RESPONSES OF YUKON THELLUNGIELLA TO A COMBINATION OF NITROGEN AND SALT STRESS

THE EFFECT OF NITROGEN CONCENTRATION ON COMPATIBLE SOLUTES DURING SALINITY STRESS IN *THELLUNGIELLA SALSUGINEA*

By

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ABSTRACT

Thellungiella salsuginea, a crucifer relative of *Arabidopsis thalinana*, tolerates extreme abiotic stresses such as salt, drought and cold (Taji et al., 2004; Inan et al., 2004; Gong et al., 2005; Wong et al., 2006; Griffith et al., 2007). Investigations into the abiotic stress tolerance of this plant have shown that the accumulation of compatible solutes in the cytosol of the cell can help restore cellular osmotic balance during periods of osmotic stress (Flowers et al., 1977; Greenway and Munns, 1980; Hasegawa and Bressan, 2000). The objective of this study was to identify qualitative and quantitative changes in polar metabolites present in *T. salsuginea* seedlings subjected to saline conditions. Seeds were germinated on Murashige and Skoog media and then *T. salsuginea* seedlings were transferred to defined tissue culture media where they were grown for a period of one to two weeks in the presence or absence of 100 mM NaCl (-S/+S) and under conditions of 0.1 mM or 1.0 mM nitrogen (designated LN and HN for low and high nitrogen, respectively). Shoot and root biomass and length determinations were taken daily and shoot tissue was extracted for analysis of polar metabolites by gas chromatography/mass spectrometry (GC/MS). Our hypothesis was that *T. salsuginea* seedlings experiencing salt stress would accumulate compatible solutes and that the level of nitrogen in the growing medium would have no influence on that outcome. Among the mass spectral tags (MSTs), proline has been proposed to perform the functions of a compatible solute (Hare et al., 1998; Zhu et al., 2001). If proline is a compatible solute in salinized plants we might expect the proline content to be the same and independent of nitrogen availability or that plants with low proline would perform poorly. However, we found the proline

content in salinized plants on LN to be 6.3-fold lower than in seedlings on HN+S media and root elongation and shoot biomass were not affected in seedlings on LN+S media relative to controls.

T. salsuginea grown under HN+S or LN+S grew at the same rate (average of 2.2 $mm \cdot d^{-1}$) and showed a 50% reduction in primary root elongation rate compared to HN or LN controls. As such, the presence of salt slowed the rate of root elongation but the level of N present had no effect. At the time of harvest, which averaged 18.7 ± 2.1 d for +S seedlings and 8.7 ± 1.5 d for –S seedlings, shoot fresh weight for plants on HN+S and LN+S media did not significantly differ from their respective controls. GC/MS analysis of metabolites showed that 49 MSTs underwent statistically significant ($p \leq 0.05$) changes for the four treatment combinations. Of these, the metabolites proline, raffinose, fructose and *myo*-Inositol were found by principal component analysis (PCA) to contribute strongly to the separation among treatment-specific datasets.

GC/MS datasets from HN, LN, HN+S, LN+S seedlings were compared with comparable datasets from mature, cabinet-grown plants (control unsalinized; C-0) and plants irrigated with solutions containing 300 mM NaCl (salinized; S-300) and mature field plants harvested over three growing seasons at a Yukon field site (2003, 2005 and 2006). In this meta-analysis, metabolic profiles were analyzed using ANOVA and of the ca. 300 metabolic compounds detected, only 52 underwent changes in abundance deemed statistically significant ($p \leq 0.05$). PCA and hierarchal cluster analysis (HCA) showed that metabolite profiles from HN, LN and C-0 were most similar to each other and metabolite profiles of HN+S seedlings more closely resembled S-300 plants. Metabolite profiles of

leaves from field plants harvested in 2005 and 2006 were similar to those of seedlings exposed to LN+S while those from 2003 were very distinct from other datasets

PCA identified proline as an MST strongly contributing to the placement of S-300 and HN+S datasets, while 2005 and 2006 field plants and LN+S plants accumulated carbohydrates, such as raffinose, fructose and several unidentifiable sugars but not proline. These results suggest that accumulation of carbohydrates may be an important stress tolerance strategy of *T. salsuginea* grown under field conditions whereas proline may be a compatible solute when nitrogen is not limiting.

Our study shows that *T. salsuginea* displays metabolic plasticity with respect to the organic solutes accumulated under osmotic stress and that nitrogen availability can influences the composition of accumulated organic solutes in seedlings grown on defined media. We propose that this plasticity may operate in the field where nitrogen content of soils is low and proline content of plants is as well. This plasticity may contribute towards *T. salsuginea*'s exceptional ability to survive and even thrive in extreme environments.

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ABBREVIATIONS

AMDIS	Automated Mass Spectral Deconvolution and Identification System
amu	atomic mass units
ANOVA	analysis of variance
Control-300	control cabinet –grown plants
d	day
dgd	digalactosyldiacylglycerol
dS/m	deci-Siemens per metre
Col-2	*Arabidopsis thaliana* genotype C2
Col-24	*Arabidopsis thaliana* genotype C24
FL	factor loading
FW	fresh weight
GASP	gas chromatography/mass spectrometry data analysis software package
GB	glycine betaine
GC/MS	gas chromatography / mass spectrometry
GolS	galactinol synthase
HCA	hierarchical cluster analysis
HN	high nitrogen (1 mM)
HN+S	high nitrogen (1 mM) and salt (100 mM)
h	hour(s)
Inps1	*myo*-Inositol 1-phosphate synthase

LN	low nitrogen (0.1 mM)
LN+S	low nitrogen (0.1 mM) and salt (100 mM)
min	minute(s)
mm	millimetres
mM	millimolars
MPa	mega pascals
MSI	Metabolomics Standards Initiative
MSTs	mass spectral tags
mtlD	mannitol-1-phosphate dehydrogenase
N_2	nitrogen gas (g) and liquid (l)
P5CS	Δ–pyrroline-5-carboxylate synthetase
PC	principal component one (1), two (2), three (3)
PCA	principal component analysis
pdh	proline dehydrogenase
RFOs	raffinose family oligosaccharides
RI	retention index
RRF	relative response factor
RT	retention time
Salt-300	cabinet-grown plants subjected to up to 300 mM NaCl stress
sdd	stomatal density development
SE	standard error
TPP	trehalose-6-phosphate phosphatise

TPS	trehalose-6-phosphate synthase
TPSP	TPS/TPP fusion gene
UDP	uracil-diphosphate
UGE	UDP-glucose 4-epimerase
WT	wild-type

LITERATURE REVIEW

Salt stress

Abiotic stresses such as drought, salinity and freezing are responsible for crop yield reductions of 50% or more per year (Boyer, 1982). High soil salinity can result either from human interference, such as irrigation with saline water, or from natural processes that result in salt deposits (Neumann, 1997). Salt affects crops by decreasing soil porosity, subsequently causing water stress which results in the loss of turgor pressure. Datta and Jong, (2002) reported salt-related impacts on agriculture in Haryana, India. According to their study the yield of wheat was reduced by 40% due to soil salinity over a five year period (Datta and Jong, 2002). During the same time, rice yield decreased by 56% (Datta and Jong, 2002). Most agricultural crops are affected by soil salinity in the range of 4-12 dS/m and are severely affected at higher concentrations (Richards, 1954; Van Hoorn and Van Alphen, 1994; Datta and Jong, 2002). By improving the ability of crop plants to cope with saline soils we can decrease yield losses and strengthen our agricultural economy. However, achieving this objective requires a greater mechanistic understanding of how plants tolerant to saline conditions withstand this stress.

Salt stress tolerance strategies used by plants

Halophytes are salt tolerant plants that grow in soils with high electrolyte concentrations (Flowers et al., 1977). These extreme environments may contain several chemically different types of salt in the soil, the most common form of which is NaCl

(Flowers et al., 1977). The extremophile plants that thrive in these environments use different strategies to maintain homeostasis and detoxify the effects of perturbing ions (Flowers et al., 1977; Hasegawa and Bressan, 2000).

One tolerance strategy used by plants involves salt exclusion or secretion. *Aeluropus litoralis* excretes accumulated Na^+ and Cl^- ions through highly specific structures called salt glands located in epidermal leaf tissue (Pollak and Waisel, 1979). NaCl that is secreted by the glands can crystallize on the leaf surface where it is no longer toxic to the plant. Another halophyte, *Rhizophora mangle*, excludes salt from its roots (Hopkins, 1999). The literature suggests that *R. mangle*'s ability to exclude salt is due to radial filtration across the cells of the root endodermis, cortex, and epidermis (Werner and Stelzer, 1989; Tomlinson, 1986).

A second salt-tolerance strategy used by halophytes involves salt uptake and accumulation. Halophytes take up Na^+ and Cl^- ions from the soil and transport these ions through the xylem where they are accumulated in the vacuoles of leaf cells (Hasegawa and Bressan, 2000). In a saline environment accumulating ions in vacuoles of leaf cells lowers the osmotic potential in aerial plant parts, which then allows for easier water uptake and transport (Flowers et al., 1977; Hasegawa and Bressan, 2000). In contrast, glycophytes (salt sensitive species) restrict ion movement and retain the majority of ions in root vacuoles (Flowers et al., 1977; Hasegawa and Bressan, 2000). Radyukina et al. (2007) reported that the halophyte *Thellungiella halophila* (C.A. Meyer) O.E. Schultz accumulated higher concentrations of Na^+ and Cl^- ions than the more salt sensitive plant *Plantago major*. *T. halophila*'s ability to effectively accumulate perturbing ions is partly

accredited to salt-responsive activation of Na^+ transport mechanisms (Hasegawa and Bressan, 2000; Vera-Estrella et al., 2005). In addition, the transport mechanisms in *T. halophila* have high Na^+/K^+ selectivity, which averts potassium (K^+) deficiency under salt conditions (Volkov et al., 2003; Vinocur and Altman, 2005; Vera-Estrella et al., 2005; Yamaguichi and Blumwald, 2005). Potassium deficiency affects enzyme activation, protein synthesis, photosynthesis, stomatal movement and turgor pressure in plants (Marschner, 1995; Kant and Kafkafi, 2001).

A third salt-tolerance strategy involves the accumulation of organic solutes (Hasagawa and Bressan, 2000). When perturbing Na^+ ions are accumulated in vacuoles, an osmotic imbalance between the vacuole and the surrounding cytoplasm occurs (Flowers et al., 1977). In order to restore balance and lower the osmotic and hence water potential of the cytoplasm, plants may actively accumulate organic solutes in the cytosol (Flowers et al., 1977; Hasegawa and Bressan, 2000). The accumulation of cytosolic solutes restores cellular osmotic balance and cellular homeostasis (Flowers et al., 1977; Greenway and Munns, 1980; Hasegawa and Bressan, 2000). The process of accumulating organic solutes (also termed compatible solutes, osmolytes or osmo-protectants) to alleviate osmotic stress is called osmoprotection (Flowers et al., 1977; Greenway and Munns, 1980; Hasegawa and Bressan, 2000). The term "compatible" refers to the requirement that organic solutes be non-toxic and not interfere with metabolism (Chen and Murata, 2002). A few well studied compatible solutes are proline, glycine-betaine and mannitol (Hayashi et al., 1997; Hasegawa and Bressan, 2000; Abebe et al., 2003).

Some scientists have called into question the classical view of osmoprotection and osmolytes (Hare et al., 1998; Hasegawa and Bressan, 2000). There is evidence that the organic solute itself may not be as important as the metabolic pathway involved in its synthesis (Hare et al., 1998; Jain and Selvaraj, 1997; Bohnert et al., 1999). For example, many metabolic pathways yield NADPH, that can then be used during photosynthesis and help negate photo-inhibition. Other researchers propose that compatible solutes are produced for scavenging reactive oxygen species, that would otherwise damage plant cells, as opposed to serving in osmotic adjustment (Hong et al., 2000; Akashi et al., 2001; Chen and Murata, 2002; Abebe et al., 2003). For example, the amino acid proline has been reported to be a key hydroxyl radical scavenger under salt stress (Smirnoff and Cumbes, 1989; Hong et al., 2000). However, regardless of the reason for osmolyte synthesis the accumulation of organic solutes under salt stress is unequivocal and thus should be addressed.

Bio-engineering salt tolerance in plants

Enhancing tolerance by genetically engineering plants is an important focus for researchers attempting to improve agricultural yields for crops experiencing saline soil conditions (Bartels and Sunkar, 2005). Several attempts have been made to bio-engineer plants to increase their production of compatible solutes (Chen and Murata, 2002; Mani et al., 2002; Jang et al., 2003; Nanjo et al., 2003; Vinocur and Altman, 2005). There are four main classes of compatible solutes: N-containing solutes, sugars, straight-chain polyhydric alcohols and cyclic polyhydric alcohols (Bartels and Sunkar, 2005).

N-containing solutes

Two nitrogenous compounds, glycine betaine (GB) and proline, are important osmoprotectants in plants subjected to salt stress (Hasegawa and Bressan, 2000; Zhu, 2001; Chen and Murata, 2002; Bartels and Sunkar, 2005; Vinocur and Altman, 2005). GB synthesis occurs via two pathways: the choline-dehydrogenation/oxidation pathway or the glycine methylation pathway (Rhodes and Hanson, 1993; Chen and Murata, 2002; Rontein et al., 2002). The choline-dehydrogenation/oxidation pathway is the dominant pathway in many organisms (Rhodes and Hanson, 1993; Rontein et al., 2002). In plants, choline is a GB precursor and the pathway involves two steps catalyzed by two enzymes: choline mono-oxygenase and betaine aldehyde dehydrogenase, respectively (Rhodes and Hanson, 1993).

To avoid the need for two separate enzymes, Huang and co-workers (2000) constitutively expressed a bacterial choline oxidase (*cox*) gene in Arabidopsis, *Brassica napus* and *Nicotiana tabacum*. The product of the *cox* gene catalyzes both steps for GB synthesis. Their purpose was to "install" an alternative GB synthesis pathway in order to test the ability of the resulting transgenic plant to survive under salt stress. However, their study showed that enhancing GB synthesis in plants has its limitations. The transgenic plants required exogenous choline to accumulate significantly more GB than levels already found in wild-type plants (Huang et al., 2000). Once transgenic Arabidopsis plants were exposed to exogenous choline, GB content in leaf tissue was 97% higher in those transgenics than in control plants (Huang et al., 2000). The difficulty of increasing GB content through a transgenic approach was reported earlier by Nuccio et al. (1998)

who also identified limitations of enhancing GB synthesis without an increased choline supply.

Proline can be synthesized via two pathways: the "glutamate pathway" and the "ornithine pathway" (Hu et al., 1993). The glutamate pathway is dominant under salt-stress (Delauney et al., 1993). Under salt stress glutamate is converted to glutamate-5-semialdehyde via Δ–pyrroline-5-carboxylate synthetase 2 (P5CS2) (Hu et al., 1992; Kishkor et al., 1995; Yoshiba et al., 1995; Zhang et al., 1995; Strizhov et al., 1997; Hong et al., 2000). Proline regulates its own production via product inhibition of P5CS2. As such, P5CS2 is considered to be the rate-limiting enzyme during proline biosynthesis (Zhang et al., 1995; Strizhov et al., 1997; Hong et al., 2000). Hong and co-workers (2000) removed the feedback inhibition property of *N. tabacum* P5CS2 using site-directed mutagenesis of a Phe residue reported earlier to be important for feedback inhibition (Zhang et al., 1995). Transgenic plants constitutively expressing the mutated version of P5CS2 accumulated significantly more proline, had reduced free radical levels and showed increased salt tolerance compared to wild-type plants (Huang et al., 2000). Other studies have over-expressed a mothbean P5CS2 in Arabidopsis (Kishor et al., 1995) and *Oryza sativa* (Zhu et al., 1998) and reported increased salt-tolerance. Transgenic Arabidopsis plants over-expressing mothbean P5CS2 had leaf osmotic potentials of -0.70 in the absence of salt stress and -0.73 MPa in the presence of 250 mM NaCl (Kishor et al., 1995). Wild-type Arabidopsis leaf osmotic potentials lowered from -0.70 to -1.11 MPa due to 250 mM NaCl stress (Kishor et al., 1995). Transgenic mothbean P5CS2 over-expressing *O. sativa* maintained significantly higher root and shoot fresh weights and root

length compared to wild-type plants, upon exposure to 100 mM NaCl five days following germination (Zhu et al., 1998).

The proline degradation pathway has also been targeted for genetically engineering salt tolerance. The enzyme proline dehydrogenase (pdh) catalyzes the first step in proline degradation (Kiyosue et al., 1996). Nanjo et al. (1999) suppressed proline degradation by transforming Arabidopsis with a *pdh* antisense gene. Under salt stress antisense transgenic plants accumulated proline and exhibited increased stress tolerance (Nanjo et al., 1999). However, as was the case for GB accumulation, proline over-production by plants is not free of challenges. An Arabidopsis *pdh* over-expressing mutant that was unable to degrade proline showed symptoms of proline toxicity (Nanjo et al., 2003). The growth of this mutant was retarded compared to wild-type plants (Nanjo et al., 2003). As well, when transgenic and wild-type plants were exposed to an exogenous supply of 2 mM proline, the *pdh* transgenics showed sensitivity while wild-type plants grew normally. Plant death of *pdh* transgenics occurred following exposure to an exogenous supply of 10 mM proline (Nanjo et al., 2003). Proline toxicity has also been reported by Hellmann et al. (2000) for proline hypersensitive Arabidopsis mutants and Deuschle et al. (2001) for yeast mutants defective in proline catabolism. Due to the potential toxicity high proline content may have on plant cells one cannot rely on proline alone to combat osmotic stress (Mani et al., 2000; Nanjo et al., 2003).

Sugars

Several reports show a strong correlation between sugar accumulation and salt stress tolerance (Ramanjulu et al., 1994; Gilmour et al., 2000; Hoekstra et al., 2001; Streeter et al., 2001; Taji et al., 2002). One such carbohydrate is trehalose, a non-reducing disaccharide of glucose that is present in many bacteria and some desiccation-tolerant plants such as *Myrothamnus flabellifolia* and *Sporobolus stapfianus* (Phillips et al., 2002). Although Arabidopsis is not a desiccation-tolerant plant its genome encodes the gene for trehalose-6-phosphate synthase (*TPS*), an enzyme involved in trehalose synthesis (Vogel et al., 1998). The physiological role for TPS in Arabidopsis was originally unknown (Vogel et al., 1998; Goddijn and van Dun., 1999). However, a study conducted by Zentella et al. (1999) showed that the Arabidopsis *TPS* gene could complement a *TPS* deficient yeast mutant. Yeast and bacteria catalyze trehalose synthesis from UDP-glucose in two steps: (1) UDP-glucose is converted to trehalose-6-phosphate via TPS and (2) trehalose-6-phosphate is converted to trehalose via trehalose-6-phosphate phosphatase (TPP) (Seo et al., 2000). Holmstrom et al. (1996) transformed tobacco with a yeast TPS gene and Yeo et al. (2000) repeated this approach using potato. In both species, transgenic *TPS*-expressing plants showed increased drought-tolerance compared to wild-type plants but also showed stunted growth and other undesirable morphological changes (Homstrom et al., 1996; Yeo et al., 2000). Goddijn et al. (1997) and Pilon-Smits et al. (1998) transformed plants with both *TSP* and *TPP* encoding genes from *E.coli* and their attempts resulted in similar morphological changes as reported by Holmstron et al. (1996). Interestingly, rice is reported to be more tolerant to trehalose accumulation than

those species mentioned above. Rice plants exposed to an exogenous supply of trehalose showed no growth inhibition or morphological differences compared to control plants (Garcia et al., 1997). Successful transformation of rice to produce trehalose by using a *TPS/TPP* (*TPSP*) fusion gene, constructed by Seo et al., (2000), was reported by Jang et al., 2003. The transgenic rice showed increased tolerance to salt and drought stress compared to wild-type plants that were unable to survive the stress treatments (Jang et al., 2003).

The raffinose family oligosaccharides (RFOs), including raffinose and stachyose, accumulate in seeds during desiccation stress (Castillo et al., 1990; Saravitz et al., 1987). A study conducted by Taji et al., 2002 examined the role of raffinose in stress-tolerance of mature Arabidopsis plants. Arabidopsis plants that were subjected to drought, salt or cold stress accumulated significantly more raffinose than control, unstressed, plants (Taji et al., 2002). These findings were supported by an increase in galactinol synthase (GolS) associated transcript levels as shown by northern blot hybridization (Taji et al., 2002). GolS catalyses the first step in the biosynthesis of RFOs (Saravitz et al., 1987). Taji et al. (2002) also reported increased drought tolerance in GolS over-expressing transgenic Arabidopsis plants compared to wild-type plants. In a complementary approach, Liu et al. (2007) transformed Arabidopsis to over-express a rice UDP-glucose 4-epimerase gene and subsequently increased the production of UDP-glucose 4-epimerase (UGE). UGE catalyzes the conversion of glucose to UDP-galactose, the substrate used by GolS to produce galactinol. After exposure to salt stress the survival rate of transgenic Arabidopsis plants was 80%, compared to 53% for salt stressed wild-type plants (Liu et

al., 2007). As well, salt-stressed transgenic plants accumulated 150-fold more raffinose than salt-stressed wild-type plants (Liu et al., 2007).

Straight chain polyhydric alcohols

Mannitol is a sugar alcohol that accumulates in some plants during periods of salt stress (Chen and Murata, 2002; Vinocur and Altman, 2005). However, evidence for a role of mannitol in stress tolerance is contradictory. Abebe et al. (2003) transformed wheat plants to express the *E. coli* mannitol-1-phosphate dehydrogenase gene (*mtlD*). They reported transgenic plant fresh weight to be unaffected by salt stress while fresh weight of control plants was reduced by 37%. However, the amount of mannitol accumulated in these transgenic plants was insufficient to account for the observed osmotic adjustment (Abebe et al., 2003). Tarczynski et al. (1993) showed that mannitol-producing transgenic tobacco plants exhibited increased tolerance to salt stress compared to control plants. Karakas et al. (1997) evaluated the contribution of mannitol to osmotic adjustment in mannitol-producing transgenic tobacco plants created by Tarczynski et al. (1993). Karakas et al. (1997) showed a 20-25% reduction in fresh weight of transgenic plants compared to wild-type plants and that the amount of mannitol accumulated in leaf tissue in transgenic tobacco plants could only account for up to 40% of the osmotic adjustment seen in the cytoplasm. A conclusion reached by both groups was that mannitol may serve as a radical oxygen scavenger during periods of osmotic stress as opposed to primarily serving as a compatible solute (Karakas et al., 1997; Abebe et al., 2003).

Cyclic polyhydric alcohols

Sugar alcohols such as pinitol, ononitol and *myo*-Inositol have been suggested to act as osmolytes in plants under salt stress (Sheveleva et al., 1997; Nelson et al., 1998). Ishitani et al. (1996) compared the regulation of the gene encoding *myo*-inositol 1-phosphate synthase (*Inps1*) in *Mesembryanthemum crystallinum* and Arabidopsis. An enzyme encoded by *Inps1* catalyzes the first step in *myo*-inositol biosynthesis. The expression of Inps1 was shown to be up-regulated in the halophyte *M. crystallinum* but not in Arabidopsis during periods of salt stress (Ishitani et al., 1996). Smart and Flores (1997) generated *Inps1*-over-expressing transgenic Arabidopsis plants in an effort to link *myo*-inositol accumulation to salt tolerance. However, the authors showed that there was no significant difference in survival between transgenic and control plants when these plants were subjected to 200 mM NaCl stress (Smart and Flores, 1997). However, Lehner et al. (2008) showed evidence in support of a possible beneficial role for this metabolite in stress tolerance for the resurrection plant *Xerophyta viscose.* The authors reported a 40-fold increase in the leaf content of *myo*-inositol during the first 6 h of salt stress (Lehner et al., 2008).

Metabolomics

In their review, Bressan et al. (2001) proposed that we have entered the "era of post-Arabidopsis genome sequence research." However, while gene sequence is no longer elusive, gene function remains as such. That is, we may have the entire

Arabidopsis genome sequence at our fingertips but as of the year 2001 the biological function for one-third of it remains unknown (Fiehn, 2001).

Comprehensive approaches to studying the genome can take place at different levels. Transcriptomics is the study of genome-level responses by assessing transcript abundance. This approach often does not meet statistical requirements, is costly and can be misleading (Fiehn, 2001; Fiehn et al., 2001). Proteomics is the study of cellular responses at the protein level and this approach often overlooks low abundance proteins that, even in small quantities, can strongly affect metabolic pathways (Fiehn et al., 2000a; Fiehn, 2001).

The metabolome of a biological system is the collection of metabolites synthesized by that system. The analysis, study and identification of these metabolites comprise the functional genomics approach called metabolomics. The addition of metabolic profiling to our transcript and proteomic "tool set" allows us to identify pleiotrophic effects including complex phenotypes resulting from single point mutations. In theory, metabolomics should enable us to acquire a greater mechanistic understanding of cellular functions. Halket et al. (1999) were able to distinguish between healthy and diseased human tissues based upon metabolites present. Fiehn et al. (2000b) analyzed the metabolites present in two Arabidopsis ecotypes, *Col-2 WT* and *Col-24 WT*, and in two mutants of each ecotype, *dgd1* and *sdd1*, respectively. The metabolic phenotypes of each ecotype were different, distinguishing *Col-2 WT* and *Col-24 WT* from each other and the mutants retained metabolic phenotypes similar but not identical to their wild-type counterparts. In the study by Fiehn et al (2000b), a greater overlap in metabolic

phenotype was seen between the *sdd1* mutant and the *Col-24 WT* than between the *dgd1* mutant and the *Col-2 WT*. Since the *sdd1* mutant is characterized by a change in stomatal density, a significant difference in metabolism compared to a wild-type plant was unexpected. However, the *dgd1* mutant is characterized by photosynthesis impairment and hypersensitivity to light (Härtel et al., 1998) and this contributes to the significant metabolic differences seen between the *dgd1* mutant and the *Col-2 WT* (Fiehn et al., 2000b). Primary metabolites have also been profiled to distinguish between mutant and wild-type potato (Roessener et al., 2001), apricot varieties (Katona et al., 1999), and stress response of Arabidopsis and its relative, *T. halophila* (Gong et al., 2005). Desbrosses et al. (2005) used metabolic profiling to distinguish between plant responses in various organs during nodulation of *Lotus japonicus*. Kim et al. (2007) profiled Arabidopsis metabolites at various time points during a 72 h salt stress study and they reported differences in short and long term stress responses. Gagneul et al. (2007) used metabolic profiling to assess compatible solute accumulation of the halophyte *Limonium latifolium* during NaCl treatments. In all of the above mentioned studies metabolite profiling was performed using gas chromatography coupled with mass spectrometry (GC/MS).

GC/MS is a robust and reliable technique used in metabolic profiling studies (Fiehn et al., 2001; Fiehn, 2001). This technique combines high chromatographic separation with a universal detector that allows for the simultaneous identification of metabolic compounds including sugars, sugar alcohols, amino acids, amines and organic acids (Fiehn, 2001). Simply put, GC/MS provides the researcher with a metabolic

"snapshot" of the organism under study. These snapshots or GC/MS profiles can be analyzed using mass spectra deconvolution software (AMDIS) (Fiehn, 2001). Deconvolution software analyses the mass spectras produced from GC/MS runs to help distinguish minor, co-eluting compounds whose presence would otherwise be disguised by chemical noise (Fiehn, 2001). Often GC/MS provides the researcher with such a large dataset that statistical analysis tools for data reduction are required.

Principal component analysis

Principal component analysis (PCA), is a multivariate, orthogonal and unsupervised statistical tool for data exploration and reduction commonly used by ecologists (Peres-Neto et al., 2003). PCA is not suitable for hypothesis testing, only for data exploration (Peres-Neto et al., 2003; Trygg et al., 2006; Fiehn et al., 2008). Recently, the Metabolomics Standards Initiative (MSI) reported PCA to be a necessary parameter for the reporting of metabolomic experiments (Fiehn et al., 2008). As a statistical tool, PCA extracts new variables termed principal components by drawing a vector through a dataset analyzed in 3D space. The first principal component extracted accounts for the greatest variance seen in the dataset (Kendall, 1980; Peres-Neto et al., 2003; Trygg et al., 2007). The second principal component extracted accounts for the second greatest variance present and so forth (Kendall, 1980; Peres-Neto et al., 2003; Trygg et al., 2007). However, as stated above PCA is not a hypothesis testing statistical tool and, as such, interpreting the biological meaning behind principal components extracted from PCA is subjective (Kendall, 1980; Cadima and Jolliffe, 1995). However, researchers do interpret

principal components in order to give a biological meaning to the statistical analysis (Jolliffe, 1986; Fiehn et al., 2000b; Fiehn et al., 2001; Desbrosses et al., 2005; Trygg et al., 2006; Kim et al., 2007; Robinson et al., 2007; Fiehn et al., 2008). For example, Desbrosses et al. (2005) applied PCA to metabolic profiles corresponding to different plants organs (roots, leaves and flowers) in *Medicago truncatula* and *L. japonicus*. The first principal component extracted from their PCA described a difference between root to shoot metabolism and the second principal component described differences between nodules and all other plant organs tested. In a study described earlier in this review, Fiehn et al. (2000b) tested metabolic changes between four Arabidopsis genotypes (two homozygous ecotypes and a mutant of each ecotype) and the authors found their first principal component to separate wild-type Arabidopsis from mutants. Since results obtained via PCA must be interpreted by the researcher, studies often couple PCA with other less subjective statistical tools, such as ANOVA and hierarchal cluster analysis (HCA) (Rossener et al., 2000; Rossener et al., 2001; Rizhsky et al., 2004; Desbrosses et al., 2005; Gong et al., 2005; Gagneul et al., 2007; Robinson et al., 2007; Sanchez et al., 2008).

Alternative genetic models for studying stress tolerance

For decades Arabidopsis has served as the model organism for plant genetics and biology (Glass, 1951; Rédei, 1992; Meinke et al., 1998; Somerville, 2000). With its small size, abundant seed production, availability of mutants, ease of transformation and rapid life cycle, Arabidopsis possesses many qualities of an ideal model organism and has

contributed new knowledge to many areas of plant biology (Meinke et al., 1998; Maes et al., 1999; Somerville, 2000; Weigel et al., 2000; Young et al., 2001). Although we have made advancements in understanding plant stress including salt response by studying Arabidopsis (Zhu et al., 1997), this plant is a glycophyte that is sensitive to water deficits and salt treatments (Hasegawa and Bressan, 2000; Bressan et al., 2001; Amtmann et al., 2005). However, salt cress mustard or *Thellungiella salsuginea* (Pallas) O.E. Schulz is a relative of Arabidopsis that is tolerant of drought, freezing and salt stress (Taji et al., 2004; Inan et al., 2004; Gong et al., 2005; Wong et al., 2006; Griffith et al., 2007). Salt cress morphologically resembles Arabidopsis and retains many of the same attractive features that made Arabidopsis a model organism including high fecundity, comparatively small genome size, and small physical size (Bressan et al., 2001; Inan et al., 2004).

Morphological and physiological responses to salt stress by *Thellungiella* species include a second endodermis in the root (Inan et al., 2004), stomatal closure (Volkov et al., 2003; Inan et al., 2004) and Na^+ transport mechanisms with high Na^+/K^+ selectivity as described above (Vera-Estrella et al., 2005). A biochemical strategy used by *Thellungiella* species for long term tolerance to salt stress is osmoprotection via the accumulation of compatible solutes (Bressan et al., 2001; Taji et al., 2004; Inan et al., 2004; Gong et al., 2005; Kant et al., 2006). As discussed in greater detail earlier in this review, one organic solute found to accumulate in many plant species undergoing salt stress is proline (Hare et al., 1998; Zhu et al., 2001). Gong et al. (2005) profiled metabolites in shoot tissue from *T. halophila* and Arabidopsis plants exposed to 150 mM NaCl. The authors reported

accumulation of proline, fructose, sucrose, *myo*-Inositol and raffinose in *T. halophila* compared to Arabidopsis (Gong et al., 2005). Other reports have implicated the involvement of those compatible solutes via transcript analysis (Taji et al, 2004; Inan et al., 2004; Wong et al., 2005; Kant et al., 2006).

Complicating research on this species is the reference to several species in the literature. References are found to *T. salsuginea* (Pallas) O.E. Schulz, *T. halophila* (C.A. Meyer) O.E. Schultz, *T. parvula* (Schrenk) Al-Shehbaz and O'Kane, and *T. botschantzevii* (German, 2008; Amtmann, 2009). In our study we used *T. salsuginea* seeds originating from the Yukon (Canada), a source now identified to be the same species as the *T. halophyla* from Shandong Province, China and used by other research groups around the world (H. Bohnert, personal communication). By convention, this plant should now be referred to as *T. salsuginea*.

Plant stress response as seen under field conditions

To date, when assessing plant stress response researchers have traditionally followed an approach involving subjecting a plant to a single stress and then determining treatment-specific changes by comparison to an unstressed control. Few studies characterize stress response in plants under multiple stresses. One study completed by Rizhsky et al. (2004) reported on the plastic metabolism in Arabidopsis under a combination of drought and heat stress. They found that under this stress combination sucrose replaces proline as the main osmolyte (Rizhsky et al., 2004). Müller et al., (2007) completed transcript profiling on Arabidopsis plants exposed to various concentrations of

sucrose and phosphate and reported on changes to transcript abundance following this combination of stresses. These two studies offer examples of how plant metabolism changes in response to multiple stresses that are more typically found in the field (Rizhsky et al., 2004; Müller et al., 2007). Robinson et al. (2007) profiled metabolites in Douglas-fir trees from 10 sub-families. The authors show that the metabolome of Douglas-fir populations is strongly correlated to field site as opposed to genetic origin. It can be argued that obtaining a greater mechanistic understanding of plant stress tolerance requires mimicking a natural environment where multiple, concurrent stresses are the norm.

Thesis objectives

The objectives of this thesis research were as follows:

- To use metabolite profiling to characterize the salt stress response of *T. salsuginea* (hereafter referred to as *Thellungiella*) seedlings, grown under high and low nitrogen conditions and in the presence and absence of salt.
- To perform a meta-analysis to compare metabolic changes between *Thellungiella* plants described in the first objective with datasets obtained from salt-stressed, mature, cabinet-grown *Thellungiella* and mature plants harvested from highly saline field sites in the Yukon.

To date only one study completed by Gong et al., 2005 has profiled *Thellungiella* leaf metabolites from plants growing under salt stress conditions (150 mM and 250 mM NaCl treatments). Gong et al. (2005) reported higher overall metabolite concentrations in

Thellungiella leaf tissue grown in the presence and absence of salt compared to Arabidopsis. Most notably, they reported a stress-related increase in various carbohydrates, sugar alcohols and in the amino acid proline.

Our laboratory has shown that *Thellungiella* plants accumulate proline when subjected to salt stress treatments in growth cabinets (Guevara and Weretilnyk, unpublished data). However, *Thellungiella* plants harvested at highly saline field sites in the Yukon do not accumulate proline (Guevara and Weretilnyk, unpublished data). To explain this contradictory behaviour we hypothesized that the availability of nitrogen under growth cabinet conditions as compared to the Yukon field site may influence the array of compatible solutes accumulated.

Null hypotheses for Seedling study:

Ho: Nutrient availability does not significantly influence the organic solute composition of *Thellungiella* seedlings growing under growth cabinet conditions.

Ho: NaCl does not significantly influence the organic solute composition of *Thellungiella* seedlings growing under growth cabinet conditions.

Ho: The interaction of NaCl and available nitrogen does not significantly influence the organic solute composition of *Thellungiella* seedlings growing under growth cabinet conditions.

Null hypotheses for Meta-analysis:

Ho: There are no MSTs found in field plants that associate with those found in seedlings subjected to different nitrogen levels and the presence and absence of NaCl.

Ho: The growth location of plant tissue has no significant influence on the composition of MSTs.

MATERIALS AND METHODS

Plant material

Thellungiella salsuginea (Pallas) O.E. Schulz seed stock was kindly collected from the Tahkini Salt-Flats in Whitehorse, Yukon, Canada by Dr. Bruce Bennett, a botanist working with Yukon Wildlife. Seeds used represented a mixed population and were not genetically homogeneous. *Arabidopsis thaliana* (CS60000) seed stock was purchased from the Arabidopsis Research Center in Ohio. *Thellungiella* and Arabidopsis seeds were bulked from plants grown at McMaster University. Seeds were collected in labelled 1.5 mL microfuge tubes (Diamed Lab Supplies, Mississauga, ON, cat. no. 50226) that were stored at room temperature in larger 50 mL polypropylene conical tubes (Becton Dickinson Labware, Franklin Lakes, NJ, cat. no. 352098) containing Drierite desiccant (W.A. Hammond, Drierite, Xenia, OH, cat. no. 23001). Seeds were surface sterilized using 50% (v/v) sodium hypochlorite (commercial bleach) and 0.1% (v/v) Triton X-100 (Sigma, Oakville, ON, cat. no. 9002-93-1) and then rinsed at least five times with 1 mL of sterile ddH_2O. The seeds were suspended in sterile 0.1% (w/v) Phytagel (Sigma, Oakville, ON, cat. no. 71010-52-1) and stored at 4^{o}C overnight.

Stress treatments

Four different stress treatments were used in this study (Table 1). Each treatment used a basic nutrient medium (Appendix 1) for plant growth to which the amount of nitrogen (nitrate) and salt (NaCl) added was varied according to Table 1. Phytagel (0.8% w/v) was added before autoclaving to solidify the medium.

Table 1. Nitrogen and NaCl concentrations tested organized according to treatment. Each treatment is represented by an abbreviation. For example, the high nitrogen (1 mM) treatment is respresented by HN. The nitrogen source used was nitrate.

Treatment	Nitrogen *mM*	NaCl *mM*
HN	1	0
LN	0.1	0
HN+S	1	100
LN+S	0.1	100

After sterilization the media was dispensed into square petri plates (100 mm^2 x 15 mm with 13 mm^2 grids, Becton Dickinson, Franklin Lakes, NJ, cat. no. 07417-1886), allowed to solidify and then plates were stored upside down, in plastic sleeves, at 4°C until needed. Directly prior to use a 0.8% (w/v) Phytagel solution was pipetted in a straight, horizontal line on the media near the top of the plate. During the seedling transfer (described below) to these media plates the shoot of the seedling would be placed on this horizontal line. This line of agar reduced seedling mortality by preventing shoots from coming into direct contact with the saline media.

Plant growth

Sterile seeds were sown onto Murashige and Skoog (MS) agar plates (100 mm x 20 mm, Fisher Scientific, Canada, cat. no. 25389-332) containing 2%(w/v) sucrose (Murashige & Skoog, 1962). The plates were sealed with micropore surgical tape (1.25 cm x 9.1 m, Fisher Scientific, Canada, cat. no. 1530) and transferred to 4°C for 48 hrs to stratify seeds. The plates were then placed in an incubator at 22°C with a 24 hour photoperiod and light intensity of approximately 200 umol/m^2·sec photon flux density. Following germination, seedlings were divided at random among the four treatments tested (Table 1) with all transfers completed under aseptic conditions in a flow bench. The plates containing seedlings were then transferred to a growth chamber set at 22°C with a 12 hour photoperiod and a light intensity of approximately 150-200 umol/m^2·sec photon flux density.

The plants were allowed to grow until their roots extended three-quarters of the way down the treatment plate. All plants (salt stressed or not) were harvested at the same root length stage of development and manipulations were completed quickly under aseptic conditions. At harvest the roots and shoots were separated and the shoot fresh weight was measured. Shoot tissue from plants representing the same treatment medium were pooled to yield a total of approximately 50 mg fresh weight and collected in sterile 1.5 mL microfuge tubes. Tubes containing tissue were flash frozen in liquid nitrogen and stored at -80°C. The experiment was repeated three times for *Thellungiella.*

Sample preparation

Previously frozen tissue was ground in a chilled mortar with a chilled pestle using liquid nitrogen to keep the tissue frozen. Before the liquid nitrogen had fully dissipated a 350 µL aliquot of 100% HPLC grade methanol (Caledon Laboratories, Georgetown, ON, cat. no. 67-56-1) was added to the mortar using a Pasteur pipette. Grinding continued until the tissue was well homogenized. A volume of 14.5 µL of 1 M NaCl (Bioshop, Burlington, ON) and 12.5 µL of 2 mg/mL ribitol (as an internal recovery standard), was added to the homogenized tissue-methanol mixture and grinding continued. The slurry was transferred to a 1.5 mL microfuge tube and placed on ice. The tubes were then placed in a shaker-water bath set to 70°C for 15 min and after incubation the tubes were centrifuged at 14,000 g for 3 min at 4°C using an Eppendorf Microfuge (Eppendorf AG, Hamburg, Germany, model no. 5415D, cat. no. 0061966). Following centrifugation the supernatants were removed using a glass pipette and transferred to a new 1.5mL

microfuge tube. A volume of 187.5 µL of 100% chloroform (ACP Chemica, Montreal, QB, cat. no. 67-66-3) was added to re-suspend the pellets by vortexing and then the tubes were placed in a water bath set to 37°C for 5 min. The tubes containing re-suspended pellets were centrifuged as above and the second supernatant was pooled with the first and 350 µL of ddH_2O was added. After vortexing to mix the contents, the tubes were centrifuged using an Eppendorf Microfuge at 5000 rpm for 15 min at room temperature. At this point three phases were apparent: the upper polar phase containing the water-soluble metabolites, the interphase of precipitated proteins and the lower non-polar phase containing lipids. The polar phase was removed using a glass pipette and two 250-µL aliquots were dispensed into Wheaton 1-mL V-vials (Wheaton, Millville, NJ, cat. no. 986254). One 250-µL aliquot was immediately evaporated to dryness under a continuous stream of N_2 (g) using an N-Evap Analytical Evaporator (Organomation, Westbury, NY, mod. no. 111). The dried sample was used for GC/MS analysis while the duplicate aliquot of 250- µL provided a back-up sample. Both aliquots were sealed with a 13mm Teflon-lined screw cap and stored at -20°C for future use. This process was repeated for all four stress treatments using duplicate samples for each experimental replicate. Each experiment of four treatment combinations yielded duplicate tissue samples (Table 1). The entire sample preparation procedure for GC/MS analysis was repeated three times for each of three separate biological replicates of the complete experiment.

Fatty acid standards

A volume of 5 μL of 10 μg/μL fatty acid standard mix (dissolved in 100% HPLC grade tetrahydrofuran, Caledon Laboratories, Georgetown, ON, cat. no. 8901-7) was added to each sample and then all samples were evaporated to dryness as described above. The fatty acid standard mix was the same as described by Roessner et al. (2000) consisting of nine standards: heptanoic acid (C_7) 3.7% v/v, nonanoic acid (C_9) 3.7% v/v, undecanoic acid (C_{11}) 3.7% v/v, tridecanoic acid (C_{13}) 3.7% v/v, pentadecanoic acid (C_{15}) 3.7% v/v, nonadecanoic acid (C_{19}) 7.4% v/v, tricosanoic acid (C_{23}) 7.4 % v/v, heptacosanoic (C_{27}) acid 22.2% v/v and hentriacontanoic acid (C_{31}) 55.5% v/v. These standards were used to calculate retention indices (RIs) using the following equation (Roessner et al., 2000):

$$RTI_x = [(100)x(C_n) + (((100)x(C_{n+1}) - (100)x(C_n))x((RT_x - RT_{Cx-1})/(RT_{Cx+1} - RT_{Cx-1})))]$$

Where,

x = the compound under analysis

C_n = the number of carbons present in the fatty acid that elute before compound x

C_{n+1} = the number of carbons present in the fatty acid that elute after compound x

RT_x = the retention time of compound x

Derivatization

Sample derivatization was completed using the protocol described by Fiehn et al., (2000b). Dried samples containing fatty acid standards were dissolved in 50 μL of 20 mg/mL methoxyamine (Sigma, Oakville, ON, cat. no. 593-56-6) prepared in 100 % pyridine (Sigma, Oakville, ON, cat. no. 110-86-1). The samples were capped and placed

in a Standard block heater (VWR Scientific Products, USA, cat. no. 13259-030) set to 30°C and incubated for 90 min. A volume of 80 µL of 100% N-methyl-N-trimethysilyl-trifluroacetamide (MSTFA; Regis Technologies, Morton Grove, IL, cat. no. 24589-78-4) was then added to the samples and the samples were placed in a Standard block heater set to 37°C and incubated for 30 min. This incubation should lead to the production of trimethylsilyl (TMS) derivatives from many of the metabolites present. The samples were then transferred to 150-µL Q-sert vials with PTFE/Silicone/PTFE Non-bonded septa (Chromatographic Specialties, Brockville, ON, cat. no. CQ2026) for analysis by GC/MS.

Gas chromatography/ mass spectrometry analysis (GC/MS)

All samples were analyzed with a TRACE Gas Chromatograph coupled to a DSQ Mass Spectrometer, tuned/calibrated with perfluorotributylamine and controlled by Thermo Finnigan data system software (Thermo Fisher Scientific, Canada). The instrument settings included: mass transfer line at 275°C, ion source at 200°C, MS multiplier voltage at approximately 1100 V and a scan rate of 2004 amu/s in the range of 50-650 amu.

Derivatized samples were diluted 5-fold with a Gerstel MPS2 auto-sampler and then a volume of 10 µL of sample and 40 µL hexane was injected into a 150 µL Q-sert vials that was sealed with a 12 mm Teflon-lined screw cap. A 1-µL volume of diluted sample was injected into the splitless injection port of the GC/MS set at 230°C. The column was comprised of a Rtx®-5M5 column (0.25 µm film thickness, 30 m length, 0.25 mm diameter and protected by a 5 m built-in guard column) with a stationary phase consisting of 5% v/v diphenyl / 95% v/v dimethyl polysiloxane was used. The mobile phase carrying the metabolites through the column was helium gas at a column head pressure of 90

kPa and 1 mL/min flow rate. For each run the GC oven was initially set to 50°C for 2.5 min, then increased to 70°C at 7.5°C/min increments and finally increased to 310°C at 5°C/min for 6 min.

Statistical analysis

Data from the GC/MS was analyzed using the Automated Mass Spectral Deconvolution and Identification System (AMDIS). The deconvolution settings included: scan direction from low to high, low deconvolution resolution, medium deconvolution sensitivity, and low shape requirement. The retention times (RTs) for ribitol and all fatty acids injected were identified in AMDIS by viewing the mass spectrum of the compound in question. Variability present in the data was corrected using the GC/MS Data Analysis program (GASP). GASP converted all RT values to retention indices (RIs) and corrected for variability in processing by relative response factors (RRFs) calculated using the recovery of the internal standard ribitol. Peaks with the same RIs, RTs and mass spectras were aligned for all three trials using GASP (Nuin, 2004). RRF values of 0 were replaced with the threshold detection of 0.00005 and all values were log10 transformed for normalization. Since the GASP software extracts and aligns data produced from multiple GC/MS runs, manual alignment of data is less time consuming and data is transformed into a format that can be analyzed by statistical packages.

The aligned data of all three trials was subjected to factorial ANOVA using Statistica 6.1 (http://www.statsoft.co. uk). The statistical significance for all of the components detected was determined using Statistica V 6.1, ANOVA was used to

identify significance among treatment combinations. Only those compounds, termed mass spectral tags (MSTs) that were statistically significant ($p \leq 0.05$) were subjected to principal component analysis (PCA) and hierarchical cluster analysis (HCA). PCA and HCA helped reveal patterns of change between components from plant samples in response to varying nitrogen and salt levels. During PCA, we considered those chemical compounds that yielded a factor loading ≥ 0.25 or ≤ -0.25 to be strongly correlated with that principal component as described by Peres-Neto et al., (2003). Factor loadings (FL) are correlation coefficients between the principal components and the variables (Gotelli and Ellison, 2004). PCA was completed using Statistica 6.1 and by using a covariance matrix. HCA was completed using Cluster (http://rana.lbl.gov/EisenSoftware.htm) and viewed using Java Treeview 1.1.0 (http://jtreeview.sourceforge.net). Clustering was completed using the complete linkage method (nearest-neighbour) and Euclidean distances.

Mass spectral tags (MSTs) selected for meta-analysis

Previously analysed chromatogram data obtained from field tissue from the 2003, 2005 and 2006 field years and data obtained for plants salinized to 300 mM NaCl as well as their corresponding controls were compared to those from the four treatments tested. The peaks from these chromatograms were aligned based on their mass spectral patterns, which were accessed and compared through AMDIS. Over 300 MSTs were subjected to ANOVA and 206 were statistically significant with a p value of ≤ 0.05 (Appendix 2). Of these 206 MSTs, 154 were present in only one of the three data sets (the four treatment

experiment, the field years or the chamber-grown salinization experiment). For the purpose of this study we have decided to focus on the trends of those 52 significant MSTs that were present in more than one of the three data sets. In order to compare stress response among the three groups (i.e. the four treatments, the three field years and mature cabinet-grown plants) we used the following formula and we standardized each dataset relative to the mean dataset for each group (all values listed in Appendices 4, 5 and 6).

$$X = (x-m)/d$$

Where,

X= the standardized log transformed relative abudance for a given MST in a given treatment

x= the relative abundance for a given MST in a given treatment

m= the mean relative abundance for a given MST across all treatments for a given experiment

d = the standard deviation associated with m

For example,

x would be the mean for a given MST using the datasets for HN, LN, HN+S **or** LN+S

m would be the mean of a given MST for the HN, LN, HN+S **and** LN+S datasets

d would be the standard deviation for the m variable

RESULTS – PART 1

Plant Growth and Stress Treatments

Thellungiella and Arabidopsis seedlings were subjected to one of four treatments: 1 mM N (HN), 0.1 mM (LN), 1 mM N and 100 mM NaCl (HN+S), or 0.1 mM N and 100 mM NaCl (LN+S) (Table 1). Figure 1 shows representative *Thellungiella* and Arabidopsis seedlings grown on the HN+S and LN+S treatments. Arabidopsis seedlings were stunted on HN+S and failed to survive LN+S treatments (Fig. 1). In contrast, *Thellungiella* seedlings subjected to the 100 mM NaCl survived and grew under both HN and LN conditions.

Thellungiella growth was slower on media with salt compared to media without added NaCl. Table 2 shows final root lengths of *Thellungiella* seedlings subjected to the four treatments tested. *Thellungiella* seedlings grown on HN and LN media were harvested approximately nine days following transfer while those growing on salt treatments required an additional ten days for their roots to reach the length needed for harvest (Table 2). At harvest the mean primary root length of LN and HN treatment plants was 44.9 ± 2.3 mm and 45.3 ± 3.2 mm, respectively (Table 2). The mean primary root lengths for LN+S and HN+S treatment plants were 35.9 ± 1.5 mm and 38.2 ± 4.3 mm, respectively (Table 2).

Primary roots of *Thellungiella* plants were measured daily and the data are shown in Figure 2. The slopes of the lines associated with the treatments indicate that the primary roots of *Thellungiella* grown in the absence of salt (LN and HN) grew at the

Figure 1. Representative *Thellungiella* (Ts) and Arabidopsis (At) seedlings grown for nineteen days on 1 mM or 0.1 mM nitrogen (HN and LN, respectively) and on 100 mM NaCl (+S) media. Arabidopsis seedlings show poor salt tolerance under both N conditions tested. See Materials and Methods for details of growth conditions and media composition. White bars = 6.5 mm.

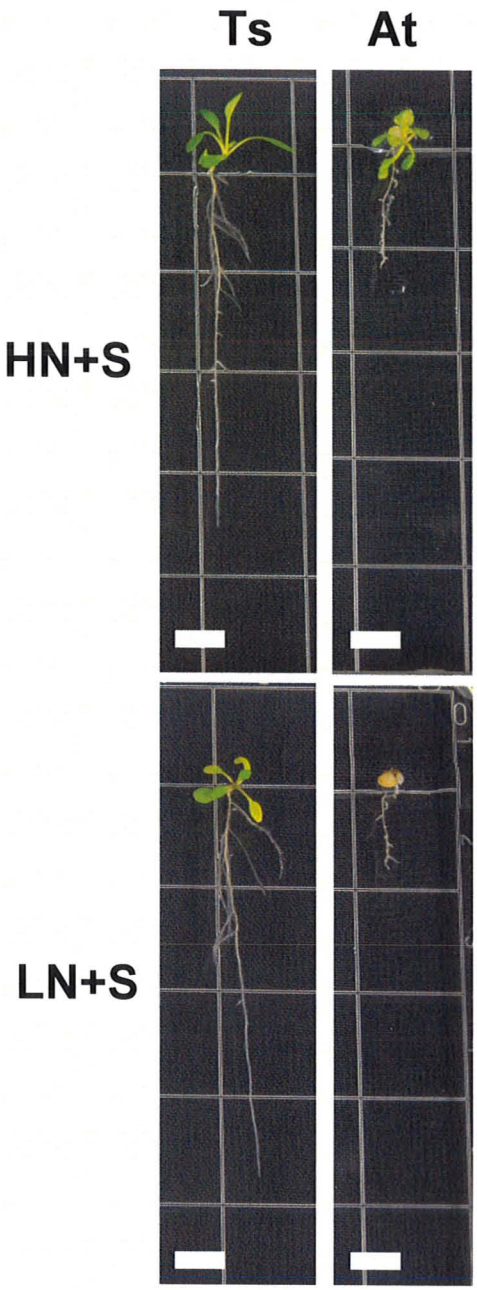
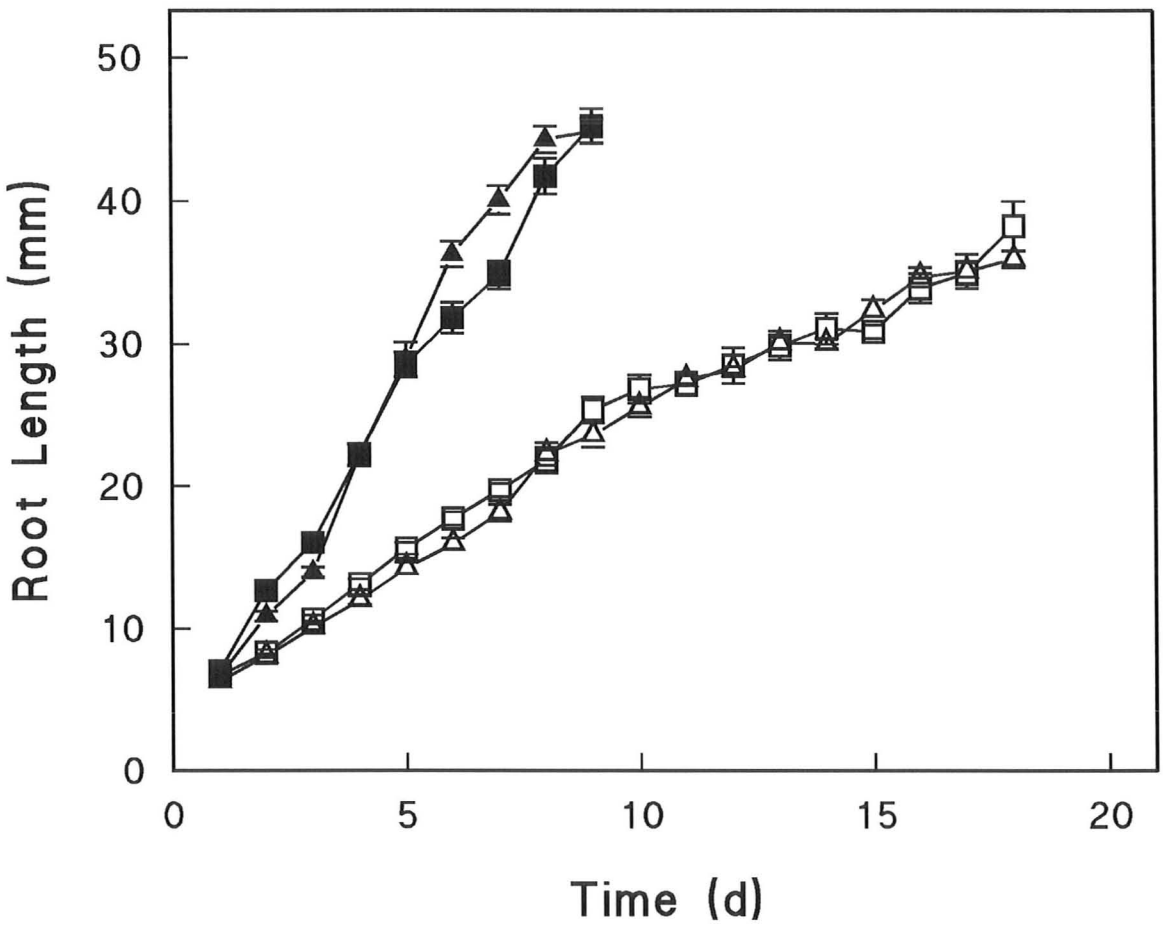

Ts
At
HN+S
LN+S

Table 2. Measurements of *Thellungiella* seedling root length recorded on the day of harvest. The treatments tested were HN, LN, HN+S, and LN+S. Seedlings grown on HN and LN treatments were harvested on approximately day nine, those grown on HN+S and LN+S treatments were harvested on approximately day nineteen. The data represents the mean ± the standard error (SE) of a minimum of 120 seedlings within three biological replicates (40 seedlings per replicate).

Treatment	Nitrogen	NaCl	Final Root Length	Time to Harvest
	mM	*mM*	*mm*	*d*
HN	1	0	45.3 ± 3.2	8.7 ± 1.5
LN	0.1	0	44.9 ± 2.3	8.7 ± 1.5
HN+S	1	100	38.2 ± 4.3	18.7 ± 2.1
LN+S	0.1	100	35.9 ± 1.5	18.7 ± 2.1

Figure 2. Salt exposure but not nitrogen treatment adversely impacts *Thellungiella* primary root growth. The four treatments shown are: HN, ■; LN, ▲; HN+S, □; LN+S, △. Root measurements were taken at the same time daily. The data represents the mean ± the standard error (SE) with a minimum of 40 seedlings from one of the three replicares. All three replicates showed the same trend with regards to root elongation. A single best-fit line for the HN and LN treatment data gives a slope of 4.6 $mm \cdot d^{-1}$ and a second best-fit line for the HN+S and LN+S treatment data gives a slope of 2.2 $mm \cdot d^{-1}$.


Root Length (mm)
50
40
30
20
10
0
0
5
10
15
20
Time (d)

same rate of 4.6 mm• d^{-1} while those exposed to 100 mM NaCl grew at 2.2 mm•d^{-1} or about half the rate of the non-saline controls (Fig. 2). These results show that the nitrogen concentration of the media did not affect primary root growth rate but the presence of added salt led to slower rates of root elongation (Table 2; Fig. 2).

Shoot fresh weight measurements were taken on the day of harvest (Fig. 3). Plants grown under HN (1 mM) conditions had shoot fresh weights that were almost two-fold greater than plants grown on LN (0.1 mM) media. The presence of 100 mM NaCl in the media had no affect on the eventual shoot biomass for plants grown on either HN or LN media. However, the growth rate was reduced by almost half in the presence of salt as shown in Figure 3. Figure 4 shows representative seedlings on each of the four media treatments tested. When adequate time was given for roots to elongate to approximately the same position on the plate (9 d for LN & HN and 19 d for LN+S & HN+S), the corresponding shoots at that time point were visually indistinguishable. These observations suggest that while the added salt slows the growth rate for *Thellungiella*, the presence of 100 mM NaCl in the media does not produce a seedling that is phenotypically different from corresponding control plants (Fig. 3 & Fig. 4). Dry weight measurements of *Thellungiella* shoots were not taken as shoots were frozen in liquid nitrogen upon harvest to preserve their state for GC/MS analysis.

Figure 3. Shoot biomass of *Thellungiella* seedlings is greatest on high nitrogen (HN) media and is unaffected by the presence of salt (HN+S). The shoot fresh weight measurements for *Thellungiella* were taken on the day of harvest (9 d for HN and LN; 19 d for HN+S and LN+S). The shoot growth rate in mg/d is displayed for each treatment. The data represents the mean ± the standard error (SE) with a minimum of 40 seedlings across three biological replicates.

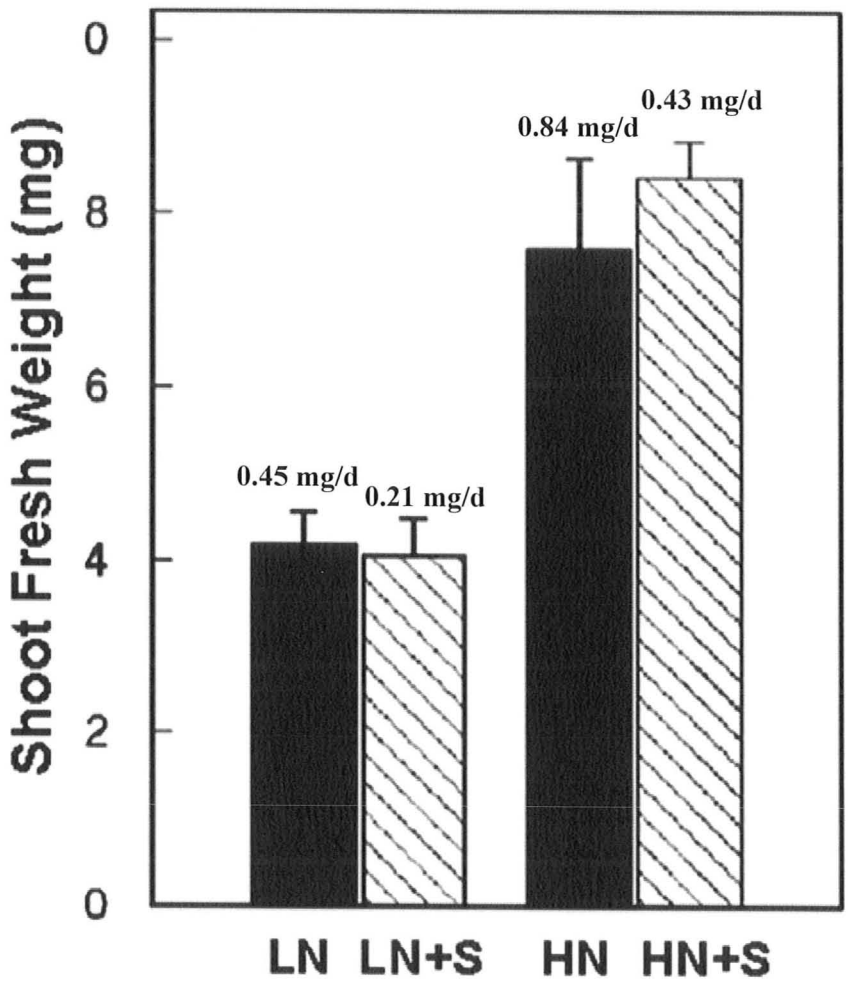
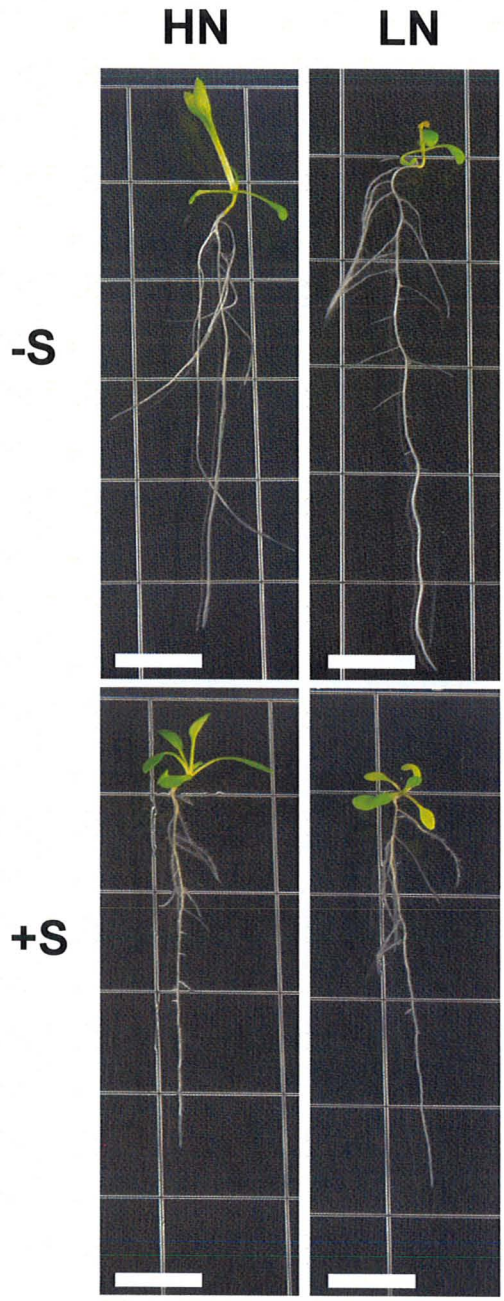

Shoot Fresh Weight (mg)
0
2
4
6
8
0
0.45 mg/d
0.21 mg/d
0.84 mg/d
0.43 mg/d
LN
LN+S
HN
HN+S

Figure 4. Representative *Thellungiella* seedlings subjected to HN and LN in the absence (-S) or presence (+S) of 100 mM NaCl. Seedlings were photographed on the day of harvest (9d for HN and LN; 19d for HN+S and LN+S). See Materials and Methods for details of growth conditions and media composition. White bars = 10 mm.


HN
LN
-S
+S

Metabolite profiling of *Thellungiella* leaves

The GC/MS methodology described by Fiehn et al. (2000b) was used to identify qualitative and quantitative changes in accumulated organic solutes extracted from shoots of *Thellungiella* seedlings grown on media varying in nitrogen and NaCl content (see Materials and Methods for details on plant material and analysis). The polar chemical components resolved by GC analysis include sugars (mono-, di-, trisaccharides), sugar alcohols, amines, amino acids, organic acids and phosphorylated compounds (Adams et al., 1998). The chromatogram for each sample yields peaks representing over 200 chemical components. Figure 5 shows representative chromatograms produced following GC/MS analysis of shoot extracts from *Thellungiella* seedlings subjected to the four treatments tested. For this study, a minimum of two chromatograms with two repeats from three biological replicates were compared for each treatment in order to identify statistically significant differences among components. MSTs were identified, where possible, by matching their corresponding retention indices (RIs) and mass spectral patterns to those of authentic and pure standards. Figure 6 shows mass spectral pattern of proline in the shoot extracts (in blue) compared with an authentic proline standard (in red). The mass spectral peak (*m/z*) 73 amu is present in all TMS-derivatized products and therefore is not specific to proline. The 142, 147 and 216 amu *m/z* peaks shown in Figure 6 help discriminate proline from other potential compounds with more conclusive evidence provided by co-injection of an authentic standard mixed with sample. Co-elution under identical conditions provides compelling evidence that a chemical

Figure 5. Representative chromatograms produced by GC/MS of polar metabolites extracted from shoots of *Thellungiella* seedlings grown on four different media treatments: HN, LN, HN+S, and LN+S. Following deconvolution, components or mass spectral tags (MSTs) are identified by their RI (based upon RT relative to internal standards) and their mass spectral pattern. Relative abundance compared to the internal standard, ribitol, allows for MST semi-quantitative comparisons between successive runs. Details regarding growth conditions, media composition, GC/MS conditions and statistical analyses are given in the Materials and Methods. Samples were analysed in duplicate with three biological replicates.

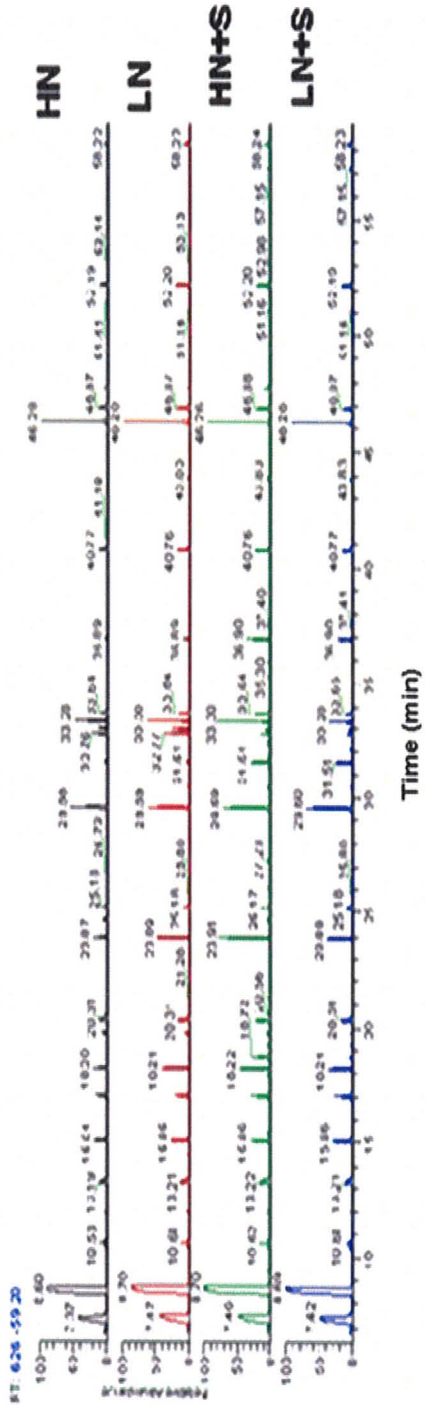
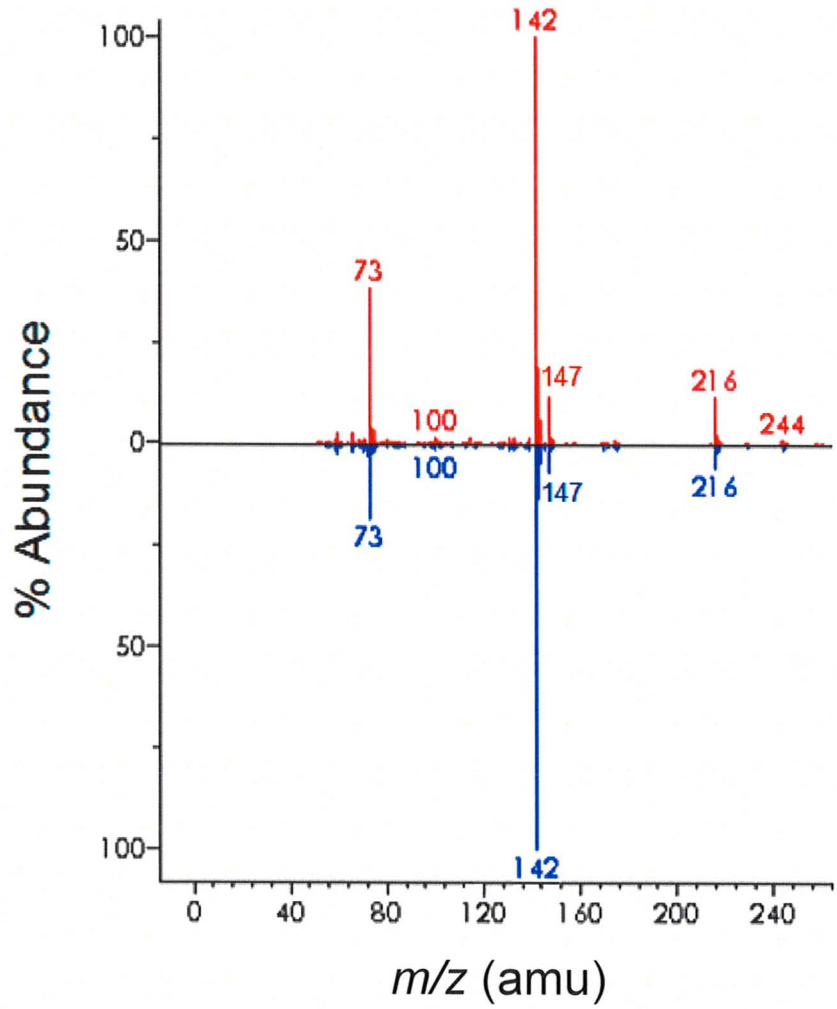

HN
LN
HN+S
LN+S
Time (min)

Figure 6. Proline detected by GC/MS analysis of extracts of *Thellungiella* leaf samples matches the mass spectral pattern of the proline standard. The mass spectral pattern of proline from *Thellungiella* is shown in blue and that of the authentic proline standard is shown in red. Proline identification was later confirmed by co-injection of leaf extracts with the proline standard where a single peak at RT= 18.73 min was found on the GC chromatogram obtained for the mixed sample. The 73 amu *m/z* peak is present in all TMS-derivatized products but 142, 147 and 216 amu *m/z* peaks help discriminate proline from other potential compounds.


142
100
50
73
147
216
244
100
0
100
147
216
73
50
100
142
0
40
80
120
160
200
240
% Abundance
m/z (amu)

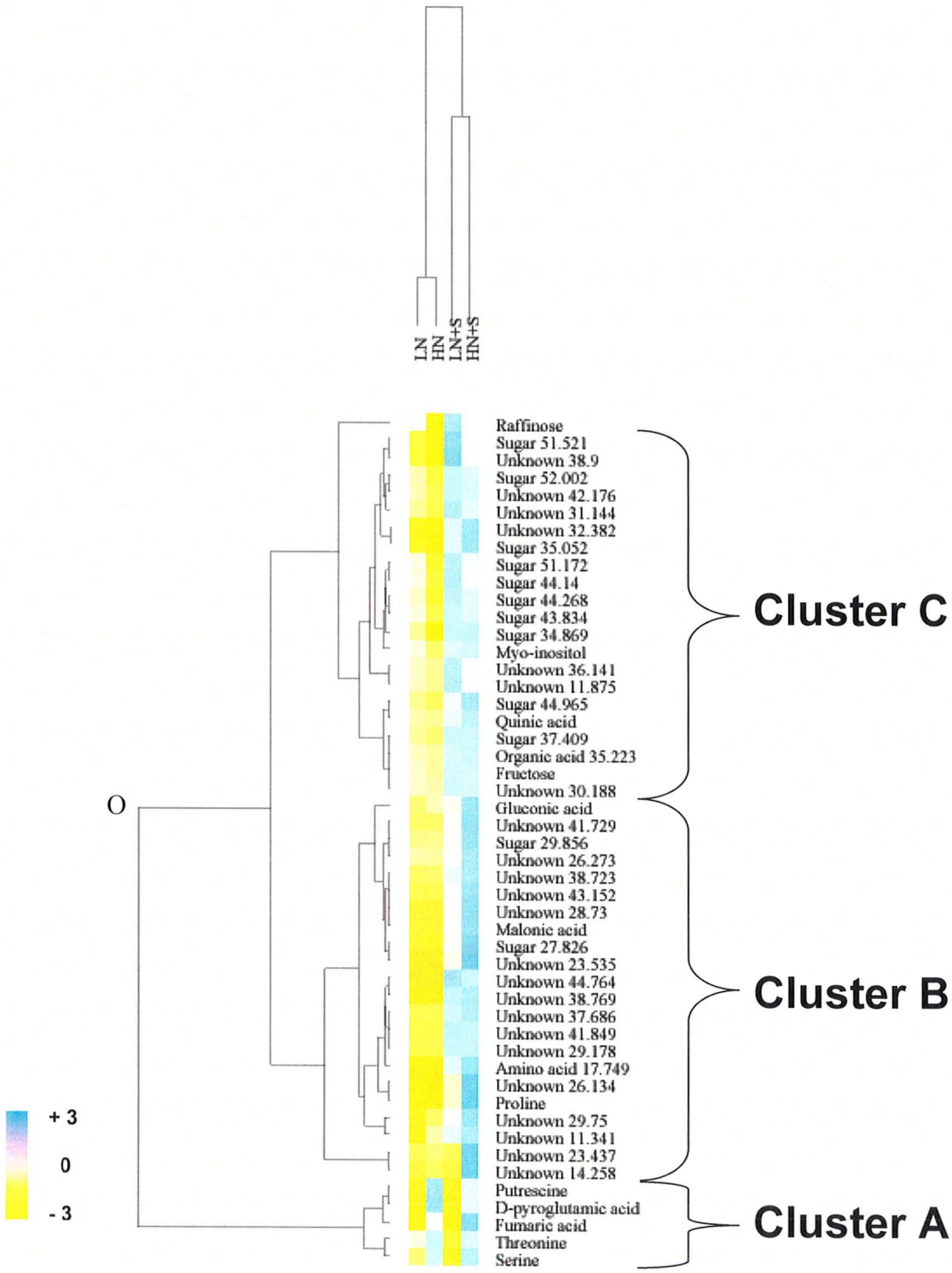
component and a standard are identical. In this study proline, raffinose and *myo*-Inositol were all co-injected to confirm their identities.

Following deconvolution and alignment of the data using GASP, ANOVA identified 49 MSTs showing statistically significant changes in relative abundance among the samples ($p \leq 0.05$; Appendix 3). These components were subjected to PCA and HCA. To minimize confusion over the use of the term "components" with respect to GC/MS chromatograms and as principal components in PCA data analysis, I will refer to the 49 chemical components as MSTs from this point on.

Hierarchal cluster analysis (HCA) of HN, LN, HN+S and LN+S datasets

HCA was used to help identify differences among MSTs detected in seedlings subjected to the four treatments (HN, LN, HN+S, LN+S). The heat-map in Figure 7 shows the abundance of a compound in a given treatment relative to its average abundance over all four treatments tested. Datasets were clustered using the complete-linkage method (nearest-neighbour) and Euclidean distances as described by Gong et al. (2005). Clustering of the datasets is shown at the level of treatments (top of heat map in Fig. 7) and at the level of MSTs. The four treatments group into two clusters with one containing datasets for control, unsalinized, plants (HN and LN) and a second containing datasets for plants exposed to 100 mM NaCl (HN+S and LN+S). The 49 MSTs showing statistically significant changes in abundance as a function of treatment are grouped into three distinct clusters and for ease of reference are arbitrarily designated A, B and C (in order of branching from the origin).

Figure 7. HCA was used to generate a heat-map showing fold-differences in the relative abundance of 49 MSTs as a function of plant exposure to HN, LN, HN+S and LN+S. The relative abundance for a given MST is expressed as a ratio between its estimated treatment abundance relative to the mean abundance for that MST over all four treatments. Fold-ratios were log10 transformed and subjected to HCA using the complete-linkage method (nearest-neighbour) and using Euclidean distances. Clustering of the datasets is shown at the level of treatments (top) and at the level of MSTs arbitrarily labelled as A, B and C in order of separation from the origin (O).


LN
HN
LN+S
HN+S
O
Raffinose
Sugar 51.521
Unknown 38.9
Sugar 52.002
Unknown 42.176
Unknown 31.144
Unknown 32.382
Sugar 35.052
Sugar 51.172
Sugar 44.14
Sugar 44.268
Sugar 43.834
Sugar 34.869
Myo-inositol
Unknown 36.141
Unknown 11.875
Sugar 44.965
Quinic acid
Sugar 37.409
Organic acid 35.223
Fructose
Unknown 30.188
Gluconic acid
Unknown 41.729
Sugar 29.856
Unknown 26.273
Unknown 38.723
Unknown 43.152
Unknown 28.73
Malonic acid
Sugar 27.826
Unknown 23.535
Unknown 44.764
Unknown 38.769
Unknown 37.686
Unknown 41.849
Unknown 29.178
Amino acid 17.749
Unknown 26.134
Proline
Unknown 29.75
Unknown 11.341
Unknown 23.437
Unknown 14.258
Putrescine
D-pyroglutamic acid
Fumaric acid
Threonine
Serine
Cluster C
Cluster B
Cluster A
+ 3
0
- 3

MSTs included in Cluster A

Cluster A includes four nitrogenous compounds and one organic acid that were found to be consistently more abundant in extracts of HN and HN+S treatment leaf tissue compared to those from LN and LN+S treated seedlings (Fig. 7). The five compounds were identified with high probability following comparison to spectra of library standards as putrescine, pyroglutamic acid, serine, threonine and fumaric acid. Of these compounds, the content of fumaric acid shows a 2.6-fold higher abundance in *Thellungiella* leaf tissue from seedlings grown under HN+S conditions compared to those grown under HN conditions (Appendix 3). In contrast, putrescine and pyroglutamic acid are about 2-fold more abundant in extracts prepared from HN than HN+S seedlings, respectively (Appendix 3).

MSTs included in Cluster B

Cluster B includes 22 compounds of which 16 are unknown with respect to their chemical class and two each showing features in common with organic acids, N-containing compounds resembling amino acids, and probable sugars (Fig. 7). Two of these compounds were tentatively identified by their high similarity to mass spectra of library standards for malonic acid and gluconic acid. Proline was also identified to be among these compounds on the basis of matches to both mass spectra and RT values with an authentic proline standard. Further support for the identity of peak at RT 18.69 min was provided by co-migration of the proline standard with the compound of interest in the sample following co-injection.

Of the MSTs comprising Cluster B, 17 are relatively more abundant in HN+S samples than those obtained from seedlings exposed to the remaining three treatments. In addition to several unknowns, the amino acid proline typified this pattern of abundance associated with media containing 1 mM nitrogen and 100 mM NaCl. In terms of absolute abundance, the proline content of leaf tissue from seedlings under HN+S conditions was 450-fold higher than the content determined for HN seedlings and 6.3-fold-higher compared to the plants grown on LN+S media (Appendix 3). In contrast to this association with nitrogen and salt, an unknown MST with RT 44.764, was found to be 1.4-fold higher in LN+S samples compared to HN+S. As well, four unidentified MSTs, were found to be in near equal abundance in HN+S and LN+S samples. These four compounds, along with unknown 44.764, group together and occupy the mid section of Cluster B (Fig. 7).

MSTs included in Cluster C

MSTs included in Cluster C are present in higher relative abundance in salt-treated seedlings and more frequently at higher levels in leaves of the plants grown on LN+S media (Fig. 7). MSTs in Cluster C include 22 compounds of which ten are probable sugars, seven are chemically unknown and two show mass spectral patterns associated with organic acids. One compound was identified through a mass spectral match with a standard in our chemical library to be fructose. In addition, following co-injection two compounds were identified as raffinose and its precursor in biosynthesis, *myo*-Inositol. The content of raffinose was about 2.6-fold higher in LN+S plants relative

to LN plants (Appendix 3). Six of the ten probable sugars in Cluster C show a similar pattern of abundance in treatment samples to that of raffinose (RTs of 51.172, 44.140, 44.268, 43.834, 52.002 and 51.521; Fig. 7).

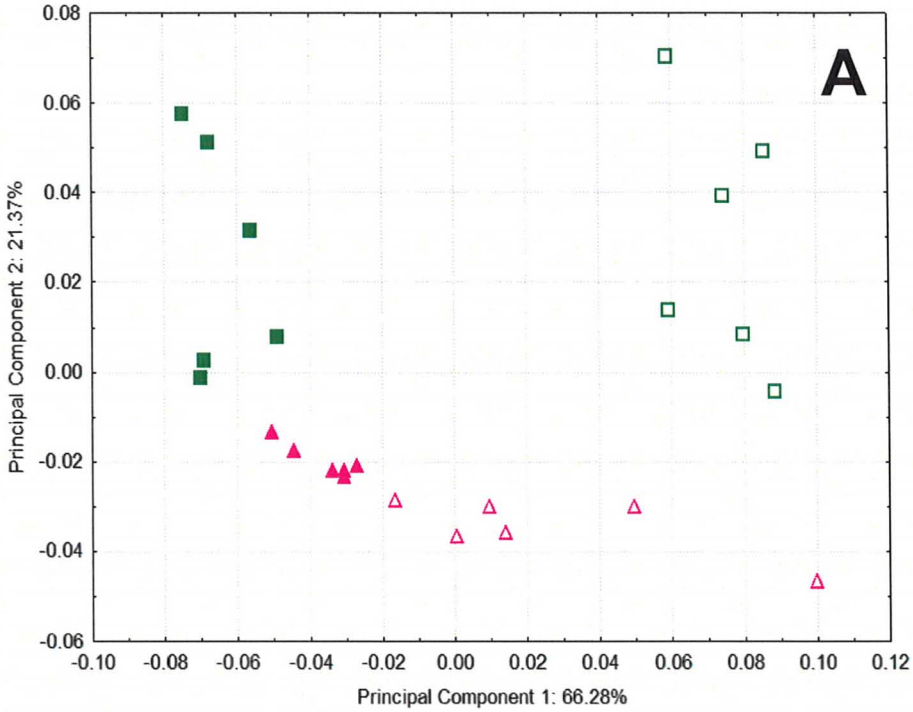
Identification of MSTs

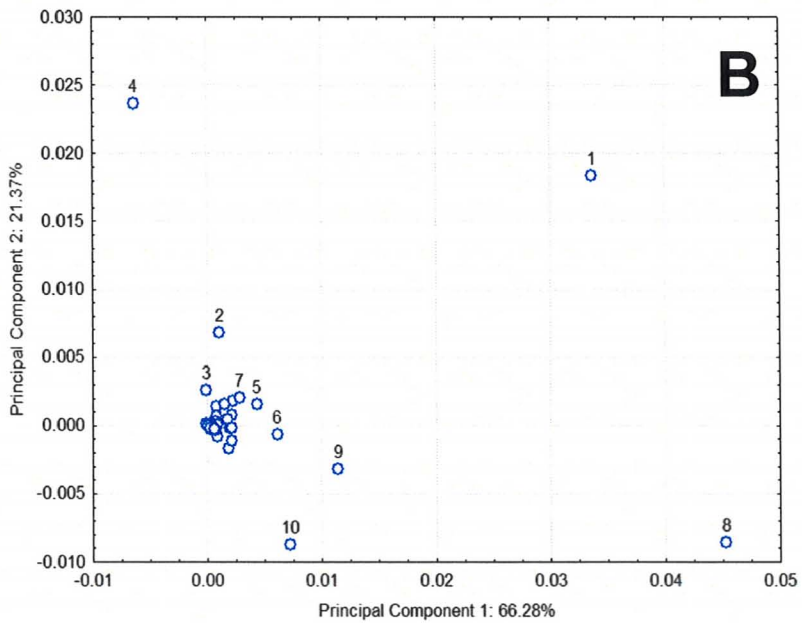
Where possible, metabolites were identified by comparing RIs and mass spectral patterns to those of authentic standards. However, of the 49 MSTs undergoing statistically significant changes in abundance, 12 showed highest probability as being carbohydrates but 23 could not be associated with any chemical class. Figure 7 shows that all 23 unidentified compounds have increased relative abundance under 100 mM NaCl media conditions with five present at higher relative abundance in LN+S treatment tissue, 12 at higher relative abundance in HN+S tissue and the remainder under saline (+S) conditions irrespective of the N content of the media.

Principal Component Analysis (PCA) of HN, LN, HN+S and LN+S datasets

Datasets comprised of the 49 statistically significant MSTs were also subjected to PCA to help identify sources of variance in the datasets. Figure 8A shows that the datasets arising from individual plants exposed to the same test media group together following application of PCA. PCA allows us to identify factors that account for variance in the datasets, with the first factor extracted accounting for the most variance. Figure 8A shows that factor one (Principal Component 1) and factor 2

Figure 8. Principal Components 1 and 2 help compare datasets from seedlings subjected to media of varying N and salt content. Panel **A** shows the PCA of log-scaled abundance data for polar metabolites resolved by GC/MS. Each point represents the dataset comprised of 49 MSTs found to undergo statistically significant changes in abundance in extracts prepared from a 50 mg leaf sample pooled from a minimum of 5 individual seedlings: ■ = HN, □ = HN+S, ▲ = LN, △ = LN+S. Panel **B** shows metabolite projections that identify MSTs contributing to the clustering seen in panel **A**. The numbers shown correspond to the following compounds: 1 = Proline, 2 = Serine, 3 = Threonine, 4 = Pyroglutamic acid, 5 = Unknown 32.382, 6 = Fructose, 7 = Sugar 35.052, 8 = *myo*-Inositol, 9 = Sugar 43.834, and 10 = Raffinose.


A
Principal Component 2: 21.37%
Principal Component 1: 66.28%
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.10
-0.08
-0.06
-0.04
-0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12


B
Principal Component 2: 21.37%
Principal Component 1: 66.28%
0.030
0.025
0.020
0.015
0.010
0.005
0.000
-0.005
-0.010
-0.01
0.00
0.01
0.02
0.03
0.04
0.05
1
2
3
4
5
6
7
8
9
10

(Principal Component 2) together account for about 87% of the variance in the datasets corresponding to the four treatments tested.

Principal Component 1: HN, LN, HN+S and LN+S

Principal component one is displayed on the x-axis of Figure 8A. At 66.28%, this factor represents the largest contribution toward variation in the data. In this figure the datasets from plants growing in the absence of salt (LN and HN) are found on the left (negative) side of principal component one while those from plants growing in the presence of salt (+S) are located further to the right side. All but two points representing metabolic profiles of LN+S plants are positioned to the right of zero (Fig. 8A).

Table 3 lists the variables that have a positive or negative relationship to principal component one. Our results show that 45 of the 49 variables examined have a strong positive relationship (factor loading ≥ 0.25) to principal component one. With respect to Figure 8A, this means that these 45 compounds contribute to the positions of those datasets to the right of the graph and, as such, influence the position of datasets from seedlings grown under +S conditions.

Principal Component 2: HN, LN, HN+S and LN+S

The second principal component is displayed on the y-axis of Figure 8A and it accounts for 21.37% of the variance in the data. Datasets from plants growing in media with high N (1 mM) are located in the plane above and separate from datasets representing plants growing on low N (0.1 mM) media.

Table 3. Factor loadings for principal component one sorted from positive to negative. Variables found to have a factor loading ≥ 0.25 or ≤ -0.25 are in bold. Of the 49 MSTs, 45 have a strong positive relationship (factor loading ≥ 0.25) to principal component one and none show a strong negative relationship (factor loading ≤ 0.25). FL= factor loading.

Variable	FL[a]	Variable	FL	Variable	FL
myo-Inositol	0.968	Unknown 29.178	0.654	Unknown 11.875	0.388
Fructose	0.886	Sugar 27.826	0.654	Fumaric acid	0.387
Unknown 32.382	0.876	Unknown 26.134	0.650	Sugar 52.002	0.374
Unknown 28.73	0.863	Sugar 44.14	0.650	Sugar 34.869	0.367
Sugar 37.409	0.863	Unknown 38.723	0.645	Sugar 44.268	0.329
Quinic acid	0.855	Sugar 51.172	0.645	Serine	0.136
Proline	0.842	Amino acid 17.749	0.644	Threonine	-0.023
Unknown 23.535	0.812	Unknown 31.144	0.632	Putrescine	-0.144
Unknown 30.188	0.808	Unknown 41.729	0.628	Pyroglutamic acid	-0.227
Unknown 43.152	0.788	Unknown 14.258	0.626		
Sugar 44.965	0.786	Unknown 42.176	0.624		
Organic acid 35.223	0.777	Gluconic acid	0.581		
Unknown 44.764	0.755	Sugar 29.856	0.571		
Unknown 29.75	0.748	Sugar 51.521	0.551		
Malonic acid	0.726	Raffinose	0.542		
Unknown 38.769	0.724	Unknown 23.437	0.513		
Unknown 26.273	0.710	Unknown 11.341	0.508		
Sugar 43.834	0.709	Unknown 36.141	0.498		
Unknown 41.849	0.696	Unknown 38.9	0.487		
Unknown 37.686	0.667	Sugar 35.052	0.432		

[a]FL, factor loading

Table 4 lists the variables influencing the relative positions of the datasets and shows that 20 of the 49 variables examined have a strong positive relationship (factor loading ≥ 0.25) to principal component two. Seven of the 20 compounds were identified as nitrogenous or acidic in nature (including proline) and six had "best-fit" mass spectra matches to putrescine, pyroglutamic acid, fumaric acid, threonine, serine and gluconic acid. Eight of the 49 variables given in Table 4 have a strong negative relationship (factor loading ≤ -0.25) to principal component two. Four of these eight variables (raffinose, sugars at RT 44.14, 51.172 and 51.521 min) were shown in the HCA heat-map of Figure 7 to be more abundant in plants exposed to the LN+S treatment.

Variables contributing to the positions of the HN, LN, HN+S and LN+S datasets

The PCA data was analyzed further to identify variables that influence the positions of the datasets given in Figure 8A. The majority of the variables yielded (x,y) values near the origin (0,0) as shown in Figure 8B. However, ten of the 49 variables were clearly separable from the rest and these include fructose (6), *myo*-inositol (8), sugar 43.834 (9) and raffinose (10). These variables lie below zero on the y axis and contribute towards the position of the LN+S datasets. In contrast, proline (1) lies above zero on the y axis and so contributes to the position of the HN+S datasets. Finally, pyroglutamic acid (4) and serine (2) lie to the left side of the x axis and above zero on the y axis where they contribute to the position of the HN datasets. These results show that a change in abundance among comparatively few MSTs can strongly influence the position and

separation of datasets produced following PCA of MST data from the *Thellungiella* seedlings used in this study.

Table 4. Factor loadings for principal component two sorted from negative to positive. Variables (MSTs) found to have a factor loading ≥ 0.25 or ≤ -0.25 are in bold. Eight of 49 MSTs have a strong negative relationship (factor loading ≥ 0.25) to principal component two while 20 show a strong positive relationship (factor loading ≤ 0.25). FL = factor loading.

Variable	FL[a]	Variable	FL	Variable	FL
Raffinose	**-0.652**	Organic acid 35.223	0.046	**Proline**	**0.459**
Unknown 38.9	**-0.416**	Sugar 34.869	0.130	**Sugar 29.856**	**0.471**
Unknown 11.875	**-0.349**	Unknown 43.152	0.135	**Unknown 23.437**	**0.472**
Sugar 44.14	**-0.343**	Unknown 11.341	0.140	**Unknown 26.134**	**0.526**
Sugar 51.172	**-0.330**	Unknown 37.686	0.167	**Gluconic acid**	**0.599**
Unknown 31.144	**-0.305**	Sugar 27.826	0.177	**Fumaric acid**	**0.690**
Sugar 51.521	**-0.277**	Quinic acid	0.203	**Threonine**	**0.740**
Unknown 36.141	**-0.265**	Malonic acid	0.226	**Serine**	**0.840**
Sugar 44.268	-0.239	Amino acid 17.749	0.234	**Pyroglutamic acid**	**0.848**
Sugar 43.834	-0.197	**Sugar 44.965**	**0.250**		
myo-Inositol	-0.183	**Unknown 29.75**	**0.264**		
Sugar 52.002	-0.161	**Unknown 38.723**	**0.284**		
Unknown 44.764	-0.123	**Putrescine**	**0.304**		
Fructose	-0.096	**Unknown 32.382**	**0.310**		
Unknown 41.849	-0.095	**Unknown 28.73**	**0.315**		
Unknown 42.176	-0.087	**Sugar 35.052**	**0.317**		
Sugar 37.409	-0.076	**Unknown 23.535**	**0.350**		
Unknown 30.188	-0.062	**Unknown 41.729**	**0.367**		
Unknown 29.178	-0.004	**Unknown 26.273**	**0.386**		
Unknown 38.769	0.026	**Unknown 14.258**	**0.416**		

[a]FL, factor loading

RESULTS – PART 2

Meta-Analysis of metabolic profiles from seedlings exposed to the four treatments, field plants and cabinet-grown Thellungiella

In this section we will focus on the results of a meta-analysis between datasets that contain metabolic profiles from seedlings exposed to four treatments (HN, HN+S, LN, LN+S) on culture media plates and from mature plants growing under field and growth cabinet conditions. In an earlier study our lab analyzed tissue collected from mature field plants collected in three different growing seasons (2003,2005,2006: J. Dedrick (2007) and D. Guevara, unpublished). Hereafter these plant samples and their datasets will be referred to by year. Mr. David Guevara also analyzed tissue collected from mature cabinet-grown plants subjected to salt stress (300 mM) and their controls (Guevara and Weretilnyk, unpublished). Hereafter these plants will be referred to as S-300 and C-0, respectively. In this thesis research we addressed the question of whether organic solute accumulation in salt-stressed seedlings was the same or different from mature plants grown in controlled environment chambers and under field conditions. A meta-analysis of metabolic profiles from plants exposed to varying environmental conditions will contribute towards our overall understanding of plant growth and stress responses under field conditions where multiple, concurrent, stresses are the norm.

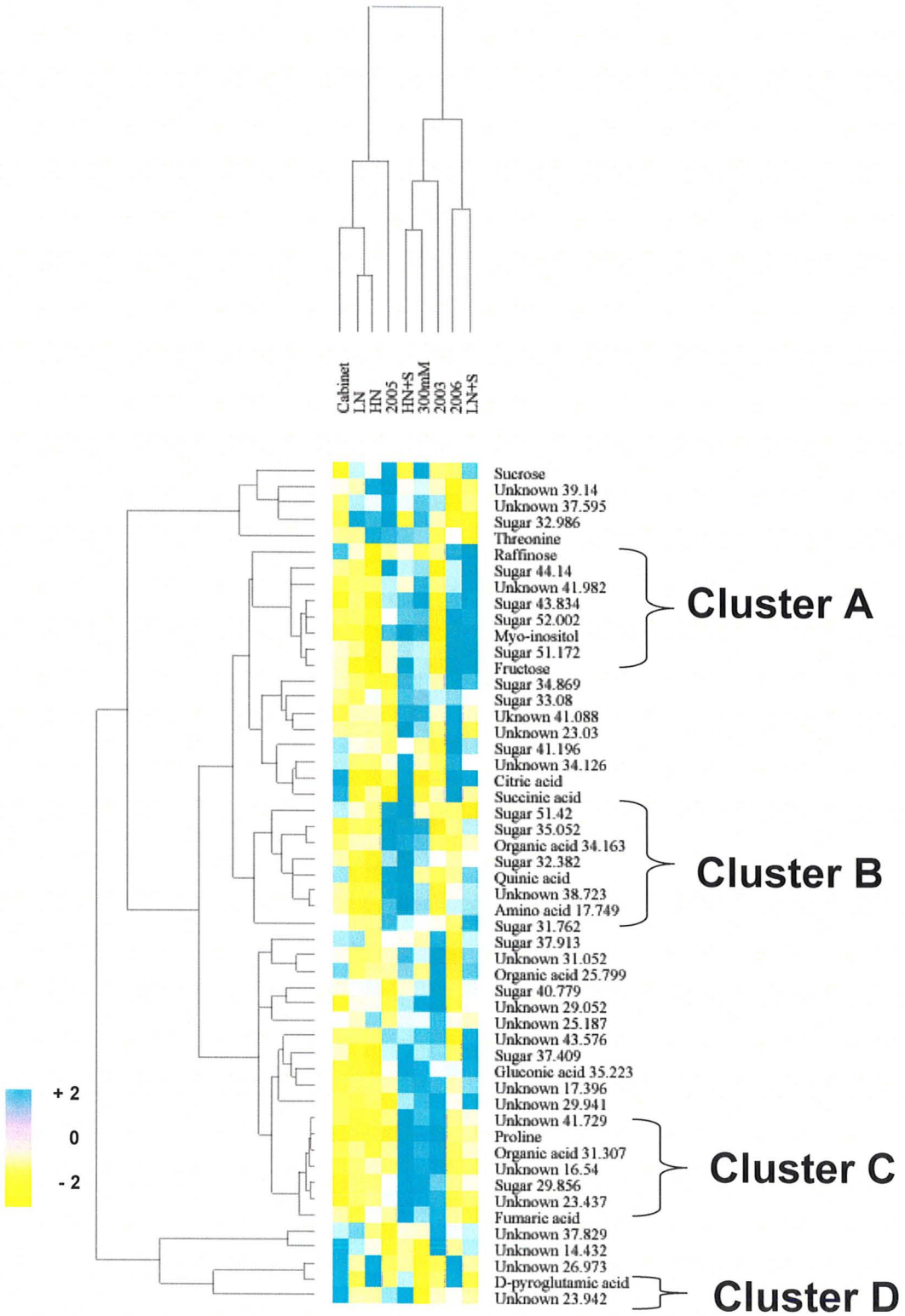
Hierarchal cluster analysis (HCA) of the four treatments, field plants and S-300 and C-0 datasets

The heat-map shown in Figure 9 shows the abundance of MSTs in a given dataset (Fig. 9). In order to perform the HCA, the datasets used in the meta-analysis were standardized separately for each of three main "treatment" groups (the four N +/-S samples, the three field years and mature cabinet-grown plants +/- S). The data transformation is described in greater detail in the Materials and Methods section of this thesis. Figure 9 shows clustering of the datasets at the level of treatments (top of the figure) and at the level of MSTs. In the heat-map shown in Figure 9, the three control datasets (HN, LN and C-0) cluster together as one of two main groups starting from the origin. The 2005 field year dataset clusters more closely to the three control datasets as opposed to those of 2003 and 2006. The HN+S and S-300 datasets cluster together while the LN+S and 2006 field year datasets comprise a separate group. The 2003 dataset clusters more closely to the HN+S and S-300 datasets than the other datasets analyzed. For ease of reference, the 52 MSTs showing statistically significant changes in relative abundance (listed in Appendices 4, 5 and 6) have been labelled clusters A through D.

MSTs in Cluster A of meta-analysis

Cluster A of Figure 9 includes eight MSTs of which one is unknown, four are probable sugars and one was identified as fructose by comparison to mass spectra and RI of a library standard. Two additional MSTs were identified as *myo*-Inositol and raffinose based on mass spectra comparison to library standards and co-migration with authentic standards.

Figure 9. Heat-map showing fold-differences in the relative abundance of 52 MSTs isolated from seedlings and mature plants obtained from field and growth cabinet environments. Leaf tissue was from seedlings cultured on defined media (HN, HN+S, LN, LN+S), rosette leaves of mature, well-watered and salinized plants under growth cabinet conditions (C-0 and S-300, respectively) and cauline leaves of mature plants harvested at a Yukon field site (2003, 2005 and 2006). For HCA the relative abundance for a given MST is expressed as a ratio between its estimated treatment-related abundance to the mean abundance for that compound over all four treatments (see Materials and Methods for details). Fold-ratios were log10 transformed and subjected to HCA using the complete-linkage method (nearest-neighbour) and using Euclidean distances. Clustering of the datasets is shown at the level of treatments (top) and at the level of MSTs (selected clusters identified for discussion in the thesis are labelled A, B, C and D).


Cabinet
LN
HN
2005
HN+S
300mM
2003
2006
LN+S
Sucrose
Unknown 39.14
Unknown 37.595
Sugar 32.986
Threonine
Raffinose
Sugar 44.14
Unknown 41.982
Sugar 43.834
Sugar 52.002
Myo-inositol
Sugar 51.172
Fructose
Sugar 34.869
Sugar 33.08
Uknown 41.088
Unknown 23.03
Sugar 41.196
Unknown 34.126
Citric acid
Succinic acid
Sugar 51.42
Sugar 35.052
Organic acid 34.163
Sugar 32.382
Quinic acid
Unknown 38.723
Amino acid 17.749
Sugar 31.762
Sugar 37.913
Unknown 31.052
Organic acid 25.799
Sugar 40.779
Unknown 29.052
Unknown 25.187
Unknown 43.576
Sugar 37.409
Gluconic acid 35.223
Unknown 17.396
Unknown 29.941
Unknown 41.729
Proline
Organic acid 31.307
Unknown 16.54
Sugar 29.856
Unknown 23.437
Fumaric acid
Unknown 37.829
Unknown 14.432
Unknown 26.973
D-pyroglutamic acid
Unknown 23.942
Cluster A
Cluster B
Cluster C
Cluster D
+ 2
0
- 2

The eight compounds comprising Cluster A show higher relative abundance in some but not all plants studied. Seedlings on LN+S, HN+S, leaves of mature plants salinzed to 300 mM NaCl and cauline leaves of 2006 plants show increased abundance of Cluster A MSTs but other patterns are less easy to discern. In contrast, controls (LN, HN and C-0), and 2003 cauline leaves show lower relative abundance of these same compounds while the 2005 field plants are somewhat intermediate with respect to this feature. Seven of the eight MSTs (the exception being unknown 41.982), were associated with salt response in *Thellungiella* seedlings grown under LN+S condition shown in Figure 7. Sugar 52.002 and sugar 51.172 are at the highest relative abundance in LN+S and 2006 samples while sugar 44.140 is at the highest relative abundance in tissue from LN+S plants and 2005 field plants. Raffinose abundance is low in LN and HN plant tissue compared to LN+S or HN+S tissue but it is 6-fold higher in C-0 than in S-300 plants (Appendix 6). In Cluster A, fructose is at the highest relative abundance in tissue from LN+S plants, HN+S plants and 2006 field plants but S-300, 2003 and 2005 field plants also experience saline conditions and yet the fructose content shows little to no increase. In summary, for MSTs in Cluster A a consistent association between their abundance and saline conditions, N content of media or field versus cabinet cannot be found making it difficult to identify underlying environmental factors responsible for their differing relative abundance in various tissue sources.

MSTs in Cluster B of meta-analysis

Cluster B of Figure 9 includes eight compounds of which one is unknown, two are likely organic acids, one a likely nitrogenous chemical resembling an amino acid and four are probable sugars. These compounds, with the exception of quinic acid and sugar 31.762, are found in the highest relative abundance in datasets of HN+S plants, S-300 plants and 2005 field plants (Fig. 9). None of these compounds show higher relative abundance in tissue harvested from 2003 field plants (Fig. 9). Out of these eight compounds only one, amino acid 17.749, is relatively elevated in 2006 field plants compared to 2003 and 2005 field plants (Fig. 9).

MSTs in Cluster C of meta-analysis

Cluster C contains seven compounds of which three are unknown, one is likely a sugar, two are probable organic acids and one is proline (Fig. 9). Compounds in cluster C were found to be present in higher relative abundance in 2003 field plants, HN+S plants and S-300 plants (Fig. 9). In terms of abundance, fumaric acid content is 1.7-fold higher in S-300 plants compared to C-0 plants (Appendix 6). Proline content is 2-fold higher in S-300 plants compared to C-0 plants (Appendix 6). It should be noted that although, proline is more abundant in cauline leaves of 2003 field plants as compared to samples from 2005 and 2006, the content of this amino acid is not significantly higher than that found for extracts prepared from cauline leaf controls of mature cabinet-grown plants (Guevara, unpublished). Also, Figure 7 shows that both proline and fumaric acid increase

in *Thellungiella* seedling tissue under salt stress when the 1.0 mM N concentration was used (HN+S).

MSTs in Cluster D of meta-analysis

Cluster D includes three MSTs, of which two are unknown and one was identified as pyroglutamic acid. All of these MSTs are present in higher relative abundance in C-0 plants. Unknown 26.975 and pyroglutamic acid are more similar in that they are also found to be more abundant in HN seedlings and 2006 field plants (Fig. 9) while this is not the case for unknown 23.942. With respect to content, pyroglutamic acid is 1.7-fold higher in C-0 tissue compared to S-300 tissue (Appendix 6).

Principal Component Analysis (PCA) for datasets from four seedling treatments, field plants and mature S-300 and C-0 plants

The 52 MSTs showing statistically significant differences in abundance by ANOVA (Appendix 4,5 and 6) were subjected to PCA. For ease of reference, these MSTs will be referred to in our PCA diagrams by numbers given in Table 5. PCA was used to help identify sources of variance in the datasets describing quantitative differences among the relative abundance of compounds extracted from seedlings grown on four treatments (HN, LN, HN+S, LN+S), mature C-0 and S-300 plants and mature plants harvested during three field years (2003, 2005, 2006). After application of PCA a scree plot showed that the first three principal components were of particular importance (data not shown). For this reason, all results are presented using at least two of the three top-

Table 5. Reference list of 52 MSTs undergoing statistically significant changes in abundance and their corresponding identification numbers. Where possible, MSTs were identified by comparison of mass spectra and retention times to those of library standards. Some MSTs were identified with respect to their most likely chemical class while others that could not are designated "unknown". All MSTs not assigned an identity are referenced by their retention time (RT).

I.D.	MST	I.D.	MST	I.D.	MST
1	Gluconic acid	21	Sugar 34.869	41	Succinic acid
2	Sugar 32.382	22	Quinic acid	42	Unknown 17.396
3	Fructose	23	Organic acid 34.163	43	Unknown 25.187
4	Sugar 37.409	24	Sugar 52.002	44	Threonine
5	Unknown 38.723	25	Citric acid	45	Sugar 33.08
6	Sugar 29.856	26	Unknown 41.982	46	Unknown 29.052
7	*myo*-Inositol	27	Sugar 51.172	47	Pyroglutamic acid
8	Sugar 43.834	28	Unknown 23.03	48	Sugar 40.779
9	Sugar 44.14	29	Unknown 16.54	49	Unknown 39.14
10	Sugar 35.052	30	Unknown 23.942	50	Sugar 32.986
11	Unknown 41.729	31	Fumaric acid	51	Unknown 26.973
12	Unknown 29.941	32	Unknown 43.576	52	Unknown 14.432
13	Unknown 31.052	33	Sucrose		
14	Unknown 23.437	34	Organic acid 25.799		
15	Unknown 34.126	35	Sugar 41.196		
16	Amino acid 17.749	36	Raffinose		
17	Organic acid 31.307	37	Unknown 37.829		
18	Uknown 41.088	38	Unknown 37.595		
19	Sugar 51.42	39	Sugar 31.762		
20	Proline	40	Sugar 37.913		

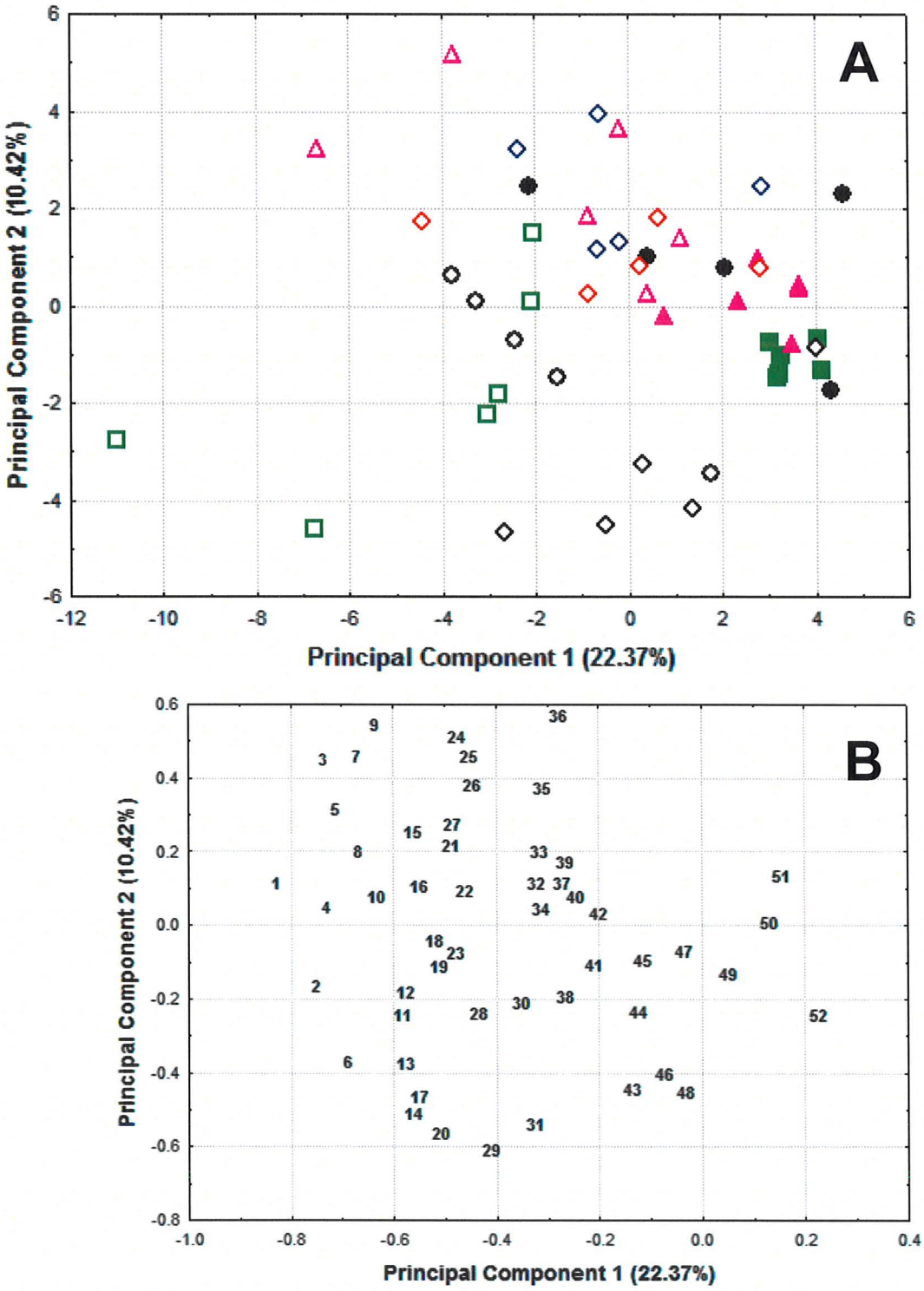
ranked principal components. Together these top three principal components account for 42.26% of the variation in the metabolic profiles of the plant tissue tested.

PCA also offered a means of determining the extent to which the 52 MSTs influenced the position of the datasets relative to each other. Analysis shows that only one of the 52 MSTs (unknown 17.396) did not have a strong influence (factor loading ≥ 0.25 or ≤ -0.25) on at least one of the first three principal components. The finding that the majority of the 52 MSTs strongly influenced the first three principal components extracted is not surprising as they show statistically significant differences ($p \leq 0.05$) across the three datasets tested and should have a strong impact on principal components.

Meta-Analysis: Principal Component 1

The first principal component, displayed on the x-axis, accounts for 22.37% of the variance in the data and represents the largest contribution towards variation in the data (Fig. 10A). On the right (positive) side of the x-axis we find all but one of the control datasets and on the left (negative) side of the x-axis we find the majority of datasets from salt-treated and field plants (Fig. 10A). Datasets from field plants, LN+S, HN+S, and S-300 samples are grouped with their respective members but the different treatment groups do not necessarily cluster closely to each other. Table 6 shows that 39 of the 52 MSTs have a strong negative correlation toward principal component one. Therefore, 39 variables contribute to the separation of datasets of plants subjected to stress in chambers and those of field plants from datasets of control plants. Variables that have a strong negative

Figure 10. PCA and Metabolite Projections for Principal Component 1 plotted against Principal Component 2. Panel **A** shows the PCA of log-scaled relative abundance data for polar metabolites resolved by GC/MS. Each point represents the significant dataset generated from a 50 mg leaf sample pooled from a minimum of 5 individual seedlings (■ = HN, □ = HN+S, ▲ = LN, △ = LN+S) or represent the profile of an individual plant (● = C-0, ○ = S-300, ◊ = 2003 field plants, ◊ = 2005 field plants, ◊ = 2006 field plants). Panel **B** shows metabolite projections found using identifies of MSTs that contribute to the clustering seen in panel **A**. See Table 5 for reference numbers for MST identification.


A
Principal Component 2 (10.42%)
Principal Component 1 (22.37%)
6
4
2
0
-2
-4
-6
-12
-10
-8
-6
-4
-2
0
2
4
6
B
Principal Component 2 (10.42%)
Principal Component 1 (22.37%)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
1
2
3
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52

Table 6. Factor loadings for principal component one sorted from negative to positive. Variables found to have a factor loading ≥ 0.25 or ≤ -0.25 are in bold. Of the 52 MSTs, 39 have a strong negative relationship (factor loading ≥ 0.25) to principal component one. No MSTs have a strong positive relationship (factor loading ≥ 0.25) to principal component one. FL= factor loading.

Variable	[a]FL	Variable	FL	Variable	FL
Gluconic acid	**-0.828**	**Sugar 34.869**	**-0.488**	Succinic acid	-0.210
Sugar 32.382	**-0.752**	**Quinic acid**	**-0.483**	Unknown 17.396	-0.202
Fructose	**-0.737**	**Organic acid 34.163**	**-0.480**	Unknown 25.187	-0.135
Sugar 37.409	**-0.729**	**Sugar 52.002**	**-0.475**	Threonine	-0.123
Unknown 38.723	**-0.713**	**Citric acid**	**-0.452**	Sugar 33.08	-0.116
Sugar 29.856	**-0.688**	**Unknown 41.982**	**-0.446**	Unknown 29.052	-0.073
***myo*-Inositol**	**-0.673**	**Sugar 51.172**	**-0.443**	Pyroglutamic acid	-0.036
Sugar 43.834	**-0.668**	**Unknown 23.03**	**-0.435**	Sugar 40.779	-0.032
Sugar 44.14	**-0.636**	**Unknown 16.54**	**-0.409**	Unknown 39.14	0.049
Sugar 35.052	**-0.634**	**Unknown 23.942**	**-0.350**	Sugar 32.986	0.129
Unknown 41.729	**-0.581**	**Fumaric acid**	**-0.324**	Unknown 26.973	0.153
Unknown 29.941	**-0.577**	**Unknown 43.576**	**-0.322**	Unknown 14.432	0.224
Unknown 31.052	**-0.577**	**Sucrose**	**-0.315**		
Unknown 23.437	**-0.563**	**Organic acid 25.799**	**-0.314**		
Unknown 34.126	**-0.561**	**Sugar 41.196**	**-0.310**		
Amino acid 17.749	**-0.549**	**Raffinose**	**-0.277**		
Organic acid 31.307	**-0.549**	**Unknown 37.829**	**-0.271**		
Unknown 41.088	**-0.520**	**Unknown 37.595**	**-0.265**		
Sugar 51.42	**-0.510**	**Sugar 31.762**	**-0.265**		
Proline	**-0.509**	Sugar 37.913	-0.244		

[a]FL, factor loading

correlation with principal component one are fructose (3), *myo*-Inositol (7), proline (20), gluconic acid (1), raffinose (36), sucrose (33), quinic acid (22), citric acid (25), fumaric acid (31), twelve sugars, fourteen unknowns and three organic acids (Fig. 10B; Table 6). Previously we showed that raffinose, proline, sugar 43.834, fructose and *myo*-Inositol had a strong influence on separating HN+S and LN+S datasets from control datasets (Fig. 8). Interestingly, Table 6 and Figure 10B show that these compounds also have a strong influence on separating datasets of plants subjected to stress in chambers and field plants from those of control plants seen in Fig. 10A.

Meta-analysis: Principal Component 2

The second principal component, shown as the y-axis in Figure 10A, accounts for 10.42% of the variance in the data. On the top (positive) side of the y-axis we find datasets from plants subjected to LN+S conditions and from plants grown in the 2005 and 2006 field years. These three datasets are distinct from those of HN+S plants, S-300 plants and 2003 field plants that group towards the bottom (negative) side of the y-axis. Proline has a strong negative correlation to principal component two, second only to an unknown chemical compound at retention time 16.54 (Fig 10B; Table 7). This means that proline (20) has a large influence on the positions of the HN+S plant dataset and the S-300 dataset shown in Figure 10A. Of note is the direction of influence by proline and the grouping of 2003 datasets near the bottom of the y axis. Cauline leaves of 2003 field plants contained higher levels of proline on average than leaves from plants collected during 2005 or 2006 (Appendix 5). The seven sugars that have the strongest positive

Table 7. Factor loadings for principal component two of the 52 MSTs subjected to PCA. Factor loadings are sorted from negative to positive. Variables found to have a factor loading ≥ 0.25 or ≤ -0.25 are in bold. Of the 52 MSTs, fourteen have a strong negative relationship (factor loading ≥ 0.25) to principal component one and ten have a strong positive relationship (factor loading ≥ 0.25) to principal component one. FL = factor loading.

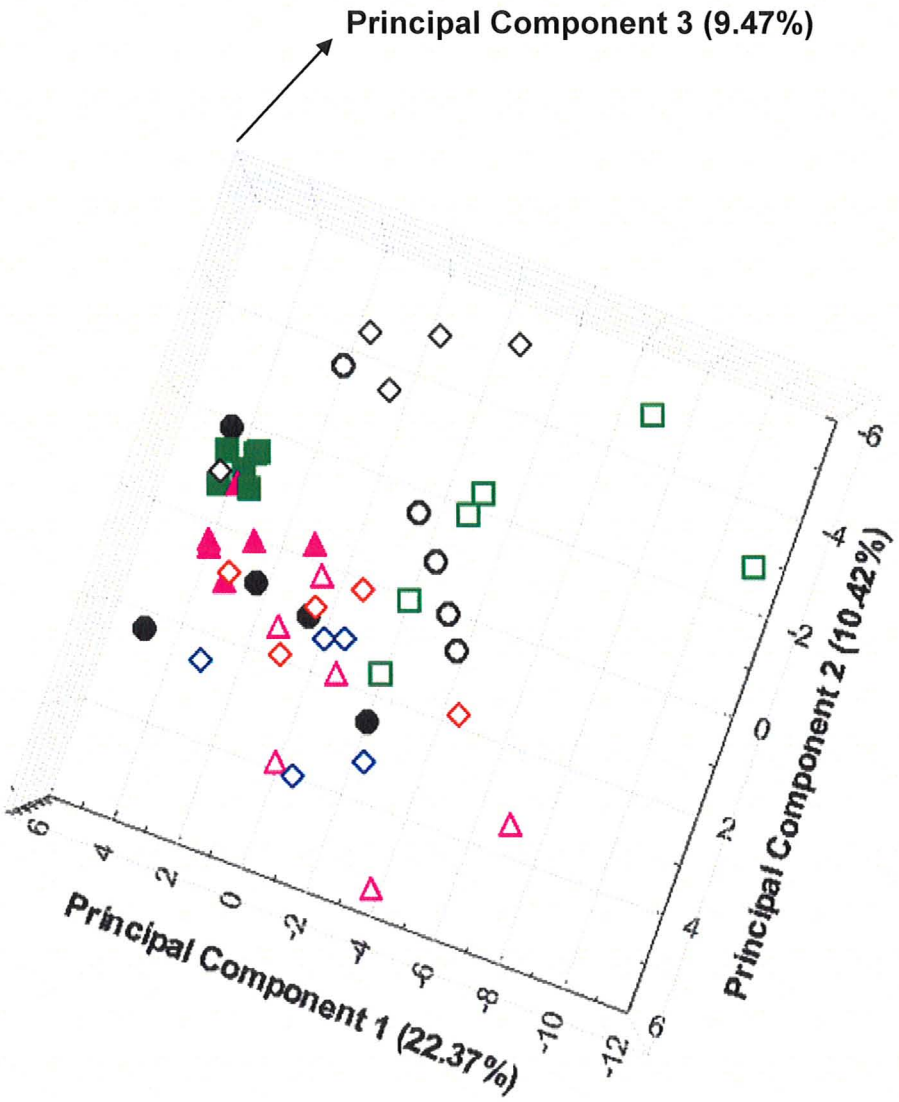
Variable	[a]FL	Variable	FL	Variable	FL
Unknown 16.54	**-0.641**	Succinic acid	-0.139	Sugar 34.869	0.186
Proline	**-0.593**	Sugar 33.08	-0.124	Unknown 34.126	0.222
Fumaric acid	**-0.567**	Organic acid 34.163	-0.104	**Unknown 38.723**	**0.284**
Unknown 23.437	**-0.540**	Pyroglutamic acid	-0.101	**Sugar 41.196**	**0.341**
Organic acid 31.307	**-0.494**	Uknown 41.088	-0.074	**Unknown 41.982**	**0.349**
Sugar 40.779	**-0.484**	Sugar 32.986	-0.022	**Sugar 51.172**	**0.350**
Unknown 25.187	**-0.474**	Unknown 17.396	0.002	**Fructose**	**0.419**
Unknown 29.052	**-0.435**	Organic acid 25.799	0.011	***myo*-Inositol**	**0.427**
Unknown 31.052	**-0.404**	Sugar 37.409	0.016	**Citric acid**	**0.428**
Sugar 29.856	**-0.402**	Sugar 35.052	0.045	**Sugar 52.002**	**0.480**
Unknown 14.432	**-0.275**	Sugar 37.913	0.048	**Sugar 44.14**	**0.514**
Unknown 41.729	**-0.275**	Amino acid 17.749	0.075	**Raffinose**	**0.540**
Unknown 23.03	**-0.269**	Gluconic acid	0.081		
Threonine	**-0.265**	Unknown 37.829	0.083		
Unknown 23.942	-0.239	Unknown 43.576	0.084		
Unknown 37.595	-0.225	Unknown 26.973	0.105		
Unknown 29.941	-0.214	Sugar 31.762	0.141		
Sugar 32.382	-0.197	Quinic acid	0.151		
Unknown 39.14	-0.163	Sugar 43.834	0.169		
Sugar 51.42	-0.140	Sucrose	0.171		

[a]FL, factor loading

correlation to principal component two include raffinose (36), sugar 44.14 (9), sugar 52.002 (24), *myo*-Inositol (7), fructose (3), citric acid (25) and sugar 51.172 (27) (Table 7). These sugars have the largest influence on the locations of the LN+S, 2005 and 2006 datasets shown in Figure 10A. Interestingly, in Figure 8A three of these sugars, raffinose (36), *myo*-Inositol (7) and fructose (6), were found to have the strongest influence in separating LN+S from HN+S datasets.

The trends related to principal components one and two are shown in 3D in Figure 11. For the purpose of discussing Figure 11, the "back" will be defined by principal components two and three. An angled top view of the first three principal components in 3D space shows overlap of control datasets in the back of the 3D plane. The datasets of LN+S, HN+S, S-300 and field conditions, with two exceptions, separate from control datasets in two different directions (Fig. 11). Datasets of LN+S, 2005 and 2006 field plants share a common plane opposite from the plane occupied by a majority of datasets from HN+S and S-300 plants. In contrast, the 2003 field plant datasets are located to the back and left in the 3D space. This grouping suggests that plants harvested during the 2003 field year have a very distinct metabolic profile, the distinctness of which was accounted for by the variance explained by the first three principal components.

Figure 11. Three-Dimensional space (top view) of the first three principal components extracted. PCA. Datasets were log-scaled MST relative abundance based upon GC/MS data. Each point represents the dataset of 52 MSTs showing statistically significant changes in abundance. Extracts were generated from a 50 mg leaf sample pooled from a minimum of five individual plants (■ = HN, □ = HN+S, ▲ = LN, △ = LN+S) or single plants (● = C-0, ○ = S-300, ◊ = 2003 field plants, ◊ = 2005 field plants, ◊ = 2006 field plants). See Table 5 for MST identification numbers.


Principal Component 3 (9.47%)
Principal Component 1 (22.37%)
Principal Component 2 (10.42%)

Meta-analysis: Principal Component 3

The third principal component, shown on the y-axis of Figure 12, accounted for 9.27% of the variance in the data. Along principal component three the 2006 dataset is located below those of other field plants. The variables contributing toward the distribution of datasets with respect to principal component three are shown in Figure 12B and listed in Table 8.

When principal component three is plotted against principal component two, datasets from all three field years are separate from each other (Fig. 13A). The 2006 field plant datasets are located on the right side of Figure 13A and below the 2005 field plant datasets. Here the LN+S datasets overlap with those of the 2005 field plants, a positioning that was also shown in Figure 12A. The datasets representing 2003 field plants are localized to the top left of Figure 13A. Across principal component two and three the plants from the three field years are most distinct.

Variables with a strong negative correlation to principal component three contribute to the position of the 2006 field plant dataset relative to datasets of the other field plants (Fig. 13B; Table 8). In contrast, variables with a strong positive correlation to principal component three contribute to the location of the 2003 and 2005 field plant datasets (Fig. 13B; Table 8).

Figure 12. Meta-analysis: PCA and metabolite projections for Principal Component 1 plotted against Principal Component 3. Panel **A** shows the PCA of log-scaled relative abundance data determined for polar metabolites by GC/MS. Each point represents the significant dataset generated from a 50 mg leaf sample pooled from a minimum of 5 individual seedlings (■ = HN, □ = HN+S, ▲ = LN, △ = LN+S) or represent the profile of an individual plant (● = C-0, ○ = S-300, ◊ = 2003 field plants, ◊ = 2005 field plants, ◊ = 2006 field plants). Panel **B** shows metabolite projections found using identifies of MSTs that contribute to the clustering seen in panel **A**. See Table 5 for reference numbers for MST identification.

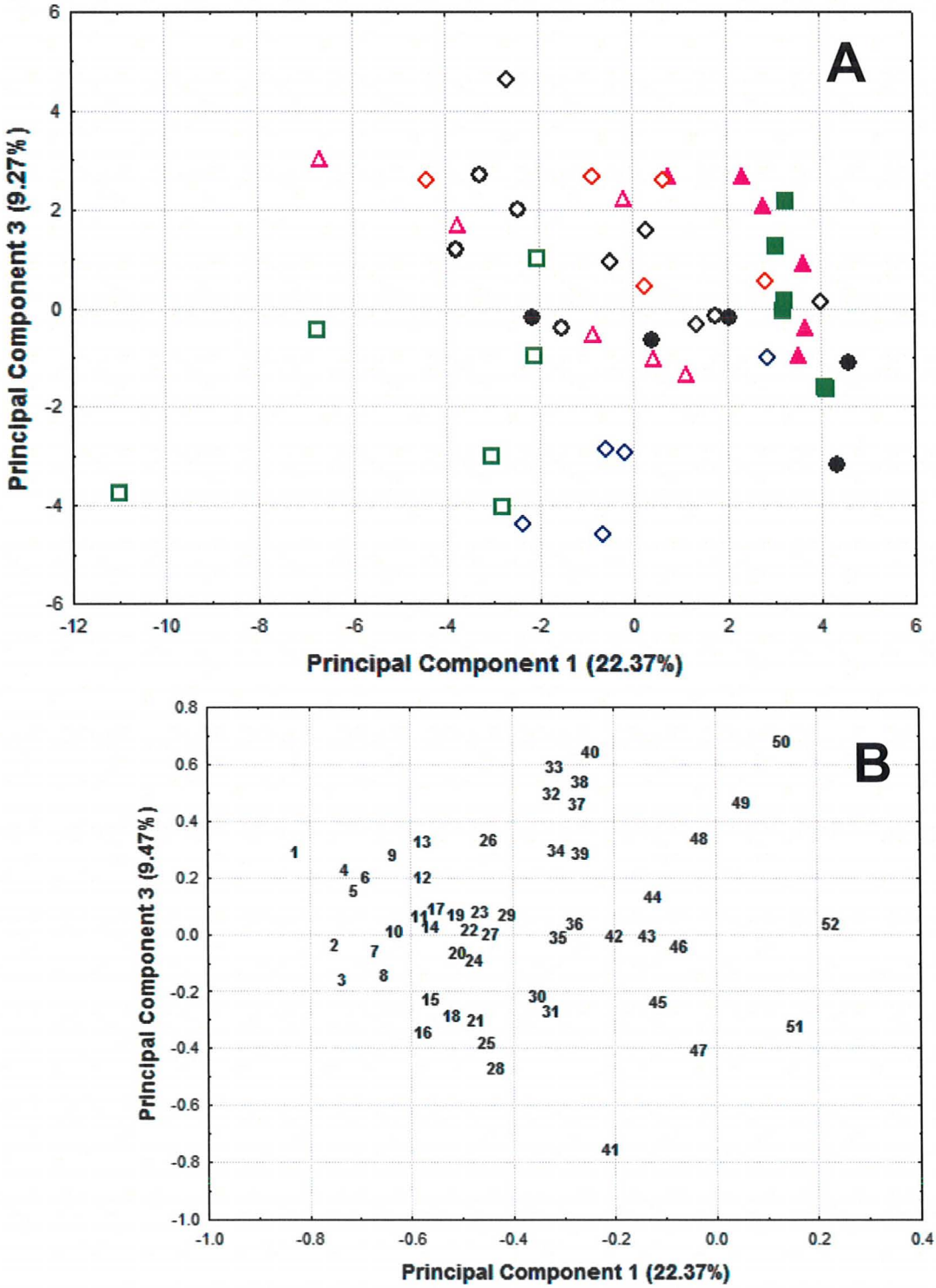
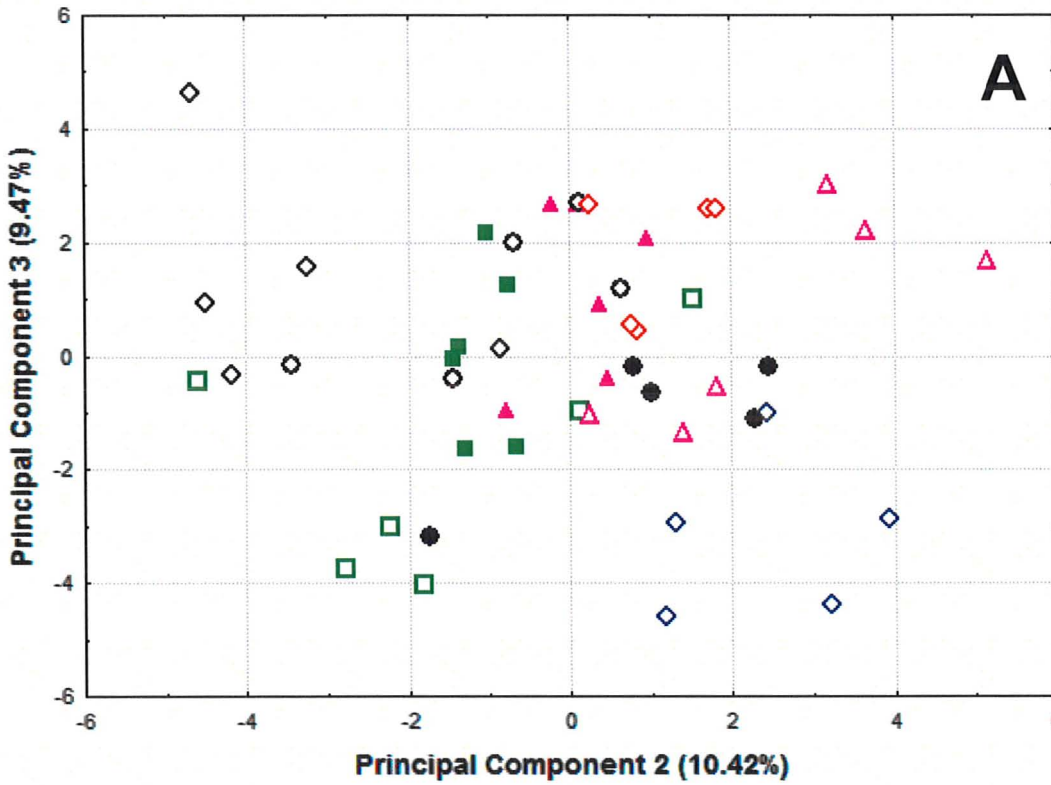

A
Principal Component 3 (9.27%)
Principal Component 1 (22.37%)
B
Principal Component 3 (9.47%)
Principal Component 1 (22.37%)

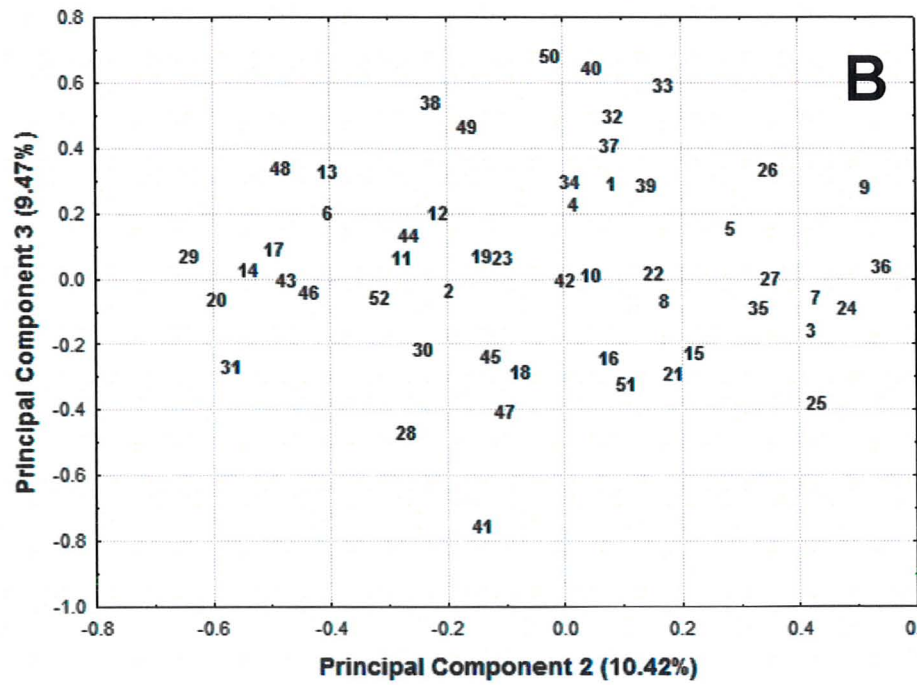
Table 8. Factor loadings for principal component two of PCA involving 52 MSTs found to be statistically significant in meta-analysis. Factor loadings are sorted from negative to positive. Variables found to have a factor loading ≥ 0.25 or ≤ -0.25 are in bold. Of the 52 variables, 12 have a strong negative relationship (factor loading ≥ 0.25) to principal component one and twelve variables have a strong positive relationship (factor loading ≥ 0.25) to principal component one. FL = factor loading.

Variable	[a]FL	Variable	FL	Variable	FL
Succinic acid	**-0.793**	Unknown 25.187	-0.044	**Gluconic acid**	**0.252**
Unknown 23.03	**-0.506**	Unknown 17.396	-0.042	**Organic acid 25.799**	**0.258**
Pyroglutamic acid	**-0.444**	Sugar 51.172	-0.038	**Unknown 31.052**	**0.287**
Citric acid	**-0.419**	Sugar 35.052	-0.030	**Unknown 41.982**	**0.292**
Unknown 26.973	**-0.362**	Quinic acid	-0.023	**Sugar 40.779**	**0.298**
Sugar 34.869	**-0.330**	Unknown 23.437	-0.015	**Unknown 37.829**	**0.418**
Uknown 41.088	**-0.322**	Raffinose	-0.004	**Unknown 39.14**	**0.425**
Fumaric acid	**-0.309**	Unknown 14.432	-0.001	**Unknown 43.576**	**0.454**
Amino acid 17.749	**-0.283**	Organic acid 34.163	0.024	**Unknown 37.595**	**0.502**
Sugar 33.08	**-0.274**	Unknown 41.729	0.025	**Sucrose**	**0.550**
Unknown 34.126	**-0.265**	Sugar 51.42	0.027	**Sugar 37.913**	**0.606**
Unknown 23.942	**-0.253**	Unknown 16.54	0.030	**Sugar 32.986**	**0.640**
Fructose	-0.199	Organic acid 31.307	0.050		
Sugar 52.002	-0.130	Threonine	0.090		
Sugar 43.834	-0.106	Unknown 38.723	0.115		
Proline	-0.100	Sugar 29.856	0.162		
myo-Inositol	-0.096	Unknown 29.941	0.163		
Unknown 29.052	-0.080	Sugar 37.409	0.185		
Sugar 32.382	-0.074	Sugar 44.14	0.239		
Sugar 41.196	-0.050	Sugar 31.762	0.246		

[a]FL, factor loading

Figure 13. Meta-Analysis: PCA and metabolite projections for Principal Component 2 plotted against Principal Component 3. Panel **A** shows PCA using log-scaled polar metabolite relative abundance GC/MS data. Each point represents the significant dataset generated from a 50 mg leaf sample pooled from a minimum of 5 individual seedlings (■ = HN, □ = HN+S, ▲ = LN, △ = LN+S) or represent the profile of an individual plant (● = C-0, ○ = S-300, ◊ = 2003 field plants, ◊ = 2005 field plants, ◊ = 2006 field plants). Panel **B** shows metabolite projections found using identities of MSTs that contribute to the clustering seen in panel **A**. See Table 5 for reference numbers for MST identification.


A
Principal Component 3 (9.47%)
Principal Component 2 (10.42%)
6
4
2
0
-2
-4
-6
-6
-4
-2
0
2
4
6


B
Principal Component 3 (9.47%)
Principal Component 2 (10.42%)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
50
40
33
38
49
32
37
48
13
34
1
39
26
9
4
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12
5
44
29
17
11
19
23
36
14
43
42
10
22
27
20
46
52
2
8
35
7
24
3
31
30
45
18
16
15
21
51
25
47
28
41

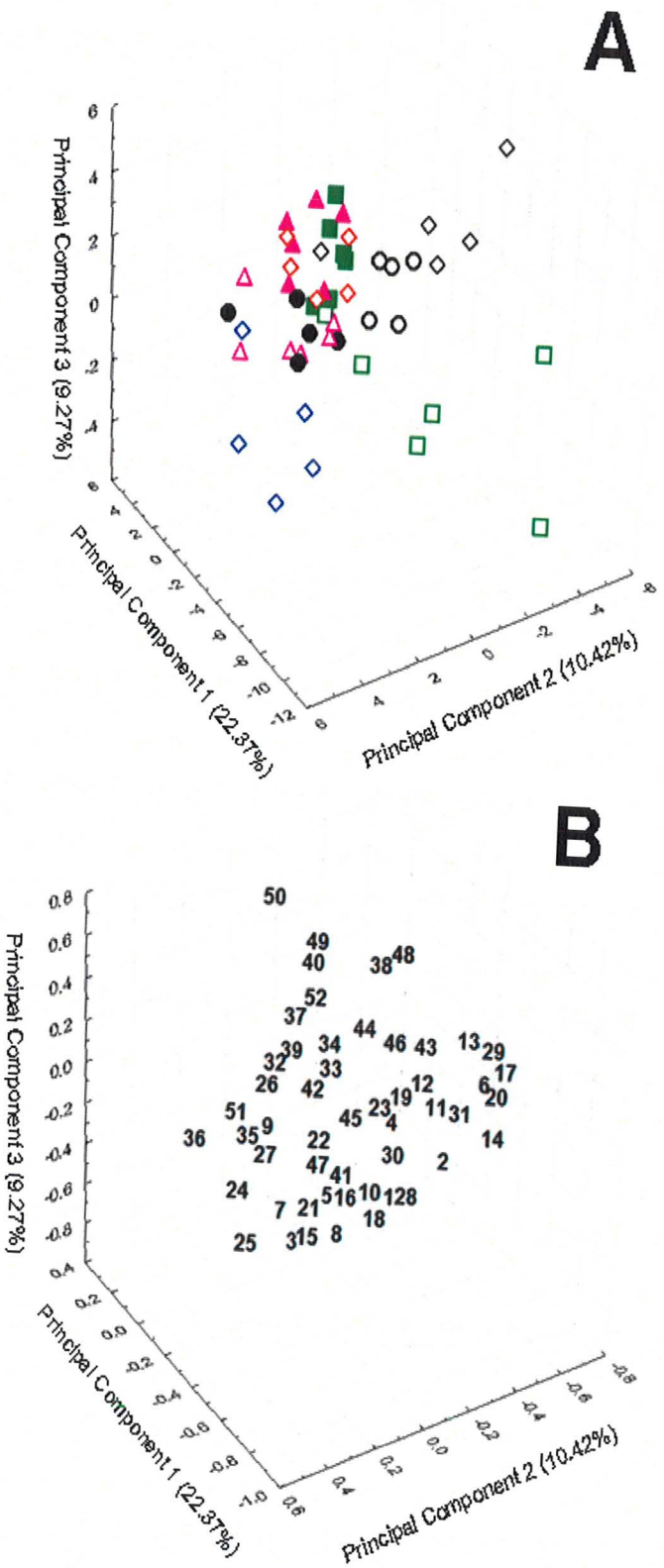
Three principal components reveal relationships between the datasets analyzed

An angled view of principal components 1, 2 and 3 in 3D space is shown in Figure 14A. For the purpose of discussing Figure 14A, the "front" will be defined by principal components one and two while the "back" is defined by principal components two and three.

Two observations seen earlier in Figure 11 are re-iterated in Figure 14A. Firstly, datasets for controls occupy a space to the back of the scatterplot and secondly, datasets for HN+S, LN+S and S-300 plants are found in two different locations in reference to control datasets (Fig. 11; Fig. 14A). However, in Figure 14A the three field years are more separate than in Figure 11. That is, datasets from 2003 samples occupy a space to the right of the control datasets while those of 2006 samples overlap with datasets from LN+S and 2005 samples (although 2006 datasets are found at a lower position with respect to principal component three). In Figure 14A the datasets can be described as forming five groups: (1) HN, LN and C-0 (2) HN+S and S-300 (3) LN+S and 2005 (4) 2006 (5) 2003.

The influence of the 52 variables on the relative position of these datasets can help identify metabolites accumulated in response to osmotic stress. Those variables present in the front, right side of the 3D plane influence the positions of datasets of HN+S plants and S-300 plants (Fig. 14B). Proline (20) occupies such a space (Fig. 14B). Surrounding proline are other metabolites that may also be important to growth in the presence of salt under sufficient nitrogen conditions. A sugar (48) at retention time 40.779 lies in the

Figure 14. Meta-analysis results showing interaction of the first three principal components in three dimensions. Panel **A** shows PCA using log-scaled polar metabolite relative abundance GC/MS data. Each point represents the significant dataset generated from a 50 mg leaf sample pooled from a minimum of 5 individual seedlings (■ = HN, □ = HN+S, ▲ = LN, △ = LN+S) or represent the profile of an individual plant (● = C-0, ○ = S-300, ◊ = 2003 field plants, ◊ = 2005 field plants, ◊ = 2006 field plants). Panel **B** shows metabolite projections found using identities of MSTs that contribute to the clustering seen in panel **A**. See Table 5 for reference numbers for MST identification.


A
Principal Component 3 (9.27%)
Principal Component 1 (22.37%)
Principal Component 2 (10.42%)
B
Principal Component 3 (9.27%)
Principal Component 1 (22.37%)
Principal Component 2 (10.42%)
50
49
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38
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12
6
20
19
11
51
45
23
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31
36
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14
27
30
2
47
41
24
5
16
10
128
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21
18
25
3
15
8

back, right of the 3D plane and shows its abundance to be in highest relative amount in 2003 field plants. Among the variables present in the back, left side of the 3D plane are four MSTs already described above as having a strong influence on the principal components. Sugar 44.140 (9), raffinose (36), sugar 52.002 (24) and sugar 51.172 (27) are the four MSTs and differences in their relative abundance in samples influence dataset positions of plants grown under LN+S conditions and 2005 field plants. The 2006 field plant datasets, located just below the LN+S and 2005 datasets, would also be affected by these variables.

DISCUSSION

A traditional approach used to assess plant response to abiotic stress under growth cabinet conditions usually entails a comparison made between a plant subjected to stress with that of an unstressed control plant. While the information obtained from such experiments can further our understanding of plant stress responses, bio-engineering crop plants with improved salt-tolerance remains difficult because abiotic stresses imposed in cabinets do not reproduce conditions in the field (Cushman and Bohnert, 2000; Mittler et al., 2001; Zhu et al., 2002). Rizhsky et al., (2004). In part, this concern can be addressed by subjecting plants to multiple concurrent stresses in controlled environments or harvesting plants grown in the field where multiple stresses are the norm. Moreover, studying a plant species that has adapted to an extreme environment, as is the case for *Thellungiella*, can help identify tolerance traits that benefit plants under stressful conditions (Bressan et al. 2001; Amtmann, 2009).

In this study we have profiled polar solutes present in leaves of *Thellungiella* seedlings grown on defined media differing in salt and nitrogen content. These profiles were then compared to those of metabolites extracted from leaves of plants harvested at a field site in the Yukon. This research led from previous work completed in our laboratory. The graduate research of D. Guevara showed that cabinet-grown *Thellungiella* accumulated proline under salt stress while tissue harvested by D. Guevara and J. Dedrick from a saline Yukon field site contained low proline content. Since a common element from both the cabinet and field included salt exposure it was not clear why some plants accumulated proline and others did not. One possibility that was considered was the

availability of nitrogen. Analyses of soil samples taken from the Yukon field site showed N to be in the range of 0.1 to 0.22 mM (Ping, unpublished). Since the plants in growth cabinets are fertilized weekly, we sought to determine if the comparatively low level of proline in field plants was a response to low nitrogen levels in the field. Access to the field tissue metabolite profiles also allowed us to determine whether the plants were accumulating any solutes in both field and laboratory growth conditions. We hypothesize that metabolites present in plants at both locations are expected to include those required by *Thellungiella* for stress tolerance under saline conditions irrespective of where the plants are grown.

Physiological response of *Thellungiella* to salt stress

Figure 4 shows representative seedlings grown under the four treatments we used for this study. The 100 mM NaCl level was first tested on the basis of several reports describing the tolerance of mature *Thellungiella* plants to varying degrees of salt (100 to 500 mM) stress (Teusink et al., 2002; Inan et al., 2004; Taji et al., 2004; Volkov et al., 2004; Gong et al., 2005; Vera-Estrella et al., 2005; Kant et al., 2006; M'rah et al., 2007; Ghars et al., 2008). However, the tolerance of *Thellungiella* seedlings to salt has not been described in previous publications and so this was carried out. Figure 1 shows Arabidopsis and *Thellungiella* seedlings tested on identical media containing100 mM NaCl and Arabidopsis is shown to have poor salt tolerance relative to *Thellungiella*. Kant et al. (2006) stated that ten-day-old Arabidopsis plants had a 58% lower shoot fresh weight when exposed to 100 mM NaCl compared to un-salinized controls. We did not

measure the shoot fresh weight of Arabidopsis seedlings grown on HN+S and LN+S media, but we observed that Arabidopsis seedlings on salt media were dead by the time *Thellungiella* seedlings reached the appropriate root length for harvest. In the end, a 100 mM NaCl content was chosen for our research because it 1) was a level high enough to be lethal to Arabidopsis seedlings yet the *Thellungiella* grew and 2) the salt content in soil at the Yukon field site approximates an osmotic potential roughly equivalent to 100 mM NaCl (Guevara and Weretilnyk, personal communication).

Many salt-stress studies use pots and salt stress is imposed by irrigating with saline liquid media. Our study involved growing *Thellungiella* seedlings on solid media in square plates. There are two advantages of this approach: (1) the concentration of both nutrients and salt is uniform throughout the plate and can be controlled and (2) we were able to observe root growth daily. We found this method allowed us to select newly germinated seedlings for uniform size and vigour and then more easily determine whether salt-stressed plants differed relative to their controls as a function of time. By ensuring that the plates were slanted vertically the roots grew on the surface of the agar allowing us to obtain a photographic record of their development and harvest tissue at the appropriate time with minimum disturbance to the roots. This approach did require a precautionary step of transferring seedlings to a bead of non-saline media so that new shoots did not come into direct contact with salt. When this bead of agar was not used the seedlings did not always recover the transfer step and seedlings often failed to thrive.

Growing *Thellungiella* seedlings in square plates allowed us to monitor root growth non-destructively and Table 2 shows that 100 mM salt-stressed seedlings

eventually reached root lengths comparable to control plants. Inan et al. (2004) reported a 50% reduction in root fresh weight of pot-grown *Thellungiella* plants exposed to 100 mM NaCl compared to their controls. Our results using seedlings on culture media are consistent with those of Inan et al. (2004) in that *Thellungiella* plants exposed to 100 mM NaCl grew at half the rate of their corresponding non-salinized controls (Table 2; Fig. 2).

Shoot fresh weight at the time of harvest (Fig. 3) does not change for *Thellungiella* exposed to 100 mM NaCl, however salt-stressed Thellungiella did require ten additional days to reach the point of harvest. Also, nitrogen content did affect shoot fresh weight. Kant et al. (2007) have reported that exposure to 0.4 mM N reduced shoot fresh weight of *Thellungiella* by 53%. Similarily, our results show that exposure to 0.1 mM N reduces shoot fresh weight of *Thellungiella* by 51% compared to *Thellungiella* grown under 1 mM N conditions (Fig. 3).

Selection of GC/MS for analysis

Profiling metabolites of organisms exposed to various abiotic conditions allows researchers to infer which metabolic pathways may be involved in different stress responses. However, when assessing changes in metabolism in plants one must consider that plant metabolism is also affected by diurnal changes (Fiehn et al., 2008). To address this concern, we standardized growth conditions and time of harvest across the four plant treatments tested and reproduced these conditions over three biological repeats of the experiment. To minimize changes to the chemicals present we flash-froze all harvested material in liquid nitrogen to preserve their state. Chemical conversions and degradation

of metabolites may occur if tissue is not well-preserved upon harvesting (Fiehn, 2001). We followed well documented procedures for sample preparation (Fiehn et al., 2000b, Rossener et al., 2000, Rossener et al., 2001; Fiehn, 2001, Kopka et al., 2004, Rizhsky et al., 2004) but it is, nonetheless, impossible to completely prevent artifacts from arising. For example, one chemical conversion reported to occur during sample preparation is the formation of pyroglutamic acid from glutamate, an outcome that has been reported by others (Meister, 1965). However, pyroglutamate has been reported by Fiehn as a metabolic signature present in extracts of Arabidopsis so its presence in our samples may reflect its natural occurrence and not artifact generation (http://fiehnlab.ucdavis.edu/ Metabolite-Library-2007/).

The amino acid glutamate is of particular interest in this study. Among the many metabolic roles for glutamate, an important one is as a precursor of the amino acid proline (Meister, 1965). A decline in glutamate content is reported to coincide with an increase in proline for plants under salinity stress (Santos et al., 2002) or drought stress (Diaz et al., 2005). Data in Figure 7 shows a similar trend for pyroglutamic acid content. ANOVA did not reveal any statistically significant change in the glutamate content of these samples. The possibility that the artifact generation may reduce glutamate and increases pyroglutamate content of samples could complicate interpreting the biological significance of the differences reported in the literature with respect to these metabolites. However, this difficulty would arise if the changes were random and not reproducible but the pattern of relative abundance is highly correlated with the sample source and

treatment combination leading us to conclude if this conversion takes place it does so with high reproducibility (Fig. 7).

Selection of transformation method

In order to analyze and compare complex datasets we first transformed the data using a $\log_{10}$ transformation (as described in Materials and Methods). This approach has been reported as useful by several investigators (Jolliffe, 1986; Fiehn et al., 2000b; Fiehn et al., 2001; Desbrosses et al., 2005; Trygg et al., 2006; Kim et al., 2007; Robinson et al., 2007; Fiehn et al., 2008). However, during our meta-analysis we found that interpreting the PCA and HCA outcomes was very difficult. More specifically, we found that PCA and HCA grouped datasets according to the experimental group. For example datasets from mature field plants, four treatment seedlings and mature cabinet-grown plants formed three distinct groups (data not shown). This made comparison of stress response between groups impossible. As such, we transformed the data further using a mean-center pre-processing strategy to standardize the data relative to the mean of each experimental group analyzed (the four treatments/mature field plants/mature cabinet-grown plants) (see Materials and Methods for details on analysis).

Mean center pre-processing of data prior to PCA has been used by others making similar comparisons of metabolite profiling data (Fiehn et al, 2000b). In this case we also subjected the transformed data to HCA. Standardizing the data relative to each group tested led to groupings that were more easily explained and the results reproduced those given when each of the experimental groups was compared independently. The value of

this approach was that it allowed us to compare multiple datasets describing the salt stress response of *Thellungiella* seedlings, field plants and mature-cabinet grown plants. This complex comparison of metabolite profiles would not have been possible otherwise.

Biological interpretation of the principal component axes

Interpretation of the axes extracted from PCA is subjective (Kendall, 1980; Cadima and Jolliffe, 1995). However, interpreting axes can offer insight into the biological relevance of statistically significant outcomes and this approach is discussed and applied frequently in the literature (Jolliffe, 1986; Fiehn et al., 2000b; Fiehn et al., 2001; Desbrosses et al., 2005; Trygg et al., 2006; Kim et al., 2007; Robinson et al., 2007; Fiehn et al., 2008).

Figures 8 and 10 offer a plausible explanation for the nature of the first principal components extracted in both of our studies as being representative of stress. We make this proposal based on the separation of control datasets (HN, LN, C-0) from those generated from plants subjected to salt stress (HN+S, LN+S, S-300). Unfortunately, there is no completely satisfactory "control" for field plants although the original data comparisons were made using cauline leaves from mature cabinet-grown plants. These control datasets were not included in this study, a consideration that will arise later in this discussion. Nonetheless, given the saline field site and influence of weather, high light and nutrient stresses, it is perhaps not surprising that datasets from field plants occupy positions closer to datasets from stressed plants than those of control plants (Fig. 8A; Fig. 10A).

The second principal component extracted from both of the analyses completed is less easy to determine but it does show a correspondence to N content of the environment. The PCA in Figure 8A shows that LN and LN+S datasets separate from HN and HN+S datasets across principal component two. We also know from soil analyses that the field plants are on low N soils while our cabinet-grown plants (C-0, S-300) are fertilized weekly with a 20-20-20 fertilizer solution. The PCA in Figure 11A shows that HN+S datasets and S-300 datasets are in close proximity to each other while 2005 and 2006 field datasets are in close proximity to LN+S datasets. However, field plants from 2003, that were also grown on low N soils, do not follow this pattern in Figure 11A.

While we cannot preclude the influence of other environmental factors in influencing the distribution of the datasets relative to each other, our proposals receive additional support through identification of MSTs responsive to stress and N treatments. If we assume that a specific stress elicits special metabolic change, then we should find MSTs with potential involvement in stress response among those showing a strong relationship toward principal component one, in the same direction as datasets from plants under stress. These metabolic changes would feature MSTs presented in bold in Tables 3 and 6. Also, if our interpretation of principal component two is correct in implicating N availability, then our results would be expected to show nitrogenous compounds influencing datasets from plants exposed to high N concentrations and non-nitrogenous compounds influencing datasets from plants exposed to low N conditions. In Figure 8B we see nitrogenous compounds, such as proline, having a strong relationship toward principal component two in the same direction as datasets from HN and HN+S

plants. In Figure 10B we also find proline having a strong relationship toward principal component two in the same direction as datasets from HN+S and S-300 plants. In contrast, Figure 8 shows carbohydrates, such as raffinose, having a strong relationship toward principal component two in the same direction as datasets from LN and LN+S plants. This feature is also shown in Figure 10B where carbohydrates, such as raffinose, fructose and several unidentifiable sugars are shown to have a strong relationship operating in the same direction toward principal component two for datasets from LN+S, 2005 and 2006 plants.

The biological significance of principal component three is unclear. However, when all three principal components were plotted together, datasets from the three field years separated from each other across principal component three (Fig. 14A). This result leads us to suggest that principal component 3 accounts for variance seen between the three field year datasets.

Statistical and biological significance of PCA and HCA results

In this study the PCA and HCA comparisons used metabolites found to be statistically significant. Although HCA is not one of the statistical techniques recommended by the Metabolomics Standards Initiative (MSI) to validate metabolomic studies (Fiehn et al., 2008), several studies have used HCA to organize both transcript and metabolic profiles as well as to confirm PCA results (Taji et al., 2004; Gong et al., 2005; Desbrosses et al., 2005; Müller et al., 2007). With one exception, the results obtained from our HCA affirm the results obtained from our PCA, Specifically, the PCA of Figure

14A shows the 2005 field datasets to overlap with LN+S datasets. This clustering using the group of statistically significant MSTs suggests that the 2005 field plants are metabolically similar to LN+S plants. In contrast, the HCA results of Figure 9 contradict this interpretation because the dataset for the 2005 field plants clusters more closely to the datasets for control plants and not those of LN+S seedlings. This apparent contradiction is likely a result of the statistical methods used. HCA clusters these plants based on their entire metabolic profile leading plants with similar profiles to cluster together (i.e. 2005 field plants and control plants). PCA, on the other hand, displays datasets across principal components that account for variance in those datasets. From this analysis we can conclude that the metabolic profile of 2005 field plants as a whole is most similar to those of control plants. However, with respect to stress (principal component one), nitrogen (principal component two) and field conditions (principal component three), 2005 field plants show greater metabolic similarity to LN+S plants and 2006 field plants.

Growth conditions of field plants corresponds to their positions on the PCA and HCA analyses

We compared metabolic data of field plants from three different field years (2003, 2005 and 2006) with those of seedlings and mature plants grown in controlled environment cabinets. As reported by Dedrick (2007), each year represents sampling under different conditions with the 2003 growing season having lower than normal precipitation resulting in a drought year. The 2005 growing season was a year of above average rainfall and the 2006 growing season had temperatures below 10°C for ten days prior to harvest (reported by Dedrick, 2007). With respect to precipitation, 8 mm of rain

with 0.6 mm snowfall occurred ten days prior to the 2006 field harvest (Dedrick, 2007). Overall the major environmental conditions that distinguish the various years include low water availability in 2003 (the drought year), higher than average water availability in 2005 (the well-watered year) and cold temperatures but otherwise normal precipitation in 2006 (the cold year) (reported by Dedrick, 2007). All field plants came from the same field location where low N soil is found along with alkaline pH and high salinity. For ease of reference, datasets from plants of the different field years will be referred to by their year of harvest.

Figure 14A shows that there is greater similarity in datasets from plants harvested in the 2005 and 2006 field years (well-watered and cold years) compared to those harvested in 2003 (drought year). Wong et al. (2005) suggested that *Thellungiella* exposed to drought or cold conditions respond by inducing similar protective mechanisms but this proposal was contradicted, in part, by microarray data in a subsequent report by Wong et al. (2006). In this later study the authors showed that only about half of cold and drought-responsive changes among transcripts are common to both stresses. The salinity dataset had little overlap with cold and drought datasets with salt-induced transcripts contributing to less than 1% of the changes in transcript abundance seen. Out of the 149 cold, salt or drought induced transcripts, only six were common to all three stresses.

If MST changes mirror the transcript abundance changes discussed above, we would predict to see the greatest similarities between the datasets arising from plants exposed to drought (2003) and cold (2006) with saline conditions a common factor (2003, 2005 and 2006). As a common factor, salinity would not be expected to separate datasets

for field years well although, in our meta-analysis, salinity may separate field years from control, unsalinized plant datasets. Figure 10A shows this to be the case. Here the field year datasets group more closely to the salt-stressed plant datasets along principal component one which we proposed earlier to identify variance in the data due to stress of stress exposure (Fig. 10A). If principal component 2 separates datasets on the basis of N as we discussed earlier, then we would expect field year datasets to separate away from many of the datasets of cabinet grown plants and perhaps cluster closer to LN+S datasets. In Figure 14A we see datasets from field years 2005 and 2006 position closer to LN+S datasets. Since salinity stress as a source of variance along principal component one is less applicable to the field samples, it is not surprising that a combination of axes with principal components 2 and 3 best separates the three field years (Fig. 13A). Figure 13A shows 2003 to be separated well from 2006 datasets. Interestingly, LN+S and 2005 datasets remain close to each other.

Our data is consistent with the interpretation proposed by Wong et. al. (2006) for transcript abundance in that the distinguishing environmental influences on metabolite abundance was cold or drought exposure and not salinity. With respect to MST abundance, salinity affords modest separation of the datasets (approximately 22% of the variance) along principal component 1 (Fig 10A) suggesting that many of the metabolic traits operating in salt-exposed plants are found when plants are not deliberately salinized. Again, this interpretation was offered by Wong et al (2006) to explain the low number of salt-responsive transcripts identified by microarray analysis. The authors stated that many salt-responsive transcripts found for other glycophytes including Arabidopsis may be

"on" constitutively in a halophyte like *Thellungiella* and our data suggests a similar conclusion for metabolic profiling traits.

Metabolic profiles of cabinet controls, stressed plants and field plants

The preceding discussion and the PCA of Figure 11A shows that the metabolic profiles of *Thellungiella* control plants (HN, LN and C-0) are more similar to one another than the metabolic profiles of stressed plants to each other. Our HCA also clusters control plants together (Fig. 9). In fact the greatest variation in control plants was found for C-0 plants whose datasets are more scattered than those of LN and HN plants (Fig. 11A). We suggest that this variation may be due to other plant conditions that we could not monitor including those relating to age.

In general, metabolic profiles of salinized, cabinet-grown *Thellungiella* and field plants (HN+S, LN+S, S-300, 2003, 2005, 2006) showed greater variation among group members than datasets for control plants (Fig. 11A). Such variation could be due to the difficulty in uniformly applying a salt treatment to individual plants and we have no reason to assume that individual plants in the field are isogenic. Therefore, some additional plant-to-plant variation in MSTs is not unexpected for salt-treated plants or those in the field. What is important, however, is that consistent, statistically significant changes among MSTs are found that are treatment and/or field specific. Overall, in our PCA shown in Figure 14A we find datasets representing stressed plants forming four subgroups based upon their proximity to each other in three dimensional space: (1) the

HN+S dataset and S-300 datasets, (2) the LN+S and 2005 datasets, (3) the 2006 field dataset, (which also associates closely to sub-group two) and (4) the 2003 field dataset.

With the sub-groups above considered and with a caveat that the methodology used offers a comparatively narrow view of the plant metabolome, HN+S stressed plants appear to be more metabolically similar to mature plants salt stressed to 300 mM NaCl (S-300) than the other plants tested (Fig. 9; Fig. 11). With the same assumptions, we can infer that metabolite profiles of seedlings growing under LN+S conditions were more similar to those of 2005 and 2006 field plants. Field site N concentration, according to soil samples, ranges from 0.1 to 0.22 mM (data not shown) with the lowest value comparable to the 0.1 mM N concentration used for our LN+S treatment. Finally, following the same reasoning, the metabolic profiles of plants from the 2003 field year are very different from all other plants tested. An extension of this meta-analysis to include data from cabinet-grown, drought-stressed *Thellungiella* could help identify the drought-responsive adjustments in MSTs for 2003 field plants. We should also consider the metabolites present in cauline leaves of well-watered cabinet-grown plants to preclude from our consideration any metabolites that are abundant in cauline as opposed to rosette leaves of well-watered plants. Nonetheless, the meta-analysis approach completed for this study already allows us to work back to the metabolic pathways operating during drought stress of *Thellungiella* under complex field conditions.

Changes among MSTs

In the four treatment study (LN, LN+S, HN, HN+S), 45 MSTs contributed to the grouping of the datasets relative to each other (Table 3). For the meta-analysis involving cabinet grown and field plant datasets, 39 MSTs were key to the association of datasets under stress relative to each other (Table 6). Of these MSTs, 17 appear as factors in both studies. While the metabolic responses to different environments may be complex, by comparing metabolite changes between different plants we can begin to identify a smaller group of MSTs that merit further study or exclude others from further consideration as targets to enhance stress tolerance.

Nitrogenous Compounds

A compatible organic solute that has received a great deal of attention in the literature is proline (Rhodes and Hanson, 1993; McNeil et al., 1999). Hu et al. (1993) showed proline to inhibit γ-glutamyl activity of the *Vigna P5CS,* a limitation to the over-production of proline by plants under stress. Since then, considerable attention has been given to bio-engineer plants to tolerate salt stress through increased proline accumulation using *P5CS2,* including an engineered version of this enzyme that is insensitive to feed-back inhibition by proline (Hong et al., 2000). Proline biosynthesis has also been examined for *T. halophila.* Using transcript profiling, Taji et al. (2004) showed that *P5CS2* was expressed in *T. halophila* at high levels in the absence of salt stress. For this plant species Kant et al. (2006) reported increasing levels of transcripts associated with *P5CS2* coupled with a rapid reduction in proline dehydrogenase enzyme activity as a

function of increasing NaCl treatment concentrations. The increase of proline under high nitrogen (1 mM) and 100 mM media conditions shows that *T. salsuginea* from the Yukon also has the same capacity to accumulate proline in response to salt exposure (Table 3, Fig. 7) and, as such, proline may perform the role of a compatible solute for these plants.

A higher proline content contributes strongly towards the separation of HN+S and S-300 datasets from corresponding controls seen in Figure 14B. A lower content of pyroglutamic acid (believed to be a glutamate derivative) in *Thellungiella* grown under HN+S conditions is consistent with more glutamate being used for proline accumulated by these seedlings. This observation would be expected if P5CS2 activity increases during conditions of salt stress as noted in the literature (Kant et al., 2006). We were unable to quantify *P5CS* mRNA (data not shown) so an association between transcript abundance and metabolite product cannot be made for these seedlings. Also, our PCAs show that proline contributes strongly towards the separation of HN+S datasets from those of LN, LN+S or HN datasets shown in Figure 8B. What is clear by virtue of the LN+S data is that proline accumulation is not an obligatory response to salt exposure for *Thellungiella*. Indeed, Table 2 shows that there appears to be no obvious growth penalty for these seedlings or the plants in the field where soils are saline but leaf proline content is also low as is soil N availability.

The nitrogenous metabolite putrescine is 2-fold higher in plant extracts prepared from tissue on HN media relative to HN+S conditions (Appendix 3). Several studies report a decline in putrescine levels to correspond with an increase in plant proline content for plants under salinity stress (Su and Bai, 2008; Tonon et al., 2004; Santa-Cruz

et al., 1999). Our study also shows this potential relationship between putrescine and proline. However, in contrast to HN and HN+S samples, changes in leaf tissue putrescine levels were not found to be statistically significant following our meta-analysis.

Two other nitrogenous compounds of interest are threonine and glycine. Gong et al. (2005) showed that both of these compounds increased during salt stress (150 mM and 250 mM NaCl) in *Thellungiella*. Our results do not show a statistically significant increase in relative abundance of glycine in *Thellungiella* tissue subjected to salt stress. Moreover, our results contradict those of Gong et al., (2005) as we found a lower threonine content in LN+S and HN+S tissue compared to control tissue (Appendix 3).

Organic acids

Four salt-responsive organic acids were identified by comparing mass spectras to those of authentic standards: citric acid, fumaric acid, succinic acid and gluconic acid. Gong et al., (2005) showed citric acid, fumaric acid and succinic acid to be constitutively higher in salt stressed (150 mM and 250 mM NaCl) *Thellungiella* compared to Arabidopsis. Citric acid and succinic acid have also been implicated as important in drought stress in *Thellungiella* by their increased abundance in plants deprived of water (Dedrick, 2007). Kim et al. (2007) showed that fumaric acid increases in Arabidopsis under 100 mM NaCl stress. We have not found a report associating gluconic acid to salt stress response in *Thellungiella*. In summary, these findings are consistent with reports in literature in that organic acids appear to play an important role in the metabolic response of plants to osmotic stress (Timpa et al., 1986; Sanchez et al., 2008).

A Role for Carbohydrates

In our study we show that *Thellungiella* subjected to a combination of salt stress and limiting nitrogen conditions accumulates carbohydrates, including raffinose, fructose, and several unidentifiable sugars (Fig. 14). As discussed earlier, the lower proline content of these samples strongly suggests that carbohydrates are accumulated to replace proline when N is limiting. Hoekstra et al. (2001) reported that under moderate osmotic stress, accumulating compatible solutes such as proline and glycine betaine will help to protect protein and membrane structure. However, under severe osmotic stress Hoekstra et al. (2001) showed that only sugars can stabilize proteins and restore osmotic equilibrium. Rizhsky et al. (2004) provided additional evidence by showing that under a combination of drought and heat stress there is a metabolic change from the production of proline to the production of sucrose in Arabidopsis.

In our study of the carbohydrates undergoing changes, raffinose may serve as an important osmoprotectant under low N and saline conditions. Raffinose content was found to increase under +S conditions and more so in LN+S stressed plants compared to HN+S stressed plants (Fig. 7). Gong et al. (2005) reported raffinose to be salt-stress responsive in *Thellungiella* and other studies have implicated raffinose in osmoprotection for Arabidopsis under salt stress (Liu et al., 2007; Nishizawa et al., 2008). Figure 9 shows raffinose to accumulate most in leaf tissue harvested from 2006 field plants. Previously, we have discussed 2006 to be a year of cold temperatures at the time of harvest. Interestingly, several studies have implicated raffinose to serve as an osmoprotectant for plants under cold stress (Taji et al., 2002; Zuther et al., 2004; Nishizawa et al., 2008).

The content of the carbohydrate *myo*-Inositol is higher under salt stress conditions, independent of N conditions (Fig. 7). *Myo*-Inositol is reported to be an important metabolite under salt stress conditions for the common ice plant *M. crystallinum* (Paul and Cockburn et al., 1989; Adams et al., 1992; Vernon et al., 1993; Chauhan et al., 2000) and in *X. viscosa* (Lenher et al., 2008). Previous work in our laboratory has shown *myo*-Inositol content to be higher in salt (Guevara and Weretilnyk, unpublished) and drought-treated *Thellungiella* grown in pots (Dedrick, 2007).

Galactinol synthase is considered to be the rate-limiting enzyme in the production of raffinose family oligosaccharides (RFOs) from substrates including *myo*-Inositol. Galactinol synthase activity is induced in Arabidopsis by drought and salt stress (Taji et al., 2002) and under heat stress (Panikulangara et al., 2004). Wong et al. (2006) showed an increase in transcripts associated with galactinol synthase in *Thellungiella* under drought conditions. We did not investigate changes in transcript levels of galactinol synthase for this thesis but this approach could offer additional insight into the role of carbohydrate metabolism under salt stress and, as such, an important continuation from this work.

Many reports in the literature connect trehalose (Holmstrom et al., 1996; Yeo et al., 2000; Zhao et al., 2000; Jang et al., 2003; Penna, 2003) and mannitol (Tarczynski et al., 1993; Thomas et al., 1995; Karakas et al., 1997; Abebe et al., 2003) accumulation to plant abiotic stress tolerance. However, this does not appear to be the case for *Thellungiella* as we found no evidence for statistically significant accumulation of trehalose or mannitol in the tissues we analyzed or subjected to meta-analysis. Gong et al.

(2005), also, did not detect elevated mannitol content in *Thellungiella* plants subjected to salt stress. However, the authors did show evidence of a salt-responsive increase of trehalose and a trehalose-like compound in *Thellungiella* (Gong et al., 2005). We did see an increase in several unidentified sugars (Sugar 43.834, Sugar 44.140, Sugar 51.521 and Sugar 51.172) under LN+S stress compared to HN+S stress conditions (Fig. 7). Our PCA revealed sugar 43.834 to be one of the ten MSTs that strongly contributed to the separating of LN+S datasets from their controls (Fig. 8B). Sugar 43.834 was also found to be present in tissue collected from 2005 and 2006 field plants (Fig. 9). Sugars 43.834, 44.14, 51.172 and 52.002 all contributed to the close grouping of LN+S, 2005 and 2006 datasets in our meta-analysis (Fig. 14). The lower levels of these sugars in 2003 field plants cannot be explained by this study. However, these unknown sugars may prove to have novel function during salt and or cold stress in *Thellungiella* given their higher relative abundance in LN+S seedlings and 2005 and 2006 field plants.

Our meta-analysis revealed that fructose also increased in S-300 plants compared to their C-0 controls and in 2006 field plants relative to all other field plants tested (Fig. 9). Fructose also accumulated in LN+S and HN+S stressed plants compared to controls during our four treatment study (Fig. 7). As such, fructose may play a role in osmoprotection in *Thellungiella*. Our PCAs showed fructose to be responsible for the positions of LN+S and HN+S datasets relative to LN and HN samples (Fig. 8) and in the position of the LN+S, 2005 and 2006 datasets (Fig. 14). Fructose has been implicated to be an important compatible solute in *Thellungiella* due to its increased abundance in plants experiencing drought stress by Dedrick (2007) and salt stress (Vinocur and

Altman, 2005). Fructose is also an important organic solute under salt stress in tobacco (Pilon-Smits et al., 1995) and in sugar beet (Pilon-Smits et al., 1999).

Several chemicals merit attention but lack of identification is problematic

Metabolomics allows us to investigate unknown compounds that may have novel function during stress. Unfortunately, progress in metabolomic studies is limited by the low number of metabolites that can be identified with certainty. Our results show that several unidentified sugars undergo changes in abundance that correlate with plants exposed to stress and, as such, may be biochemically important to *Thellungiella* under stress conditions. Identification of these sugars is not trivial but this activity may represent an important continuation of this work. We have also only tested polar metabolites amenable to analysis by GC-MS (see Materials and Methods) and many higher molecular mass compounds, lipids and other non-polar compounds, were not analyzed. In particular, lipids have been shown to play a role in stress response of plants (Somerville and Browse, 1991; Farmer, 1994; Nishiuchi and Iba, 1998; Rubio et al., 2008; Darwish et al., 2009). An investigation into changes of lipid composition during salt stress in may offer further insight into *Thellungiella* stress response under field conditions.

CONCLUSION

Thellungiella seedlings exposed to 100 mM NaCl and varying nitrogen availability showed no discernable adverse consequences at the level of root elongation or shoot biomass. However, a comparison of the metabolites present shows many notable differences. In general, plants on 1.0 mM N had a higher content of nitrogenous solutes with proline content significantly higher when salt was in the media. This shows that *Thellungiella* has a capacity to alter its metabolite profile in response to its environment. It is possible that this plasticity at the level of metabolites confers an advantage to *Thellungiella* under field conditions where multiple, concurrent stresses are the norm.

To evaluate the metabolic plasticity of this plant, a meta-analysis was carried out using metabolite profiling datasets from leaves of plants harvested under field and growth cabinet conditions. In this study we show that *Thellungiella* plants grown under limiting nitrogen conditions with salt stress metabolically resemble plants harvested from Yukon field sites, particularly plants harvested in 2005 and 2006. Among the metabolites profiled, carbohydrates, including raffinose, are enriched in LN+S plants and field plants, while proline content is reduced. This suggests that Thellungiella can use a broad variety of metabolites under stress conditions and the reduced dependence upon proline may be advantageous when N is limiting.

GC/MS analysis of leaf metabolite extracts coupled with statistical analyses (ANOVA, PCA and HCA) have proven to be invaluable methods for this study. By using these statistical tools, large datasets of metabolic information can be reduced to a workable number of data points to serve as leads meriting further study. Several

metabolites showing important correlations with stress remain unidentified and this will be a challenge to future characterization of their roles in stress tolerance.

An important contribution of this work is that it provides needed information about the behaviour of *Thellungiella* under controlled environment and field conditions. The methodologies used in this study and the information we obtained will help develop *Thellungiella* as an exceptional model plant for the study of abiotic stress

Appendix 1. Nutrient recipe used for the culture media. Nitrogen and salt concentrations that distinguish the four treatments (HN, LN, HN+S and LN+S) from each other are reported in Table 1.

Instructions to make nutrient media:

1) Four 1-L flasks were labelled with the one of the following: HN, LN, HN+S and LN+S.

2) The macro and micro nutrients were added to the all four flasks (see working volumes below). The macro and micro nutrients are listed below.

Macro-Nutrient Solutions:

Nutrient	[Stock]	[Final]	Working Volumes			
			mL			
	M	*mM*	100	200	250	500
$MgSO_4$	0.5	0.5	0.1	0.2	0.25	0.5
$CaCl_2$	0.5	0.5	0.1	0.2	0.25	0.5
Na_2HPO_4/NaH_2PO_4	0.5	0.5	0.1	0.2	0.25	0.5

Micro-Nutrient Solutions:

Nutrient	[Stock]	[Final]	Working Volumes			
	M	*mM*	*mL*			
			100	200	250	500
$FeSO_4$	10	100	1	2	2.5	5
H_3BO_3	10	100	1	2	2.5	5
$MnCl_2$	2	20	1	2	2.5	5
$CuSO_4$	0.1	1	1	2	2.5	5
$ZnSO_4$	0.1	3	3	6	7.5	15
Na_2MoO_4	0.02	0.4	2	4	5	10
$CoCl_2$	0.001	0.01	1	2	2.5	5

3) KNO_3 and KCl were added to the HN and HN+S flasks as described in the table titled "1 *mM* N Solution." KNO_3 and KCl were added to the LN and LN+S flasks as described in the table titled "0.1 *mM* N Solution."

1 *mM* N Solution:

Nutrient	[Stock]	Working Volumes			
		mL			
	M	100	200	250	500
KNO_3	0.5	0.2	0.4	0.5	1.0
KCl	0.5	0.2	0.4	0.5	1.0

0.1 *mM* N Solution:

Nutrient	[Stock]	Working Volumes			
		µL			
	M	100	200	250	500
KNO_3	0.5	20	40	50	100
KCl	0.5	380	760	950	1900

4) NaCl was added to the HN+S and LN+S flasks as described in the table titled "100 mM NaCl Solution." A 2 M stock solution of NaCl was made by dissolving 11.69 g of NaCl in 100 mL of ddH_2O.

100 mM NaCl Solution:

Nutrient	[Stock]	Working Volumes			
		mL			
	M	100	200	250	500
NaCl	2	5	10	15	25

5) The volume of each nutrient solution was increased to the desired volume using ddH_2O. (i.e. If creating a 500 mL nutrient solution increase the volume to 500 mL)

6) Prior to autoclaving 0.8% (w:v) of phytagel and 2%(w:v) of sucrose was added to each flask. To calculate the amount of phytagel and sucrose to add multiple the percentage by the initial volume. (i.e. A 500 mL nutrient solution would require 10 g of sucrose)

7) All flasks were autoclaved using the setting labelled liquid 1 (30 minute cycle). The top of all flasks was covered with tinfoil prior to autoclaving.

Appendix 2. 206 MSTs that were statistically significant as described in the Materials and Methods.. Levels of metabolites shown as expressing statistically significant ($P \leq 0.05$) changes in their abundance for *Thellungiella* seedlings as compared to the mean metabolite levels of all four treatments. Values are expressed as average RRF ± standard error. SE = standard error. n = 6

MST	C-0	SE	S-300	SE	HN	SE	LN	SE	HN+S	SE	LN+S	SE
14.432	0.003	0.000	0.000	0.000	0.008	0.001	0.010	0.001	0.007	0.000	0.010	0.001
15.389	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.001	0.000
16.540	0.005	0.001	0.011	0.001	0.001	0.000	0.001	0.000	0.003	0.000	0.001	0.000
17.396	0.000	0.000	0.008	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
16.939	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.003	0.001
18.771	0.402	0.016	0.657	0.019	0.001	0.000	0.001	0.000	0.090	0.003	0.014	0.003
19.211	0.008	0.001	0.001	0.000	0.010	0.001	0.008	0.001	0.021	0.001	0.010	0.001
20.069	0.001	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.004	0.000	0.000	0.000
20.862	0.107	0.008	0.104	0.002	0.010	0.001	0.002	0.000	0.012	0.002	0.000	0.000
20.991	0.043	0.002	0.052	0.004	0.005	0.001	0.002	0.000	0.004	0.001	0.000	0.000
23.030	0.000	0.000	0.001	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
23.437	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23.535	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
23.778	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23.903	0.154	0.037	0.002	0.000	0.188	0.012	0.195	0.005	0.252	0.029	0.209	0.010
23.942	1.021	0.008	0.819	0.010	0.000	0.000	0.000	0.000	0.114	0.030	0.000	0.000
24.668	0.075	0.005	0.043	0.001	0.046	0.006	0.000	0.000	0.023	0.004	0.000	0.000
24.775	0.017	0.004	0.042	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
25.187	0.145	0.010	0.150	0.011	0.087	0.002	0.074	0.004	0.081	0.003	0.074	0.005
25.341	0.018	0.000	0.020	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
25.799	0.039	0.000	0.034	0.002	0.005	0.001	0.004	0.000	0.009	0.001	0.009	0.001
26.713	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
26.973	0.118	0.005	0.079	0.002	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
28.200	0.000	0.000	0.001	0.000	0.002	0.000	0.003	0.001	0.003	0.000	0.007	0.001
29.052	0.002	0.001	0.013	0.001	0.001	0.000	0.001	0.000	0.002	0.000	0.001	0.000
29.856	0.019	0.001	0.035	0.001	0.000	0.000	0.000	0.000	0.002	0.000	0.001	0.000
29.941	0.005	0.000	0.009	0.001	0.002	0.000	0.002	0.000	0.004	0.000	0.005	0.001
30.080	0.014	0.001	0.018	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
30.367	0.068	0.015	0.099	0.012	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
31.052	0.002	0.000	0.002	0.000	0.001	0.000	0.000	0.000	0.002	0.000	0.001	0.000
31.209	0.004	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
31.307	0.001	0.000	0.006	0.001	0.000	0.000	0.001	0.000	0.005	0.001	0.001	0.000
31.519	0.169	0.009	0.058	0.004	0.052	0.002	0.055	0.002	0.164	0.005	0.154	0.010
31.762	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
32.096	0.068	0.004	0.121	0.006	0.002	0.000	0.003	0.000	0.006	0.001	0.006	0.001
32.382	0.015	0.001	0.017	0.001	0.001	0.000	0.002	0.000	0.012	0.001	0.005	0.001
32.459	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.005	0.000	0.004	0.000
32.687	0.001	0.000	0.009	0.002	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
32.872	0.049	0.002	0.052	0.001	0.005	0.000	0.006	0.000	0.018	0.000	0.018	0.001
32.986	0.003	0.001	0.017	0.003	0.093	0.009	0.104	0.004	0.045	0.003	0.040	0.003
33.080	0.004	0.002	0.021	0.007	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
34.126	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
34.163	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
34.195	0.003	0.001	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
34.372	0.001	0.000	0.001	0.000	0.001	0.000	0.001	0.000	0.002	0.000	0.001	0.000
34.869	0.023	0.002	0.031	0.004	0.000	0.000	0.000	0.000	0.002	0.001	0.002	0.000
35.052	0.015	0.004	0.109	0.016	0.000	0.000	0.000	0.000	0.008	0.002	0.005	0.001
35.223	0.001	0.000	0.002	0.000	0.001	0.000	0.001	0.000	0.002	0.000	0.002	0.000
35.514	0.012	0.002	0.014	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
35.883	0.003	0.001	0.022	0.002	0.006	0.001	0.003	0.001	0.004	0.001	0.002	0.000
36.448	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
36.911	0.213	0.009	0.270	0.008	0.019	0.002	0.046	0.002	0.119	0.003	0.106	0.006
37.409	0.034	0.001	0.038	0.002	0.001	0.000	0.001	0.000	0.005	0.000	0.005	0.000
37.595	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
37.686	0.002	0.001	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
37.829	0.006	0.001	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
37.913	0.002	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
38.022	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
38.723	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000
38.900	0.001	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.003	0.000
39.140	0.018	0.003	0.022	0.003	0.003	0.001	0.001	0.000	0.002	0.000	0.001	0.000
40.779	0.172	0.007	0.185	0.011	0.124	0.007	0.124	0.006	0.116	0.005	0.124	0.007
41.088	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
41.196	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000	0.002	0.000	0.002	0.000
41.484	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
41.729	0.002	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
41.982	0.001	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
42.503	0.001	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
42.652	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
43.576	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
43.834	0.030	0.002	0.062	0.004	0.003	0.000	0.010	0.001	0.024	0.002	0.028	0.004
44.140	0.004	0.001	0.006	0.001	0.000	0.000	0.001	0.000	0.001	0.000	0.003	0.000
46.086	0.003	0.000	0.004	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

46.196	0.003	0.001	0.082	0.035	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000
44.268	0.897	0.015	1.067	0.011	0.352	0.020	0.384	0.016	0.295	0.016	0.408	0.022
51.172	0.006	0.001	0.009	0.002	0.000	0.000	0.002	0.000	0.004	0.001	0.006	0.001
51.420	0.003	0.001	0.002	0.000	0.002	0.000	0.002	0.000	0.005	0.000	0.001	0.000
52.002	0.000	0.000	0.002	0.001	0.000	0.000	0.001	0.000	0.002	0.000	0.002	0.000
52.206	0.149	0.009	0.159	0.010	0.096	0.005	0.104	0.008	0.092	0.007	0.104	0.007
57.162	0.005	0.001	0.001	0.000	0.000	0.000	0.012	0.001	0.012	0.001	0.031	0.002

RT	2003	SE	2005	SE	2006	SE	HN	SE	LN	SE	HN+S	SE	LN+S	SE
13.214	0.458	0.024	0.550	0.027	0.582	0.030	0.118	0.009	0.122	0.007	0.141	0.009	0.116	0.014
13.349	0.419	0.022	0.519	0.026	0.925	0.060	0.068	0.005	0.051	0.005	0.058	0.005	0.057	0.011
14.273	0.000	0.000	0.000	0.000	0.005	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
14.432	0.079	0.008	0.004	0.001	0.019	0.001	0.008	0.001	0.010	0.001	0.007	0.000	0.010	0.001
15.389	0.000	0.000	0.012	0.001	0.009	0.000	0.003	0.000	0.005	0.000	0.007	0.001	0.007	0.001
16.540	0.004	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.003	0.000	0.001	0.000
16.939	0.000	0.000	0.000	0.000	0.061	0.006	0.020	0.001	0.021	0.001	0.017	0.002	0.024	0.001
17.396	0.016	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
17.749	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000	0.003	0.001
17.875	0.021	0.002	0.015	0.003	0.018	0.002	0.009	0.000	0.005	0.000	0.007	0.000	0.006	0.000
18.226	0.004	0.001	0.000	0.000	0.001	0.000	0.119	0.011	0.153	0.006	0.182	0.009	0.168	0.014
18.447	0.002	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
18.771	0.338	0.024	0.014	0.004	0.072	0.009	0.000	0.000	0.000	0.000	0.090	0.003	0.014	0.003
19.211	0.018	0.001	0.006	0.001	0.041	0.002	0.010	0.001	0.008	0.001	0.021	0.001	0.010	0.001
20.069	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.004	0.000	0.000	0.000
20.862	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
20.991	0.000	0.000	0.001	0.000	0.014	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000
21.293	0.000	0.000	0.001	0.000	0.000	0.000	0.005	0.001	0.002	0.000	0.004	0.001	0.000	0.000
21.803	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
23.030	0.000	0.000	0.000	0.000	0.008	0.002	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
23.437	0.010	0.001	0.007	0.001	0.004	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
23.942	0.544	0.027	0.634	0.013	0.569	0.048	0.000	0.000	0.000	0.000	0.114	0.030	0.000	0.000
24.668	0.000	0.000	0.000	0.000	0.008	0.002	0.046	0.006	0.000	0.000	0.023	0.004	0.000	0.000
24.722	0.027	0.003	0.000	0.000	0.002	0.001	0.003	0.001	0.001	0.000	0.005	0.001	0.002	0.000
25.187	0.254	0.011	0.211	0.004	0.225	0.010	0.087	0.002	0.074	0.004	0.081	0.003	0.074	0.005
25.799	0.072	0.005	0.039	0.002	0.028	0.001	0.005	0.001	0.004	0.000	0.008	0.001	0.009	0.001
26.134	0.001	0.000	0.002	0.001	0.031	0.005	0.000	0.000	0.000	0.000	0.006	0.001	0.001	0.000
26.273	0.001	0.000	0.002	0.000	0.003	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
26.519	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
26.973	0.069	0.005	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
27.072	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
27.256	0.000	0.000	0.000	0.000	0.003	0.001	0.000	0.000	0.000	0.000	0.009	0.002	0.001	0.000
27.826	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
28.002	0.003	0.001	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.000	0.000
28.111	0.001	0.000	0.002	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
28.579	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
28.932	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
29.052	0.026	0.004	0.019	0.002	0.013	0.001	0.001	0.000	0.001	0.000	0.002	0.000	0.001	0.000
29.178	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
29.451	0.000	0.000	0.001	0.000	0.003	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
29.750	0.006	0.001	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
29.856	0.009	0.001	0.001	0.000	0.004	0.001	0.000	0.000	0.000	0.000	0.002	0.000	0.001	0.000
29.941	0.048	0.003	0.018	0.002	0.002	0.000	0.002	0.000	0.002	0.000	0.004	0.000	0.005	0.001
30.188	0.000	0.000	0.012	0.001	0.005	0.000	0.002	0.000	0.002	0.000	0.005	0.000	0.006	0.000
30.433	0.110	0.011	0.125	0.014	0.066	0.005	0.007	0.000	0.009	0.000	0.009	0.000	0.016	0.001
30.668	0.080	0.006	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
30.823	0.001	0.000	0.002	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000
31.052	0.005	0.001	0.002	0.001	0.001	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.001	0.000
31.144	0.000	0.000	0.001	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000
31.307	0.000	0.000	0.004	0.001	0.272	0.034	0.000	0.000	0.001	0.000	0.005	0.001	0.001	0.000
31.519	0.532	0.033	0.642	0.036	0.471	0.028	0.052	0.002	0.055	0.002	0.164	0.005	0.154	0.010
31.693	0.000	0.000	0.002	0.000	0.000	0.000	0.001	0.000	0.003	0.000	0.006	0.000	0.005	0.001
31.762	0.002	0.000	0.002	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
31.957	0.002	0.000	0.000	0.000	0.007	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
32.300	0.010	0.001	0.001	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.001	0.000
32.382	0.000	0.000	0.009	0.002	0.001	0.000	0.001	0.000	0.002	0.000	0.012	0.001	0.005	0.001
32.459	0.000	0.000	0.021	0.003	0.062	0.008	0.001	0.000	0.001	0.000	0.005	0.000	0.004	0.000
32.473	0.012	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
32.768	0.473	0.038	0.346	0.024	0.481	0.032	0.132	0.013	0.160	0.006	0.074	0.006	0.070	0.004
32.872	0.022	0.004	0.045	0.003	0.012	0.001	0.004	0.000	0.006	0.000	0.018	0.000	0.018	0.001
32.986	0.329	0.028	0.244	0.019	0.351	0.026	0.093	0.009	0.104	0.004	0.045	0.003	0.040	0.003
33.080	0.016	0.003	0.027	0.003	0.054	0.011	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000
33.151	0.017	0.003	0.009	0.002	0.481	0.042	0.002	0.000	0.004	0.000	0.005	0.000	0.005	0.001
33.308	0.458	0.030	0.325	0.021	0.237	0.019	0.236	0.012	0.244	0.008	0.239	0.010	0.212	0.019

33.644	0.252	0.012	0.129	0.011	0.434	0.014	0.126	0.006	0.130	0.011	0.160	0.007	0.128	0.008
34.035	0.003	0.000	0.002	0.001	0.000	0.000	0.001	0.000	0.002	0.000	0.001	0.000	0.004	0.001
34.126	0.000	0.000	0.005	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
34.163	0.009	0.001	0.002	0.000	0.031	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
34.498	0.004	0.000	0.000	0.000	0.013	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
34.869	0.001	0.000	0.059	0.006	0.013	0.002	0.000	0.000	0.000	0.000	0.002	0.001	0.002	0.000
34.932	0.037	0.005	0.116	0.049	0.556	0.046	0.004	0.001	0.004	0.001	0.005	0.001	0.003	0.000
35.052	0.065	0.015	0.059	0.009	0.067	0.004	0.000	0.000	0.000	0.000	0.008	0.002	0.005	0.001
35.223	0.001	0.000	0.009	0.000	0.010	0.001	0.001	0.000	0.001	0.000	0.002	0.000	0.002	0.000
35.309	0.000	0.000	0.001	0.000	0.002	0.000	0.001	0.000	0.001	0.000	0.005	0.001	0.001	0.000
35.535	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
35.597	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
35.651	0.000	0.000	0.000	0.000	0.001	0.000	0.002	0.000	0.000	0.000	0.001	0.000	0.000	0.000
36.141	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000
36.230	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
36.911	0.314	0.021	0.298	0.011	0.239	0.017	0.019	0.002	0.046	0.002	0.119	0.003	0.106	0.006
37.409	0.038	0.007	0.050	0.003	0.001	0.000	0.001	0.000	0.001	0.000	0.005	0.000	0.005	0.000
37.595	0.003	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
37.829	0.007	0.001	0.004	0.001	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
37.913	0.001	0.000	0.007	0.001	0.004	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
38.723	0.003	0.001	0.007	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000
38.769	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000
38.941	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.002	0.000	0.001	0.000
39.140	0.008	0.000	0.001	0.000	0.002	0.000	0.003	0.001	0.001	0.000	0.001	0.000	0.001	0.000
40.779	0.190	0.008	0.156	0.006	0.257	0.012	0.124	0.007	0.124	0.006	0.116	0.005	0.124	0.007
41.088	0.006	0.001	0.010	0.002	0.048	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
41.196	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.002	0.000	0.002	0.000	0.002	0.000
41.729	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
41.849	0.009	0.002	0.007	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
41.982	0.027	0.002	0.025	0.003	0.007	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
42.084	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
42.176	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
43.576	0.000	0.000	0.002	0.001	0.004	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
43.834	0.040	0.004	0.098	0.014	0.112	0.013	0.003	0.000	0.010	0.001	0.024	0.002	0.028	0.004
43.994	0.008	0.002	0.006	0.001	0.013	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
44.140	0.000	0.000	0.023	0.002	0.015	0.002	0.000	0.000	0.001	0.000	0.001	0.000	0.002	0.000
44.268	0.002	0.000	0.010	0.002	0.027	0.003	0.000	0.000	0.000	0.000	0.001	0.000	0.001	0.000
44.466	0.000	0.000	0.010	0.001	0.003	0.000	0.002	0.000	0.001	0.000	0.000	0.000	0.000	0.000
44.965	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
45.436	0.000	0.000	0.002	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
46.297	0.856	0.057	1.169	0.026	0.846	0.027	0.352	0.020	0.384	0.016	0.295	0.016	0.408	0.022
46.882	0.148	0.006	0.174	0.007	0.225	0.011	0.114	0.007	0.113	0.005	0.104	0.004	0.103	0.006
45.608	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
47.768	0.000	0.000	0.009	0.001	0.000	0.000	0.019	0.003	0.008	0.001	0.025	0.003	0.008	0.000
47.965	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
47.996	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
48.193	0.000	0.000	0.002	0.001	0.005	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
48.693	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
48.984	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
49.158	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
51.172	0.002	0.000	0.007	0.002	0.017	0.002	0.000	0.000	0.002	0.000	0.004	0.001	0.006	0.001
51.420	0.000	0.000	0.003	0.001	0.000	0.000	0.002	0.000	0.002	0.000	0.005	0.000	0.001	0.000
51.521	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000
52.002	0.000	0.000	0.005	0.001	0.009	0.001	0.000	0.000	0.001	0.000	0.002	0.000	0.002	0.000
52.990	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.004	0.000	0.008	0.001
57.162	0.000	0.000	0.000	0.000	0.004	0.002	0.000	0.000	0.012	0.001	0.012	0.001	0.031	0.002

Appendix 3. 49 statistically significant MSTs subject to analysis in Results Section 1. Levels of metabolites shown as expressing statistically significant ($P \leq 0.05$) changes in their abundance for *Thellungiella* seedlings as compared to the mean metabolite levels of all four treatments. Values are expressed as average RRF ± standard error. SE = standard error. n = 6 for HN, LN, HN+S and LN+S and n = 5 for C-0, S-300, 2003, 2005 and 2006

metabolites	HN	SE	LN	SE	HN+S	SE	LN+S	SE
Unknown 11.341	0.0002	0.0001	0.0000	0.0000	0.0012	0.0005	0.0008	0.0003
Unknown 11.875	0.0018	0.0008	0.0026	0.0011	0.0057	0.0023	0.0100	0.0041
Unknown 14.258	0.0000	0.0000	0.0000	0.0000	0.0005	0.0002	0.0000	0.0000
Malonic acid	0.0001	0.0000	0.0001	0.0000	0.0018	0.0007	0.0008	0.0003
Amino acid 17.749	0.0000	0.0000	0.0000	0.0000	0.0064	0.0026	0.0033	0.0014
Proline	0.0002	0.0001	0.0000	0.0000	0.0903	0.0369	0.0144	0.0059
Fumaric acid	0.0014	0.0006	0.0000	0.0000	0.0039	0.0016	0.0000	0.0000
Serine	0.0100	0.0041	0.0015	0.0006	0.0119	0.0049	0.0003	0.0001
Threonine	0.0053	0.0021	0.0018	0.0008	0.0044	0.0018	0.0004	0.0002
Unknown 23.437	0.0000	0.0000	0.0000	0.0000	0.0004	0.0002	0.0000	0.0000
Unknown 23.535	0.0000	0.0000	0.0000	0.0000	0.0009	0.0004	0.0002	0.0001
Pyroglutamic acid	0.0456	0.0186	0.0001	0.0000	0.0229	0.0093	0.0000	0.0000
Unknown 26.134	0.0000	0.0000	0.0000	0.0000	0.0063	0.0026	0.0010	0.0004
Unknown 26.273	0.0001	0.0001	0.0002	0.0001	0.0008	0.0003	0.0004	0.0001
Sugar 27.826	0.0000	0.0000	0.0000	0.0000	0.0010	0.0004	0.0003	0.0001
Unknown 28.73	0.0001	0.0000	0.0001	0.0000	0.0014	0.0006	0.0005	0.0002
Unknown 29.178	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001	0.0002	0.0001
Putrescine	0.0008	0.0003	0.0000	0.0000	0.0004	0.0002	0.0000	0.0000
Unknown 29.75	0.0001	0.0000	0.0000	0.0000	0.0009	0.0004	0.0003	0.0001
Sugar 29.856	0.0003	0.0001	0.0003	0.0001	0.0024	0.0010	0.0008	0.0003
Unknown 30.188	0.0016	0.0007	0.0018	0.0007	0.0053	0.0022	0.0057	0.0023
Unknown 31.144	0.0001	0.0000	0.0002	0.0001	0.0006	0.0002	0.0009	0.0004
Quinic acid	0.0011	0.0004	0.0013	0.0005	0.0054	0.0022	0.0037	0.0015
Fructose	0.0045	0.0018	0.0061	0.0025	0.0176	0.0072	0.0177	0.0072
Sugar 34.869	0.0000	0.0000	0.0004	0.0002	0.0022	0.0009	0.0022	0.0009
Sugar 35.052	0.0000	0.0000	0.0002	0.0001	0.0083	0.0034	0.0046	0.0019
Organic acid 35.223	0.0006	0.0002	0.0007	0.0003	0.0024	0.0010	0.0022	0.0009
Gluconic acid	0.0009	0.0004	0.0005	0.0002	0.0051	0.0021	0.0015	0.0006
Unknown 36.141	0.0001	0.0000	0.0002	0.0001	0.0003	0.0001	0.0006	0.0002
myo-Inositol	0.0193	0.0079	0.0464	0.0189	0.1186	0.0484	0.1061	0.0433
Sugar 37.409	0.0008	0.0003	0.0011	0.0005	0.0049	0.0020	0.0050	0.0020
Unknown 37.686	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001	0.0002	0.0001
Unknown 38.723	0.0001	0.0000	0.0001	0.0000	0.0010	0.0004	0.0006	0.0002
Unknown 38.769	0.0000	0.0000	0.0000	0.0000	0.0007	0.0003	0.0006	0.0003
Unknown 38.9	0.0000	0.0000	0.0002	0.0001	0.0009	0.0004	0.0030	0.0012
Unknown 41.729	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001	0.0001	0.0000
Unknown 41.849	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001	0.0002	0.0001
Unknown 42.176	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001	0.0002	0.0001
Sugar 43.834	0.0031	0.0013	0.0102	0.0042	0.0238	0.0097	0.0281	0.0115
Sugar 44.268	0.0001	0.0000	0.0004	0.0001	0.0010	0.0004	0.0013	0.0005
Sugar 44.14	0.0002	0.0001	0.0008	0.0003	0.0014	0.0006	0.0025	0.0010
Unknown 43.152	0.0000	0.0000	0.0000	0.0000	0.0003	0.0001	0.0001	0.0001
Unknown 44.764	0.0000	0.0000	0.0000	0.0000	0.0005	0.0002	0.0006	0.0003
Sugar 44.965	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001	0.0001	0.0001
Sugar 51.172	0.0004	0.0001	0.0021	0.0008	0.0039	0.0016	0.0063	0.0026
Sugar 51.521	0.0000	0.0000	0.0001	0.0000	0.0004	0.0001	0.0009	0.0004
Sugar 52.002	0.0002	0.0001	0.0006	0.0002	0.0019	0.0008	0.0023	0.0009
Raffinose	0.0001	0.0000	0.0116	0.0047	0.0119	0.0049	0.0314	0.0128

Appendix 4. 52 statistically significant MSTs subject to meta-analysis in Results Section 2. Levels of metabolites shown as expressing statistically significant ($P \leq 0.05$) changes in their abundance for *Thellungiella* seedlings as compared to the mean metabolite levels of all four treatments. Values are expressed as average RRF ± standard error. SE = standard error. n = 6

metabolites	HN	SE	LN	SE	HN+S	SE	LN+S	SE
Unknown 14.432	0.0078	0.0032	0.0098	0.0040	0.0068	0.0028	0.0098	0.0040
Unknown 16.54	0.0014	0.0006	0.0011	0.0005	0.0026	0.0011	0.0011	0.0004
Unknown 17.396	0.0000	0.0000	0.0000	0.0000	0.0003	0.0001	0.0002	0.0001
N-compound 17.749	0.0000	0.0000	0.0000	0.0000	0.0064	0.0026	0.0034	0.0014
Proline	0.0002	0.0001	0.0009	0.0004	0.0903	0.0369	0.0144	0.0059
Succinic acid	0.0105	0.0043	0.0076	0.0031	0.0207	0.0084	0.0101	0.0041
Fumaric acid	0.0014	0.0006	0.0000	0.0000	0.0039	0.0016	0.0000	0.0000
Threonine	0.0053	0.0022	0.0019	0.0008	0.0044	0.0018	0.0004	0.0002
Unknown 23.03	0.0003	0.0001	0.0003	0.0001	0.0011	0.0004	0.0001	0.0001
Unknown 23.437	0.0000	0.0000	0.0000	0.0000	0.0004	0.0002	0.0000	0.0000
Unknown 23.942	0.0000	0.0000	0.0001	0.0000	0.1137	0.0464	0.0000	0.0000
Pyroglutamic acid	0.0456	0.0186	0.0001	0.0000	0.0229	0.0093	0.0001	0.0000
Unknown 25.187	0.0870	0.0355	0.0742	0.0303	0.0806	0.0329	0.0742	0.0303
Organic acid 25.799	0.0048	0.0020	0.0041	0.0017	0.0085	0.0035	0.0090	0.0037
Unknown 26.973	0.0014	0.0006	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Unknown 29.052	0.0012	0.0005	0.0012	0.0005	0.0017	0.0007	0.0013	0.0005
Sugar 29.856	0.0003	0.0001	0.0003	0.0001	0.0024	0.0010	0.0008	0.0003
Unknown 29.941	0.0021	0.0009	0.0020	0.0008	0.0039	0.0016	0.0052	0.0021
Unknown 31.052	0.0005	0.0002	0.0005	0.0002	0.0015	0.0006	0.0014	0.0006
Organic acid 31.307	0.0004	0.0002	0.0011	0.0005	0.0046	0.0019	0.0014	0.0006
Citric acid	0.0517	0.0211	0.0547	0.0223	0.1638	0.0669	0.1540	0.0629
Sugar 31.762	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0001	0.0000
Sugar 32.382	0.0014	0.0006	0.0022	0.0009	0.0120	0.0049	0.0051	0.0021
Quinic acid	0.0011	0.0005	0.0013	0.0005	0.0055	0.0022	0.0038	0.0015
Fructose	0.0045	0.0018	0.0061	0.0025	0.0177	0.0072	0.0177	0.0072
Sugar 32.986	0.0930	0.0380	0.1039	0.0424	0.0448	0.0183	0.0399	0.0163
Sugar 33.08	0.0003	0.0001	0.0000	0.0000	0.0005	0.0002	0.0003	0.0001
Unknown 34.126	0.0000	0.0000	0.0000	0.0000	0.0004	0.0001	0.0001	0.0001
Organic acid 34.163	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001	0.0000	0.0000
Sugar 34.869	0.0000	0.0000	0.0004	0.0002	0.0022	0.0009	0.0022	0.0009
Sugar 35.052	0.0000	0.0000	0.0002	0.0001	0.0083	0.0034	0.0046	0.0019
Gluconic acid 35.223	0.0006	0.0002	0.0007	0.0003	0.0024	0.0010	0.0023	0.0009
myo-Inositol	0.0194	0.0079	0.0464	0.0190	0.1186	0.0484	0.1061	0.0433
Sugar 37.409	0.0008	0.0003	0.0011	0.0005	0.0049	0.0020	0.0050	0.0020
Unknown 37.595	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000
Unknown 37.829	0.0001	0.0000	0.0002	0.0001	0.0001	0.0001	0.0002	0.0001
Sugar 37.913	0.0002	0.0001	0.0003	0.0001	0.0002	0.0001	0.0003	0.0001
Unknown 38.723	0.0001	0.0000	0.0001	0.0000	0.0010	0.0004	0.0006	0.0002
Unknown 39.14	0.0029	0.0012	0.0012	0.0005	0.0015	0.0006	0.0009	0.0004
Sugar 40.779	0.1244	0.0508	0.1238	0.0506	0.1157	0.0472	0.1239	0.0506
Uknown 41.088	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000
Sugar 41.196	0.0017	0.0007	0.0018	0.0007	0.0019	0.0008	0.0023	0.0010
Unknown 41.729	0.0000	0.0000	0.0000	0.0000	0.0003	0.0001	0.0001	0.0000
Unknown 41.982	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0001
Unknown 43.576	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0001	0.0001
Sugar 43.834	0.0031	0.0013	0.0102	0.0042	0.0238	0.0097	0.0281	0.0115
Sugar 44.14	0.0002	0.0001	0.0008	0.0003	0.0014	0.0006	0.0025	0.0010
Sucrose	0.3524	0.1438	0.3836	0.1566	0.2946	0.1203	0.4076	0.1664
Sugar 51.172	0.0004	0.0002	0.0021	0.0009	0.0039	0.0016	0.0063	0.0026
Sugar 51.42	0.0020	0.0008	0.0024	0.0010	0.0051	0.0021	0.0012	0.0005
Sugar 52.002	0.0002	0.0001	0.0006	0.0003	0.0020	0.0008	0.0023	0.0009
Raffinose	0.0001	0.0001	0.0116	0.0047	0.0119	0.0049	0.0314	0.0128

Appendix 5. Relative abundance of 52 MSTs over three years tested. Levels of metabolites shown as expressing statistically significant ($P \leq 0.05$) changes in their abundance for *Thellungiella* seedlings as compared to the mean metabolite levels of all four treatments. Values are expressed as average RRF ± standard error. SE = standard error. n = 5

metabolites	2003	SE	2005	SE	2006	SE
Unknown 14.432	0.0790	0.0353	0.0037	0.0017	0.0193	0.0086
Unknown 16.54	0.0038	0.0017	0.0003	0.0001	0.0001	0.0000
Unknown 17.396	0.0160	0.0072	0.0001	0.0000	0.0002	0.0001
N-compound 17.749	0.0000	0.0000	0.0002	0.0001	0.0002	0.0001
Proline	0.3378	0.1511	0.0144	0.0065	0.0716	0.0320
Succinic acid	0.0183	0.0082	0.0059	0.0027	0.0410	0.0184
Fumaric acid	0.0002	0.0001	0.0000	0.0000	0.0000	0.0000
Threonine	0.0000	0.0000	0.0008	0.0004	0.0004	0.0002
Unknown 23.03	0.0000	0.0000	0.0000	0.0000	0.0080	0.0036
Unknown 23.437	0.0096	0.0043	0.0073	0.0033	0.0045	0.0020
Unknown 23.942	0.5441	0.2433	0.6344	0.2837	0.5693	0.2546
Pyroglutamic acid	0.0000	0.0000	0.0000	0.0000	0.0084	0.0038
Unknown 25.187	0.2543	0.1137	0.2114	0.0945	0.2245	0.1004
Organic acid 25.799	0.0724	0.0324	0.0388	0.0174	0.0278	0.0124
Unknown 26.973	0.0011	0.0005	0.0021	0.0009	0.0315	0.0141
Unknown 29.052	0.0686	0.0307	0.0000	0.0000	0.0000	0.0000
Sugar 29.856	0.0259	0.0116	0.0188	0.0084	0.0132	0.0059
Unknown 29.941	0.0091	0.0041	0.0008	0.0004	0.0042	0.0019
Unknown 31.052	0.0484	0.0217	0.0177	0.0079	0.0019	0.0009
Organic acid 31.307	0.0051	0.0023	0.0018	0.0008	0.0013	0.0006
Citric acid	0.0002	0.0001	0.0044	0.0020	0.2718	0.1216
Sugar 31.762	0.5321	0.2380	0.6420	0.2871	0.4712	0.2107
Sugar 32.382	0.0020	0.0009	0.0024	0.0011	0.0017	0.0007
Quinic acid	0.0000	0.0000	0.0093	0.0042	0.0014	0.0006
Fructose	0.0000	0.0000	0.0213	0.0095	0.0617	0.0276
Sugar 32.986	0.0216	0.0097	0.0451	0.0202	0.0122	0.0054
Sugar 33.08	0.3286	0.1469	0.2437	0.1090	0.3507	0.1568
Unknown 34.126	0.0157	0.0070	0.0269	0.0121	0.0542	0.0242
Organic acid 34.163	0.0004	0.0002	0.0053	0.0023	0.0002	0.0001
Sugar 34.869	0.0094	0.0042	0.0017	0.0007	0.0305	0.0137
Sugar 35.052	0.0014	0.0006	0.0593	0.0265	0.0131	0.0059
Gluconic acid 35.223	0.0653	0.0292	0.0587	0.0263	0.0672	0.0301
myo-Inositol	0.0008	0.0004	0.0087	0.0039	0.0098	0.0044
Sugar 37.409	0.3136	0.1402	0.2978	0.1332	0.2394	0.1071
Unknown 37.595	0.0376	0.0168	0.0497	0.0222	0.0007	0.0003
Unknown 37.829	0.0034	0.0015	0.0004	0.0002	0.0021	0.0009
Sugar 37.913	0.0066	0.0030	0.0043	0.0019	0.0019	0.0008
Unknown 38.723	0.0011	0.0005	0.0072	0.0032	0.0036	0.0016
Unknown 39.14	0.0032	0.0014	0.0074	0.0033	0.0009	0.0004
Sugar 40.779	0.0085	0.0038	0.0009	0.0004	0.0016	0.0007
Uknown 41.088	0.1901	0.0850	0.1565	0.0700	0.2572	0.1150
Sugar 41.196	0.0059	0.0026	0.0098	0.0044	0.0484	0.0217
Unknown 41.729	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000
Unknown 41.982	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Unknown 43.576	0.0274	0.0122	0.0252	0.0113	0.0072	0.0032
Sugar 43.834	0.0400	0.0179	0.0976	0.0437	0.1120	0.0501
Sugar 44.14	0.0001	0.0000	0.0227	0.0102	0.0146	0.0065
Sucrose	0.8564	0.3830	1.1691	0.5228	0.8460	0.3783
Sugar 51.172	0.0022	0.0010	0.0066	0.0029	0.0166	0.0074
Sugar 51.42	0.0000	0.0000	0.0031	0.0014	0.0000	0.0000
Sugar 52.002	0.0000	0.0000	0.0046	0.0020	0.0088	0.0039
Raffinose	0.0000	0.0000	0.0000	0.0000	0.0042	0.0019

Appendix 6. Relative abundance of 52 MSTs in C-0 and S-300 leaf samples analyzed in Results – Section 2. Levels of metabolites shown as expressing statistically significant ($P \leq 0.05$) changes in their abundance for *Thellungiella* seedlings as compared to the mean metabolite levels of all four treatments. Values are expressed as average RRF ± standard error. SE = standard error. n = 5

metabolites	C-0	SE	S-300	SE
Unknown 14.432	0.0030	0.0014	0.0000	0.0000
Unknown 16.54	0.0049	0.0022	0.0114	0.0051
Unknown 17.396	0.0000	0.0000	0.0080	0.0036
N-compound 17.749	0.0000	0.0000	0.0003	0.0001
Proline	0.4020	0.1798	0.8566	0.2937
Succinic acid	0.0077	0.0034	0.0013	0.0006
Fumaric acid	0.0007	0.0003	0.0012	0.0006
Threonine	0.0429	0.0192	0.0523	0.0234
Unknown 23.03	0.0000	0.0000	0.0012	0.0005
Unknown 23.437	0.0003	0.0001	0.0017	0.0008
Unknown 23.942	1.0213	0.4567	0.8186	0.3661
Pyroglutamic acid	0.0746	0.0334	0.0433	0.0194
Unknown 25.187	0.1452	0.0649	0.1500	0.0671
Organic acid 25.799	0.0392	0.0175	0.0340	0.0152
Unknown 26.973	0.1185	0.0530	0.0794	0.0355
Unknown 29.052	0.0020	0.0009	0.0125	0.0056
Sugar 29.856	0.0194	0.0087	0.0345	0.0154
Unknown 29.941	0.0053	0.0023	0.0085	0.0038
Unknown 31.052	0.0018	0.0008	0.0020	0.0009
Organic acid 31.307	0.0013	0.0006	0.0057	0.0025
Citric acid	0.1694	0.0758	0.0581	0.0260
Sugar 31.762	0.0020	0.0009	0.0019	0.0009
Sugar 32.382	0.0148	0.0066	0.0166	0.0074
Quinic acid	0.0002	0.0001	0.0000	0.0000
Fructose	0.0491	0.0220	0.0522	0.0234
Sugar 32.986	0.0030	0.0013	0.0175	0.0078
Sugar 33.08	0.0038	0.0017	0.0212	0.0095
Unknown 34.126	0.0004	0.0002	0.0001	0.0001
Organic acid 34.163	0.0001	0.0000	0.0015	0.0007
Sugar 34.869	0.0233	0.0104	0.0309	0.0138
Sugar 35.052	0.0149	0.0067	0.1086	0.0486
Gluconic acid 35.223	0.0008	0.0003	0.0022	0.0010
myo-Inositol	0.2134	0.0955	0.2695	0.1205
Sugar 37.409	0.0337	0.0151	0.0379	0.0169
Unknown 37.595	0.0006	0.0003	0.0014	0.0006
Unknown 37.829	0.0057	0.0026	0.0048	0.0021
Sugar 37.913	0.0016	0.0007	0.0010	0.0004
Unknown 38.723	0.0009	0.0004	0.0014	0.0006
Unknown 39.14	0.0182	0.0081	0.0216	0.0097
Sugar 40.779	0.1719	0.0769	0.1847	0.0826
Uknown 41.088	0.0000	0.0000	0.0014	0.0006
Sugar 41.196	0.0002	0.0001	0.0000	0.0000
Unknown 41.729	0.0020	0.0009	0.0038	0.0017
Unknown 41.982	0.0011	0.0005	0.0020	0.0009
Unknown 43.576	0.0000	0.0000	0.0002	0.0001
Sugar 43.834	0.0302	0.0135	0.0615	0.0275
Sugar 44.14	0.0043	0.0019	0.0061	0.0027
Sucrose	0.8967	0.4010	1.0671	0.4772
Sugar 51.172	0.0060	0.0027	0.0086	0.0039
Sugar 51.42	0.0026	0.0012	0.0017	0.0008
Sugar 52.002	0.0000	0.0000	0.0017	0.0008
Raffinose	0.0046	0.0021	0.0008	0.0003

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