Salt and Cold Tolerance in Alfalfa

Rokebul Anower

Department of Biology & Microbiology
South Dakota State University
Introduction

Alfalfa and Importance

Stress tolerance in alfalfa

A. Salt Stress
   i. Growth and Biomass production
   ii. Physiological Analysis
   iii. Potential Mechanisms

B. Cold Stress
   i. Screening
   ii. Physiological Analysis
   iii. Expression of cold responsive genes
Introduction: Alfalfa & Importance

- Alfalfa (Medicago sativa L.) is one of the most important forage legume crops in the world.

- Total value ~$27 billion/yr in the US after considering export and the benefit to ruminant livestock etc.

- 2011
  South Dakota (Alfalfa/Alfalfa Mixtures Hay)
  Area Harvested: 2350x1000 acres
  Production: 6345x1000 tones
  Rank: Top 5 in the US

*USDA: Crop production 2011 summary, January 2012.*
Effect of Cold & Salt Stress

Cold & Salinity

Affects both yield and quality of alfalfa

50 to 75% of the agriculture yield is lost

Objectives

• Understand how plants sense and respond to abiotic stress, such as salinity and cold

• Improve plant performance and production under stress conditions
Salt Stress

Characterization of physiological responses of two alfalfa half-sib families with improved salt tolerance
Dramatic differences between a population of alfalfa (right) that has undergone three cycles of selection for ability to survive at 18.0 dS m\(^{-1}\) compared to unselected (left) alfalfa. Surviving plants were allowed to cross and subsequent generations were subjected to the selection protocol. (Drs. Mott & Peel)
Salt-tolerant selections stayed mostly green while the original populations from which they were selected showed senescence one week after 12.0 dS m⁻¹ treatment.
7 Days After 12 DS (eq. 120 mM NaCl) Salt Treatment
HS-B VS P-B After 12 DS Treatment

Selection: HS-B

Parents: P-B
Improved Shoot & Root Biomass

Shoot

Root

Dry Weight (g / plant)
Maintained Stem Length in HS-B

[Graphs showing stem length measurements at different time points (0, 21, 63, 126) for Ctrl and Salt conditions in P-A and HS-A, P-B and HS-B.]
Maintained Leaf Number in Selections

Leaf Number

Ctrl Salt Ctrl Salt Ctrl Salt Ctrl Salt
P-A HS-A P-B HS-B

A
B
C
D
E
F
G
H
Higher Chlorophyll Content in Selections

Chlorophyll Content Index

0 10 20 30

10 20 30 40

0 10 20

21 24

63

126

Ctrl Salt Ctrl Salt Ctrl Salt Ctrl Salt

P-A HS-A P-B HS-B

Chlorophyll Content Index
Inorganic Solutes Accumulation in Shoots

- **Na⁺**
- **Ca²⁺**
- **K⁺**
- **Mg²⁺**
- **Si⁺**
Maintained Relative Water Content

A

B

RWC (%)
HS-B VS P-B Root After 7d at 12 DS
Root System After 7d at 12 DS
Summary: Salt Tolerance

Under salt stress, the selected lines HS-A & HS-B:

- Greater leaf number (72 & 84%)
- Better stem elongation (44%)
- Higher accumulation of chlorophyll (78 & 208%)
- Maintenance of RWC
- HS-B appeared to exclude Na⁺
- Better root growth and biomass production in HS-A, HS-B, and HS-C
Physiological Mechanisms in Salt Tolerance

Under salt stress, the selected lines HS-A & HS-B:

- Higher accumulation of chlorophyll - less reactive oxygen species (ROS)?
Physiological Mechanisms in Salt Tolerance

Under salt stress, the selected lines HS-A & HS-B:

- Higher accumulation of chlorophyll - less reactive oxygen species (ROS) ?
- Maintenance of RWC - accumulation of osmotic solutes ?
Physiological Mechanisms in Salt Tolerance

Under salt stress, the selected lines HS-A & HS-B:

- Higher accumulation of chlorophyll - less reactive oxygen species (ROS)?
- Maintenance of RWC - accumulation of osmotic solutes?
- HS-B appeared to exclude Na\(^+\) - Na is located outside of the cell?
Less amount of ROS in selected genotypes

Roots

Shoots

\[ \text{H}_2\text{O}_2 \text{ (mM / g FW)} \]

Ctrl
Salt

P-C  HS-C  P-A  HS-A  P-B  HS-B

P-C  HS-C  P-A  HS-A  P-B  HS-B
More soluble sugars in selected genotypes

Roots

Shoots

Soluble Sugar (mg/g FW)

P-C  HS-C  P-A  HS-A  P-B  HS-B

P-C  HS-C  P-A  HS-A  P-B  HS-B
Proline Accumulation

Roots

Shoots

Proline Content (µM/g FW)

P-C HS-C P-A HS-A P-B HS-B

P-C HS-C P-A HS-A P-B HS-B
Na Localization using fluorescence dye

Root section W/O Salt treatment [FITC & UV, 10x] under confocal microscopy after 12 hrs staining with CoroNa-Green. ($\lambda_{\text{exc}} = 543$ nm, $\lambda_{\text{em}} = 500-540$ nm, XYZ scanning mode, image volume= 150 $\mu$m, thickness= 3$\mu$m)

- 10 $\mu$M Cell-permeant CoroNa-Green Sodium Indicator (C-36676, Invitrogen)
Staining was done 12 hours after 9 dS/m (~90 mM NaCl) salt treatment [FITC & UV, 10x] In parental line (P-B) Na can enter root parenchyma cells (red arrow) and accumulated highly in xylem tissue. Selected plants (HS-B) however showed strong accumulation in cell walls (yellow arrow), especially high in the intercellular junction area of adjacent cells.
Summary: Salt Tolerance

Under salt stress, the selected lines HS-A & HS-B:

- Less ROS amount in roots and shoots
- Greater accumulation of soluble sugar in roots and shoots
- Greater proline accumulation in roots
- $\text{Na}^+$ are seemingly localized in cell walls and intercellular space.
Cold Stress Tolerance

- Screening
- Physiological characteristics - electrolyte leakage assay
- Expression of cold responsive genes
Plant Materials

- Alfagraze
- Wind River
- Don
- SD-201
- River Side
  - Bcbb-04
  - Chbb-04
  - Mt-0
- Foster Ranch
  - Apica
  - Caribou
  - Cuf-101
1. Screening of Cold Tolerance in Alfalfa
Freezing Test Program

May 23, 2010

Freezing Test Program: The freezing test program shown on sketch bellow.

Step 1#: Setpoint 1=25°C, 1 second. Starts program at 25°C with 1 second as a starting point.

Step 2#: Setpoint 1=-2°C, 30 min. Decline the temperature down to -2°C in 30 min.


Step 4#: Setpoint 1=-12°C, 2.5 hrs. Decline the temperature down to -12°C (at -2°C/30 min).

Step 5#: Setpoint 1=-12°C, 90 min. Holds at -12°C for 90 min.

Step 6#: Setpoint 1=4°C, 30 min. Ramps program up to 4°C in 30 min.

Step 7#: Setpoint 1=4°C, 24 hrs. Holds at 4°C for 24 hrs.

Step 8#: Stop program
Freezing survival test of alfalfa seedlings. Temperatures were gradually dropped to -5°C and kept at the temperature for 1.5 h. Freezing treated plants were thawed at 4°C for 24 h before returned to greenhouse. A few green ones (River side- “RS” and Foster ranch- “FR”) on the right survived the freezing test.
<table>
<thead>
<tr>
<th>Cultivars</th>
<th>NA (-5°C)</th>
<th>CA (-5°C)</th>
<th>DA (-5°C)</th>
<th>NA (-10°C)</th>
<th>CA (-10°C)</th>
<th>NA (-10°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1991</td>
<td>44</td>
<td>100</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alfagraze</td>
<td>56</td>
<td>100</td>
<td>67</td>
<td>89</td>
<td>78</td>
<td>33</td>
</tr>
<tr>
<td>BCBB-04</td>
<td>44</td>
<td>100</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHBB-04</td>
<td>22</td>
<td>100</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foster Ranch</td>
<td>56</td>
<td>100</td>
<td>67</td>
<td>78</td>
<td>100</td>
<td>61</td>
</tr>
<tr>
<td>Riverside</td>
<td>56</td>
<td>100</td>
<td>67</td>
<td>78</td>
<td>89</td>
<td>47</td>
</tr>
<tr>
<td>Wind River</td>
<td>44</td>
<td>100</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT-0</td>
<td>44</td>
<td>100</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DON</td>
<td>11</td>
<td>78</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD-201</td>
<td>11</td>
<td>89</td>
<td>22</td>
<td>0</td>
<td>78</td>
<td>33</td>
</tr>
<tr>
<td>Apica</td>
<td>44</td>
<td>100</td>
<td>44</td>
<td>78</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>Caribou</td>
<td>11</td>
<td>100</td>
<td>44</td>
<td>56</td>
<td>89</td>
<td>33</td>
</tr>
<tr>
<td>Ameristand</td>
<td>22</td>
<td>100</td>
<td>67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CUF-101</td>
<td>11</td>
<td>67</td>
<td>22</td>
<td>11</td>
<td></td>
<td>33</td>
</tr>
</tbody>
</table>
Cold treatment at -10°C (Non-Acclimated)

Survival rate (%). Each Value represents the mean ± SE. The Different letter indicate significant differences (p<0.05) between treatments. The data combination of three freezing treatments (-5, -10 and -12 °C) of 24 tests (p<0.05).
Selected VS Control 7d After -10°C Treatment

Selected Line

Control (PC, NC)
Leaf electrolyte leakage of non-cold acclimated plants

Electrolyte Leakage (%) vs Temperature (°C)

Temperature (°C):
0  -2  -4  -6  -8

Electrolyte Leakage (%):
0  20  40  60  80  100

Graph showing electrolyte leakage as a function of temperature for different plant species: FR-5, RS-6, CUF, Apica, and Caribou.
Leaf electrolyte leakage of cold acclimated plants

Electrolyte Leakage (%) vs. Temperature (°C)

- FR-5
- RS-6
- CUF
- Apica
- Caribou
50% Killing Point ($T_{k50}$ or $LD_{50}$)
Change in CBF1 transcripts levels after cold treatment (2°C) in different genotypes.
RS: Riverside, FR: Foster Ranch, API: Apica, CUF: CUF101
Change in CBF2 transcripts levels after cold treatment (2°C) in different genotypes. RS: Riverside, FR: Foster Ranch, API: Apica, CUF: CUF101
Gene Expression: *Cas15B*

Change in *cas15B* transcripts levels after cold treatment (2°C) in different genotypes.
RS: Riverside, FR: Foster Ranch, API: Apica, CUF: CUF101
Salt Stress

Physiological analysis showed that the selected genotypes are more salt tolerant than their parental plants: better growth and biomass production, greener, and capable of maintaining RWC.

The salt tolerance is associated with lower ROS levels, greater accumulation of osmotic solutes, and limiting Na to enter the cells.
Summary

- **Cold Stress**

  - Our freezing tests suggested that two genotypes (River Side and Foster Ranch) have greater freezing tolerance as they have higher survival rate (%) $T_{k50}$, lower EL (%) after freezing.

  - Gene expression analysis revealed that the selected genotypes showed more rapid and higher induction of known cold-responsive genes.
Cold Stress

While CBF genes may play important role in freezing tolerance in the selected genotypes, specific genes involved and their regulation varied among genotypes.
Acknowledgements

Dr. Wu  Dr. Mott  Dr. Peel  Dr. Fennell  Dr. Boe

USDA  FRRL  SDSU
Thank You!