The Freezing Tolerance of Alfalfa Nodules Depends on *Sinorhizobium Meliloti* Strains

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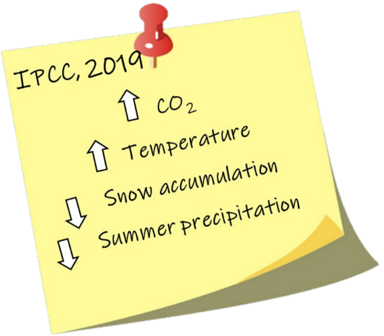
Alfalfa

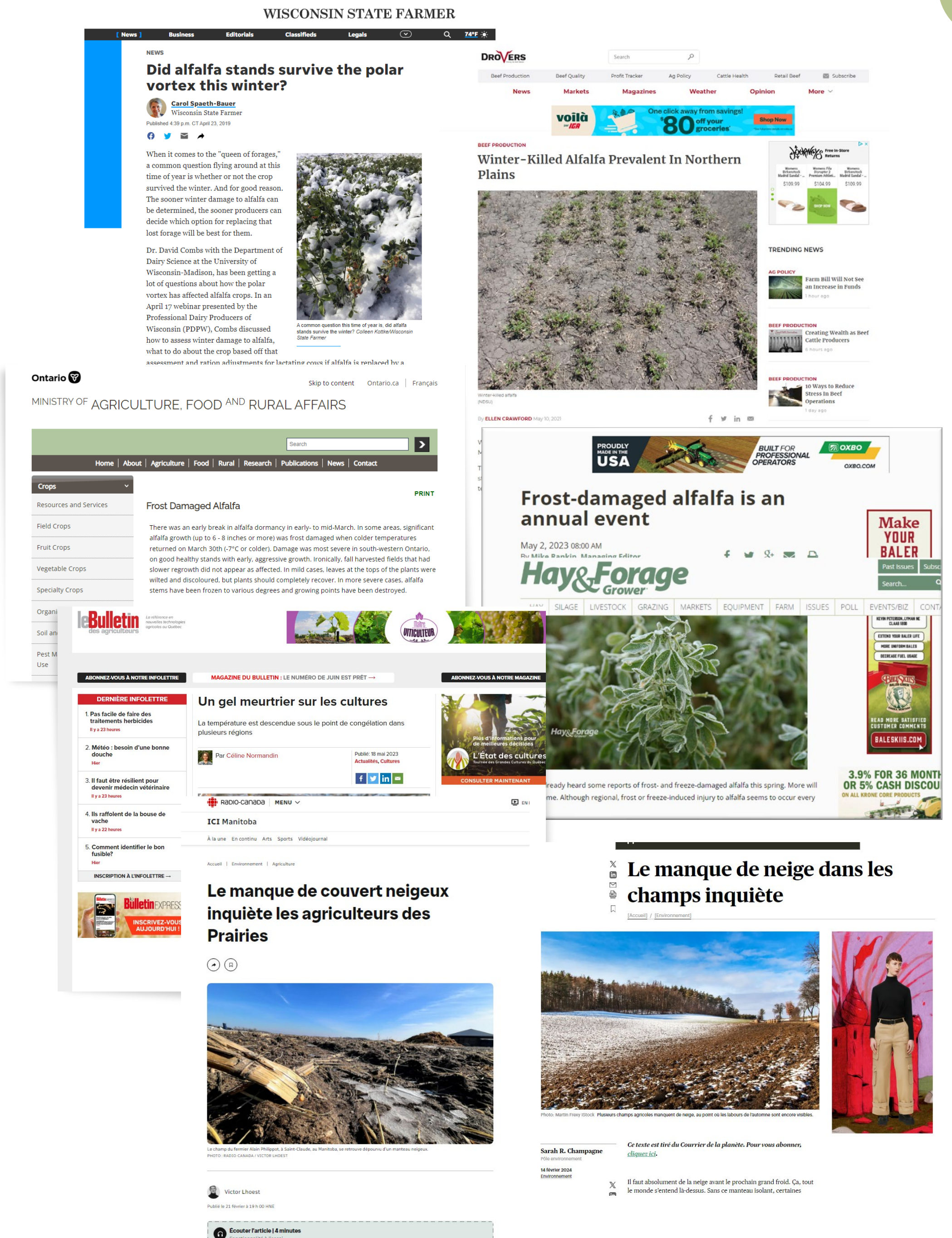
Winter Survival

- Single most important factor is the **level of freezing tolerance** that the plant can achieve during winter (McKenzie et al. 1988; Castonguay et al. 2006; Bertrand et al. 2014).

- More freeze-thaw episodes with climate change!



Winter 2024



Symbiosis

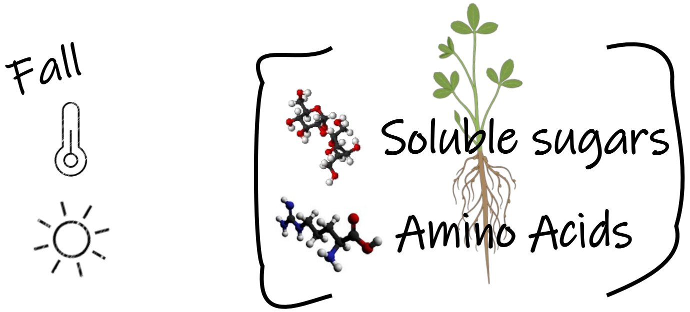
Host-Rhizobia Specific

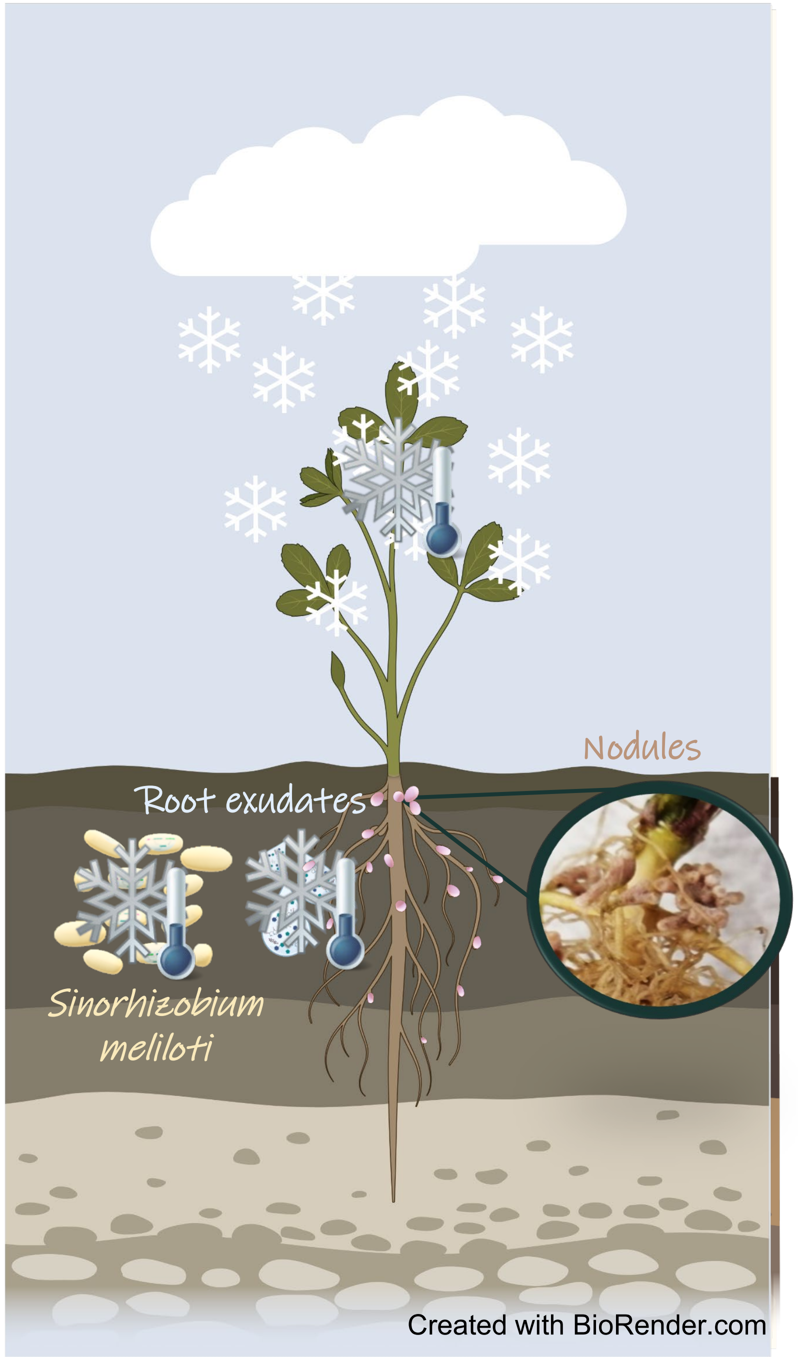
- Crosstalk between the plant and the rhizobia involving root exudation of specific flavonoids

- Optimum temperatures : **25-30°C** (Alexandre and Oliveira, 2013).

- **Cold stress** affects both plant and microorganisms and the crosstalk between the two symbiotic partners

- **Cold acclimation process** promotes the accumulation of cryoprotective and reserve metabolites, increase the tolerance to freezing and support spring regrowth



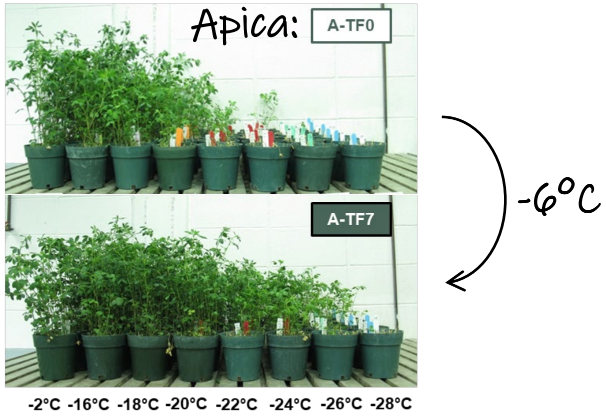


Main objective: To improve alfalfa production under low temperatures by identifying the best associations between alfalfa populations and rhizobial strains in terms of higher tolerance to freezing of both partners and greater crop yield in cold soil, and to study the underlying mechanisms.

- *Identifying the best freezing tolerant symbiotic association*
- *Study the underlying mechanisms of a freezing tolerant symbiotic association*

Alfalfa populations A-TF0 et A-TF7

Recurrent selection method performed under controlled conditions (Castonguay et al. 2009).



A-TF0

A-TF7

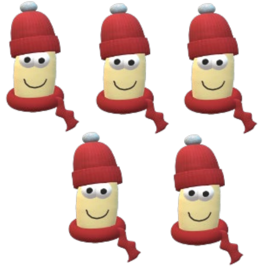
VS

Sinorhizobium (Ensifer) meliloti strains

Strains isolated from different regions

Commercial strain with high efficiency under various conditions

5 cold tolerant strains



VS

B399

Symbiosis efficiency after freezing stress

There will be an additive positive effect on the biomass regrowth after a freezing stress when combining a freezing-tolerant alfalfa population (A-TF7) with freezing tolerant strains of *Sinorhizobium meliloti*

Symbiosis efficiency after freezing stress

Treatments:

Cone-tainers

8 rep

X

Alfalfa populations

A-TF0

VS

A-TF7

X

Inoculation

5 cold tolerant strains

VS

B399

X

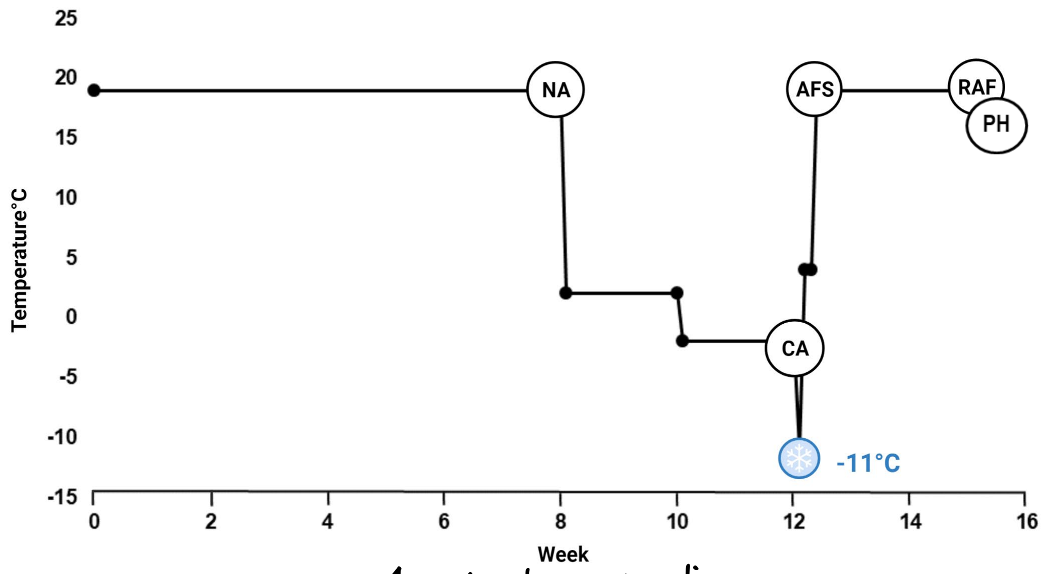
4 events sampling + 1 phenotype sampling

=

Total: 480 plants

Growth chamber:

Fall + Freeze- thaw + Regrowth



4 events sampling

Measurements:

NA
CA
AFS
RAF

Root biomass

+

Nodules biomass

NA
RAF

Shoot biomass

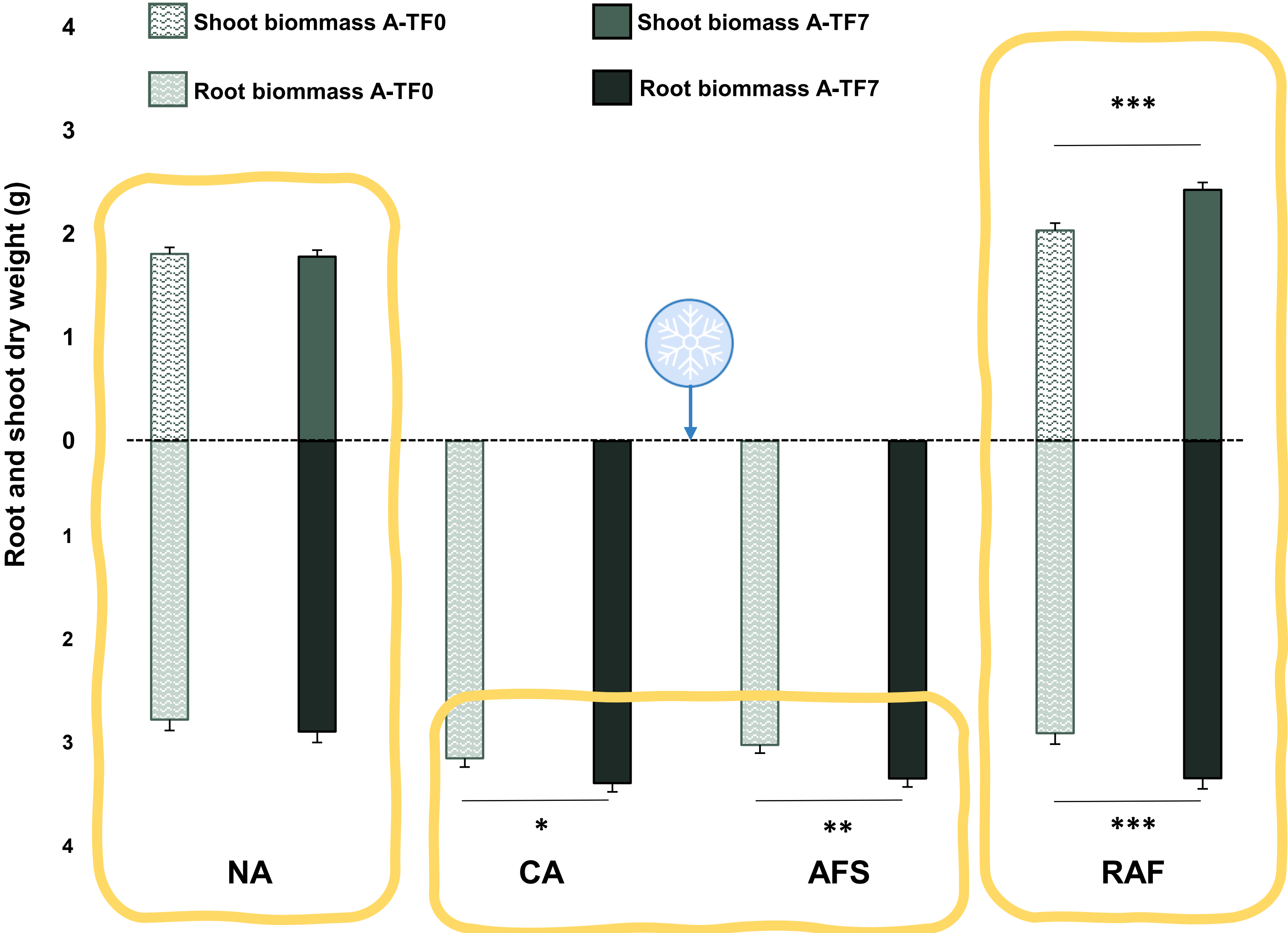
Nodules characterizations

PH

Results:

Roots and shoot biomass: *Alfalfa populations effect*

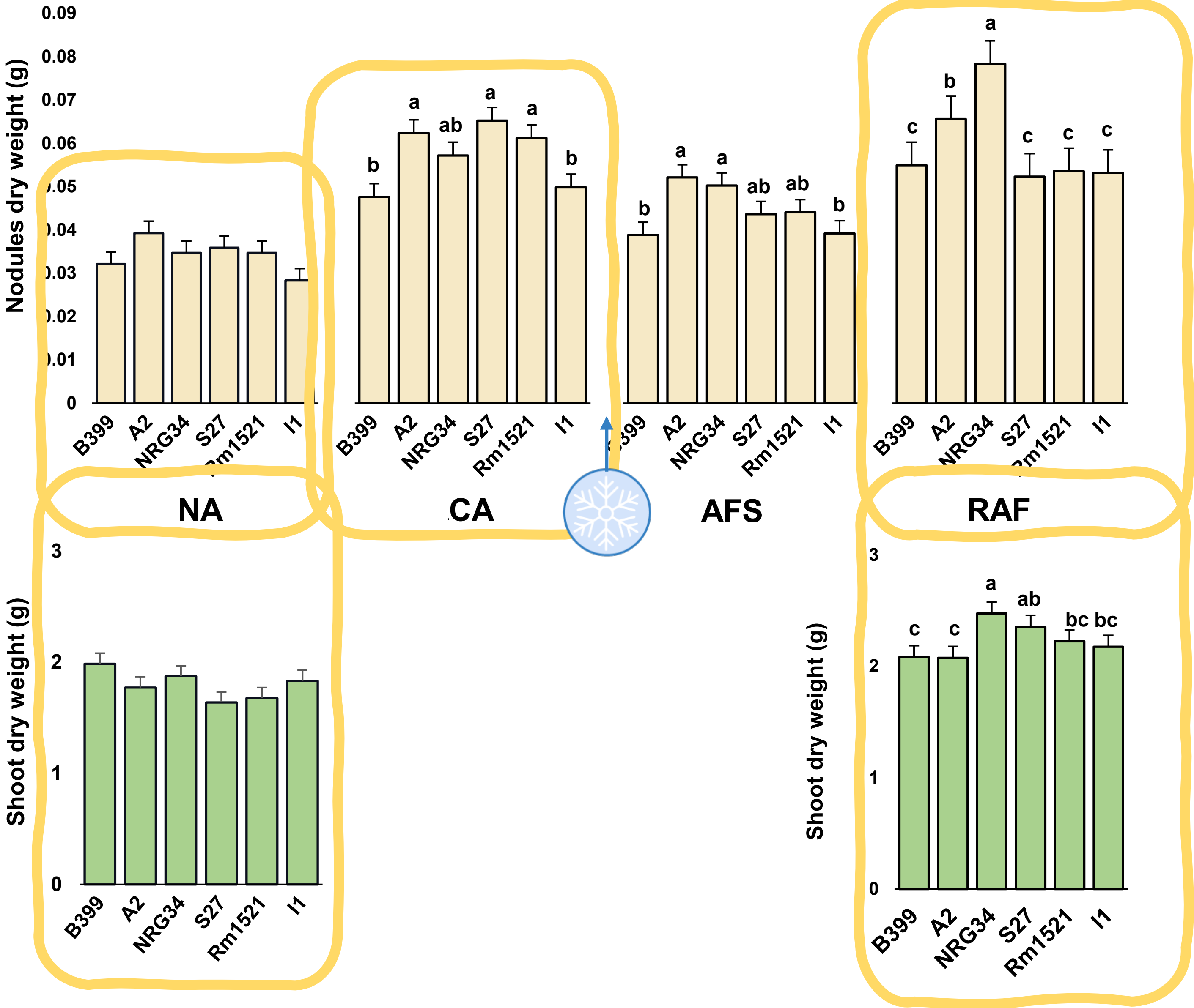
- No effect in growth optimal conditions (NA)
- 16% increase in root biomass with cold acclimation (CA).
- **A-TF7** 10% (average) root biomass (CA and AFS)
- 3 weeks regrowth after freezing stress
  - **A-TF7** 15% root biomass;
  - **A-TF7** 19% de shoot biomass.



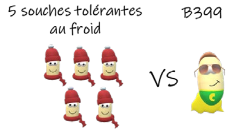
Results:

Nodules and shoot biomasses: *S. meliloti strain effect*

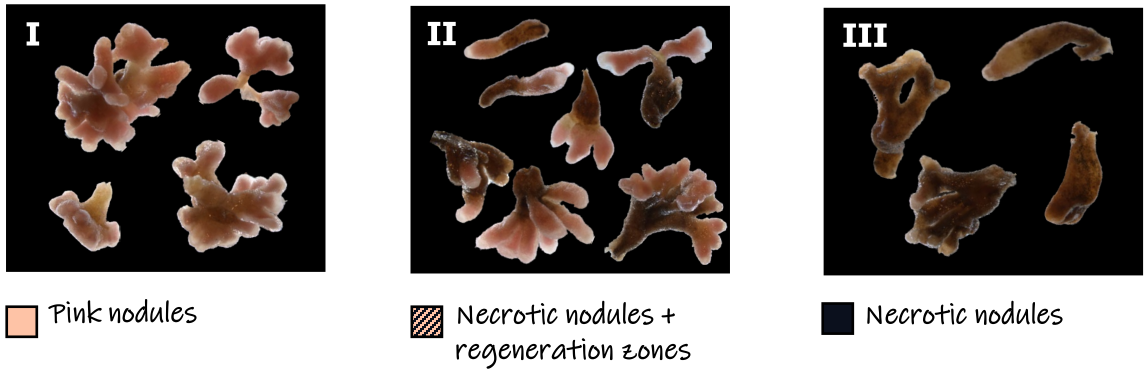
- No effect under optimal growth conditions (NA).
- with cold acclimation (CA):
  - nodule biomass (+68%);
  - differences between strains.
- 48 h after freezing stress (AFS) 22% nodule biomass.
- 3 weeks after freezing stress (RAF) :
  - **NRG34** 19% nodule biomass than A2;
  - **NRG34** 46% nodule biomass than the other 4 strains;
  - **NRG34** 19 % shoot biomass than strains B399 and A2.

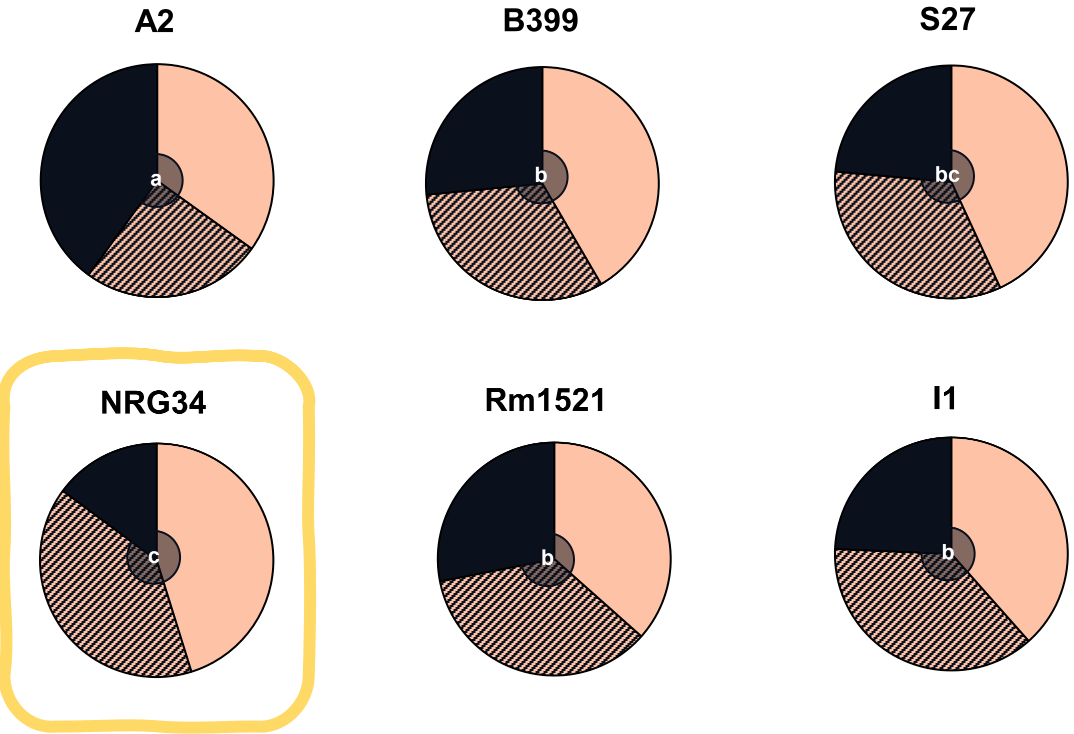


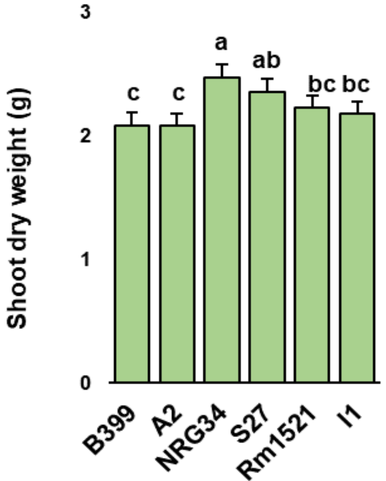
Results:
Nodules characterization 3 weeks after freezing: *S. meliloti* strain effect



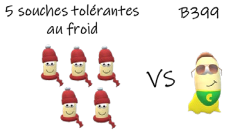
- Observation of nodule damage caused by freezing on the root system (PH)
- 3 categories:
  - I : No damage, pink nodules;
  - II: Necrotic nodules at the base of the root and showing a pink regeneration zone;
  - III: Completely necrotic nodules.
- Distinct distribution profile between strains:
  - **NRG34** 85% nodules without damage and showing areas of regeneration.





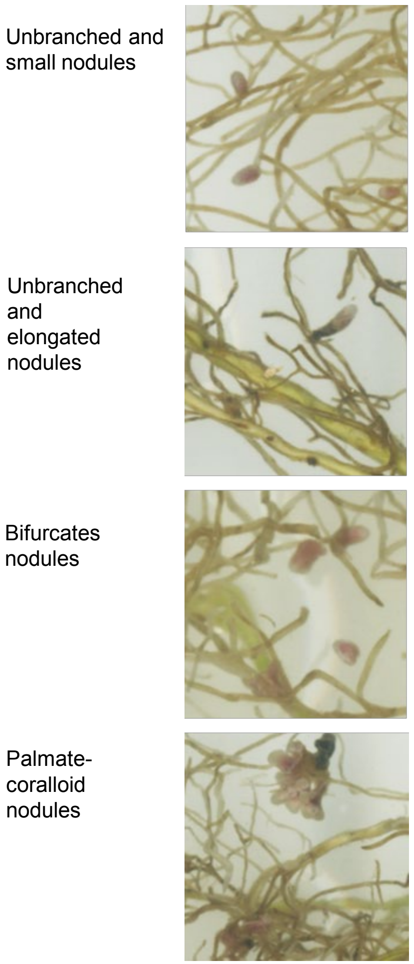


Results:
Nodules characterization 3 weeks after freezing: *S. meliloti* strain effect



- Observation of nodule shape after freezing (PH)
- 4 categories:
  - **NRG34** 65% palmate-coralloid nodules
  - Larger nodules could store more resources to ensure the regrowth after freezing and represent a stronger carbon sink to sustain spring regrowth than smaller nodules

Nodules shapes



Palmate-coralloid nodules

B399: 30% c
S27: 33% bc
A2: 15% d
RM1521: 40% b
NRG34: 65% a
I1: 30% c

Contribution to Knowledge

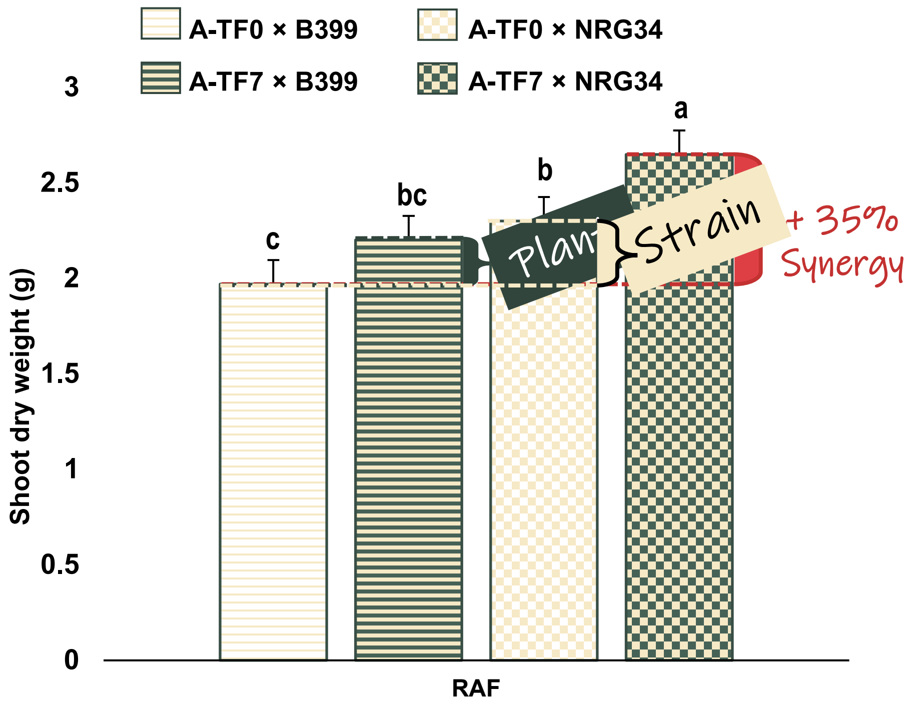
- ✓ Alfalfa regrowth after freezing is positively linked with the proportion of nodules showing less freezing damages which in turn depends on rhizobial strains.
- ✓ First in vivo observations of variability in nodule freezing damages and of nodules regeneration zones induced by *S. meliloti* strains differing in their level of freezing tolerance.
- ✓ Both the choice of alfalfa populations and of rhizobial strains adapted to stress are complementary to increase the persistence of alfalfa in cold and temperate regions.

Study underlying mechanisms of a freezing tolerant symbiotic association

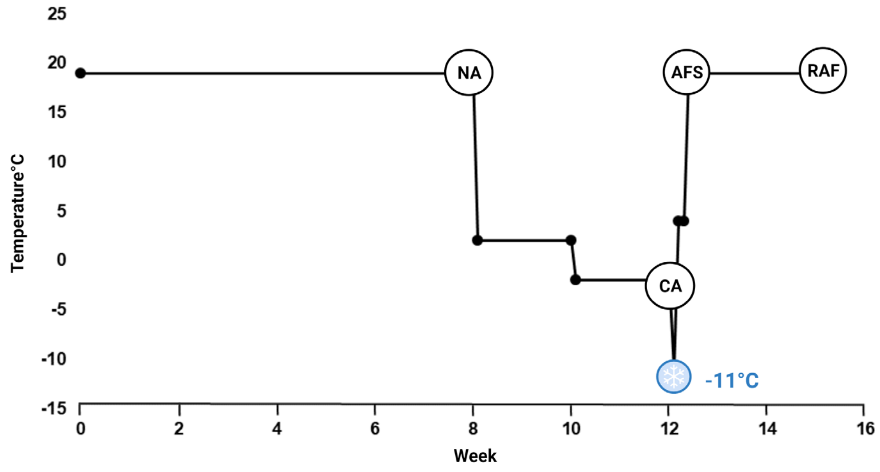
Cold acclimation induces metabolic and genetic changes in alfalfa overwintering perennial organs and in the flavonoid concentration of root exudates that differ between the most performant and the less performant host/strains associations.

Study underlying mechanisms of a freezing tolerant symbiotic association

Treatments:



Growth chamber: Fall + Freeze- thaw + Regrowth



4 events sampling

Measurements:

- Root exudates
- Collect alfalfa crowns, roots and nodules

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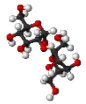
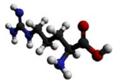
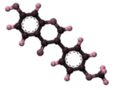

Soluble sugars


Amino Acids


Flavonoids

Biochemical analysis

Contribution to Knowledge

- ✓ Nodules play a pivotal role in the acquisition of freezing tolerance and regrowth of alfalfa.
- ✓ New insights into the molecular dialog of alfalfa-rhizobia associations in response to cold acclimation, freezing stress and deacclimation as well as a comprehensive understanding of the contribution of each cold-tolerant partner for a successful symbiosis in alfalfa.

Conclusions:

- Investment by the plants in **nodules!**

  Major strain effect observed

- **Rhizobia and plants** play complementary roles to ensure enhanced regrowth after freezing

  N storage and remobilization

- Importance to **include the rhizobial symbiosis** in strategies aimed at improving stress tolerance in legumes.

- Freezing strain NRG34 has a good potential in cold soil

  Need field experiments with other cultivars

"In the long history of humankind (and animal kind, too) those who learned to collaborate and improvise most effectively have prevailed."
-Charles Darwin

Thanks for you attention!
And thanks to the team:

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Fièrement propulsée par les FRQ

Québec

McGill

Agriculture and Agri-Food Canada

Agriculture et Agroalimentaire Canada

Results:

34 metabolites

- 7 sugars
- 21 amino acids
- 6 flavonoids

***Investment in nodules

Concentration increases compared to NA

Concentration decreases compared to NA

Alfalfa organs

Metabolites	Exudates NA	Exudates CA	Exudates AFS	Exudates RAF	Nodules NA	Nodules CA	Nodules AFS	Nodules RAF	Roots NA	Roots CA	Roots AFS	Roots RAF	Crowns NA	Crowns CA	Crowns AFS	Crowns RAF
Sugars	mg/g Root DM				mg/g Nodules DM				mg/g Root DM				mg/g Crown DM			
Sucrose	0.514	0.529	0.223	0.129	58.18	158.74	67.64	48.64	49.80	174.25	143.63	73.26	43.70	183.19	124.98	66.08
Glucose	0.361	0.396	0.382	0.691	2.79	3.72	3.47	3.10	2.16	2.13	1.72	1.72	5.22	4.32	2.51	3.72
Stachyose	0.011	0.068	0.038	0.015	0.00	9.12	2.54	0.00	0.22	3.77	2.04	0.27	0.46	4.47	2.16	0.51
Raffinose	0.002	0.082	0.009	0.008	0.01	16.73	3.18	0.00	0.11	4.06	1.12	0.16	0.12	3.05	0.79	0.15
Fructose	0.402	0.398	0.401	0.754	0.79	0.77	2.57	2.25	3.68	3.44	2.90	1.86	4.51	4.51	2.76	4.26
Pinitol	0.269	0.123	0.117	0.202	29.54	26.47	12.94	13.08	9.58	8.56	8.64	7.30	10.89	9.16	9.85	7.64
SSTot	1.560	1.597	1.168	1.798	91.24	215.55	92.34	67.06	65.55	196.21	160.05	84.58	64.91	208.70	143.05	82.36
Starch					1.62	4.64	1.48	1.46	321.78	114.05	119.36	209.32	272.32	95.46	101.04	151.24
NSC					89.81	220.19	93.82	68.52	387.33	310.26	279.41	293.90	337.23	304.16	244.10	233.60
Amino Acids	µmol/g Root DM				µmol/g Nodule DM				µmol/g Root DM				µmol/g Crown DM			
Glu	0.181	0.170	0.165	0.193	16.05	18.15	12.95	14.93	5.93	7.87	9.59	5.42	7.50	10.82	13.25	7.95
Gln	0.038	0.005	0.002	0.044	2.45	3.00	3.56	3.73	1.37	0.93	0.78	1.58	1.77	0.87	0.87	2.44
Pro	0.722	0.134	0.128	0.641	25.10	24.73	21.07	22.50	28.83	19.14	21.49	22.63	25.95	22.85	25.10	25.67
Orn	0.017	0.007	0.005	0.019	0.24	2.44	1.03	0.36	0.28	0.49	0.37	0.18	0.34	0.64	0.56	0.42
Arg	0.009	0.011	0.009	0.010	2.15	19.30	22.44	2.54	6.22	11.59	11.47	3.68	8.32	13.92	13.84	4.72
His	0.011	0.003	0.005	0.010	3.06	7.02	9.11	3.13	1.99	2.68	3.10	0.99	2.83	4.20	4.68	1.68
Asp	0.186	0.174	0.153	0.222	4.99	4.97	4.59	4.28	1.22	2.84	2.28	1.49	3.74	6.63	3.48	4.23
Asn	0.867	0.043	0.004	0.628	416.32	359.93	248.41	278.54	83.01	63.19	62.19	73.56	69.14	44.83	40.54	83.43
Ala	0.078	0.042	0.064	0.071	12.89	20.24	15.77	9.87	3.70	3.14	6.51	2.96	2.64	1.64	6.29	2.53
Thr	0.039	0.015	0.023	0.036	1.68	1.45	5.33	1.98	1.09	0.82	1.79	0.89	0.89	0.65	1.51	0.97
Lys	0.008	0.006	0.008	0.011	0.82	0.84	3.06	0.89	0.08	0.13	0.43	0.08	0.11	0.12	0.37	0.09
Met	0.004	0.005	0.005	0.006	0.07	0.08	0.24	0.15	0.03	0.03	0.16	0.03	0.10	0.09	0.06	0.04
Ile	0.009	0.006	0.013	0.013	0.72	0.91	6.07	1.07	0.15	0.23	1.17	0.12	0.16	0.20	0.87	0.14
Leu	0.011	0.009	0.017	0.014	0.79	0.76	8.74	0.85	0.17	0.22	1.46	0.12	0.16	0.15	1.08	0.13
Val	0.019	0.012	0.021	0.021	1.51	1.86	9.39	2.21	0.37	0.57	1.43	0.32	0.33	0.52	1.15	0.36
Ser	0.087	0.036	0.043	0.082	3.47	11.08	9.21	3.99	2.61	4.44	5.93	1.93	2.71	4.38	5.68	2.68
Gly	0.024	0.011	0.017	0.029	2.33	3.34	6.35	2.09	0.39	0.29	0.59	0.37	0.42	0.29	0.61	0.46
GABA	0.175	0.071	0.094	0.137	27.00	25.38	23.51	16.07	5.56	7.50	6.91	3.92	4.25	3.34	3.57	3.61
AABA	0.001	0.000	0.000	0.001	0.39	0.29	0.09	0.10	0.04	0.06	0.05	0.05	0.09	0.13	0.16	0.11
Tyr	0.004	0.003	0.004	0.006	0.49	1.00	1.71	0.30	0.11	0.31	0.55	0.20	0.11	0.17	0.43	0.21
Phe	0.009	0.006	0.007	0.010	0.44	0.34	1.73	0.68	0.27	0.27	0.67	0.19	0.30	0.19	0.60	0.23
AATot	2.50	0.77	0.79	2.20	523.42	507.12	414.36	370.26	143.42	126.75	138.94	120.72	131.84	116.63	124.72	142.09
Flavonoids	µg/g Root DM				µg/g Nodule DM				µg/g Root DM							
Naringenin	0.038	0.025	0.013	0.020	1.23	1.24	1.82	1.80	0.06	0.57	0.03	0.57				
Luteolin	0.045	0.026	0.027	0.094	1.08	1.43	2.24	2.65	0.00	0.23	0.63	0.44				
Echinatin	0.236	0.091	0.070	0.363	2.64	5.86	12.34	9.33	0.01	1.39	0.76	1.06				
Coumestrol	0.115	0.077	0.061	0.117	6.14	17.67	21.70	28.49	0.05	0.00	0.01	0.46				
Formononetin	2.044	2.262	1.722	1.237	39.48	22.45	22.61	36.43	84.12	74.04	59.98	24.96				
Medicarpin	0.170	0.504	0.178	0.134	2.48	8.10	5.33	8.55	4.57	10.96	8.33	3.04				
FlaTot	2.651	2.985	2.070	1.965	51.85	56.76	66.18	87.83	88.81	87.18	69.74	30.55				

log2 Fold changes (Event sampling/NA)

decrease	Increase
<	>
-0.00	0.00
-0.25	0.25
-0.50	0.50
-1.00	1.00
-2.00	2.00
-3.00	3.00

Chapter 2: Study the underlying mechanisms of a freezing tolerant symbiotic association

Treatments:

Alfalfa populations

A-TF0 A-TF7

VS

X

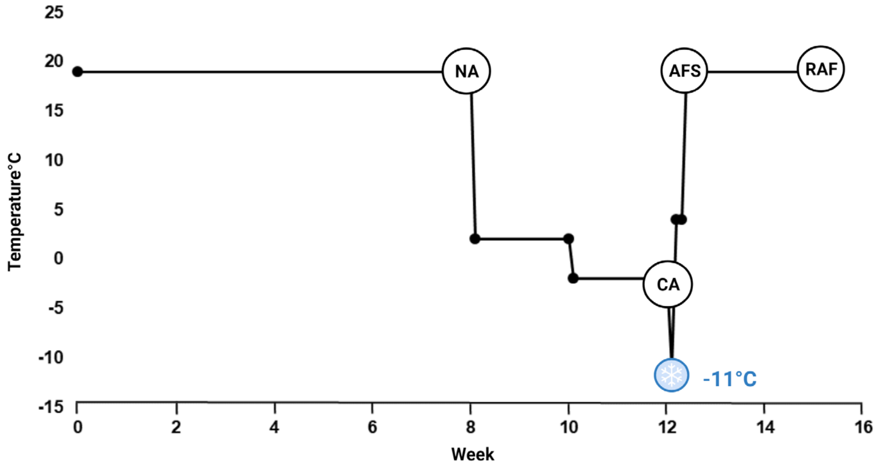
Inoculation

B399 NRG34

VS

Most contrasting strains

Growth chamber: Fall + Freeze- thaw + Regrowth



4 events sampling

Measurements:

- Root exudates
- Collect alfalfa crowns, roots and nodules

Biochemical analysis

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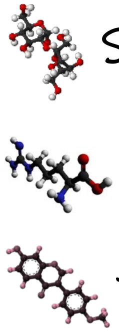


Soluble sugars

Amino Acids

Flavonoids

Chapter 3: Competitive ability of cold tolerant strain in two soils

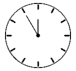
1. There is a variability among *Sinirhizobium meliloti* strains in their ability to nodulate and fix N under low temperatures when associated with alfalfa populations differing in their levels of freezing tolerance.

2. Rhizobial strains selected for their efficiency at low temperatures have the competitive ability to establish an efficient symbiosis with alfalfa in cold soil as compared to natives strains, particularly with freezing-tolerant population (A-TF7).

Chapter 3: Competitive ability of cold tolerant strain in two soils

Growth chambers:

Spring T°
15/10° CD/N


8 weeks

X

Cone-tainers

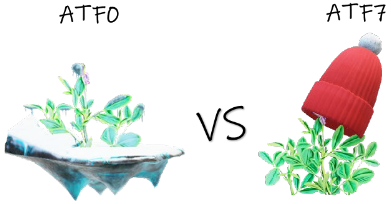
5 rep

X

Soil sites collection

X

Alfalfa populations

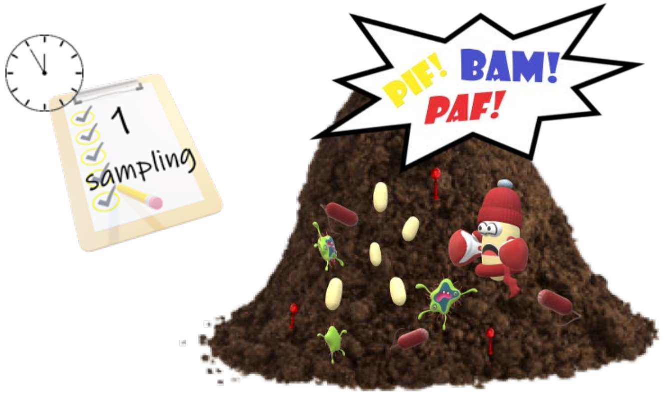


X

Inoculation

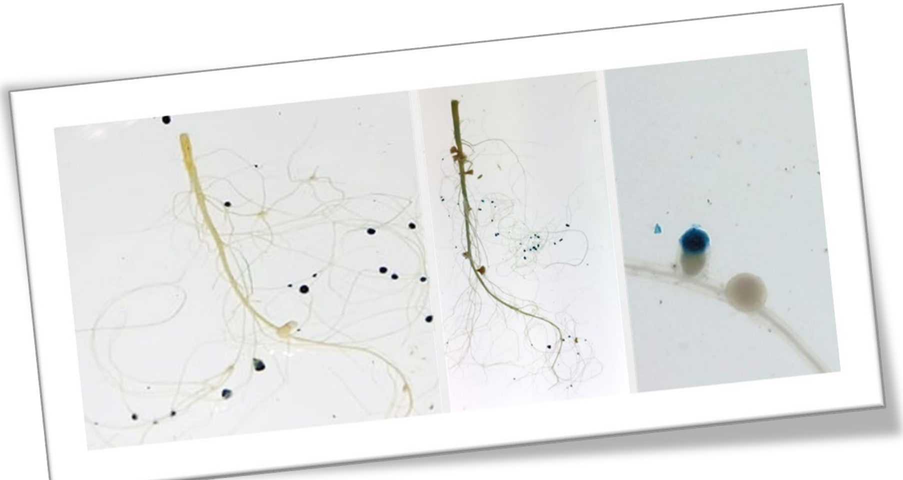
3 strains tagged with reporter gene *gusA*

Measurements:



Total nodules number and % of occupancy

- Tagged strain
- Soil natives strain

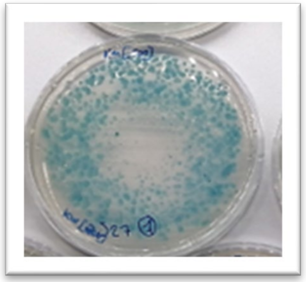


Biomass (shoot & root)

Results: Interaction soils x alfalfa populations

X

	Total nodule number per plant		% occupancy (# of tagged/ total nodules × 100)		Shoot dry weight (mg) per plant	
Effect	Northern soil	Southern soil	Northern soil	Southern soil	Northern soil	Southern soil
Populations						
A-TF0	9.7^{c}	48.9^{b}	33.5	51.9	37.3^{c}	166.3^{b}
A-TF7	10.4^{c}	**69.4^{a}**	31.7	48.4	37.6^{c}	**198.4^{a}**
Strains**						
Non inoculated	5.1^{f}	35.9^{c}	0^{d}	0^{d}	25.3^{d}	120.6^{b}
NRG34	**15^{d}**	**74^{a}**	**64.7^{b}**	**79.7^{a}**	**45.5^{c}**	**212.9^{a}**
Rm1521	11.3^{e}	60^{ab}	32.4^{c}	70.8^{b}	**43.56^{c}**	**187.8^{a}**
S27	8.9^{e}	55.2^{b}	nd***	nd	35.8^{cd}	**209.3^{a}**



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Selection of effective and competitive *Sinorhizobium meliloti* strains that nodulate alfalfa under low temperature

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Contribution to Knowledge

- ✓ Confirmed that strain NRG34, isolated from northern Canada, is cross-adapted to cold and freezing stress and have higher competitive ability to nodulate alfalfa in two soils with different physicochemical and biological properties.
- ✓ Revealed the potential of that strain to be commercialized as inoculant for alfalfa in northern environments.
- ✓ Reports that a freezing-tolerant alfalfa population is more effective in the recruitment of rhizobia under low temperature as shown by a larger number of nodules which translated into larger plant biomass compared to a less freezing-tolerant population.

Results:

Nodules biomass: *Alfalfa populations effect*

- No effect under optimal growth conditions (NA).

- **A-TF0** 10% of nodule biomass 48 h after freezing stress (AFS).