66780	7.944997	expressed protein

254823_at	AT4G12580	5.4238	expressed protein
263881_at	AT2G21820.1	3.254599	expressed protein
255527_at	AT4G02360	3.217197	expressed protein
253401_at	AT4G32870	2.85058	expressed protein
249454_at	AT5G39520	2.444388	expressed protein

We also observed an increase in the expression of phospholipase D delta (AT4g35790). Phospholipase D catalyses the hydrolysis of a structural phospholipid, phosphatidylcholine (PtdCho), and other phospholipids, to form phosphatidic acid (PtdOH) (Liscovitch et al., 2000).

Many of the genes involved in cellular biogenesis, mainly Lipid transfer proteins were induced. (LTPs) are small, abundant basic proteins in higher plants. Ethyl acetate subfraction treatment induced transcription of a number of LTPs (AT4g33550; AT4g15910; AT5g59310.1AT1G62510.1; AT3g18280; AT5g59320). LTPS function by binding fatty acids and by transferring phospholipids between membranes *in vitro*. Other genes that were induced include GST, Annexin, Glutathione S-transferases, transcription factors like RING zinc finger proteins, MYBs and rd29B.

CATEGORY 3: Down-regulated genes in ethyl actetate subfraction treatment on day 1

Genes that were down-regulated by ethyl acetate fraction on day 1 are listed in Table 3. A number of cellular organization and biogenesis genes were repressed that include cellulose synthase family protein (AT1G55850; AT4G24000); xyloglucan:xyloglucosyl transferase and invertase/pectin methylesterase inhibitor family protein (AT1g62760). A group of genes encoding xyloglucan:xyloglucosyl transferase are also present which are responsible for cell-wall construction in plants. Both these groups of genes are down-regulated by ethyl acetate subfraction treatment. Besides, an auxin-responsive gene (AT2g23170) and several heat shock proteins were also repressed.

Table 3: Microarray data for selected genes repressed by salinity stress in *Arabidopsis thaliana by* ethyl acetate extract treatment after Day 1 of treatment.

Array Element	**Locus Identifier**	**Fold (log_2)**	**Annotation**

CELLULAR ORGANIZATION AND BIOGENESIS			
260592_at	AT1G55850	**0.6467134**	cellulose synthase family protein
254189_at	AT4G24000	**0.6013953**	cellulose synthase family protein
247866_at	AT5G57550	**0.4196655**	xyloglucan:xyloglucosyl transferase / xyloglucan endotransglycosylase / endo-xyloglucan transferase (XTR3)
262640_at	AT1G62760	**0.4770471**	invertase/pectin methylesterase inhibitor family protein
METABOLISM			
257216_at	AT3G14990	**0.658962**	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein, putative
261081_at	AT1G07350	**0.6519919**	transformer serine/arginine-rich ribonucleoprotein, putative
245076_at	AT2G23170	**0.6454968**	auxin-responsive GH3 family protein
259418_at	AT1G02390	**0.6417782**	phospholipid/glycerol acyltransferase family protein
260567_at	AT2G43820	**0.6411763**	UDP-glucoronosyl/UDP-glucosyl transferase family protein
249869_at	AT5G23050	**0.6395717**	acyl-activating enzyme 17 (AAE17)
256598_at	AT3G30180	**0.6338174**	cytochrome P450, putative
258063_at	AT3G14620	**0.6321929**	cytochrome P450, putative
247348_at	AT5G63810	**0.6255499**	beta-galactosidase, putative / lactase, putative
258336_at	AT3G16050	**0.6242512**	stress-responsive protein, putative
253101_at	AT4G37430,	**0.6178195**	cytochrome P450 81F1 (CYP81F1) (CYP91A2)
259694_at	AT1G63180	**0.5897521**	UDP-glucose 4-epimerase, putative / UDP-galactose 4-epimerase, putative / Galactowaldenase, putative
248918_at	AT5G45890	**0.587878**	senescence-specific SAG12 protein (SAG12) / cysteine proteinase, putative
266578_at	AT2G23910	**0.5510421**	cinnamoyl-CoA reductase-related
254764_at	AT4G13250	**0.5501392**	short-chain dehydrogenase/reductase (SDR) family protein
255943_at	AT1G22370	**0.5122407**	UDP-glucoronosyl/UDP-glucosyl transferase family protein
SIGNAL TRANSDUCTION			
266743_at	AT2G02990	**0.6475001**	ribonuclease 1 (RNS1)
248429_at	AT5G51770	**0.6437611**	protein kinase family protein
251432_at	AT3G59820	**0.6388801**	calcium-binding mitochondrial protein-related
256965_at	AT3G13450	**0.6259579**	2-oxoisovalerate dehydrogenase / 3-methyl-2-oxobutanoate

			dehydrogenase / branched-chain alpha-keto acid dehydrogenase E1 beta subunit (DIN4)
248046_at	AT5G56040	**0.5736343**	leucine-rich repeat protein kinase, putative
246028_at	AT5G21170	**0.5517346**	5'-AMP-activated protein kinase beta-2 subunit, putative
TRANSCRIPTION FACTORS			
251575_at	AT3G58120	**0.6544735**	bZIP transcription factor family protein
260431_at	AT1G68190	**0.633786**	zinc finger (B-box type) family protein
249234_at	AT5G42200	**0.6324005**	zinc finger (C3HC4-type RING finger) family protein
249862_at	AT5G22920	**0.631574**	zinc finger (C3HC4-type RING finger) family protein
252475_s_at	AT5G59570	**0.6210956**	myb family transcription factor /// myb family transcription factor
258133_at	AT3G24500	**0.6148897**	ATMBF1C/MBF1C (MULTIPROTEIN BRIDGING FACTOR 1C); DNA binding / transcription coactivator/ transcription factor
266839_at	AT2G25930	**0.6140176**	ELF3 (EARLY FLOWERING 3)
248606_at	AT5G49450	**0.5904502**	bZIP family transcription factor
260266_at	AT1G68520	**0.5843768**	zinc finger (B-box type) family protein
261265_at	AT1G26800	**0.5620626**	zinc finger (C3HC4-type RING finger) family protein
252367_at	AT3G48360	**0.3535679**	BT2 (BTB and TAZ domain protein 2); protein binding / transcription regulator
259244_at	AT3G07650	**0.3175566**	COL9 (CONSTANS-LIKE 9); transcription factor/ zinc ion binding
257985_at	AT3G20810	**0.2453973**	transcription factor jumonji (jmjC) domain-containing protein
CELLULAR RESCUE AND DEFENSE			
263374_at	AT2G20560	**0.6543058**	DNAJ heat shock family protein
265471_at	AT2G37130	**0.6189507**	peroxidase 21 (PER21) (P21) (PRXR5)
249850_at	AT5G23240	**0.6070001**	DNAJ heat shock N-terminal domain-containing protein
256245_at	AT3G12580	**0.5724005**	heat shock protein 70, putative / HSP70, putative
248332_at	AT5G52640	**0.5616923**	heat shock protein 81-1 (HSP81-1) / heat shock protein 83 (HSP83)
258957_at	AT3G01420	**0.4513215**	pathogen-responsive alpha-dioxygenase, putative
248045_at	AT5G56030	**0.6028267**	heat shock protein 81-2 (HSP81-2)
UNKNOWN GENES			
253630_at	AT4G30490	**0.6661879**	AFG1-like ATPase family protein

254574_at	AT4G19430	**0.6644498**	expressed protein
267178_at	AT2G37750	**0.6620409**	expressed protein
261608_at	AT1G49650	**0.6582341**	cell death associated protein-related
263210_at	AT1G10585	**0.6547906**	expressed protein
260933_at	AT1G02470	**0.6540852**	expressed protein
265478_at	AT2G15890	**0.6520669**	expressed protein
258647_at	AT3G07870	**0.649577**	F-box family protein
245433_at	AT4G17110	**0.6486495**	expressed protein
247443_at	AT5G62720	**0.6427957**	integral membrane HPP family protein
265387_at	AT2G20670	**0.6365452**	expressed protein
260688_at	AT1G17665	**0.629737**	expressed protein
262229_at	AT1G68620	**0.6251304**	expressed protein
249454_at	AT5G39520	**0.6247977**	expressed protein
245434_at	AT4G17120	**0.621736**	expressed protein
253322_at	AT4G33980	**0.6192145**	expressed protein
258262_at	AT3G15770	**0.6189757**	expressed protein
247293_at	AT5G64510	**0.6097934**	expressed protein
249190_at	AT5G42750	**0.588055**	expressed protein
249377_at	AT5G40690	**0.5853797**	expressed protein
253874_at	AT4G27450	**0.5333842**	expressed protein
249174_at	AT5G42900	**0.5323932**	expressed protein
258939_at	AT3G10020	**0.5271035**	expressed protein
258225_at	AT3G15630	**0.5155414**	expressed protein
249923_at	AT5G19120	**0.4857222**	expressed protein
267364_at	AT2G40080	**0.4846057**	expressed protein
251293_at	AT3G61930	**0.4634457**	expressed protein
257615_at	AT3G26510	**0.5881068**	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein
259382_s_at	AT3G16430	**0.5787531**	jacalin lectin family protein /// jacalin lectin family protein
259502_at	AT1G15670	**0.5692633**	kelch repeat-containing F-box family protein
260101_at	AT1G73260	**0.5661098**	trypsin and protease inhibitor family protein / Kunitz family protein

256060_at	AT1G07050	**0.5603096**	CONSTANS-like protein-related
253024_at	AT4G38080	**0.5514374**	hydroxyproline-rich glycoprotein family protein
267238_at	AT2G44130	**0.5333408**	kelch repeat-containing F-box family protein
249174_at	AT5G42900	**0.5323932**	expressed protein
245668_at	AT1G28330	**0.377387**	dormancy-associated protein, putative (DRM1)
OTHER GENES			
259037_at	AT3G09350	**0.6384012**	armadillo/beta-catenin repeat family protein
250217_at	AT5G14120	**0.633485**	nodulin family protein
245136_at	AT2G45210	**0.6271569**	auxin-responsive protein-related
255345_at	AT4G04460	**0.6041092**	aspartyl protease family protein
260427_at	AT1G72430	**0.5536881**	auxin-responsive protein-related
252698_at	AT3G43670	**0.5290776**	copper amine oxidase, putative
267461_at	AT2G33830	**0.5287762**	dormancy/auxin associated family protein
246114_at	AT5G20250	**0.5174247**	raffinose synthase family protein / seed imbibition protein, putative (din10)
247668_at	AT5G60100	**0.4680666**	pseudo-response regulator 3 (APRR3)
245076_at	AT2G23170	**0.6454968**	auxin-responsive GH3 family protein

CATEGORY 4: Down-regulated genes in ethyl actetate sub-fraction treatment on day 5

The classification of repressed genes on day 5 of ethyl acetate fraction treatment. Notably, only a small subset of abiotic factors (1.6%) was down-regulated. Table 4 lists the genes that were down-regulated by ethyl acetate fraction treatment on day 5. Many of the abiotic stress genes, including AT4g19120; AT3g30775 were down-regulated. It has been shown that reciprocal regulation of P5CS and PDH genes appears to regulate the concentration of proline under osmotic stress. The induction of PDH by proline, however, was inhibited by salt stress (Peng, Z et al., 1996). In the cellular biogenesis group, cellulose synthetase and a few pectinesterases were inhibited. Similarly, transcripts of a wall-associated kinase (WAK1), RNA binding proteins AT5G61030 and AT4G39260 were also reduced.

Table 4: Microarray data for selected genes repressed by salinity stress in *Arabidopsis thaliana by* ethyl acetate extract treatment after Day 5 of treatment

Array Element	Locus Identifier	Fold (log_2)	Annotation
ABIOTIC STRESS			
254563_at	AT4G19120	**0.647701**	early-responsive to dehydration stress protein (ERD3)
257315_at	AT3G30775	**0.5572566**	proline oxidase, mitochondrial / osmotic stress-responsive proline dehydrogenase (POX) (PRO1) (ERD5)
245736_at	AT1G73330	**0.5882807**	protease inhibitor, putative (DR4)
Cellular Organization and Biogenesis			
254185_at	AT4G23990	**0.6028183**	cellulose synthase family protein
258750_at	AT3G05910	**0.64114**	pectinacetylesterase, putative
261055_at	AT1G01300	**0.6151036**	aspartyl protease family protein
248419_at	AT5G51550	**0.5840732**	phosphate-responsive 1 family protein
258764_at	AT3G10720	**0.5772374**	pectinesterase, putative
262225_at	AT1G53840	**0.6164894**	pectinesterase family protein ENERGY
259507_at	AT1G43910	**0.5697036**	AAA-type ATPase family protein
Metabolism			
266338_at	AT2G32400	**0.6572218**	glutamate receptor family protein (GLR3.7) (GLR5)
259763_at	AT1G77630	**0.6472076**	peptidoglycan-binding LysM domain-containing protein
262414_at	AT1G49430	**0.6407058**	long-chain-fatty-acid--CoA ligase / long-chain acyl-CoA synthetase
264339_at	AT1G70290	**0.6406543**	trehalose-6-phosphate synthase, putative
248404_at	AT5G51460	**0.5753056**	trehalose-6-phosphate phosphatase (TPPA
264956_at	AT1G76990	**0.5620685**	ACT domain containing protein
254687_at	AT4G13770	**0.6489285**	cytochrome P450 family protein
262793_at	AT1G13110	**0.6155948**	cytochrome P450 71B7 (CYP71B7)
246949_at	AT5G25140	**0.6032137**	cytochrome P450 family protein
266996_at	AT2G34490	**0.5750135**	cytochrome P450 family protein
245676_at	AT1G56670	**0.6613705**	GDSL-motif lipase/hydrolase family protein
267162_s_at	AT2G37690	**0.6585301**	phosphoribosylaminoimidazole carboxylase family protein / AIR carboxylase family protein /// phosphoribosylaminoimidazole carboxylase family protein / AIR carboxylase family protein

267126_s_at	AT2G23600	**0.6117982**	hydrolase, alpha/beta fold family protein /// hydrolase, alpha/beta fold family protein
254835_s_at	AT4G12320	**0.6572362**	cytochrome P450, putative /// cytochrome P450, putative
254163_s_at	AT4G24340	**0.6179798**	phosphorylase family protein /// phosphorylase family protein
261804_at	AT1G30530	**0.6109135**	UDP-glucoronosyl/UDP-glucosyl transferase family protein
264100_at	AT1G78970	**0.608777**	lupeol synthase (LUP1) / 2,3-oxidosqualene-triterpenoid cyclase
254328_at	AT4G22570	**0.5959715**	adenine phosphoribosyltransferase, putative
249777_at	AT5G24210	**0.5699643**	lipase class 3 family protein
267138_s_at	AT2G38230.1	**0.6213904**	ethylene-responsive protein, putative /// ethylene-responsive protein, putative
255692_at	AT4G00400	**0.5554115**	phospholipid/glycerol acyltransferase family protein
246330_at	AT3G43600	**0.6589094**	aldehyde oxidase, putative
SIGNAL Transduction			
261479_at	AT1G14380	**0.6509323**	calmodulin-binding family protein
264693_at	AT1G69970	**0.6496384**	CLE26, putative
256769_at	AT3G13690	**0.6486765**	protein kinase family protein
259560_at	AT1G21270	**0.6477601**	wall-associated kinase 2 (WAK2)
260774_at	AT1G78290	**0.6378505**	serine/threonine protein kinase, putative
259561_at	AT1G21250	**0.6225038**	wall-associated kinase 1 (WAK1)
252280_at	AT3G49260	**0.6222309**	calmodulin-binding family protein
245765_at	AT1G33600	**0.6144454**	leucine-rich repeat family protein
265467_at	AT2G37050	**0.6126747**	leucine-rich repeat family protein / protein kinase family protein
259348_at	AT3G03770	**0.6075288**	leucine-rich repeat transmembrane protein kinase, putative
247383_at	AT5G63410	**0.6046734**	leucine-rich repeat transmembrane protein kinase, putative
266078_at	AT2G40670	**0.6002468**	two-component responsive regulator / response regulator 16 (ARR16)
259080_at	AT3G04910	**0.5973995**	protein kinase family protein
251714_at	AT3G56370	**0.5956617**	leucine-rich repeat transmembrane protein kinase, putative
255344_s_at	AT4G04570	**0.6244325**	protein kinase family protein /// protein kinase family protein
253949_at	AT4G26780	**0.594573**	co-chaperone grpE family protein
256516_at	AT1G66150	**0.5707533**	leucine-rich repeat protein kinase, putative (TMK1)
247153_at	AT5G65700	**0.5580592**	leucine-rich repeat transmembrane protein kinase, putative
257964_at	AT3G19850	**0.5827569**	phototropic-responsive NPH3 family protein

257206_at	AT3G16530	**0.5523409**	legume lectin family protein
TRANSCRIPTION FACTORS			
251365_at	AT3G61310	**0.6660593**	DNA-binding family protein
252033_at	AT3G51950	**0.6645191**	zinc finger (CCCH-type) family protein / RNA recognition motif (RRM)-containing protein
251374_at	AT3G60390	**0.6628526**	homeobox-leucine zipper protein 3 (HAT3) / HD-ZIP protein 3
255636_at	AT4G00730	**0.662729**	anthocyaninless2 (ANL2)
247575_at	AT5G61030	**0.6626609**	RNA-binding protein, putative
261254_at	AT1G05805	**0.6612244**	basic helix-loop-helix (bHLH) family protein
253996_at	AT4G26110	**0.6570448**	nucleosome assembly protein (NAP), putative
265718_at	AT2G03340	**0.6538019** **0.6469632**	WRKY family transcription factor
260664_at	AT1G19510	**0.6414992**	myb family transcription factor
252885_at	AT4G39260	**0.6268705**	glycine-rich RNA-binding protein 8 (GRP8) (CCR1)
260395_at	AT1G69780	**0.6146252**	homeobox-leucine zipper protein 13 (HB-13) / HD-ZIP transcription factor 13
253806_at	AT4G28270	**0.6070816**	zinc finger (C3HC4-type RING finger) family protein
261603_at	AT1G49600	**0.6065655**	RNA-binding protein 47 (RBP47), putative
250582_at	AT5G07580	**0.6017309**	ethylene-responsive element-binding family protein
250167_at	AT5G15310	**0.6046402**	myb family transcription factor
245183_at	AT5G12440	**0.5924099**	zinc finger (CCCH-type) family protein
245925_at	AT5G28770	**0.5880778**	bZIP transcription factor family protein
253061_at	AT4G37610	**0.5638107**	TAZ zinc finger family protein / BTB/POZ domain-containing protein
246522_at	AT5G15830	**0.5627193**	bZIP transcription factor family protein
260618_at	AT1G53230	**0.5603034**	TCP family transcription factor 3 (TCP3)
258890_at	AT3G05690	**0.547895**	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein
252483_at	AT3G46600	**0.633563**	scarecrow transcription factor family protein
247029_at	AT5G67190	**0.6326514**	AP2 domain-containing transcription factor, putative
245029_at	AT2G26580	**0.6236421**	plant-specific transcription factor YABBY family protein
251373_at	AT3G60530	**0.6207959**	zinc finger (GATA type) family protein
250694_at	AT5G06710	**0.612039**	homeobox-leucine zipper protein 14 (HAT14) / HD-ZIP protein 14
260070_at	AT1G73830	**0.6469632**	basic helix-loop-helix (bHLH) family protein
CELLULAR RESCUE and DEFENSE			

252712_at	AT3G43800	**0.5759795**	glutathione S-transferase, putative
259102_at	AT3G11660	**0.5984216**	harpin-induced family protein / HIN1 family protein / harpin-responsive family protein
TRANSPORTERS			
250248_at	AT5G13740	**0.6640223**	sugar transporter family protein
261143_at	AT1G19770	**0.6625168**	purine permease-related
245399_at	AT4G17340	**0.6549112**	DELTA-TIP2/TIP2;2 (tonoplast intrinsic protein 2;2); water channel/ major intrinsic family protein / MIP family protein
247923_at	AT5G57490	**0.6536791**	porin, putative
262797_at	AT1G20840	**0.6239806**	transporter-related
259185_at	AT3G01550	**0.6186855**	triose phosphate/phosphate translocator, putative
245891_at	AT5G09220	**0.5838115**	amino acid permease 2 (AAP2)
251906_at	AT3G53720	**0.5760856**	cation/hydrogen exchanger, putative (CHX20)
267305_at	AT2G30070	**0.5658897**	potassium transporter (KUP1)
264348_at	AT1G12110	**0.564492**	nitrate/chlorate transporter (NRT1.1) (CHL1)
262883_at	AT1G64780	**0.5623503**	**ammonium transporter 1, member 2 (AMT1.2)**
262456_at	AT1G11260	**0.5612672**	glucose transporter (STP1)
263319_at	AT2G47160	**0.5597867**	anion exchange family protein
247304_at	AT5G63850	**0.5419114**	amino acid transporter 4, putative (AAP4)
252594_at	AT3G45680	**0.6578252**	proton-dependent oligopeptide transport (POT) family protein
250123_at	AT5G16530	**0.6332869**	auxin efflux carrier family protein
254862_at	AT4G12030	**0.6125951**	bile acid:sodium symporter family protein
259680_at	AT1G77690	**0.604502**	amino acid permease, putative
263867_at	AT2G36830	**0.5610635**	major intrinsic family protein / MIP family protein
Unknown genes			
253165_at	AT4G35320	**0.64601**	expressed protein
253891_at	AT4G27720	**0.5921742**	expressed protein
261247_at	AT1G20070	**0.5855067**	expressed protein
249190_at	AT5G42750	**0.540648**	expressed protein
256675_at	AT3G52170	**0.6653361**	expressed protein
257076_at	AT3G19680	**0.6647934**	expressed protein
256396_at	AT3G06150	**0.6637115**	expressed protein
263632_at	AT2G04795	**0.6620487**	expressed protein

257860_at	AT3G13062	**0.6602042**	expressed protein
245501_at	AT4G15620	**0.6581931**	integral membrane family protein
245229_at	AT4G25620	**0.6570833**	hydroxyproline-rich glycoprotein family protein
265726_at	AT2G32010	**0.6551995**	endonuclease/exonuclease/phosphatase family protein
251793_at	AT3G55580	**0.6540455**	regulator of chromosome condensation (RCC1) family protein
259520_at	AT1G12320	**0.6510951**	expressed protein
248238_at	AT5G53900	**0.6482072**	expressed protein
265716_at	AT2G03350	**0.6473129**	expressed protein
261377_at	AT1G18850	**0.6417601**	expressed protein
267199_at	AT2G30990	**0.6411912**	expressed protein
247417_at	AT5G63040	**0.6409489**	expressed protein
250107_at	AT5G15330	**0.6354472**	SPX (SYG1/Pho81/XPR1) domain-containing protein
261439_at	AT1G28395	**0.632307**	expressed protein
248302_at	AT5G53160	**0.6304484**	expressed protein
250696_at	AT5G06790	**0.6289911**	expressed protein
266473_at	AT2G31110	**0.6287217**	expressed protein
250307_at	AT5G12170	**0.628706**	expressed protein
258460_at	AT3G17330	**0.6281968**	expressed protein
264609_at	AT1G04530	**0.6182555**	expressed protein
264590_at	AT2G17710	**0.6173761**	expressed protein
262868_at	AT1G64980	**0.6157789**	expressed protein
262446_at	AT1G49310	**0.6151057**	expressed protein
245479_at	AT4G16140	**0.6147034**	proline-rich family protein
255224_at	AT4G05400	**0.608637**	expressed protein
252471_at	AT3G46820	**0.6040995**	serine/threonine protein phosphatase PP1 isozyme 5 (TOPP5) / phosphoprotein phosphatase 1
256570_at	AT3G19540	**0.6035768**	expressed protein
262094_at	AT1G56110	**0.5976843**	nucleolar protein Nop56, putative
263662_at	AT1G04430	**0.5972668**	dehydration-responsive protein-related

264160_at	AT1G65450	**0.5951164**	transferase family protein
245984_at	AT5G13090	**0.5952409**	expressed protein
265025_at	AT1G24575	**0.5896628**	expressed protein
249378_at	AT5G40450	**0.5886615**	expressed protein
248186_at	AT5G53880	**0.588162**	expressed protein
261056_at	AT1G01360	**0.5767976**	expressed protein
251355_at	AT3G61100	**0.5742316**	expressed protein
266474_at	AT2G31110	**0.5736725**	expressed protein
251476_at	AT3G59670	**0.5734383**	expressed protein
267339_at	AT2G39870	**0.5630156**	expressed protein
OTHER GENES			
244937_at	ATCG01110	**0.658965**	49KDa plastid NAD(P)H dehydrogenase subunit H protein
267288_at	AT2G23680	**0.6535307**	stress-responsive protein, putative
266460_at	AT2G47930	**0.6526838**	hydroxyproline-rich glycoprotein family protein
264605_at	AT1G04550	**0.591109**	auxin-responsive protein / indoleacetic acid-induced protein 12 (IAA12)
265872_at	AT2G01670	**0.6485412**	MutT/nudix family protein
250546_at	AT5G08180	**0.6573014**	ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
256862_at	AT3G23940	**0.6160713**	dehydratase family
258549_at	AT3G06930	**0.6563085**	protein arginine N-methyltransferase family protein
264770_at	AT1G23030	**0.6530662**	armadillo/beta-catenin repeat family protein / U-box domain-containing protein
251740_at	AT3G56070	**0.6414133**	peptidyl-prolyl cis-trans isomerase, putative / cyclophilin, putative / rotamase, putative
262162_at	AT1G78020	**0.5921358**	senescence-associated protein-related
250633_at	AT5G07460	**0.5904073**	peptide methionine sulfoxide reductase, putative
259955_s_at	AT1G75080	**0.5876056**	brassinosteroid signalling positive regulator, putative /// brassinosteroid signalling positive regulator, putative
267517_at	AT2G30520	**0.5829322**	signal transducer of phototropic response (RPT2)
260101_at	AT1G73260	**0.5764861**	trypsin and protease inhibitor family protein / Kunitz family protein
257748_at	AT3G18710	**0.6546239**	U-box domain-containing protein
257300_at	AT3G28080	**0.5693235**	nodulin MtN21 family protein

265962_at	AT2G37460	**0.5684019**	nodulin MtN21 family protein
263679_at	AT1G59990	**0.5578415**	DEAD/DEAH box helicase, putative (RH22)
260739_at	AT1G15000	**0.5560959**	serine carboxypeptidase S10 family protein
248467_at	AT5G50800	**0.5472308**	nodulin MtN3 family protein
252992_at	AT4G38520	**0.6484653**	protein phosphatase 2C family protein / PP2C family protein
248427_at	AT5G51750	**0.6480575**	subtilase family protein
255578_at	AT4G01450	**0.6418294**	nodulin MtN21 family protein
251853_at	AT3G54790	**0.6406978**	armadillo/beta-catenin repeat family protein / U-box domain-containing protein
253566_at	AT4G31210	**0.6394941**	DNA topoisomerase family protein
255127_at	AT4G08300	**0.6350847**	nodulin MtN21 family protein
245063_at	AT2G39795	**0.6219979**	mitochondrial glycoprotein family protein / MAM33 family protein
264340_at	AT1G70280	**0.6108837**	NHL repeat-containing protein
252327_at	AT3G48740	**0.6102671**	nodulin MtN3 family protein
258155_at	AT3G18130	**0.6089905**	guanine nucleotide-binding family protein / activated protein kinase C receptor (RACK1)
258708_at	AT3G09580	**0.6023964**	amine oxidase family protein
257288_at	AT3G29670	**0.5823003**	transferase family protein
259444_at	AT1G02370	**0.5748528**	pentatricopeptide (PPR) repeat-containing protein
256222_at	AT1G56210	**0.5745925**	copper chaperone (CCH)-related
265672_at	AT2G31980	**0.6177624**	cysteine proteinase inhibitor-related
246701_at	AT5G28020	**0.6582037**	cysteine synthase, putative / O-acetylserine (thiol)-lyase, putative / O-acetylserine sulfhydrylase, putative
261406_at	AT1G18800	**0.5675847**	nucleosome assembly protein (NAP) family protein

Validation of Microarrays using Semi-quantitative RT-PCR:

To confirm the validity of the microarray data, we conducted semi quantitative RT-PCR of selected genes that showed differential expression upon treatment with ethyl actetate subfraction. 18S rDNA were used as a control to ensure loading of equal concentrations of cDNA. The first set of genes that we studied were those involved in proline metabolism. We did not find increase in expression of P5CS1 and P5CS2 on day 1 of NaCl treatment. On the other hand, in day 5, transcripts of P5CS1 and P5CS2 increased. As seen in the microarray results, RT-PCR also confirmed that PDH expression levels decreased upon treatment with ethyl acetate extract

fractions (Figure 12).

Salt stress tolerance is promoted by the upregulation of stress responsive genes. We selected several stress genes as shown by Microarray result and other stress induced genes (DREB 2A, DREB 1A, 1C, Cor 15A, RD 29A, RD 29B, RAB and LEA) and transcript levels were analyzed using RT-PCR. DREB 2A, encoding a transcription factor activated in the early stages of abiotic stress, was significantly induced on day 1 than on day 5 of the NaCl treatment. However, there were no clear differences between treatment with ethyl acetate extract fractions and NaCl treated plants. A similar expression profile was observed for COR15A, which encodes a chloroplast targeted LEA-protein (Artus et al., 1996). DREB 1A, 1C increased in day 1 of the extract treatment in comparison to NaCl treated controls like in microarray. rD29A and rd29B (Responsive to desiccation), which are tandemly organized in the *Arabidopsis* genome have been shown to differ in the expression kinetics during salt stress treatments. While rd29A mRNA levels could be detected in the early stages of salt stress, rd29B mRNA accumulated at a much slower rate. We also obtained similar results after NaCl treatment. rd29A mRNA accumulation was much more pronounced than rd29B at day 1 of NaCl treatment. We found increased expression of rd29A and rd29B mRNA in treatments with ethyl acetate extract fractions than in NaCl treated plants. rd 29B showed increased expression on day 5 of the treatment and the pattern mirrored the microarray results. Again, RAB18 and LEA, Di 21, RNABP, Annexin and AmmT, which showed increased expression in microarrays, were also confirmed by RT-PCR.

Effect of Extract on the Alleviation of Salt Stress in a Variety of Crop Plants:

Seeds of pea, lettuce, mung bean, cucumber, cauliflower, barley, and cabbages were placed in Petri dishes on filter paper soaked with water (control), 150mM NaCl or 150Mm NaCl + organic fraction of *A. nodosum*. For each treatment there were 5 replications. The Petri dishes were incubated and percent germination was observed periodically (Table 5).

Table 5 Organic components of *A. nodosum* extract alleviates salt stress (by increased germination and vigour) in a number of crop plants

Crop	Control	NaCl (200mM)	NaCl (200mM) + *A. nodosum* extract
Cauliflower	96	76	92
Pea	96	56	74
Lettuce	96	22	42
Barley	62	20	30
Mungbean	62	20	30

Induction of Salt Tolerance by *A. nodosum* in Lettuce and Beet

Abiotic stresses exert oxidative damage through reactive oxygen species (ROS) causing harm to cellular components including membrane lipids (Smirnoff, 1995). High salinity has shown to be the most severe factor, limiting plant growth in the salt affected areas. Saline conditions have shown to increase ROS causing membrane damage and hence it has been the major cause of the cellular toxicity by salinity in C3 and C4 plants (Greenway and Munns, 1980; Hasegawa et al., 2000). Antioxidant enzymes are related to the resistance of various abiotic stresses including salinity. Estimating antioxidant enzyme response of glycophytes, garden lettuce (Lactuca sativa L.) and sugarbeet (Beta vulgaris L.) to salt treatments can provide important clues to the mechanisms of saline stress tolerance in crop plants. Among several antioxidant enzymes, catalase has been found to be greatly enhanced under saline stress in barley (Kim et al., 2005).

Extracts from *Ascophyllum nodosum* were tested to ascertain whether they could enhance plant antioxidant enzyme response to salt stress and retain chlorophyll levels in saline stress.

Estimation of Catalase activity: Circular leaf discs of (diameter 2-3 cm) sugar beet and lettuce weighing ~ 200 mg were floated in Petridishes containing 20 ml of 1.0 g/L 1186 MeOH extracted Soluble Seaweed Extract Powder (SSEP), 1186 $CHCl_3$ extracted SSEP, 1186 Ethyl acetate extracted SSEP, NaCl 100 and 150 mM and in combinations. The treatments were replicated 4 times and the experimental design followed was completely randomized design. The treatments include:

1. Control 1(NaCl 100mM)

2. Control 2 (NaCl 150mM)

3. 1186 Me-OH extracted SSEP

4. 1186 $CHCl_3$ extracted SSEP

5. 1186 Ethyl acetate extracted SSEP

6. 100mM NaCl + 1186 Me-OH extracted SSEP

7. 100mM NaCl + 1186 $CHCl_3$ extracted SSEP

8. 100mM NaCl + 1186 Ethyl acetate extracted SSEP

9. 150mM NaCl + 1186 Me-OH extracted SSEP

10. 150mM NaCl + 1186 $CHCl_3$ extracted SSEP

11. 150mM NaCl + 1186 Ethyl acetate extracted SSEP

Catalase activity was assayed using the methodology described by Havir and McHale (1987). Ten to twenty discs were cut from the tip-half region of the fully expanded leaves with sharp punch. The leaves were washed in distilled water, randomized, and floated in groups of five (~ 200 mg) in 30 ml control and treated solutions. The leaves were immediately placed in an ice-cold microfuge tube. To each microfuge tubes in the ice bath, 0.4 ml of freshly prepared ice cold buffer (potassium phosphate buffer, 50 mM at pH 7.4, containing 10 mm dithiothreitol) was added. Catalase enzyme was extracted by repeatedly inserting and rotating a tight-fitting plastic pestle into a microfuge tube for about 30 sec. The homogenate was centrifuged in a cold microcentrifuge at 12,000x g for 3 min. The tubes were stored in a ice bath. Catalase activity assay was carried out by adding 15 µl of the supernatant (crude enzyme extract) in 3.0 ml of assay medium (freshly prepared 12.5 mM hydrogen peroxide in 50 mM potassium phosphate, pH 7.0) in a 1 cm cuvette at 30°C. Catalase activity was measured by assaying the rate of decrease in absorbance at 240 nm to determine the initial rate (60 sec) of H_20_2 breakdown. One unit is defined as the amount of enzyme catalyzing the decomposition of 1 µmol of hydrogen peroxide per minute under standard conditions at 30°C. Results are shown in Figures 13 and 14. As can be seen, extracts from *A. nodosum* enhance plant catalase activity in response to salt stress.

Percent leaf area: The percentage of leaf area affected by salt treatments in comparison with total leaf area was calculated using Sigma scan Pro® software package. Digital pictures of individual leaf bits in the treatments were calibrated in Sigma scan Pro® and leaf areas were measured. Results are shown in Figure 15. As can be seen, extracts from *A. nodosum* reduce the percentage of leaf area affected by salt stress.

Estimation of chlorophyll content: Chlorophyll concentration in the leaf discs was measured using the protocol described by Arnon (1949). Approximately, 500 mg of the leaf discs per replication were taken and macerated with 80% acetone using a pestle and mortar. The macerated sample was then centrifuged at 3000 rpm for 10 min. The supernatant solution was decanted in a 25 ml volumetric flask and the volume was made up to 25 ml with 80% acetone. Using a spectrophotometer (Beckman Spectrophotometer Model; DU-65 S/n 20017), the optical density (OD) of the solution was recorded at 645 nm, 663 nm and 652 nm. The wavelength required for measurements was selected and the instrument was calibrated using a blank

containing acetone at that respective wavelength before making measurements using the method described by Arnon, (1949). Results of measuring chlorophyll content in lettuce after 48 h under 100 mM NaCl and 150 mM NaCl conditions are shown in Figure 16, and results of measuring chlorophyll content in sugarbeet after 48 h under 100 mM NaCl and 150 mM NaCl conditions are shown in Figure 17. As can be seen, plants treated with extracts from *A. nodosum* retain chlorophyll levels even at 150 mM NaCl testing levels.

Effect of Fucosterol and Different Organic Extracts of *A. nodosum* on Root Length

The code numbers in Figure 18 refer to different organic extracts. As observed, plants treated with fucosterol and extract RS5-45C have a longer root length than untreated plants under salt stress (compared to Na^+).

Ethyl Acetate Fraction Reduced Na^+ Uptake by Roots

Ion Depletion Experiments: Net Na^+ uptake and K^+ loss from *Arabidopsis* roots was studied according to the method of Chen et al., 2007 (Chen CN, Chu CC, Zentella R, Pan SM, Ho TH (2002) AtHVA22 gene family in Arabidopsis: phylogenetic relationship, ABA and stress regulation, and tissue-specific expression. Plant Mol Biol **49:** 633–644). Briefly, roots of 1 week-old *Arabidopsis* plants were immersed in glass vials containing 11 mL NaCl solution (80 mM NaCl, 0.5 mM KCl, and 0.1 mM $CaCl_2$) prepared using Millipore deionized water. Seedlings were kept at 25°C in the dark for 24 h. At the end of incubation, roots were blotted dry, cut, and weighed. Na^+ and K^+ concentrations in the remaining solution were determined using Atomic absorption spectrophotometer, and net Na^+ uptake and K^+ loss were calculated on a fresh weight basis. This experiment was conducted with three replicates (5 plants per replicate).

NaCl treatments and Sodium and potassium estimation: Arabidopsis plants were grown as described above in a green house. For NaCl treatments, *Arabidopsis* plants grown in peat pellets were placed in individual plastic trays (5 cm diameter) at the rate of 15 plants. Each tray (constituting a replicate) was irrigated with 150 mM NaCl solution (prepared in distilled water) for 24 hours. For ethyl acetate fraction (EAA) treatments, 20 mL EAA was added subsequently. Additionally, the plants were irrigated on alternate days with distilled water to maintain uniform moisture for optimum growth of *Arabidopsis* plants. Control, NaCl treated plants received no EAA treatment, while, on the other hand, control plants received only water during the whole experiment. After the 5th day, leaf samples from two sets of replicates were harvested.

Arabidopsis leaf tissue (1g) was flash frozen and ground in mortar and pestle. For Na^+ and K^+ ions measurement of ashed leaf samples, method as described in AOAC 968.08 (Association of

Offical Analytical Chemists, standard protocol) was performed using NaCl and KCl as standards. Briefly, 1 g of the ground leaf tissue was kept in a furnace, at 550°C for 4 h. The samples were then cooled, 10 mL 3M HCl added and boiled gently for 10 min. The solution was then filtered in a 100 mL volumetric flask, and diluted to final volume with deionized water. Subsequently, dilutions with 0.1–0.5M HCl was done to bring the samples in range with the NaCl and KCl standards.

Results: Differences in Na+ uptake after treatment with EAA treatment

Plants exposed to high concentration of NaCl accumulate elevated concentration of Na^+ in the tissue leading to disruption of ionic balance and ultimately cellular function. *Arabidopsis* plants were treated with EAA after 24 h exposure to 150mM NaCl. As expected, sodium content in leaves of NaCl treated plants (150 mM) increased by 84% in comparison to control plants that were not irrigated with NaCl. Interestingly, plants treated with EAA caused a decreased accumulation of Na^+ in the leaves; it reduced the concentration of Na^+ in the leaf tissue by 53%. Moreover, EAA treatments also decreased potassium content by 56%. On the other hand, nitrogen and phosphorous content differed only slightly between untreated and treated controls (see Figure 9). Ion depletion experiments showed that *Arabidopsis* plants treated with EAA were not only able to reduce net root Na^+ uptake by 41% compared with NaCl treated seedlings, but also loose 25% less K^+ from the cytosol (Figure 19).

It will be understood that numerous modifications to the above described invention will appear to those skilled in the art. Accordingly, the above description and accompanying drawings should be taken as illustrative of the invention and not in a limiting sense. It will further be understood that it is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth, and as follows in the scope of the appended claims. All references cited herein are hereby incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A process for preparing an organic extract useful as a treatment for inducing salinity tolerance in plants, said process comprising the steps of:

suspending dried *A. nodosum* or extracts of *A. nodosum* in methanol,

mixing the suspension, and

separating any remaining solid material from the resulting methanol extract,

wherein said methanol extract is useful as a treatment for inducing salinity tolerance in plants.

2. The process of claim 1, further comprising the steps of:

removing the solvent from said methanol extract to form a dried residual,

resuspending the dried residual from said methanol extract in water,

adding chloroform to the resuspended residual from the methanol extract,

mixing and allowing the phases to separate into water and chloroform extracts,

collecting the chloroform extract, and

removing the solvent from said chloroform extract to form a dried residual,

wherein the dried residual of said chloroform extract is useful as a treatment for inducing salinity tolerance in plants.

3. The process of claim 2, further comprising the steps:

resuspending the dried residual from said chloroform extract in water,

adding ethyl acetate to the resuspended residual from the chloroform extract,

mixing and allowing the phases to separate into water and ethyl acetate extracts,

collecting the ethyl acetate extract, and

removing the solvent from said ethyl acetate extract to form a dried residual,

wherein the dried residual of said ethyl acetate extract is useful as a treatment for inducing salinity tolerance in plants.

4. The process of any one of claims 1 to 3, wherein the *A. nodosum* is suspended in the methanol in a ratio of *A. nodosum* to methanol from about 1:1 to about 1:50 volume/volume.

5. The process of claim 4, wherein the *A. nodosum* is suspended in the methanol in a ratio of *A. nodosum* to methanol of 1:3 volume/volume.

6. The process of claim 2, wherein the steps of adding chloroform to the resuspended residual from the methanol extract, mixing and allowing the phases to separate into water and chloroform extracts, and collecting the chloroform extract, are repeated up to 3 times.

7. The process of claim 3, wherein the steps of adding ethyl acetate to the resuspended residual from the chloroform extract, mixing and allowing the phases to separate into water and ethyl acetate extracts, and collecting the ethyl acetate extract, are repeated up to 3 times.

8. A method of inducing salinity tolerance in plants, comprising obtaining an organic extract as defined in any one of claims 1 to 7, and administering said organic extract to a plant under salt stress in an amount effective to ameliorate said salt stress in said plant.

9. Use of an organic extract as defined in any one of claims 1 to 7 for inducing salinity tolerance in plants.

10. A composition for inducing salinity tolerance in plants, comprising as active agent at least one phytosterol, fungal sterol, terpenoid or fatty acid, or combinations thereof, derived from *Ascophyllum nodosum*.

11. The composition according to claim 10, wherein said fungal sterol is derived from *Mycosphaerella ascophylli*.

12. The composition according to claim 10, wherein said phytosterol is fucosterol.

13. A method of inducing salinity tolerance in plants, comprising administering a composition as defined in any one of claims 10 to 13 to a plant under salt stress in an amount effective to ameliorate said salt stress in said plant.

14. Use of a composition as defined in any one of claims 10 to 13 for inducing salinity tolerance in plants.

15. A method of inducing salinity tolerance in plants, comprising extracting at least one phytosterol, fungal sterol, terpenoid or fatty acid, or combinations thereof from *Ascophyllum nodosum*, and administering said organic extract to a plant under salt stress in an amount effective to ameliorate said salt stress in said plant.

16. The method according to claim 15, wherein said fungal sterol is derived from *Mycosphaerella ascophylli.*

17. The method according to claim 15 or 16, wherein said phytosterol is fucosterol.

18. A composition of matter comprising at least one phytosterol, fungal sterol, terpenoid, fatty acid, or combinations thereof, from *Ascophyllum nodosum*, for alleviating salinity stress in plants.

19. The composition of matter according to claim 18, wherein said at least one phytosterol, fungal sterol, terpenoid, fatty acid, or combinations thereof elicit coordinated expression of multiple genes in said plant to induce salinity tolerance.

20. The composition of matter according to claim 18 or 19, wherein said fungal sterol is derived from *Mycosphaerella ascophylli.*

21. The composition of matter according to any one of claims 18 to 20, wherein said phytosterol is fucosterol.

22. A composition useful as a treatment for inducing salinity tolerance in plants, said composition obtained according to a process comprising the steps of:

suspending dried *A. nodosum* or extracts of *A. nodosum* in methanol,

mixing the suspension, and

separating any remaining solid material from the resulting methanol extract,

wherein said methanol extract is provided as a composition useful as a treatment for inducing salinity tolerance in plants.

23. The composition of claim 22, wherein the methanol extract is further processed by the steps of:

removing the solvent from said methanol extract to form a dried residual,

resuspending the dried residual from said methanol extract in water,

adding chloroform to the resuspended residual from the methanol extract,

mixing and allowing the phases to separate into water and chloroform extracts,

collecting the chloroform extract, and

removing the solvent from said chloroform extract to form a dried residual,

wherein the dried residual of said chloroform extract is provided as a composition useful for inducing salinity tolerance in plants.

24. The composition of claim 23, wherein the dried residual of said chloroform extract is further processed by the steps:

resuspending the dried residual from said chloroform extract in water,

adding ethyl acetate to the resuspended residual from the chloroform extract,

mixing and allowing the phases to separate into water and ethyl acetate extracts,

collecting the ethyl acetate extract, and

removing the solvent from said ethyl acetate extract to form a dried residual,

wherein the dried residual of said ethyl acetate extract is provided as a composition useful for inducing salinity tolerance in plants.

25. The composition of any one of claims 22 to 24, wherein the *A. nodosum* is suspended in the methanol in a ratio of *A. nodosum* to methanol from about 1:1 to about 1:50 volume/volume.

26. The composition of claim 25, wherein the *A. nodosum* is suspended in the methanol in a ratio of *A. nodosum* to methanol of 1:3 volume/volume.

27. The composition of claim 23, wherein the steps of adding chloroform to the resuspended residual from the methanol extract, mixing and allowing the phases to separate into water and chloroform extracts, and collecting the chloroform extract, are repeated up to 3 times.

28. The composition of claim 24, wherein the steps of adding ethyl acetate to the resuspended residual from the chloroform extract, mixing and allowing the phases to separate into water and ethyl acetate extracts, and collecting the ethyl acetate extract, are repeated up to 3 times.

29. The composition according to any one of claims 10 to 12 and 22 to 28, formulated as a liquid for spray or root irrigation or as a solid for seed treatment.

30. A liquid formulation for spray or root irrigation useful for inducing salinity tolerance in plants, said formulation comprising a composition as defined in any one of claims 10 to 12 and 22 to 28 and an acceptable carrier.

31. A solid formulation for seed treatment useful for inducing salinity tolerance in plants, said formulation comprising a composition as defined in any one of claims 10 to 12 and 22 to 28 and an acceptable carrier.

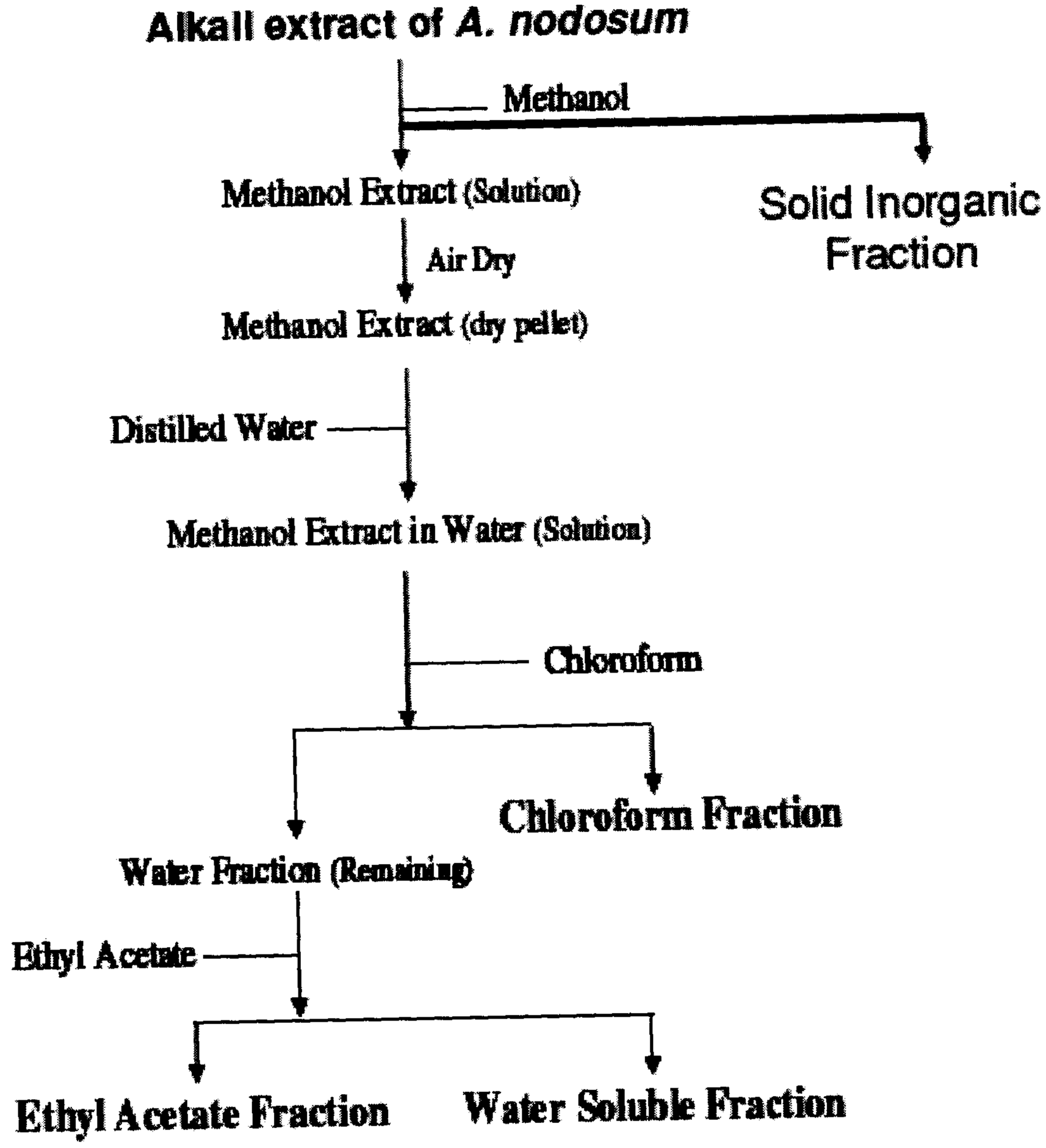

Alkali extract of A. nodosum
Methanol
Methanol Extract (Solution)
Solid Inorganic Fraction
Air Dry
Methanol Extract (dry pellet)
Distilled Water
Methanol Extract in Water (Solution)
Chloroform
Chloroform Fraction
Water Fraction (Remaining)
Ethyl Acetate
Ethyl Acetate Fraction
Water Soluble Fraction

Figure 1

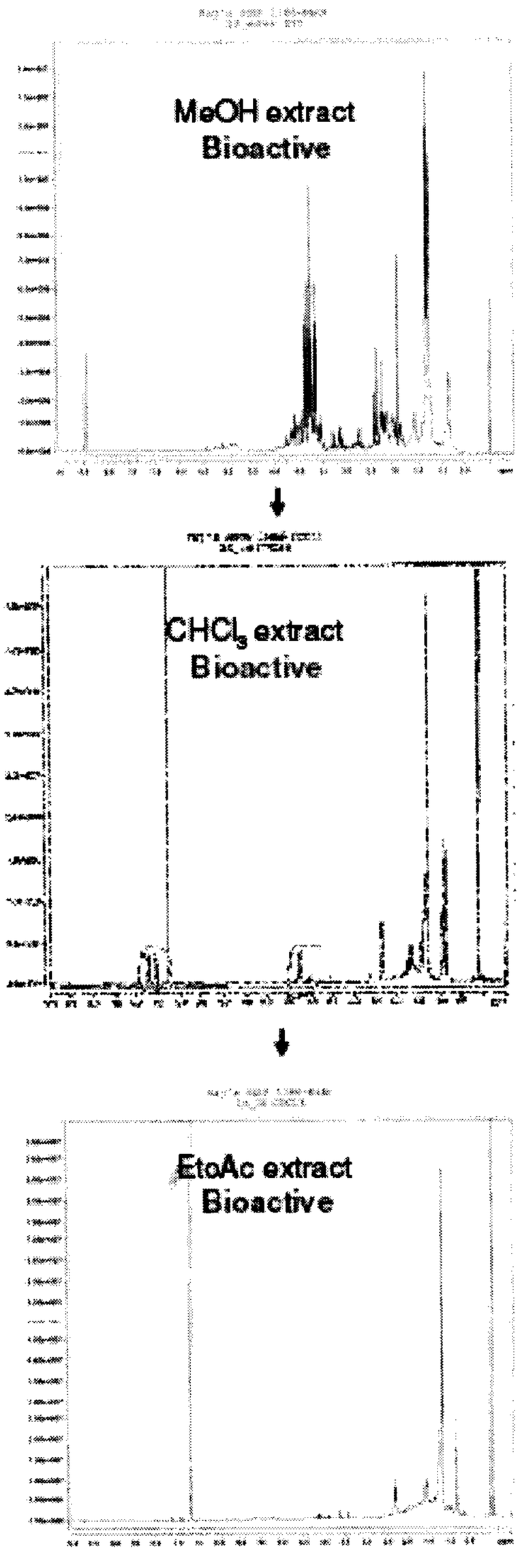

MeOH extract
Bioactive
CHCl3 extract
Bioactive
Charcoal
CHCl3 extract
Not active
EtoAc extract
Bioactive
Charcoal
EtoAc extract
Not active

Figure 2

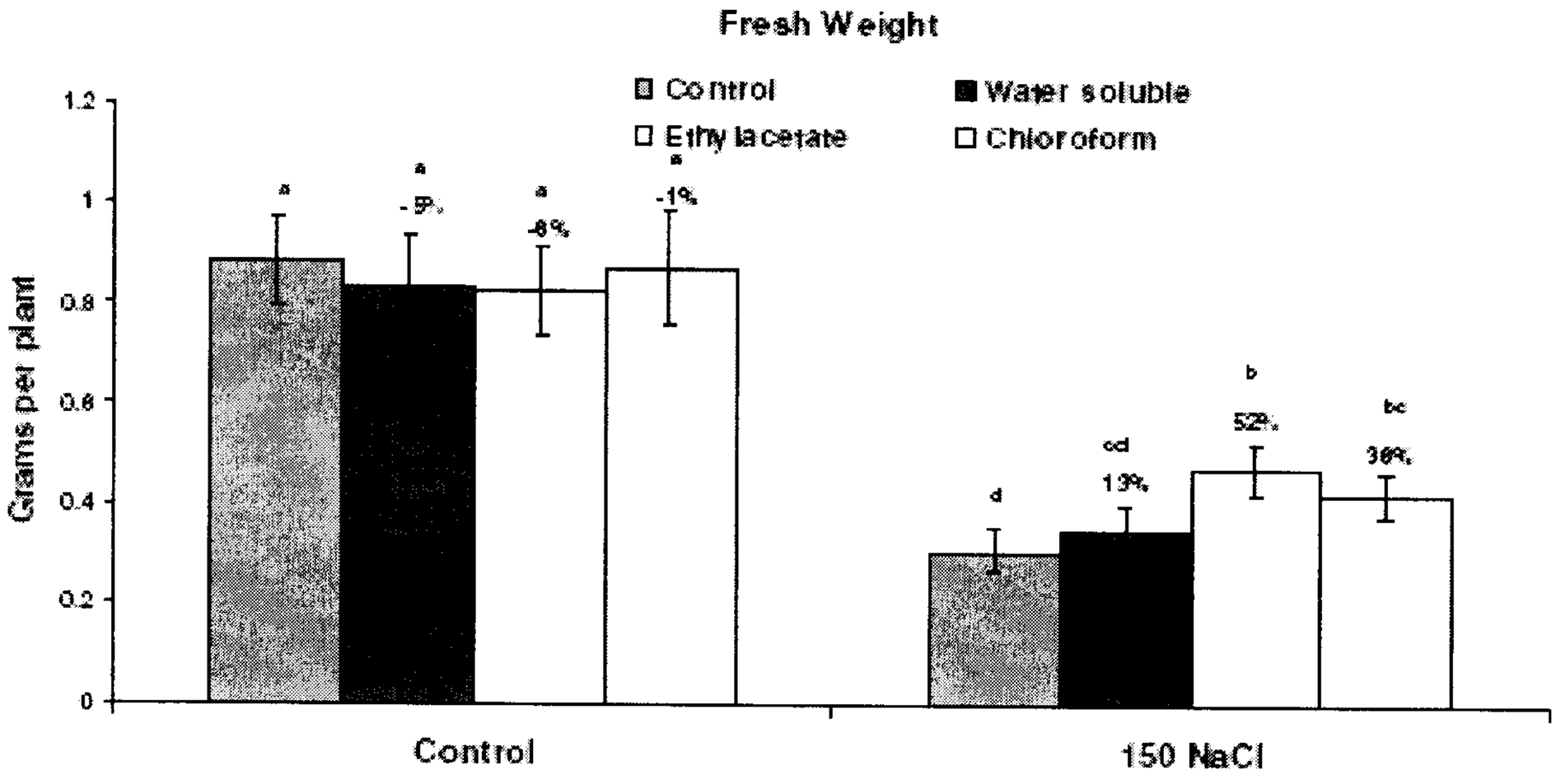

Fresh Weight
Control
Water soluble
Ethylacetate
Chloroform
Grams per plant
1.2
1
0.8
0.6
0.4
0.2
0
a
a
-9%
a
-8%
a
-1%
b
52%
bc
36%
cd
13%
d
Control
150 NaCl

Figure 3

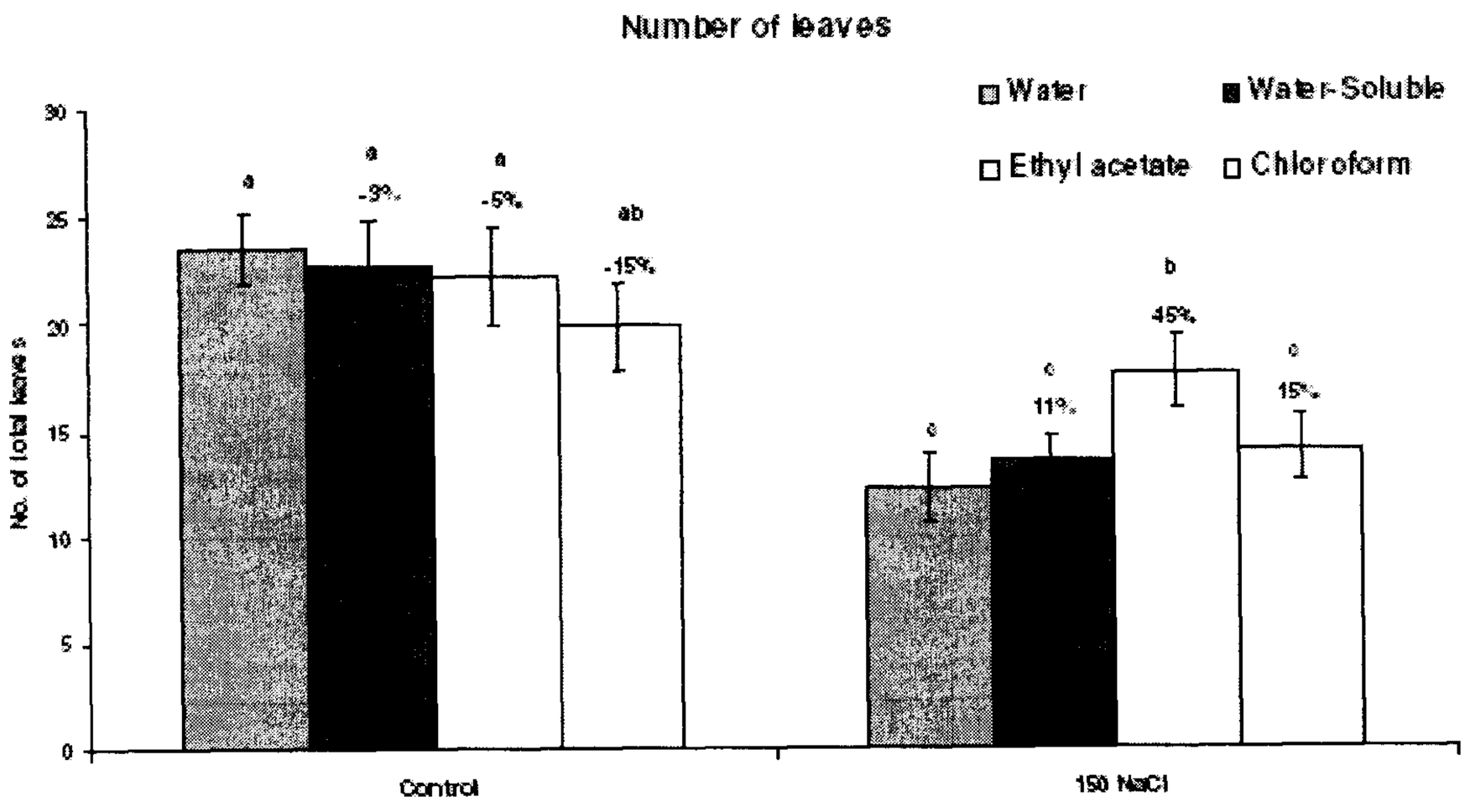

Number of leaves
Water
Water-Soluble
Ethyl acetate
Chloroform
No. of total leaves
30
25
20
15
10
5
0
a
a
-3%
a
-5%
ab
-15%
b
45%
c
11%
c
c
15%
Control
150 NaCl

Figure 4

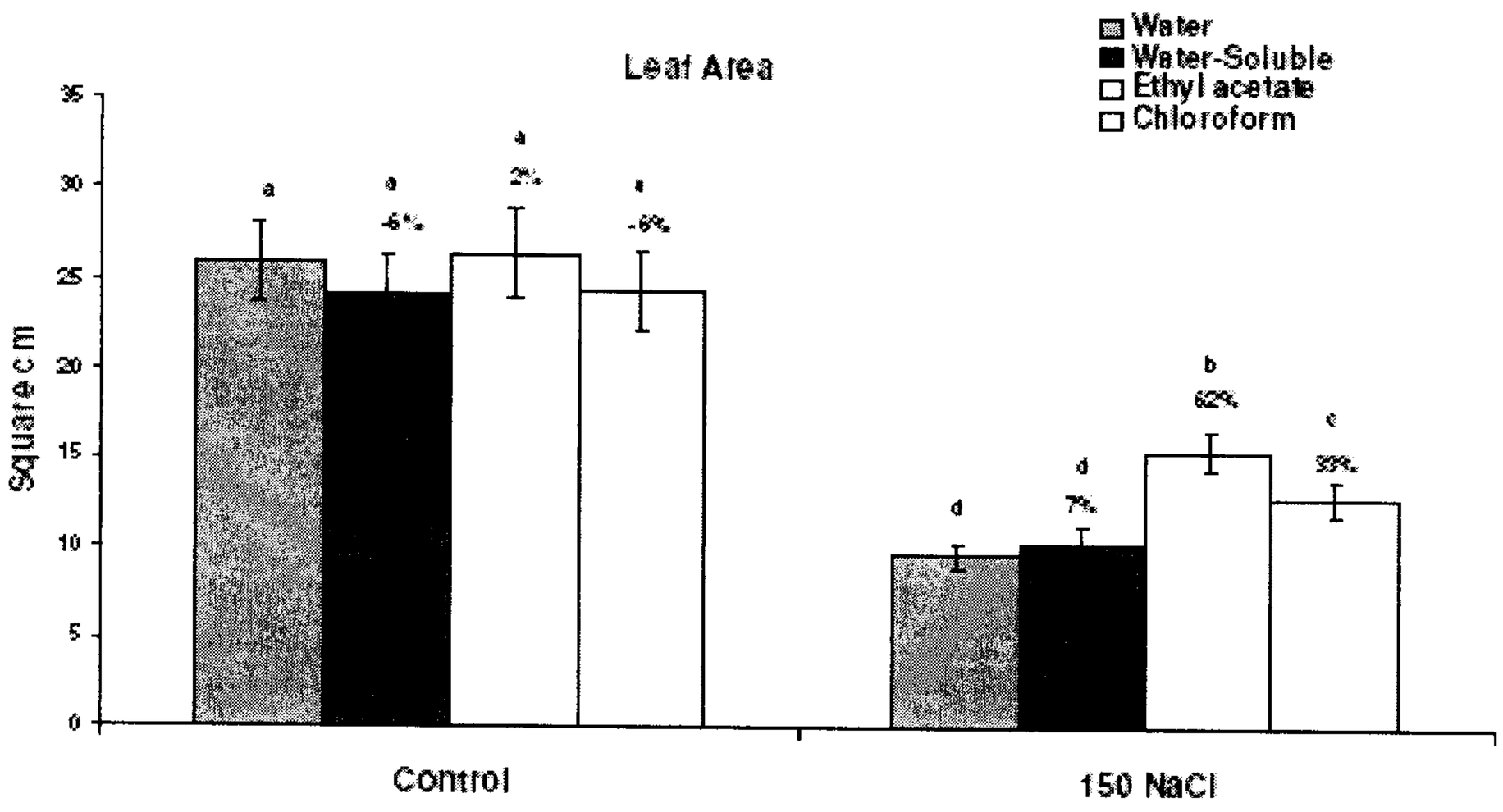

Leaf Area
Water
Water-Soluble
Ethyl acetate
Chloroform
Square cm
35
30
25
20
15
10
5
0
a
a
-5%
a
2%
a
-6%
b
62%
c
39%
d
d
7%
Control
150 NaCl

Figure 5

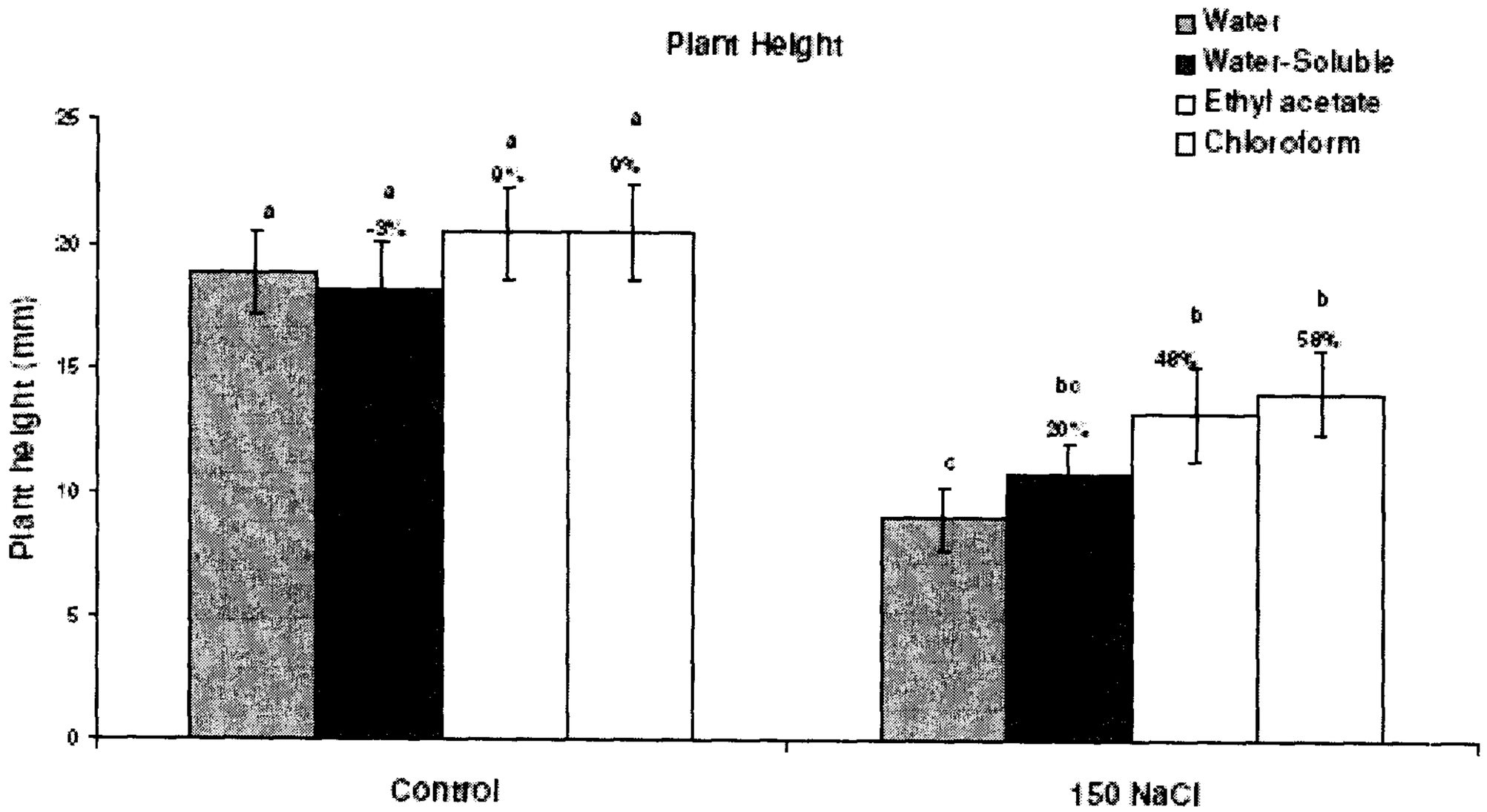

Plant Height
Water
Water-Soluble
Ethyl acetate
Chloroform
Plant height (mm)
25
20
15
10
5
0
a
a
a
a
0%
0%
-9%
b
b
bc
c
40%
58%
20%
Control
150 NaCl

Figure 6

150mM NaCl
150mM NaCl + Methanol extract

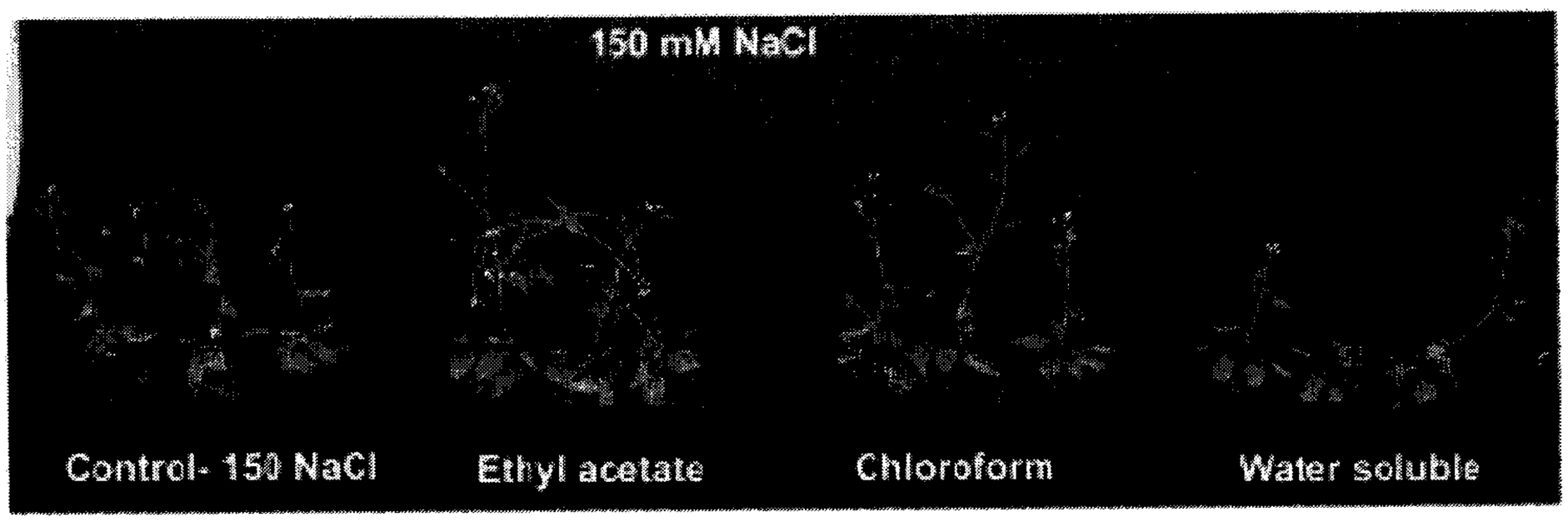

150 mM NaCl
Control- 150 NaCl
Ethyl acetate
Chloroform
Water soluble

Figure 7

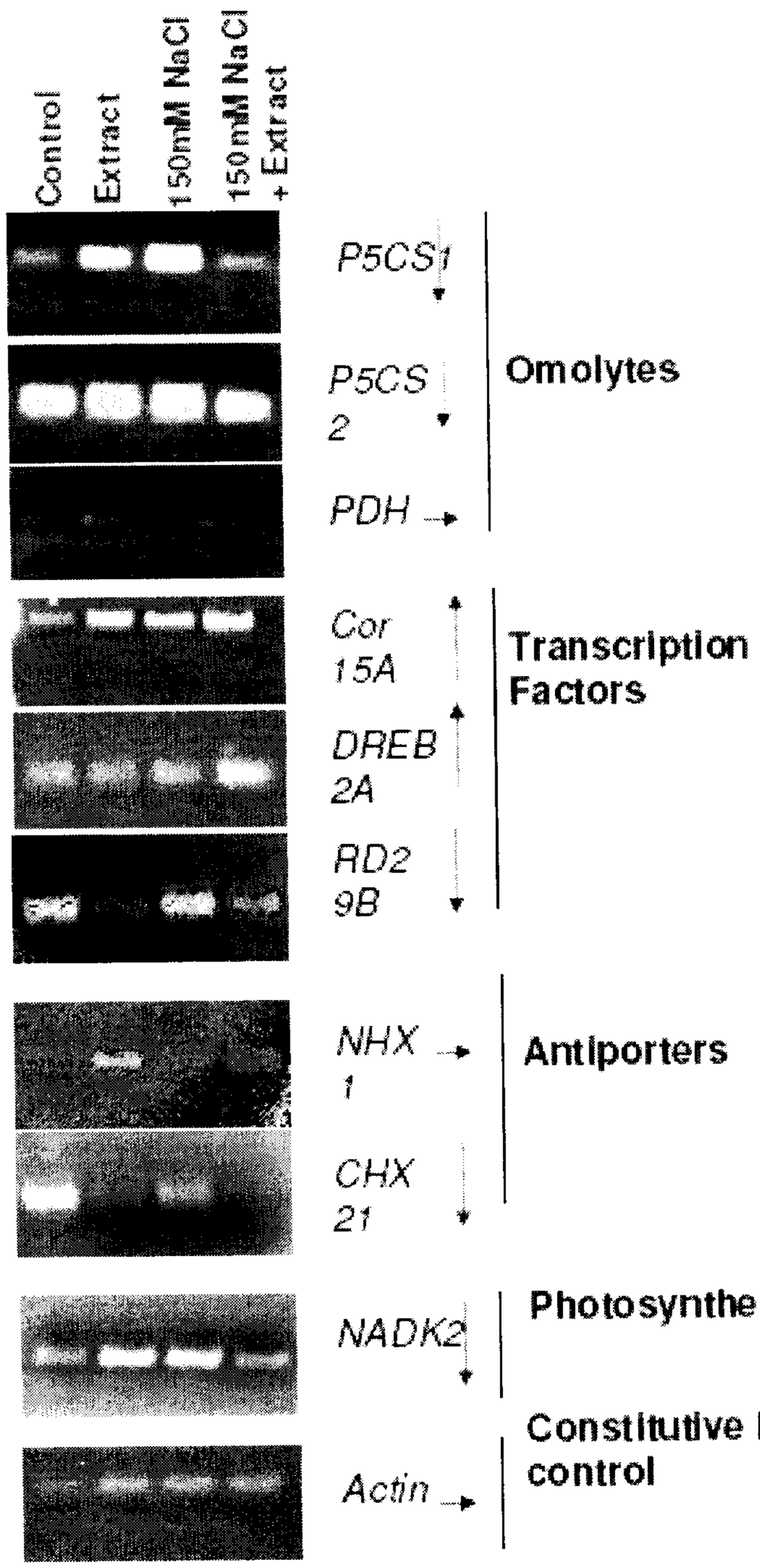

Control
Extract
150mM NaCl
150mM NaCl + Extract
P5CS1
P5CS 2
PDH
Omolytes
Cor 15A
DREB 2A
RD2 9B
Transcription Factors
NHX 1
CHX 21
Antiporters
NADK2
Photosynthesis
Actin
Constitutive Internal control

Figure 8

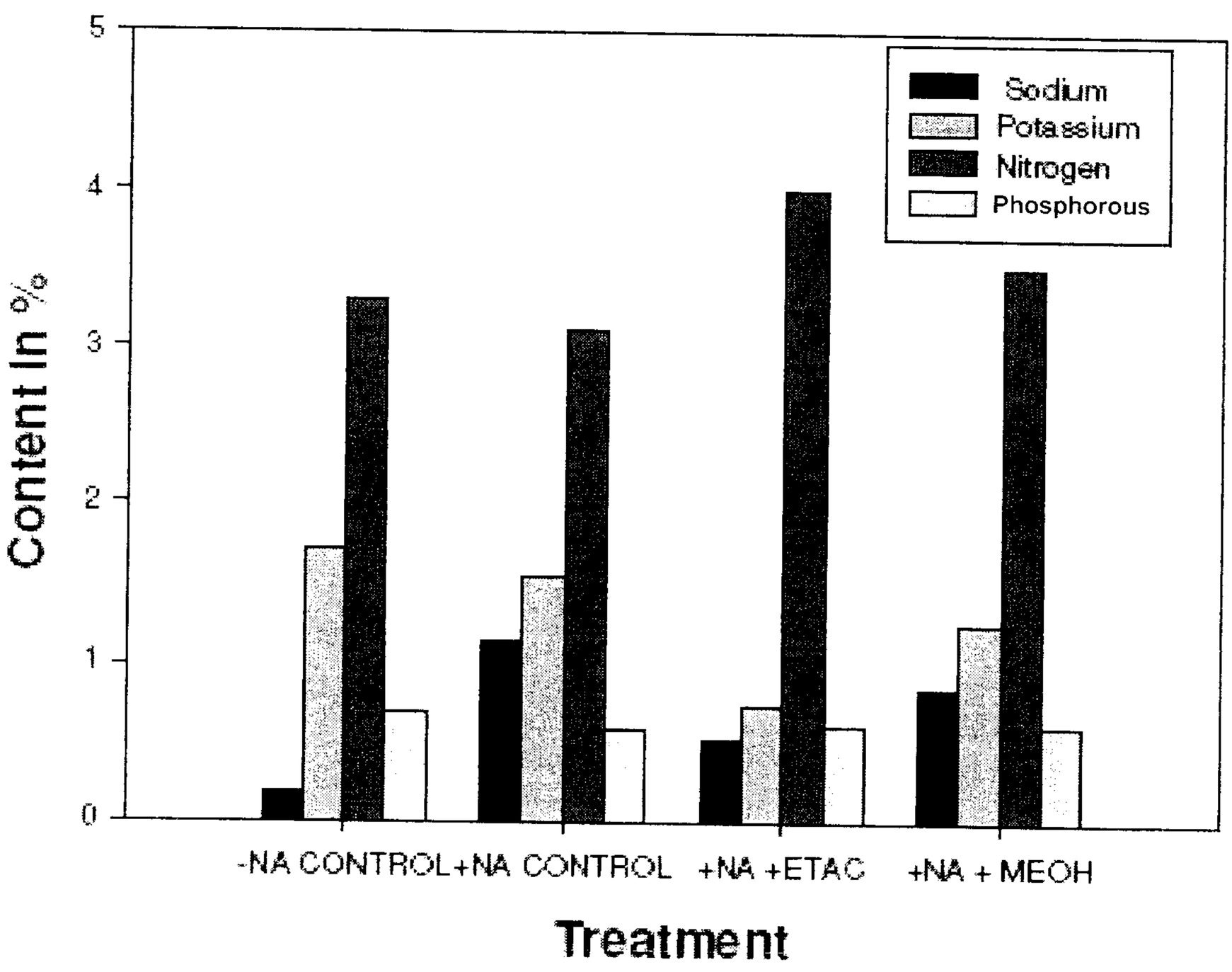
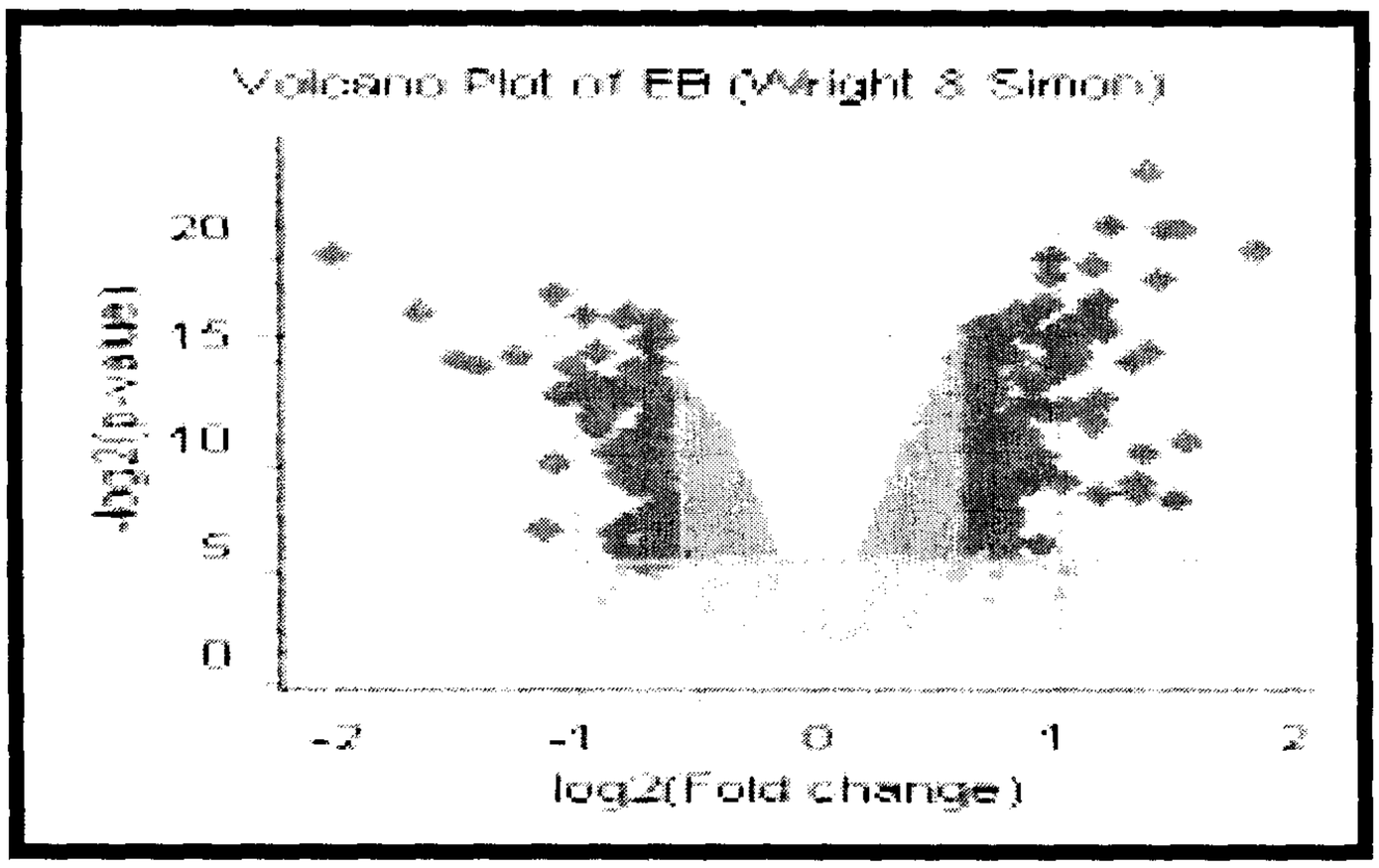
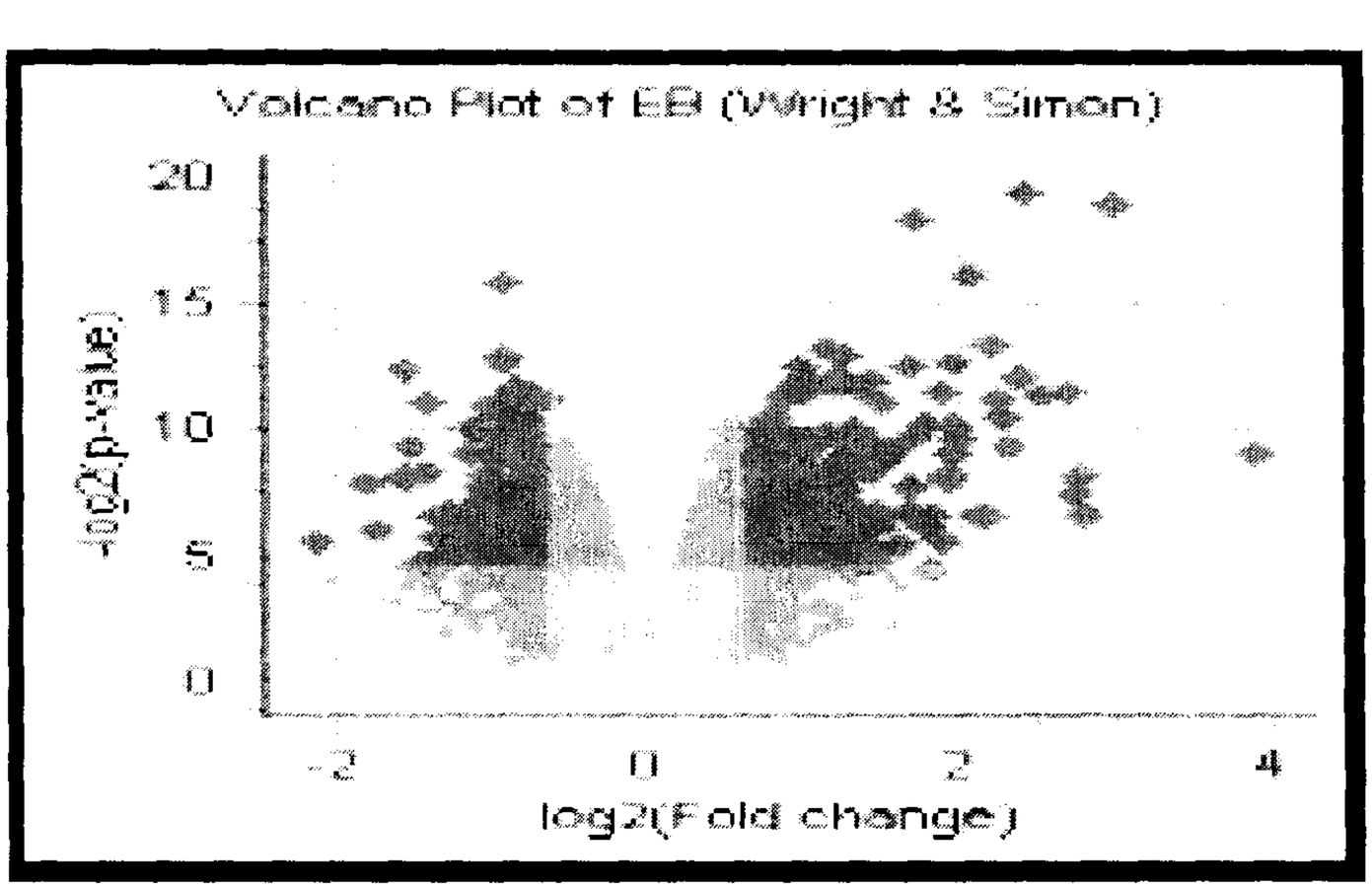

Sodium
Potassium
Nitrogen
Phosphorous
Content In %
0
1
2
3
4
5
-NA CONTROL
+NA CONTROL
+NA +ETAC
+NA + MEOH
Treatment

Figure 9

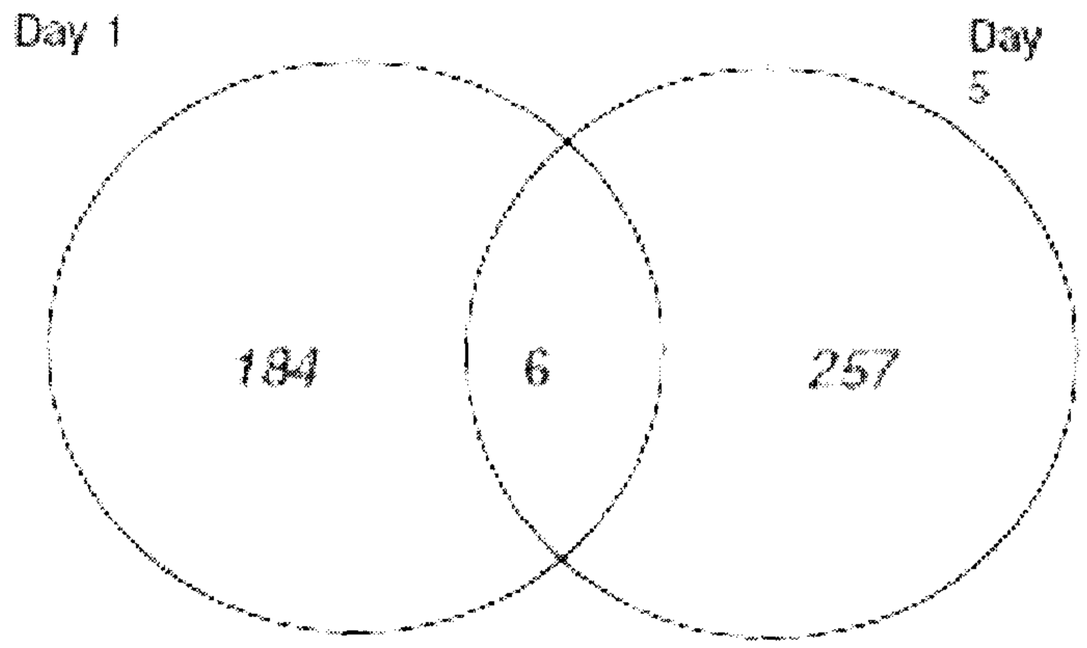


EtAc/NaCl controls –Day 1



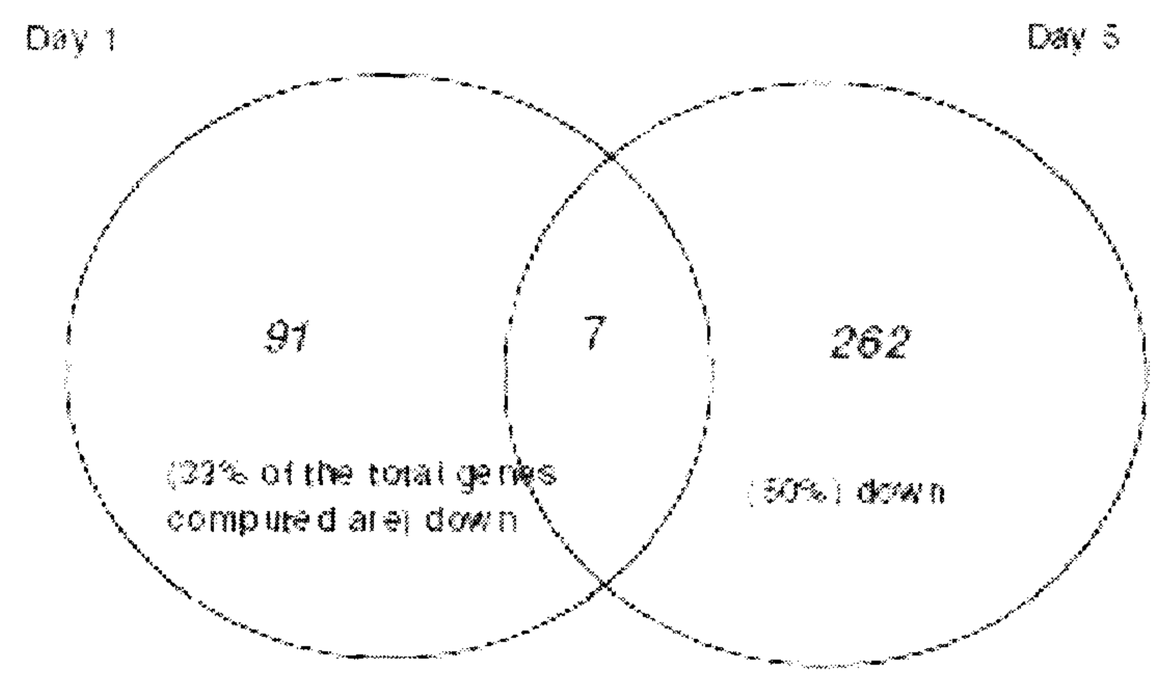
EtAc/NaCl controls –Day 5

Figure 10



1d- 5d stress period comparison

Distribution of stress response (inducible) (>1.5 fold) with Ethyl acetate extract treatment



1d- 5d stress period comparison

Distribution of stress response (repression) < 1.5 fold with Ethyl acetate extract treatment

Figure 11

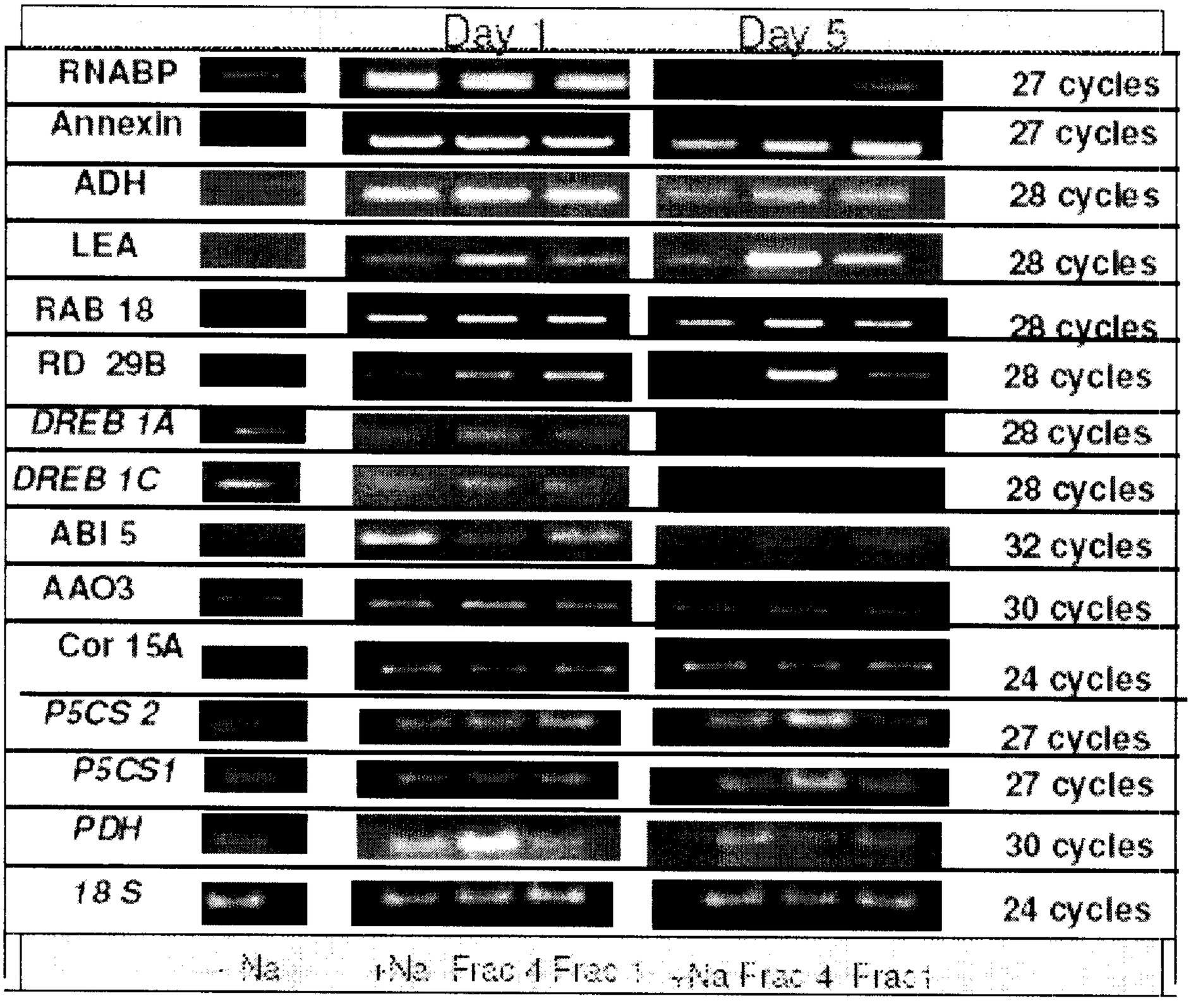

Day 1
Day 5
RNABP
27 cycles
Annexin
27 cycles
ADH
28 cycles
LEA
28 cycles
RAB 18
28 cycles
RD 29B
28 cycles
DREB 1A
28 cycles
DREB 1C
28 cycles
ABI 5
32 cycles
AAO3
30 cycles
Cor 15A
24 cycles
P5CS 2
27 cycles
P5CS1
27 cycles
PDH
30 cycles
18 S
24 cycles
- Na
+Na Frac 4 Frac 1
+Na Frac 4 Frac1

Figure 12

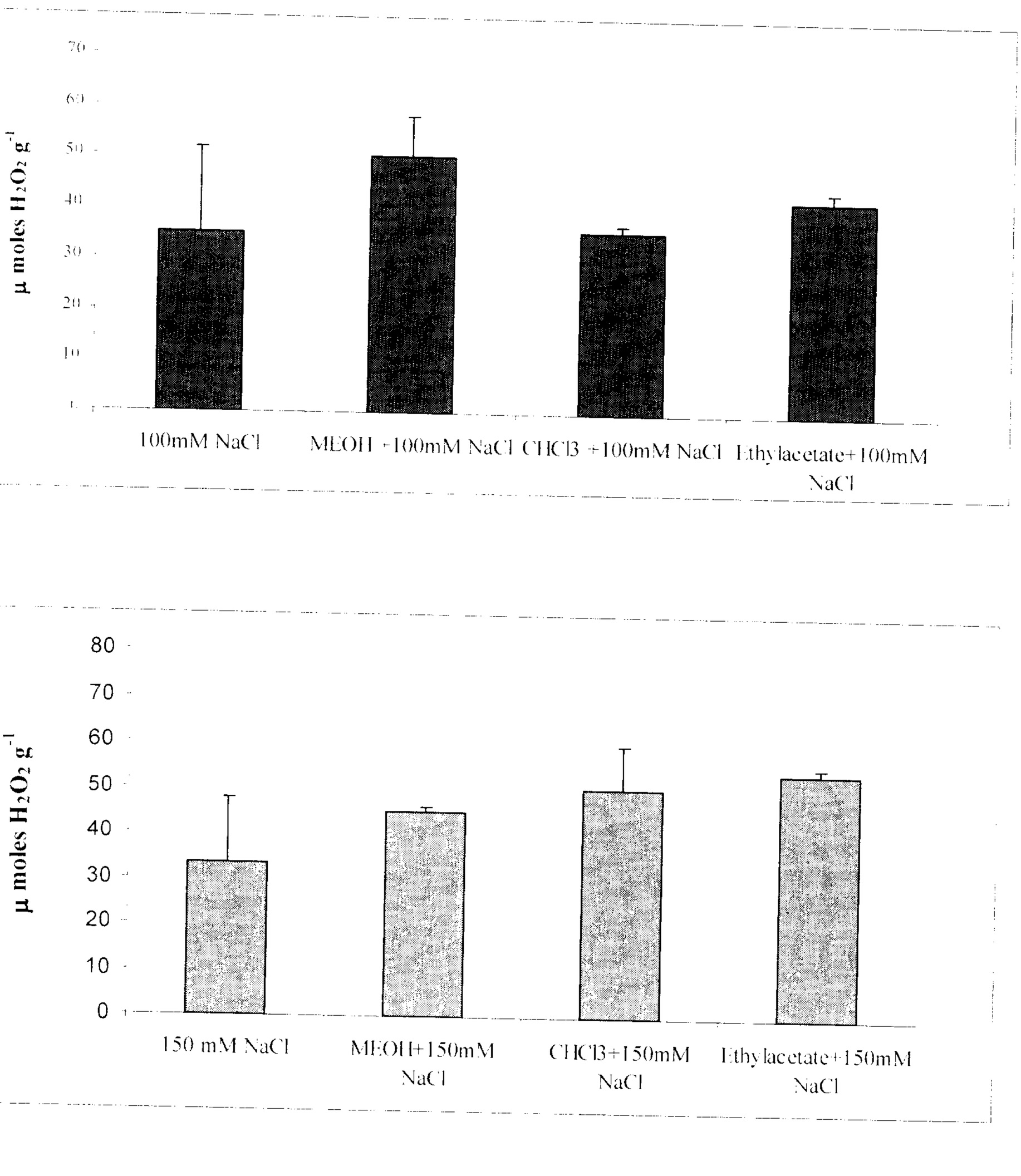

µ moles H_2O_2 g^{-1}
0
10
20
30
40
50
60
70
80
100mM NaCl
MEOH +100mM NaCl
CHCl3 +100mM NaCl
Ethylacetate+100mM NaCl
150 mM NaCl
MEOH+150mM NaCl
CHCl3+150mM NaCl
Ethylacetate+150mM NaCl

Figure 13

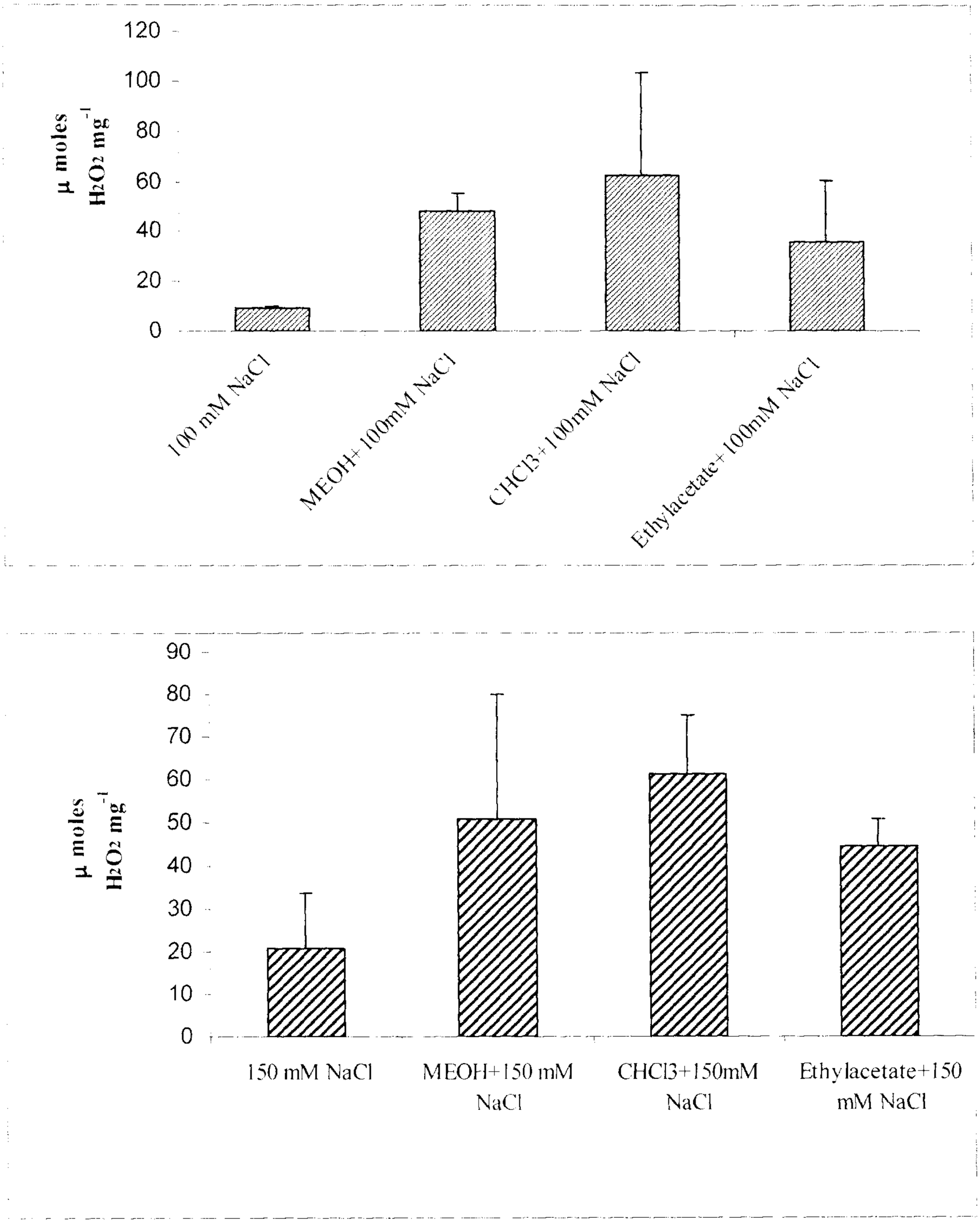

120
100
80
60
40
20
0
μ moles H_2O_2 mg^{-1}
100 mM NaCl
MEOH+100mM NaCl
CHCl3+100mM NaCl
Ethylacetate+100mM NaCl
90
80
70
60
50
40
30
20
10
0
μ moles H_2O_2 mg^{-1}
150 mM NaCl
MEOH+150 mM NaCl
CHCl3+150mM NaCl
Ethylacetate+150 mM NaCl

Figure 14

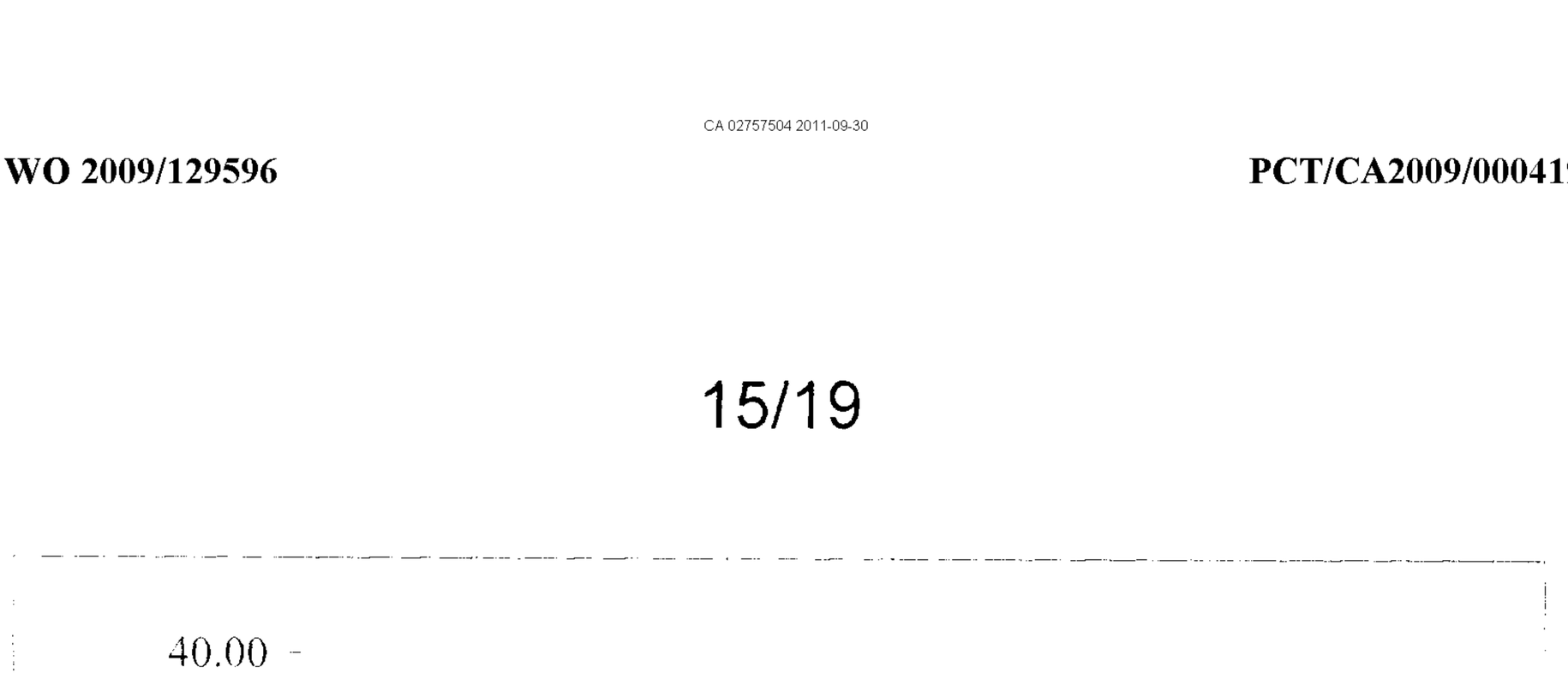
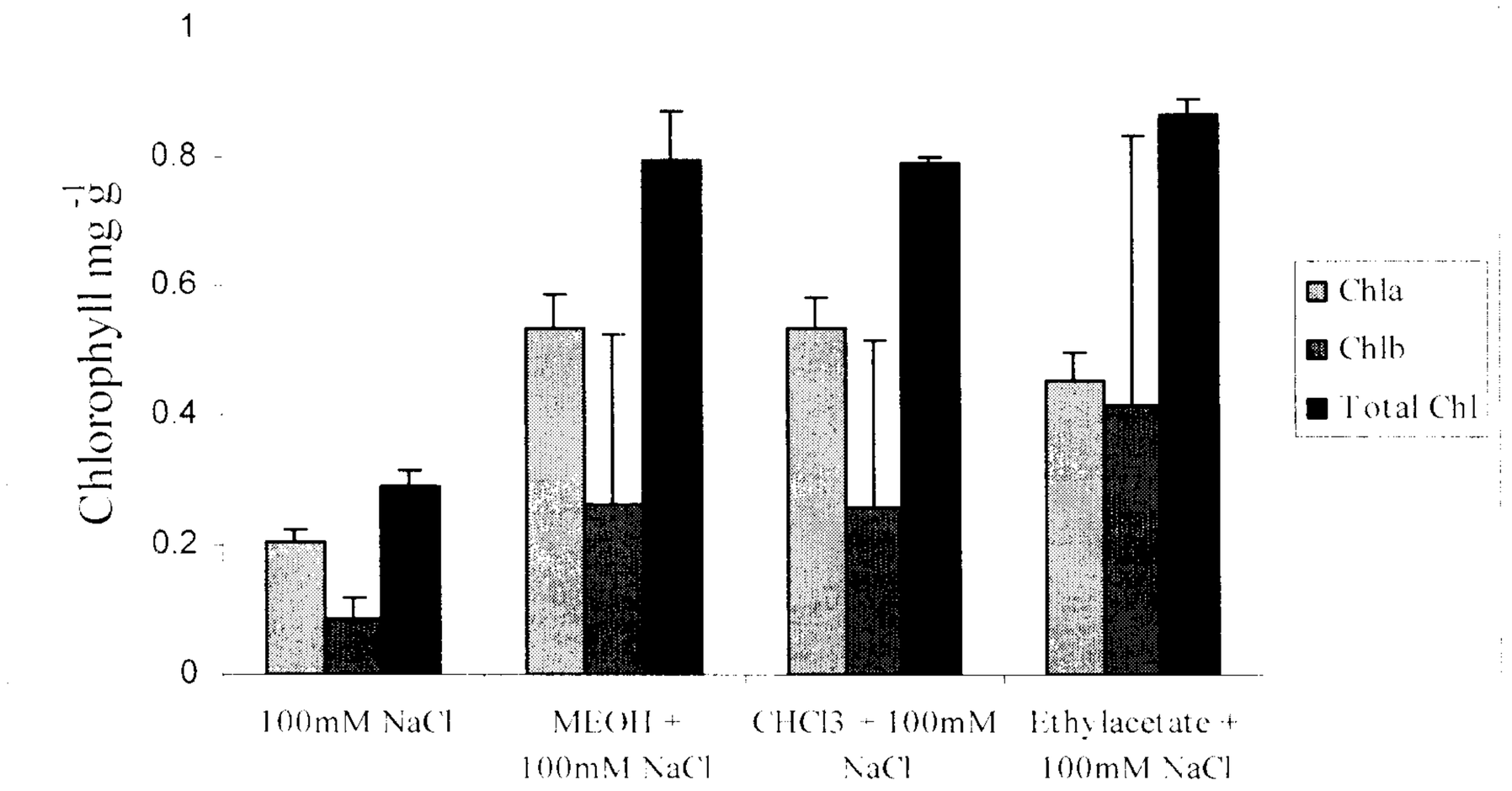

Percent leaf area affected
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
100 mM NaCl
MEOH+100mM NaCl
CHCl3-100mM NaCl
Ethace+100mM NaCl
Percent leaf area affected
120.00
100.00
80.00
60.00
40.00
20.00
0.00
150 mM NaCl
MEOH+150 mM NaCl
CHCl3+150mM NaCl
Ethylacetate+150 mM NaCl

Figure 15


Chlorophyll mg g-1
0
0.2
0.4
0.6
0.8
100mM NaCl
MEOH + 100mM NaCl
CHCl3 + 100mM NaCl
Ethylacetate + 100mM NaCl
Chla
Chlb
Total Chl

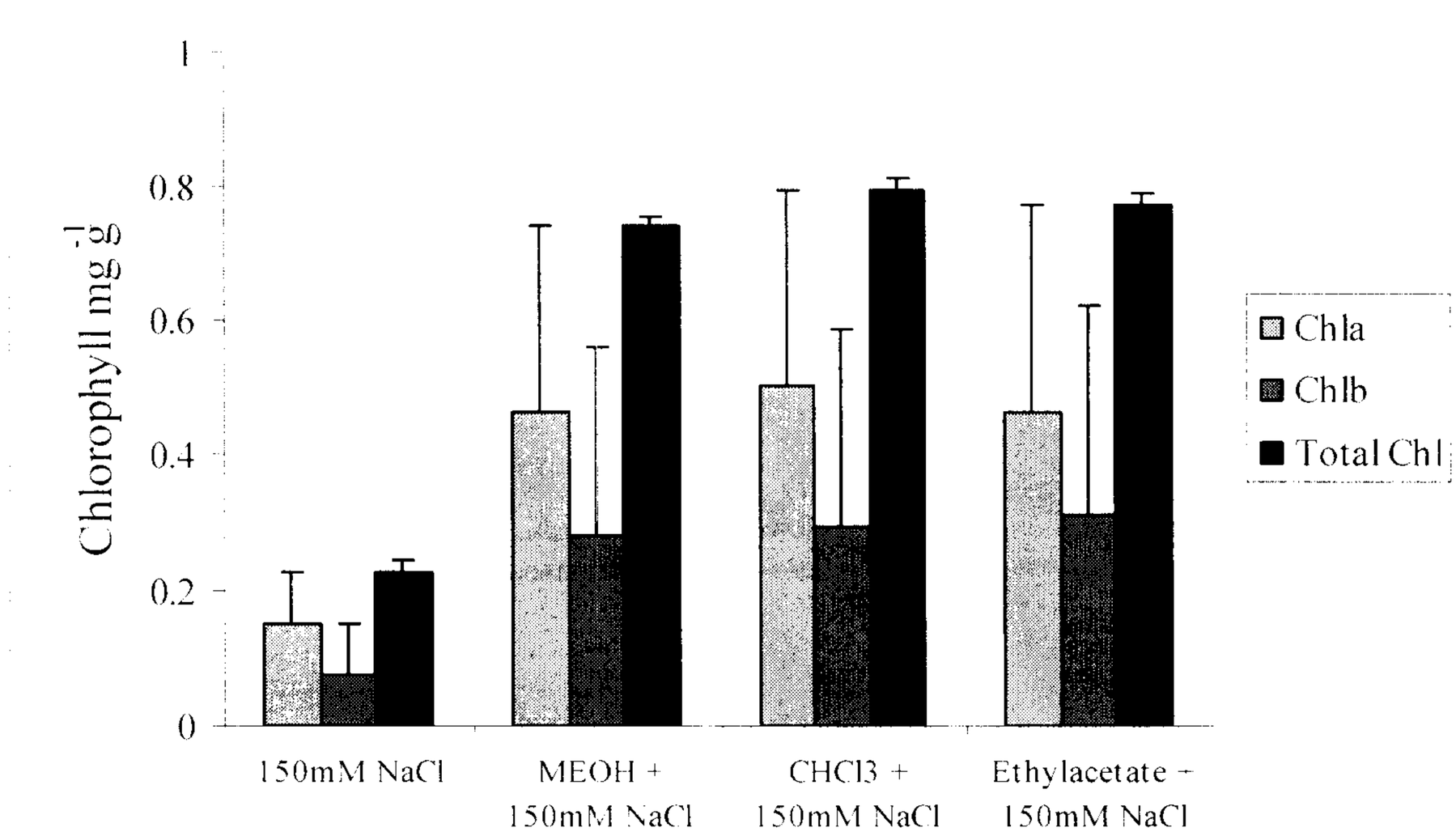
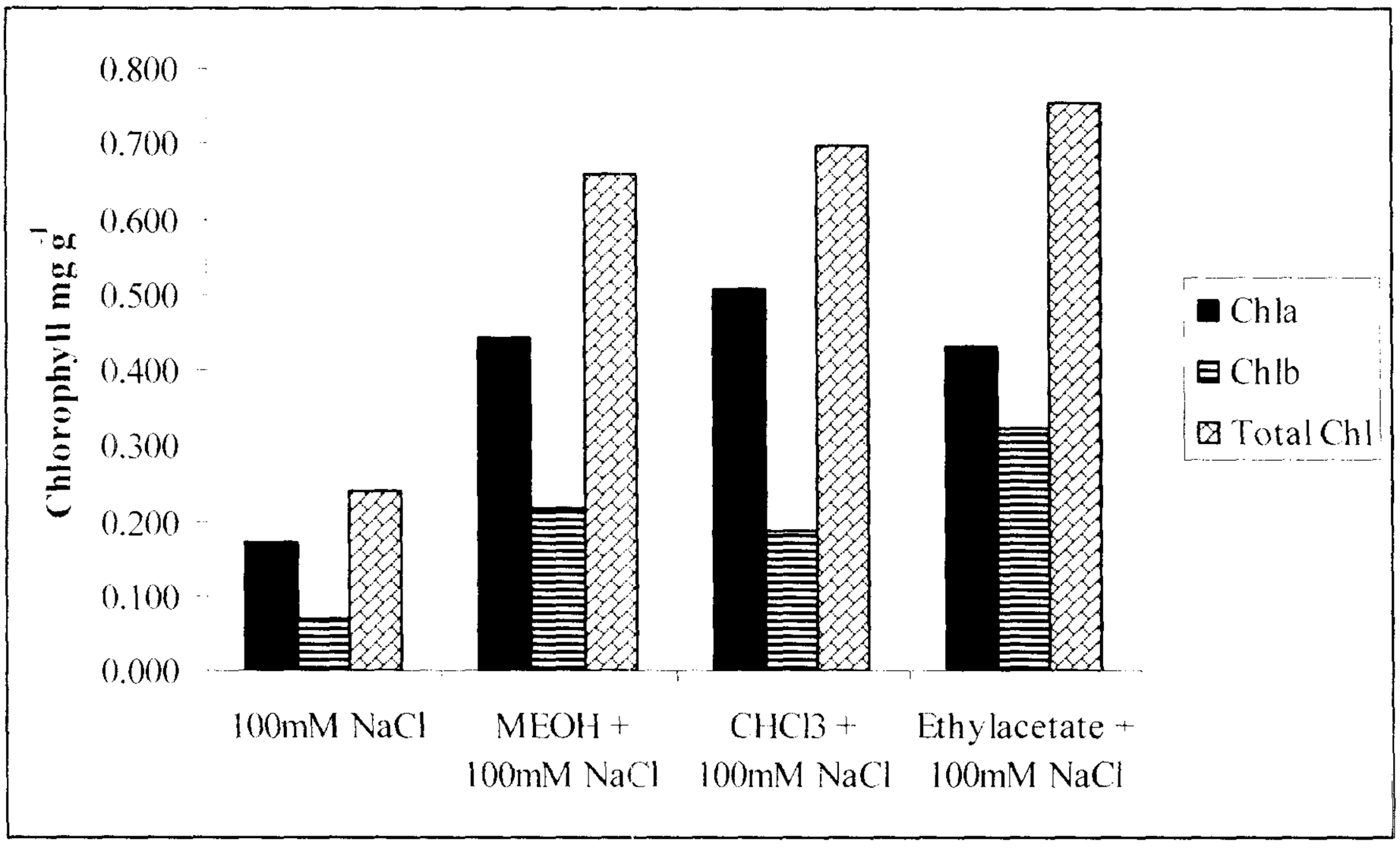

Chlorophyll mg g-1
0
0.2
0.4
0.6
0.8
1
150mM NaCl
MEOH + 150mM NaCl
CHCl3 + 150mM NaCl
Ethylacetate + 150mM NaCl
Chla
Chlb
Total Chl

Figure 16


0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Chlorophyll mg g-1
100mM NaCl
MEOH + 100mM NaCl
CHCl3 + 100mM NaCl
Ethylacetate + 100mM NaCl
Chla
Chlb
Total Chl

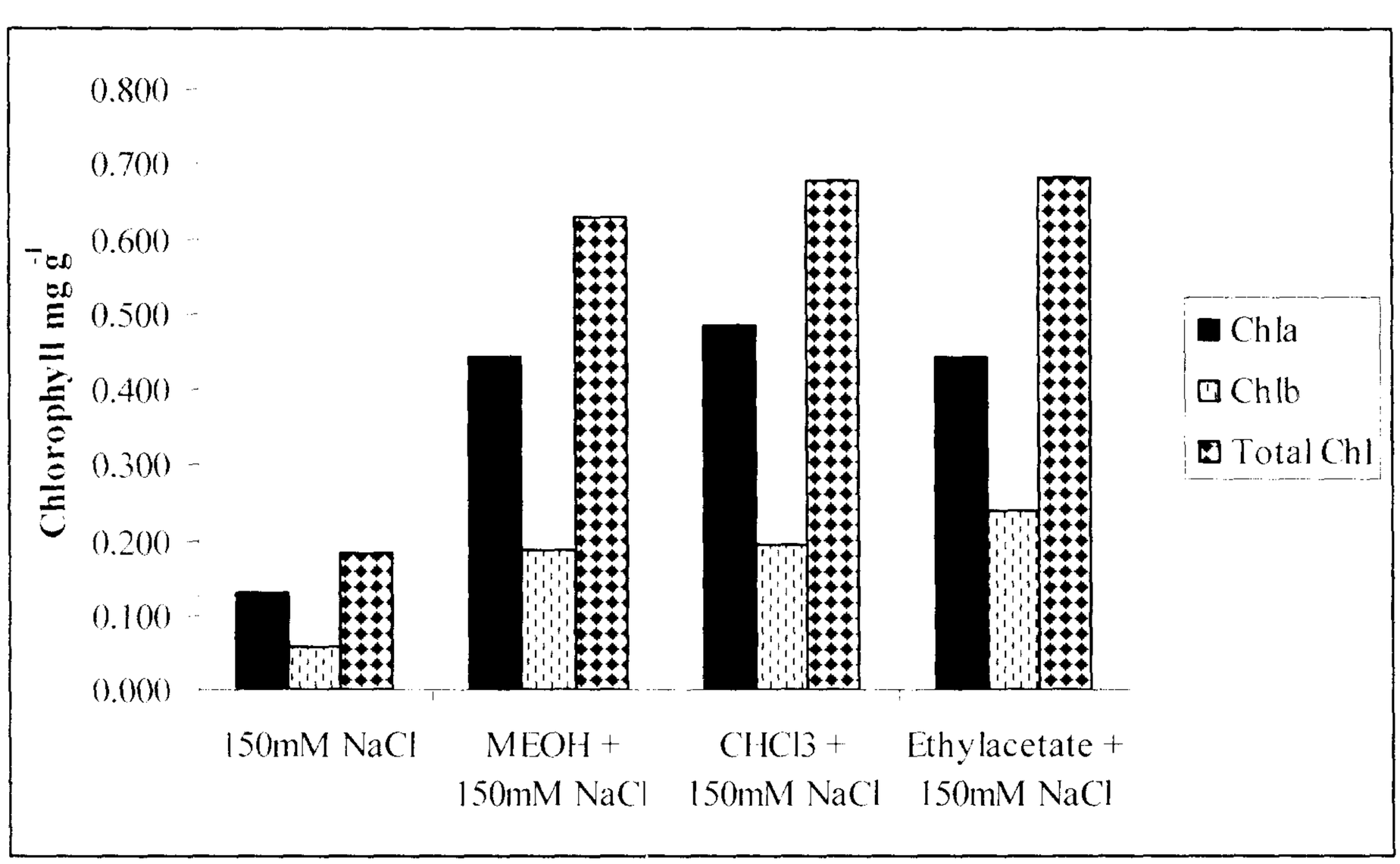

0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Chlorophyll mg g-1
150mM NaCl
MEOH + 150mM NaCl
CHCl3 + 150mM NaCl
Ethylacetate + 150mM NaCl
Chla
Chlb
Total Chl

Figure 17

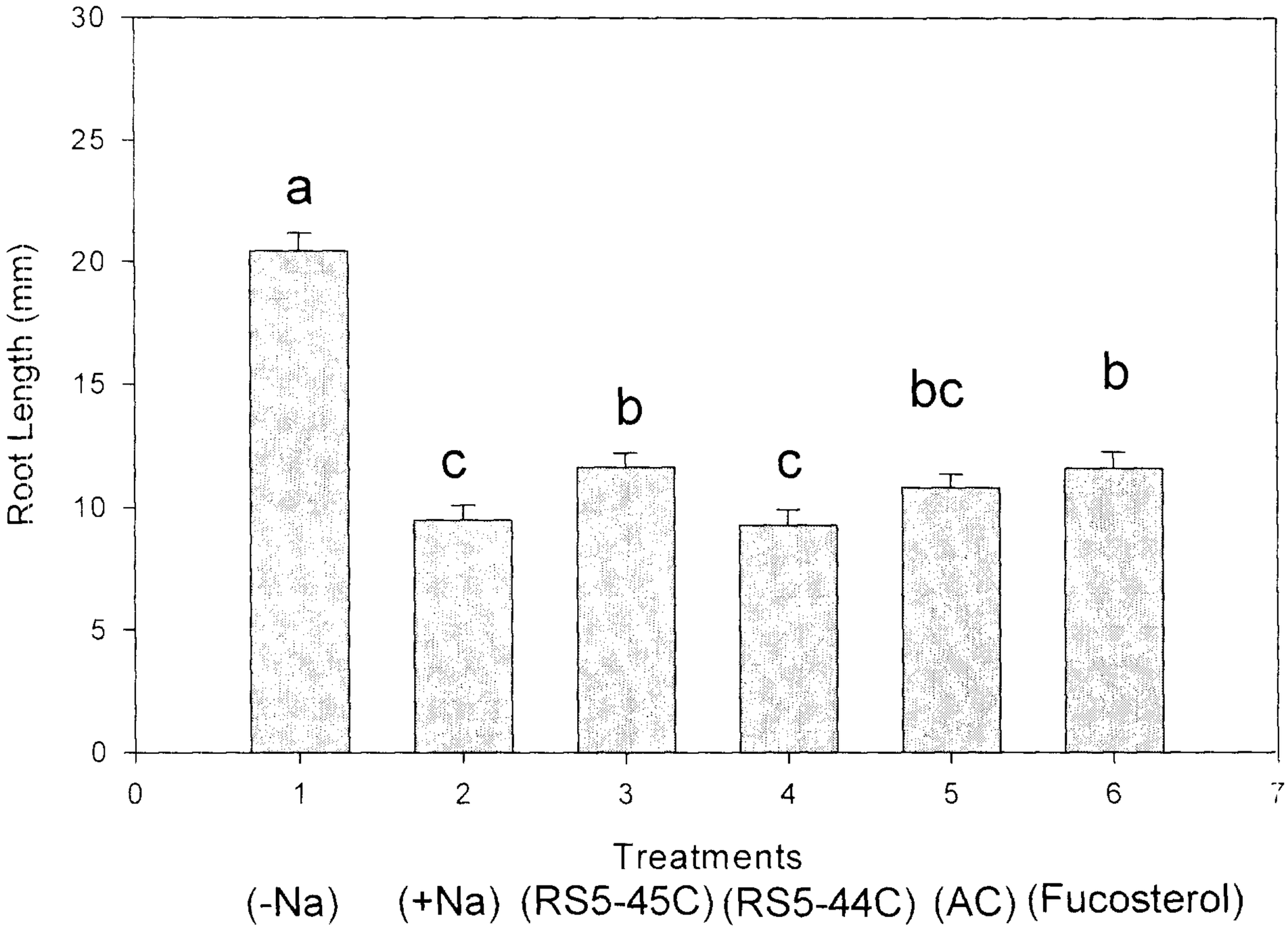

30
25
20
15
10
5
0
Root Length (mm)
a
c
b
c
bc
b
0
1
2
3
4
5
6
7
Treatments
(-Na)
(+Na)
(RS5-45C)
(RS5-44C)
(AC)
(Fucosterol)

Figure 18

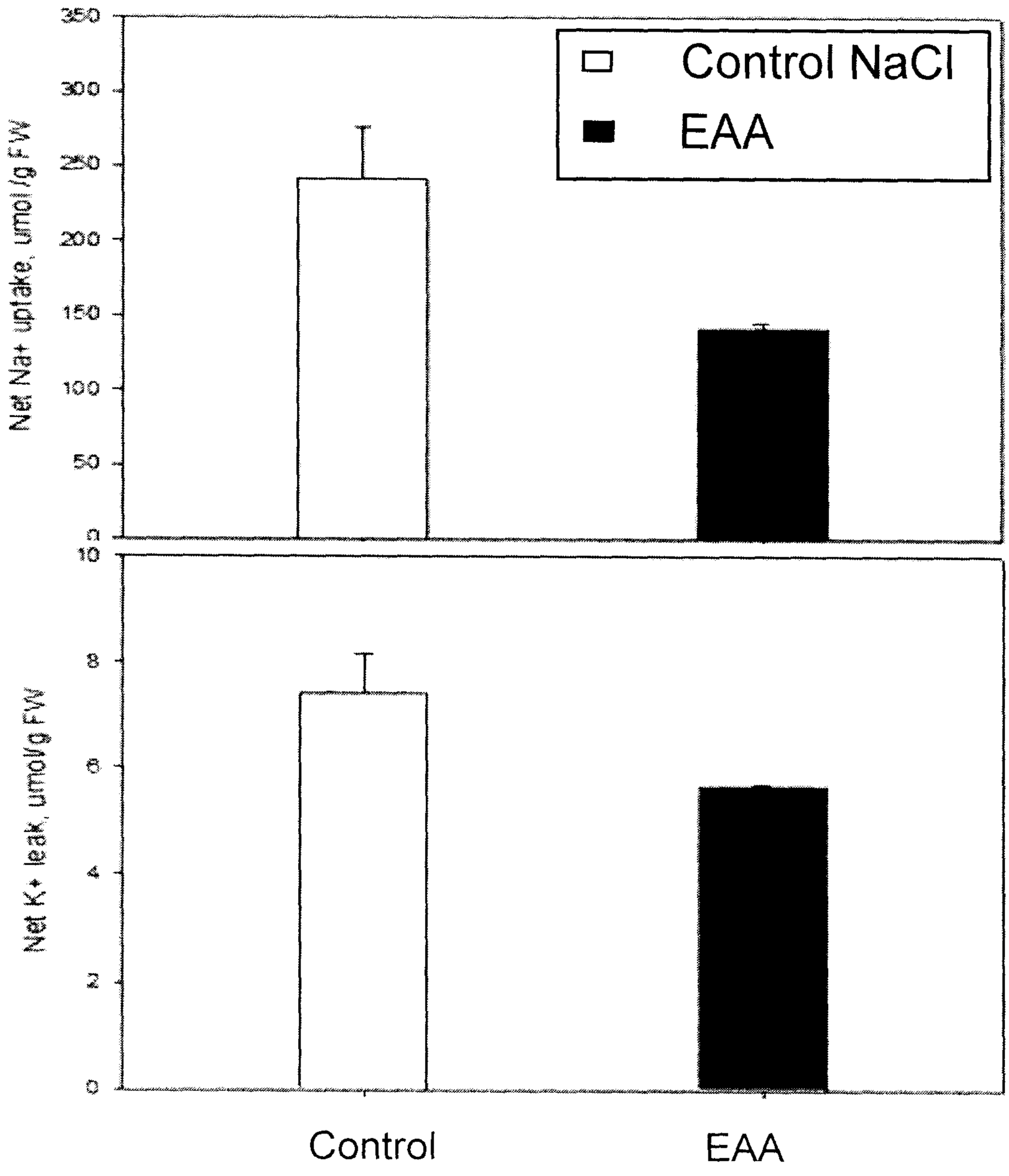

Control NaCl
EAA
Net Na+ uptake, umol/g FW
350
300
250
200
150
100
50
0
Net K+ leak, umol/g FW
10
8
6
4
2
0
Control
EAA

Figure 19

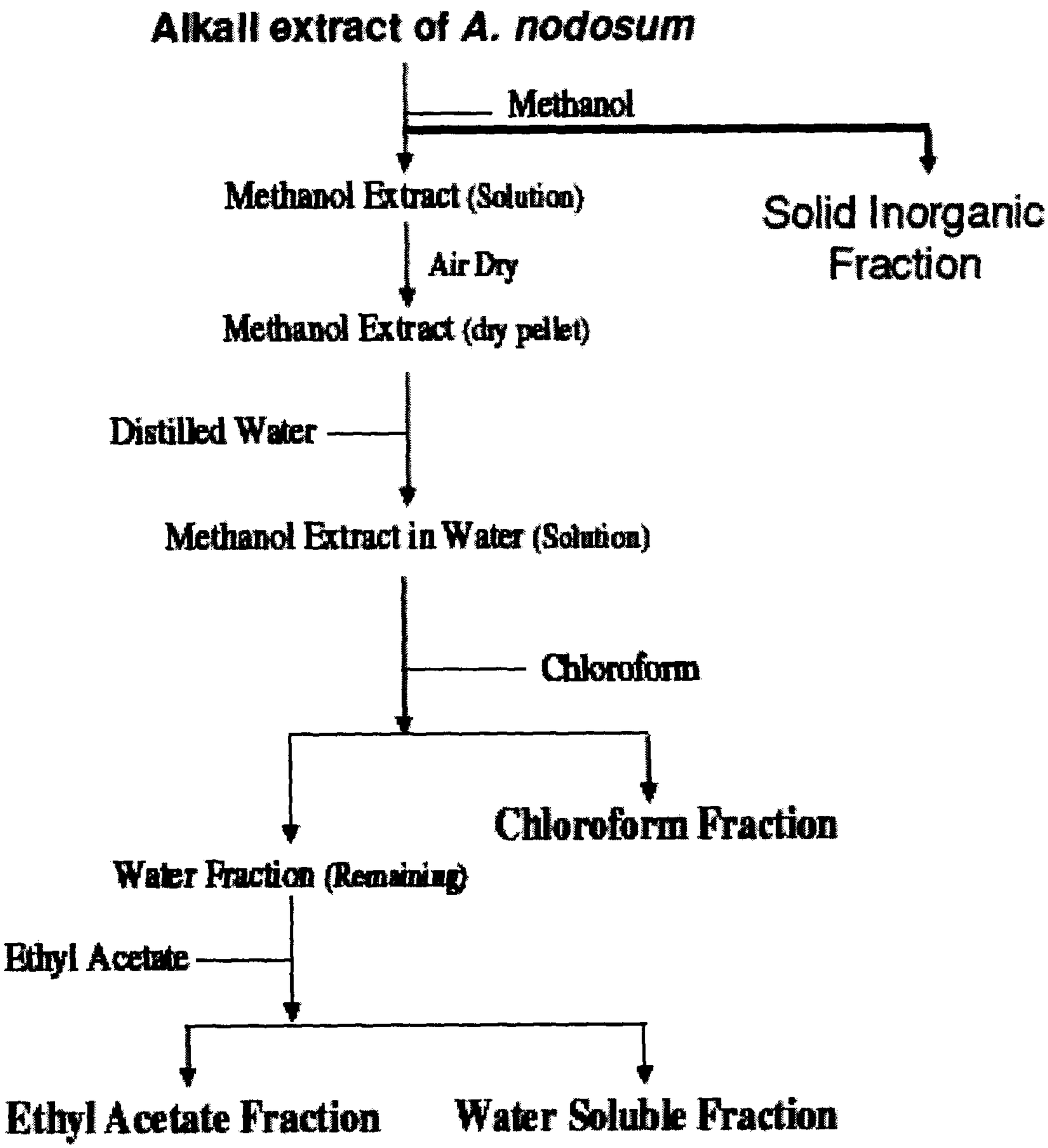

Alkali extract of A. nodosum
Methanol
Methanol Extract (Solution)
Solid Inorganic Fraction
Air Dry
Methanol Extract (dry pellet)
Distilled Water
Methanol Extract in Water (Solution)
Chloroform
Chloroform Fraction
Water Fraction (Remaining)
Ethyl Acetate
Ethyl Acetate Fraction
Water Soluble Fraction

Figure 1