Characterization of metabolic and genetic processes involved in cold tolerant symbioses between alfalfa and rhizobia

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February 2024

A thesis submitted to McGill University in partial fulfillment
of the requirements of the degree of Doctor of Philosophy

À Claunia,

« Sonje lapli ki leve mayi ou »

-proverbe haïtien

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AABA	α-aminobutyric acid
AATot	total amino acids
AFS	forty-eight hours after freezing stress
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Aptase	vacuolar H+- ATPase A subunit
CA	cold-acclimated
C_2H_2	acetylene
C_2H_4	ethylene
C4H	cinamic acid 4-hydoxylase
CO_2	carbon dioxide
COR	cold-related
DM	dry matter
DNA	deoxyribonucleic acid
DW	dry weight
eEF-1α	eukariotic elongation factor 1-alphas
Formo	formononetin
FlaTot	total flavonoids
GABA	γ-aminobutyric acid
GaS	galactinol synthase
GHG	greenhouse gas
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Ile	isoleucine

IOMT	isoflavone-O-methytransferase
Leu	leucine
Lys	lysine
Met	methionine
N	nitrogen
NA	non-acclimated
N_2O	nitrous oxide
NSC	non structural carbohydrates
nod genes	nodulation genes that control host specificity, infection
Nod factors	signaling molecule produced by bacteria-lipochitooligosaccharides
Phe	phenylalanine
Pro	proline
qPCR	quantitative polymerase chain reaction
RAF	three weeks regrowth after freezing stress
RFO	raffinose-family oligosaccharides
ROS	reactive oxygen species
RNA	ribonucleic acid
Ser	serine
SPS	sucrose phosphatase synthase
SSTot	total soluble sugars
SuSy	sucrose synthase
Thr	threonine
Tyr	tyrosine
UBQ2	Ubiquitin 2
Val	valine
Orn	ornithine

Abstract

Alfalfa (*Medicago sativa* L.) is the most important forage crop species in Canada and with its ability to symbiotically fix nitrogen with a rhizobial partner *Sinorhizobium (Ensifer) meliloti,* contributes positively to a more sustainable agriculture. Winter survival, a major determinant of alfalfa yield and low temperatures, affects both symbiotic partners. A large range of cold tolerance has been reported for both alfalfa and rhizobial strains, and a symbiosis involving two freezing-tolerant partners could have a synergistic effect resulting in a greater winter survival of alfalfa. Selected freezing tolerant strains also require a high capacity to compete with native soil rhizobia under various stresses to prevent nodule occupancy by competitive but low-efficient native strains. The main objective was to improve alfalfa production under low temperatures by identifying superior rhizobial strains in association with alfalfa populations differing in their levels of freezing tolerance.

In a first study, six *S. meliloti* strains were tested in combination with two alfalfa populations bred to differ in their levels of freezing tolerance (A-TF0 and A-TF7). Plants were grown for eight weeks in a growth chamber before being exposed to temperatures promoting their cold acclimation. Plants were then exposed to a freezing stress and regrown for three weeks. Shoot, root and nodule biomass were measured before cold acclimation and three weeks after the freezing stress. After freezing, A-TF7 selected for better freezing tolerance had shoot and root biomasses respectively 19 and 15% larger than A-TF0. Alfalfa inoculated with strain NRG34, showed both a larger shoot biomass and a higher nodule dry weight than plants inoculated with any other strains and also presented the largest proportion of undamaged nodules or of nodules with a regeneration zone.

In a second study, we compared the regrowth after a freezing stress of four symbiotic associations of contrasted adaptation to cold: populations A-TF0 and A-TF7 inoculated with *S. meliloti* strains B399 (commercial) and NRG34 (cold-adapted). To better understand the contribution of each partner of the symbiosis to a better regrowth after freezing, we identified molecular traits having major roles in cold acclimation, freezing tolerance, and involved in crosstalk between alfalfa and its symbiont. Alfalfa regrowth after exposure to a freezing stress was 35 % larger in the A-TF7 × NRG34 than in the A-TF0 × B399 association. The study of metabolites in roots, crowns, and nodules revealed profound changes in these organs, switching from a sink to

support cold acclimation to a source of reserves enabling regrowth after deacclimation. Stachyose and raffinose increased markedly in alfalfa during cold acclimation confirming their protective roles in overwintering alfalfa. Both cold-adapted symbiotic partners contributed to increases in arginine concentration in nodules during cold acclimation and deacclimation underscoring the importance of N storage and remobilization to increase the persistence and regrowth of alfalfa.

In a final study, three highly-efficient strains were selected under simulated spring conditions, based on nodulation speed, nitrogenase activity, and alfalfa biomass yield. Competitive ability of these three strains was assessed by determining the percent of alfalfa nodules occupancy by the selected tagged strains. Two soils originating from south and north of Quebec containing natives rhizobia as well as A-TF0 and A-TF7 were tested. Strain NRG34 proved to be very competitive as shown by a greater nodule occupancy than any other strain in two cold soils.

Overall, this work demonstrates a relationship between nodule biomass and shoot regrowth after a freezing stress, the latter being associated with the proportion of nodules showing less freezing damage. Both choice of alfalfa populations and *S. meliloti* strains adapted to stress are complementary to increase the persistence of alfalfa.

Résumé

La luzerne (*Medicago sativa* L.) est l'espèce de culture fourragère la plus importante au Canada et, grâce à sa capacité à fixer l'azote de manière symbiotique avec un partenaire rhizobium *Sinorhizobium (Ensifer) meliloti*, elle contribue positivement à une agriculture plus durable. La survie hivernale est un déterminant majeur du rendement de la luzerne et les basses températures affectent les deux partenaires symbiotiques. Une large gamme de tolérances au froid a été rapportée autant pour les populations de luzerne que pour les souches rhizobiennes et une symbiose impliquant deux partenaires tolérants au gel pourrait avoir un effet synergique entraînant une meilleure survie hivernale de la luzerne. Les souches tolérantes au gel sélectionnées nécessitent également une grande capacité à rivaliser avec les souches natives de rhizobium du sol sous divers stress afin d'empêcher l'occupation des nodules par des souches natives et compétitives, mais peu efficaces. L'objectif principal de cette thèse était d'améliorer la production de luzerne à basses températures en identifiant des souches rhizobiennes bien adaptées au froid en association avec des populations de luzerne différant par leurs niveaux de tolérance au gel.

Dans une première étude, six souches de *S. meliloti* ont été testées en combinaison avec deux populations de luzerne sélectionnées pour leurs niveaux contrastés de tolérance au gel (A-TF0 et A-TF7). Les plants ont été cultivés pendant huit semaines en condition contrôlées avant d'être exposés à des températures favorisant leur acclimatation au froid. Les plants ont ensuite été exposés à un stress de gel et remis en conditions initiales pour le regain pendant trois semaines. Les biomasses aérienne, racinaire et des nodules ont été mesurées avant l'acclimatation au froid et trois semaines après le stress de gel. Après le stress de gel, la population A-TF7 sélectionnée pour une meilleure tolérance au gel avait une biomasse aérienne et racinaire de 19 % et 15 % respectivement, supérieures à celles de A-TF0. Les plants de luzernes inoculés avec la souche NRG34 présentaient à la fois une plus grande biomasse aérienne et un poids sec de nodules plus élevé que les plants inoculés avec toute autre souche et présentaient également une plus grande proportion de nodules non endommagés par le gel ou de nodules avec une zone de régénération.

Dans une seconde étude, nous avons comparé le regain après un stress de gel de quatre associations symbiotiques d'adaptation contrastée au froid : les populations A-TF0 et A-TF7 inoculées avec les souches *S. meliloti* B399 (commerciale) et NRG34 (adaptée au froid). Pour mieux comprendre la contribution de chaque partenaire de la symbiose à un meilleur regain après

un stress de gel, nous avons analysé les changements moléculaires associés à l'acclimatation au froid, à la tolérance au gel, et dans la communication entre la luzerne et son symbiote. La biomasse aérienne de la luzerne après exposition à un stress de gel était 35 % plus élevée dans l'association A-TF7 × NRG34 que dans l'association A-TF0 × B399. L'étude des métabolites dans les racines, les couronnes et les nodules a révélé de profondes modifications dans le rôle métabolique de ces organes, passant d'un puits pour supporter l'acclimatation au froid à une source de réserves permettant le regain après la désacclimatation. Les concentrations de stachyose et de raffinose ont augmenté de façon marquée pendant l'acclimatation au froid, confirmant ainsi leur rôle de protection cellulaire menant à une meilleure survie hivernale de la luzerne. Les deux partenaires symbiotiques adaptés au froid ont contribué à l'augmentation de la concentration d'arginine dans les nodules lors de l'acclimatation et de la désacclimatation au froid, soulignant l'importance du stockage et de la remobilisation de l'azote pour augmenter la persistance et le regain de la luzerne.

Dans une étude finale, trois souches très efficaces ont été sélectionnées dans des conditions simulées de printemps, sur la base de la vitesse de nodulation, de l'activité de la nitrogénase et du rendement en biomasse de la luzerne. La capacité compétitive de ces trois souches a été évaluée en déterminant le pourcentage d'occupation des nodules de luzerne par les souches marquées sélectionnées. Deux sols originaires du sud et du nord du Québec contenant des souches natives ainsi que les luzernes A-TF0 et A-TF7 ont été testés. La souche NRG34 s'est révélée très compétitive, montrant une plus grande occupation des nodules que toute autre souche dans deux sols froids.

Globalement, ce travail montre une relation entre la biomasse des nodules et le regain de la luzerne après un stress de gel, cette dernière étant associée à la proportion de nodules présentant moins de dommages dus au gel. Le choix des populations de luzerne et celui des souches de *S. meliloti* adaptées au stress sont complémentaires pour augmenter la persistance de la luzerne.

Remerciements

«*Sonje lapli ki leve mayi ou*». Songe à la pluie qui a fait pousser ton maïs. Ce proverbe haïtien rappelle d'être reconnaissant pour l'aide qui nous a été apportée depuis le début et qui a été vitale à la réussite d'un accomplissement. Si mon nom figure seul sur la première page de cette thèse, il cache pourtant celui de nombreuses actrices et nombreux acteurs, qui sans eux, aucun des mots qui suivront à ce présent texte n'auraient pu s'aligner. Si je ne peux les joindre au mien sur cette première page, je les porte tous néanmoins dans mon cœur. D'Amours ne s'écrit pas au pluriel pour rien, à vous tous, merci !

Il y a cinq ans, je fonçais bien naïvement et tête baissée dans cette nouvelle aventure. En amour avec l'Argentine depuis plusieurs années, un congrès sur la luzerne à Córdoba auquel ma future codirectrice de doctorat allait assister avait suscité mon intérêt. Serait-il possible de joindre plusieurs de mes amours en même temps? Poursuivre mes intérêts pour la recherche, continuer d'apprendre sur la fixation symbiotique qui me fascine tant et retourner en Argentine pour y faire un stage doctoral? Je pourrais peut-être m'y établir ensuite? Ce serait sans doute plus facile pour mon mari et mes beaux-enfants de m'y rejoindre et d'y immigrer que d'entamer les démarches de parrainage pour cette forteresse inaccessible qu'est devenue la province de Québec. Comme rien ne demeure chimérique dans les mains d'Annick, l'idée est devenue opportunité, quel plan fantastique de carrière et de vie ! J'étais bien loin de me douter que tous mes rêves allaient sombrer, noyés par un tsunami de cinq, six ou peut-être sept, je ne sais plus combien, vagues dévastatrices de Covid-19… *¡Chau Argentina, holà pandemia!* Bon, je dramatise un brin, le stage en Argentine aura tristement péri, le reste cependant, le plus important, aura su apprendre à nager. Et vous êtes beaucoup à m'avoir aidée à garder la tête hors de l'eau, à m'avoir guidée et calmée à travers la tempête, à m'avoir motivée et poussée à atteindre la rive. Je me sens énormément privilégiée d'avoir eu ce luxe de continuer à apprendre sur un sujet qui me passionne autant et d'avoir pu compter sur tout l'amour et le soutien de mon entourage dans toute cette aventure.

Je tiens d'abord et avant tout à remercier mes directeurs de thèse, Prof. Philippe Seguin et Annick Bertrand Ph.D.. Je suis extrêmement reconnaissante d'avoir eu la chance d'être dirigée et épaulée par des chercheurs avec d'autant grandes qualités humaines que professionnelles. Je tiens à remercier sincèrement mon directeur de thèse, Philippe Seguin pour son encadrement, sa grande rigueur scientifique, ses commentaires toujours bien réfléchis et constructifs ainsi que pour son

soutien tout au long de cette thèse. Merci de m'avoir offert cette chance de poursuivre mon parcours académique à l'Université McGill et d'avoir contribué grandement à améliorer mes capacités à communiquer en anglais. Merci pour cette grande patience, ce calme rassurant et pour les encouragements constants. Bien que physiquement plus loin, les rencontres virtuelles auront toujours été des plus enrichissantes et motivantes.

Annick, je suis sincèrement reconnaissante pour tout ce que tu as fait pour moi. Merci de m'avoir offert l'occasion de travailler dans ton équipe comme adjointe de recherche sur la symbiose des légumineuses en 2018 et d'avoir ravivé mon intérêt sur le sujet. Merci d'avoir accepté de me prendre sous ton aile en codirigeant ce projet de thèse, de m'avoir fait confiance et de m'avoir guidé et appuyé tout au long de mes études doctorales. Merci infiniment d'avoir investi autant de temps et d'énergie en moi comme dans ce projet. Merci d'avoir rebondi pour trouver maintes et maintes solutions aux nombreuses fermetures du centre pendant la pandémie alors que nos expériences étaient en cours et que j'avais envie de tout laisser tomber. Observer cette habileté à résoudre les problèmes rationnellement et efficacement aura été à la fois très formatrice pour moi et aura su calmer mes angoisses, me garder motivée et me pousser à ne pas abandonner malgré les difficultés. Ton dévouement, ton dynamisme, ta passion pour la recherche et ton grand leadership m'ont profondément marqué et ces qualités resteront pour moi un modèle vibrant et des plus inspirant.

Formidable équipe, tout cet immense travail des cabinets de croissance aux analyses statistiques en passant par le laboratoire n'aurait pu être réalisé sans votre aide, plus qu'appréciée. Josée Bourassa, Josée Michaud, Jean Cloutier et Sandra Delaney, je tiens à vous remercier pour votre patience et votre grande générosité d'avoir partagé avec moi vos innombrables connaissances et de m'avoir appris tout ce que je sais aujourd'hui sur les techniques d'analyse en microbiologie, en biologie moléculaire et en biochimie des plantes. Je me sens extrêmement privilégiée d'avoir réalisé mes expériences au Centre de Recherche d'Agriculture et Agroalimentaire Canada qui se démarque par ses infrastructures et ses nombreux équipements facilitants, mais surtout par son équipe d'adjoints de recherches des plus compétents. Merci pour tout votre travail, merci pour tous ces petits nodules prélevés avec soins et merci pour votre soutien dans les moments de découragement. Je tiens à remercier spécialement Josée Bourassa, pour son dévouement particulier, pour son courage et son aide inestimable tout au long de ces cinq dernières années et de façon encore plus marquée pendant la pandémie. Armée de son masque N95, confinée dans ton

laboratoire et moi dans un autre, courant derrière nos chariots, je n'aurais pu souhaiter meilleure coéquipière pour affronter ce défi d'échantillonnage marathonien lors d'une des multiples fermetures du centre. Un défi un brin stressant, qui restera à la fois un souvenir un peu surréaliste, mais une fierté commune d'y être arrivée ! Un grand merci aussi à David Gagné et Mario Laterrière pour l'aide en bio-informatique, à Normand Bertrand pour les analyses de gaz, à Julie Lajeunesse pour le sol prélevé à Normandin ainsi qu'à Steve Methot pour l'accompagnement dans les analyses statistiques.

Je voudrais également remercier les membres de mes comités de supervision de doctorat de l'Université McGill, les professeurs Suha Jabajiet Jean-Benoît Charronainsi que d'Agriculture et Agroalimentaire Canada, les chercheuses scientifiques Annie Classens, Ph. D. et Solen Rocher, Ph. D. pour vos conseils judicieux et aiguillant. Un merci tout spécial aussi à Mary-Cathrine Leewis, Ph. D. et à Yves Castonguay, Ph. D. d'avoir révisé un article de cette thèse. Vos commentaires constructifs auront grandement bonifié ces deux articles.

J'aimerais aussi adresser un merci particulier à Prof Geneviève Lajoie et à Prof Donald Smith d'avoir accepté d'évaluer cette thèse et sa soutenance.

Plus personnellement, j'aimerais dire merci à ma famille et à tous mes ami(e)s pour votre soutien pendant ces cinq dernières années. De façon particulière, je remercie mes précieuses Justine Le Blanc, Vicky Lévesque et Annie Robichaud. Votre appuie à tous mes projets fous, nos grandes conversations, nos rigolades et tous ces instants magiques passés en votre compagnie auront été autant des bouées de sauvetage lors des tempêtes que de grandes voiles qui m'auront permis de voguer doucement lors des journées plus ensoleillées. Votre amitié est inestimable et m'aura permis de garder le cap et de rester saine dans toute cette aventure.

Un mot enfin pour ceux à qui je dois tout, mes parents. Je suis pleinement consciente qu'on ne naît pas tous égaux et que d'accéder aux études graduées est un immense privilège. Quelle chance d'être née ici, dans une famille aimante qui a toujours valorisé l'éducation ! Papa, merci pour tout ce que tu as fait pour moi et de m'avoir ouvert à toutes ces possibilités. Ton regard tout aussi bienveillant que critique à mon égard m'aura poussé à me dépasser et à ne pas abandonner. Maman, merci à l'infini d'être ma plus grande fan depuis toujours et de me supporter, quels que soient mes choix. Merci pour tes encouragements constants, ton appui émotionnel et ton amour inconditionnel. Merci de m'avoir permis de concrétiser ce rêve de finir mes études doctorales tout en accomplissant mes engagements de parraine pour mon mari et mes beaux-enfants. Merci pour

ce modèle de femme courageuse et vaillante qui sait défendre brillamment ce en quoi elle croit. Sans ton aide, je n'aurais jamais pu mener mes deux barques, celle du doctorat et du parrainage en immigration à bon port. Sans toi, je ne serais pas qui je suis aujourd'hui.

Enfin, merci à vous mes chéris, mon doux et encourageant mari ainsi qu'à mes merveilleux beaux-enfants, Boyson et Claunia. *Ak tout kè mwen, mesi ampil!* Merci pour votre patience et votre compréhension, merci de m'avoir permis de me réaliser professionnellement malgré les sacrifices que cela a impliqué pour notre famille. Merci d'avoir célébré les victoires et pleuré les défaites avec moi. « *Sonje lapli ki leve mayi ou* ». Des averses, des torrents, des déluges pendant ces cinq dernières années on en aura connu et nous ne les oublierons pas. Malgré leur rudesse, nous les avons vécus et ressentis ensemble, physiquement ou non. Je n'ai pas de doute que toute cette pluie aura su augmenter notre résilience et notre capacité à nous adapter aux différents stress et à tous les autres défis que la vie nous apportera. Votre amour inébranlable, vos encouragements et votre appui tout au long de cette aventure auront été source de motivation et de dépassement de soi. Vous êtes, tous les trois, ma force. Vous riez alors que tous auraient envie de pleurer et de s'effondrer, vous vous relevez et regardez devant avec enthousiasme et espoir, vous vous adaptez et affrontez l'adversité, comment ne pas rester inspirée à vos côtés? Luckson, merci de croire en moi plus que je ne sais le faire. Merci de m'aimer sans limites malgré la distance et les nombreux défis et de partager les mêmes valeurs dans nos mondes contrastés. Tes sages paroles et ton intelligence émotionnelle auront su me calmer, m'apprendre à relativiser et me garder motivée dans les bonnes comme dans les mauvaises journées. Je n'aurais pu souhaiter meilleur allié pour me pousser à avancer. Boyson et Claunia, notre relation a commencé presque en même temps que cette thèse. Malgré votre jeune âge, vous qui avez déjà tellement connu de souffrance et d'injustice, c'est sans aucune méfiance ni appréhension que vous m'avez ouvert grand les bras et que vous m'avez appelé : « Maman ». Un titre que je ne mérite assurément pas, mais dont j'essaierai d'honorer toute la profondeur et l'importance. En réalisant cette thèse, en allant jusqu'au bout, j'ai voulu vous donner le modèle d'une femme aimante pour sa famille qui désire se réaliser pleinement, tout en se battant pour vous avoir près d'elle avec la même volonté et détermination. Ce défi, je l'ai surmonté avec vous et pour vous. De la même façon, ensemble, nous surmonterons le prochain. Comme dit si bien Luckson : « *Tout sa ki bon pwan tan* », gardez confiance. Claunia, si c'est à toi que je dédie cette thèse c'est pour te remercier pour l'amour et la confiance que tu me portes et pour te dire à quel point je considère ton affection à mon égard. Merci d'être fière de moi

et de par tous tes efforts quotidiens de vouloir me rendre si fière en retour. Je le suis et je me sens extrêmement privilégiée de pouvoir contribuer à cette magnifique et valeureuse jeune femme que tu deviens. Merci de valoriser pleinement le peu que je pouvais t'offrir pour l'instant, tout mon amour et une éducation. Je ne sais pas encore où le vent nous mènera, mais ensemble tous les quatre, je sais que l'on réussira. Du plus profond de mon cœur, je vous souhaite à tous les trois d'avoir la même chance que moi, celle de pouvoir choisir de réaliser vos rêves et d'aller au bout de vos ambitions quelles qu'elles soient.

Songe à la pluie. Je songe à ces épreuves et à ces réussites des cinq dernières années qui m'ont rendue plus forte et qui m'ont fait grandir. Aujourd'hui, je songe à la pluie et c'est le cœur débordant de gratitude que je réalise que bien entourée et avec assez de volonté et de détermination, j'ai la capacité d'y arriver. Aujourd'hui, je songe à la pluie et je suis impatiente de voir ce qui poussera demain.

Une dernière fois et du fond du cœur, à vous tous, merci !

Contributions of Authors

The following thesis was prepared according to the "Thesis Guidelines" of McGill University. The thesis contains seven chapters. The first two chapters are a general introduction and literature review (Chapters 1-2), the three following chapters represent three separate research manuscripts (Chapters 3-5), and two additional chapters present a general discussion, conclusions and future research perspectives (Chapters 6-7). Chapter 3 was published in Plant and Soil journal, Chapter 4 was published in Symbiosis journal, and Chapter 5 was published in Rhizosphere journal.

Author Contributions:

Emmanuelle D'Amours was the primary researcher for each chapter and designed the research, planned, and conducted experiments and analyses with the guidance of Prof Philippe Seguin, Department of Plant Science of McGill University and Annick Bertrand, Ph.D. of Agriculture and Agri-Food Canada.

Prof Philippe Seguin provided supervision and guidance for the research performed in all chapters and revised all the manuscripts.

Annick Bertrand Ph.D., provided co-supervision, guidance and funding for the research performed in all chapters and revised all the manuscripts.

Jean Cloutier Ph.D., Agriculture and Agri-Food Canada, contributed to laboratory work for all the chapters and help to the conception of the studies and revised Chapters 3-4-5.

Annie Classen Ph.D., Agriculture and Agri-Food Canada, provide the alfalfa germplasm used in the studies and revised Chapters 3-4-5.

Solen Rocher Ph.D., Agriculture and Agri-Food Canada provided studies of phylogenetic trees of the *Sinorhizobium meliloti* strains selected for the researches and guidance in the analysis of gene expression. She revised Chapters 3-4-5.

Contribution to Knowledge

The chapters of this thesis represent significant contributions to the knowledge of the role that play the symbiotic association between alfalfa and rhizobia to increase alfalfa persistence under low temperature. New insights on the relationship between the two partners of the symbiosis and on their impact on plant adaptation to stress are provided.

Chapter 3

- The study demonstrates that alfalfa regrowth after freezing is positively linked with the proportion of nodules showing less freezing damages which in turn depends on rhizobial strains.
- These are the 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 in symbiosis with alfalfa.
- This chapter reports that 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.

Chapter 4

- This is the first study that monitored the independent effects of each partner of the alfalfa-rhizobia symbiotic association on the metabolites profile modifications in response to cold acclimation and deacclimation.
- This study provided new evidences that nodules play a pivotal role in the acquisition of freezing tolerance and regrowth of alfalfa.
- This targeted metabolites and gene expression approach yielded 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.

Chapter 5

- This study 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.
- These results revealed the potential of that strain to be commercialized as inoculant for alfalfa in northern environments.
- This study also 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.

Chapter 1: General Introduction

1.1 Alfalfa-rhizobia symbiosis for sustainable agriculture in northern environments

The green revolution initiated in the 1940's in response to a major food crisis affecting mainly developing countries was based on the use of chemicals fertilizers and pesticides and large scale irrigation along with intensive breeding and monoculture-type management. Although these approaches allowed to double and triple crop yield, it is now obvious that gains obtained by the green revolution were made in part at the expense of soil degradation, increased water consumption, and chemical runoff causing significant environmental damages. Over time, the loss of traditional crops and growing techniques further decreased the resilience of the global food system. Today, greenhouse gas (GHG) emissions associated with industrial agriculture accelerates climate change, further exposing the vulnerabilities of the modern food system. The actual increase in food demand due to the increase in human population and the pressure exerted by agricultural practices on the environment, urge the need to initiate the next green revolution based on sustainable and resilient agricultural practices (Finigan, 2018). This essential change will have to rely on renewable practices based mainly on the plant itself in relation with its close allies, namely the soil, the atmosphere, the rhizosphere, and the microbiome to increase yield with a concomitant improvement of the agroecosystem. Perennial forage legumes are well known for their ecosystemic services to the environment. With their nitrogen (N) fixation and N soil storage capacities, they reduce GHG emissions, increase carbon sequestration, and improve soil fertility and soil health (reviewed in Lazali and Devron, 2023).

Alfalfa (*Medicago sativa* L.) is the most important forage crop species and the fourth largest crop by area in Canada with 3 million hectares of pure stands or alfalfa-based mixtures (Statistics Canada 2022). The ability of alfalfa to establish a symbiosis with the N fixing rhizobium partner *Sinorhizobium (Ensifer) meliloti* reduces the need for N fertilization of this crop as well as for subsequent crops making it a key contributor to the current efforts to reduce agriculture reliance on fossil fuels (Cummings, 2005). The addition of alfalfa in rotations has reduced N_2O emissions by 15-25% and N fixation may replace up to 20% of fertilizer-N in agriculture as alfalfa leaves an N credit in the soil for the subsequent crops reducing the amount of fertilizer-N required in the following years due to a legacy effect (Sheaffer and Seguin, 2003). Among all perennial legume

species used in Canada, alfalfa has the highest N fixation rate reaching up to 386 kg/ha/year (Issah et al., 2020). This level can be further increased by using improved rhizobia strains, particularly those adapted to cold soils that could initiate symbiosis and N fixation early in the spring thus increasing the competitiveness of alfalfa against weeds, enhancing annual yield and, consequently, the economic sustainability of the beef and dairy sectors that depends on forage yield.

Crop production is intrinsically linked with the environment and is dependent on weather and climate. In return, crop production and agricultural management have an impact on the environment by inducing changes in biological, chemical and physical interactions between the soil and the atmosphere. In northern latitudes, winter survival, field persistence and yield of alfalfa depend on its ability to tolerate low freezing temperatures (Bélanger et al., 2006; Seppänen et al., 2018). Global climate models show growing agreement within the scientific community that the future Canadian climate will be warmer with a high incidence on winter temperature and precipitations (Derksen et al., 2018; Bush et al., 2019). Higher temperatures in the fall can interfere with the cold acclimation process and cause a decrease of the insulating snow cover exposing the belowground parts of the plant to low freezing temperatures causing winter injuries and mortality (Bertrand et al., 2003; Ambroise et al., 2020). Thus, the improvement of freezing tolerance through breeding remains critical to increase the persistence of this species under current and future climatic conditions in northern regions (Castonguay et al., 2006; Bertrand and Castonguay 2013). The large reservoir of genetic diversity in alfalfa allowed the development of a breeding method by recurrent selection consisting in a cyclical repeated exposure of a large number of genotypes to a freezing stress. The method successfully improved the freezing tolerance and the field persistence of alfalfa (Castonguay et al., 2009). Research efforts toward the selection of high-performing rhizobia strains in terms of nodulation and N fixation in cold soils is now needed to reveal the real potential of the new freezing-tolerant alfalfa germplasms that has been developed.

The selection of low-temperature efficient strains is all the more important as multiple studies have shown that the symbiosis between the legume-host and rhizobia can have a positive effect on plant persistence under stressed conditions by improving tolerance to various abiotic stresses such as drought, high temperatures, salinity and non-optimal soil pH (Alexandre and Oliveira, 2011; Staudinger et al., 2016; Song et al., 2017; Bertrand et al., 2020a, Knežević et al., 2022). A study based on 4500 alfalfa plants highlighted the important role played by the rhizobial symbiosis in low temperatures stress tolerance (Liu et al., 2019). The authors have shown that under subfreezing temperatures (-6 °C) more plants with active nodule survived and showed less

cell membrane damage than those without nodules or with inactive nodules (Liu et al., 2019). Those results suggest that root nodules provide more benefits to the legumes-host than those linked with supplying N through biological fixation, such as increases in winter survival and spring regrowth. However, the effects of the overwintering period on the survival or senescence of nodules is very little documented and more research is needed to better understand their role in the persistence of the plant. The formation of nodules and the transfer of carbohydrates to the bacteroids are very energy-intensive and the host-plant has to exert a tight control on the number of nodules as well as on resources allocation to nodules, depending on resources availability and environmental conditions (Oldroyd et al., 2011; Friel and Friesen 2019). As a counterpart, the level of tolerance of the rhizobial partner can also exert a control through its sink strength for metabolites to maintain nodule activity under stressed conditions (Bertrand et al., 2015; 2016; Marquez-Garcia et al., 2015). For instance, the use of stress-adapted rhizobial strains has been shown to improve survival and yield performance of alfalfa (Prévost and Bromfield, 2003; Bertrand et al., 2007b; 2015; 2016) and other legumes crops (Prévost et al., 2010; Bertrand et al., 2011; D'Amours et al., 2023) Therefore, the choice of freezing tolerant *Sinorhizobium meliloti* strain in association with a freezing tolerant alfalfa population could present synergistic positive effects on the winter survival and spring regrowth.

The establishment of the symbiosis is initiated by the exudation by the roots of the host-plant of various organics compounds and secondary metabolites including flavonoids (Oldroyd et al., 2011). Those signaling molecules play an essential role in the legume-rhizobia symbiosis as chemoattractant and *nod* gene inducers (Gifford et al., 2018; Poole et al., 2018). Only few and specific flavonoids excreted by the legume- host will activate the expression of a group of bacterial *nod* genes, leading to the synthesis of the Nod factors (lipochitooligosaccharides) by the bacteria. Nod factors are essential for initiating nodules formation and to maintain symbiotic activity (Reviewed in Liu and Murray 2016). Environmental stresses induce molecular changes in the host-plant that can affect the signaling cascade that lead to an effective symbiosis. The rhizobia partner is also affected by various stresses simultaneously occurring from soil conditions, the rhizosphere, and the plant leading to modifications in the production of biochemical compounds or to changes in their lifestyle allowing to survive and to establish a functional symbiosis (Lira et al., 2015; Hawkins and Oresnik, 2022). Studies on various legumes crops have shown that cold temperatures can delay nodule formation and the efficiency of the biological N fixation (Jones and Tisdale, 1921; Zhang et al.,1995; Prévost et al., 2003; Lira et al., 2005; Alexandre and Oliveira 2013;

Thurston et al., 2022). Indeed, cold temperatures has been shown to decrease lipochitooligosaccharides production by rhizobia while these Nod factors are essential for the infection process (Zhang et al., 1996; Duzan et al., 2006). The allocation of metabolites between host-plants and nodules also depends on the symbiotic rhizobial strains (Marquez-Garcia et al., 2015; Bertrand et al., 2016; Khambani et al., 2023) but the mechanisms regulating alfalfa/strains interrelation under cold stress remain to be elucidated.

While the ability of a rhizobial strain to adapt to soil conditions strongly influences the nodulation and the efficiency to fix N, its ability to compete with native soil rhizobia under various abiotic stress is also a major selection criterion (Checcucci et al., 2017). Indeed, efficient strains are not necessarily competitive and the opposite is also true (Mendoza-Suarez et al., 2021). Commercial rhizobial inoculants can be very efficient but in specific pedoclimatic conditions or under environmental stresses these selected strains could be overtaken by highly competitive but low-efficient native strains that will occupy a significant portion of the nodule thereby reducing the positive impacts of inoculation and their benefits on host-plants growth (Denieson and Kier 2004; Ji et al., 2017; diCenzo et al., 2018; Mwenda et al., 2023; Rahman et al., 2023). Thus, strain selection scheme should take into account both the efficiency of the strain in terms of nodulation and N fixation under regional climatic conditions, and the competitiveness of the strain in local soils containing natives strains (Irisarri et al., 2019). Moreover, nodules occupancy by different strains seems to be also driven by host genotype suggesting a co-evolution in both symbiotic partner or changes in the molecular profiles of roots exudates resulting in disparities in rhizobial roots colonization (Vargas and Graham 1989; Boivin et al., 2020; Burghardt et al., 2022).

The symbiosis between legumes and rhizobia is well documented but important knowledge gaps still remain. Interactions between plant genotypes, rhizobia strains, and stressful environments could change the symbiotic efficiency and the synergetic benefits on plants tolerance. The profound consequences of climate change on agriculture, highlights the need to implement sustainable and resilient practices that will bring to play the plant and its beneficial surroundings. More studies are needed to better understand the molecular mechanisms driven by those interactions to maximize symbiotic success and performance under abiotic stress conditions.

1.2 Objectives and Hypotheses

The identification of associations between rhizobial strains and alfalfa populations that are highly performant under cold conditions would increase alfalfa yield in Canada. Major Canadian agricultural sectors such as the beef and dairy industries would benefit from these findings as well the Canadian public increasingly concerned by the environmental footprint of these productions.

Main Objective: 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 study the underlying mechanisms to improve alfalfa production under low temperatures.

1.2.1 Chapter 3

Freeze-thaw episodes are very damaging, compromising the persistence of alfalfa and are predicted to occur more often under climate change. The goal of this chapter was to identify the most performant strains/alfalfa associations by testing two alfalfa populations [initial cultivar Apica (A-TF0) and a population obtained after seven cycles of recurrent selection for freezing tolerance (A-TF7)] in association with five cold-tolerant rhizobial strains and a commercial strain. The performance of the associations was based on alfalfa regrowth after exposure to low sub-freezing temperature.

Hypothesis

There will be an additive positive effect on the biomass regrowth after a freezing stress when combining a freezing-tolerant alfalfa population (A-TF7) to strains of *Sinorhizobium meliloti* selected for growth at low temperatures compared to the same strains combined with the initial cultivar Apica (A-TF0).

1.2.2 Chapter 4

Climate change will increase the risks of freezing damages in alfalfa due to sub-optimal cold acclimation conditions in the fall and untimely plant deacclimation in the spring. In this chapter the two most contrasted alfalfa/strain associations in their responses to freezing identified in the previous chapter were exposed to simulated fall, winter and spring conditions. Metabolites

and genes expression were analyzed in plants, nodules and root exudates to understand the contribution of each partner in the adaptive response to acclimation and deacclimation as well as in the crosstalk between the plant and rhizobia in cold soil. We wanted to explore how the symbiosis is affected by the cold acclimation and deacclimation of the plant, and if some association could improve alfalfa regrowth potential after a freezing stress.

Hypothesis

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.

1.2.3 Chapter 5

Selection of strains that induce abundant nodulation while having a high N fixation capacity in cold soils is important for alfalfa to benefit from an extended growing season. However, to be used for alfalfa inoculation, selected strains also have to be competitive to avoid a situation where low-efficient but competitive native strains already present in soil occupy a significant portion of the nodule thereby reducing the impact of the inoculant strain on the host plant. The first objective of this chapter was to identify *Sinorhizobium meliloti* strains able to initiate nodulation early and showing good efficiency to fix N under cold temperatures. The second objective was to assess the competitive ability of the selected strains in two soils originating from southern and northern Québec containing natives strains. The experiments also allowed to compare nodule occupancy between initial cultivar A-TF0 and freezing-tolerant population A-TF7 and to relate it with yield performance of both alfalfa populations.

Hypotheses

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 2: Literature Review

2.1 Importance of Alfalfa

Forage crops including pastures and cultivated forages represent Canada's largest crop by production volume (Bonnefield, 2016). Alfalfa (*Medicago sativa* L.), a perennial legume crop, is the most important forage species and the fourth largest crop by area in Canada representing approximately three million hectares of alfalfa pure stand or alfalfa-based mixtures (Statistics Canada, 2022). Considered to be the "queen of forages" alfalfa is cultivated worldwide for its high-quality forage (Li and Brummer, 2012) and its ability to establish a symbiosis with nitrogen (N)-fixing rhizobia. Alfalfa can improve environmental health and soil conservation by providing carbon (Angers et al., 1992) and N (Harris and Hester, 1990) to soils. Studies have estimated that the amount of N fixed annually by alfalfa varies between 62 and 462 kg N ha^{-1}, this large variability resulting from the diversity in pedoclimatic conditions between the studies and the methodology used (e.g., Yang et al., 2010; Nimmo et al., 2013; Anglade et al., 2015). As a perennial crop cultivated in northern environments, it is critical for alfalfa to establish a symbiosis with a strain that is adapted to cold conditions. The bacteria released from senescent nodules during overwintering needs to have a high capacity to re-establish the symbiotic relationship with their host in the spring through crosstalk or other mechanisms. Furthermore, the establishment of an early symbiosis in cold soils in the spring that continues to be efficient in the fall will allow alfalfa to benefit from an extended growing season and potentially from an additional cut and higher yields (Thivierge et al., 2017). Finally, adapted rhizobial strains have been shown to directly affect alfalfa physiology and enhance its tolerance to freezing stress (Bertrand et al., 2007b).

2.1.1 Challenges in alfalfa production

Alfalfa being cultivated all over the world, several studies have reviewed the negative effects of environmental stresses, such as extreme temperatures, drought, elevated CO_2 levels, and soil salinity on yield, nutritive value, and persistence of alfalfa grown in different areas of the world (Kulkarni et al., 2018). In Canada, harsh winter conditions are the principal threats limiting the persistence of alfalfa. Winter in Western Canada is characterized by dry conditions and shallow snow covers while Eastern Canada has a wet and cold climate, variable snow covers and high

incidence of freezing rain during the winter. In addition to low sub-freezing temperatures, factors affecting winter survival of alfalfa vary across the country with higher incidence of freezing dessication in the west and ice encasement in the east. As a perennial, alfalfa survives winter because of its capacity to profoundly change its physiology in response to declining temperature and photoperiod in the fall through a process called cold acclimation. Afterward, alfalfa remains dormant and freezing tolerant throughout winter followed by deacclimation and regrowth in the spring (Li et al., 2022). While the cold acclimation process has been extensively studied in alfalfa, there is a knowledge gap regarding the deacclimation process although increasing evidences shows that spring temperatures that determine deacclimation are as important as cold acclimation conditions in the fall for shaping the cold-range limits of a plant species (Rapacz et al., 2017). Deacclimation occurs rapidly upon the return to warm temperatures in the spring and a premature transition involves that plants are more susceptible to damage by freeze–thaw cycles (Kalberer et al., 2006). However, the single most important factor favoring winter survival of alfalfa across Canada is the level of freezing tolerance that the plant can achieve during winter (Castonguay et al., 2009). To develop alfalfa germplasm with improved freezing tolerance is even more important today because the predicted global climate change will likely be conducive to higher incidence of winterkill of perennial crops.

2.1.2 Impact of climate changes

Global climate change has an impact on terrestrial ecosystems including agricultural crop productivity. In Canada, expected increases in CO_2 concentration, warmer temperature, and longer growing season may benefit crop productivity (Thivierge et al., 2017). However, since most forages grown in Canada are perennials, these crops are strongly affected by both summer and winter climatic changes. Whereas the extended growing season could increase yield, higher fall temperature will likely delay the process of cold acclimation and, as such, will increase the risk of winter damages and jeopardise forage crops persistence (Bertrand, 2012). Warmer temperatures in the fall are sub-optimal for plant cold acclimation while freeze-thaw events during overwintering can expose the plants to low sub-freezing temperatures (Vyse et al., 2019). Higher incidence of warm spells above 0°C causing plant deacclimation and loss of freezing tolerance followed by extreme cold are likely to increase in the future and to cause severe damages in alfalfa during both winter and spring (Bertrand, 2012). Freeze-thaw episodes are very damaging because

deacclimation is a rapid process compared to cold acclimation (Vyse et al., 2019; Xu et al., 2020; Li et al., 2022). Since perennials will likely be exposed to more frequent and larger temperature fluctuations in springtime due to climate change (Pagter and Arora, 2013), a better understanding of the deacclimation process is needed (Adhikari et al., 2022). Two recent reports examined the mechanisms of cold deacclimation in annual winter cereals and concluded that deacclimation tolerance is genetically different from cold-acclimation capacity (Horvath et al., 2020; Wójcik-Jagła et al., 2021) and that different mechanisms are involved in cold acclimation and deacclimation. This emerging knowledge on the mechanisms and genetic control of deacclimation tolerance will be crucial for breeding plants that are better adapted to the changing climate (Li et al., 2022; Rapacz et al., 2022; Vaitkevičiūtė et al., 2022). Altogether, winter survival of perennials forage crops will likely be compromised by inadequate snow cover during the winter, and/or ice encasement of plants and anoxia damages stresses, that are predicted to occur more often under climate change (Castonguay et al., 2006). Research is needed to better understand the adaptation of perennial crops to stresses associated with climate change to ensure their long-term persistence (Bertrand, 2012).

2.2 Mechanism of cold acclimation, freezing tolerance and deacclimation in alfalfa

As already mentioned, winter survival is a major factor affecting the persistence of perennial crops such as alfalfa and it will become a major issue with climate change (Bélanger et al., 2002). When freezing damage occurs, it is mainly the result of osmotic dehydration triggered by extracellular ice crystallization that leads to the diffusion of water from the cells to the growing ice crystals (Castonguay et al., 2006). Cold acclimation is a relatively long-time process involving a cascade of molecular events to evoke the adaptive responses. Plants need to perceive the environmental signals (mainly low temperature and short days), to transduce the signals to activate or repress the expression of cold-related (COR) genes, and to translate these transcriptomic changes into biochemical and ultrastructural modifications that will confer superior tolerance to freezing at the cellular level (Smallwood and Bowles, 2002; Kaur and Gupta, 2005). Cold deacclimation on the other hand, occurs promptly with the increase of temperatures causing loss of plant hardiness and thereby initiating plant regrowth (Vyse et al., 2019).

2.2.1 Biochemical and molecular changes

In general, the concentration of soluble sugars in perennial tissues, such as alfalfa crowns, rises during autumn/cold acclimation and decreases during spring/deacclimation, while starch content shows the opposite behavior (Dhont et al., 2002; Poirier et al., 2010; Pagter et al., 2011; Lee et al., 2012; Shin et al., 2015; Andersen et al., 2017). Thus the mobilization of soluble sugars from storage carbohydrates to achieve maximum freezing tolerance and re-mobilisation of carbohydrate reserves to support spring regrowth seems essential. Specifically, a study on the biochemical changes occurring in overwintering alfalfa showed a rapid decline of starch concentration in crowns during the first weeks of acclimation concomitant with a marked accumulation of sucrose concentration (Castonguay et al., 2011). The accumulation of the sucrosyl-galactosides raffinose and stachyose was, however, the most striking biochemical changes observed in overwintering alfalfa crowns. The increase in concentrations of these two sugars having cryoprotective properties reached their peaks in January when alfalfa achieved its maximum level of freezing tolerance. Proline, asparagine and arginine known to intervene in cold acclimation also reached their peak concentration in January when alfalfa was the most freezing tolerant (Castonguay et al., 2011). At low temperatures, changes in gene expression can be observed and, up to now, a great diversity of COR genes have been isolated and characterized including the up regulation of galactinol synthase and dehydrin and the down regulation of sucrose synthase (Castonguay et al., 2011). It has been shown that the kinetics of deacclimation is rapid, with most changes already taking place during the first 24 h, with the transcriptome responding faster to the increase in temperature than the metabolome (Pagter et al., 2013). Deacclimation is characterized by a reduction of protective metabolites compounds such as carbohydrates, amino acids, and proteins accumulated during cold acclimation which increased susceptibility of plants to subfreezing temperatures (Svenning et al., 1997; Aurora et al., 2004; Pagter et al., 2011; Hoffman et al., 2014). Deacclimation has also been associated with a decrease in abundance of specific stress-related proteins including dehydrins (Aurora et al., 2004).

2.2.2 Breeding for freezing tolerance in alfalfa

Alfalfa is a cross pollinated, polyploid, heterozygous species, in which every plant is genetically different. As such, there is a large genetic diversity in alfalfa which opens the door to

genetic improvement while in the same time the complexity of its genome impedes breeding for complex traits such as freezing tolerance. Field selection for freezing tolerance represent a long and costly process due to the unpredictability of test winters. To avoid repeating field selection during many years at multiple sites, a recurrent selection method performed under controlled conditions has been developed (Castonguay et al., 2009; Bertrand et al., 2014). Recurrent selection is a cyclical breeding method involving repeated exposures of a large number of genotypes to a given stress followed by the selection of genotypes showing tolerance to the stress. The recurrent selection method for the improvement of freezing tolerance in alfalfa, entirely performed indoors, exploits the large reservoir of genetic diversity in alfalfa by boosting the number of functional alleles responsible for a better survival under a repeated exposure to low freezing temperatures (Castonguay et al., 2009; Bertrand et al., 2020b). The method improved the tolerance to freezing stress from a lethal temperature of -27 °C in initial cultivar Apica (A-TF0) to around -31° C in A-TF6 obtained after six cycles of selection within Apica (Castonguay et al., 2011; Bertrand and Castonguay 2013). Biochemical compounds responsible for alfalfa freezing tolerance also responded to recurrent selection and, in the heart of winter, when plants were at their maximum freezing tolerance, sucrose, raffinose and stachyose, all contributing to stabilize membrane of frost-induced dessicated cells, were more abundant in A-TF6 than in A-TF0. The concentration of total free amino acids and, particularly of asparagine and arginine were also higher in A-TF6 than in ATF-0, confirming the involvement of these compounds in the acquisition of freezing tolerance of alfalfa. Comparison of polymorphism of DNA fragments between A-TF0 and A-TF6 confirms that recurrent selection exerts its action at the genome level and that dehydrin variants are one of the target of the selection conferring a significant increase in freezing tolerance in populations preferentially enriched in the polymorphic dehydrin (Rémus-Borel et al., 2010).

2.3 Symbiosis with rhizobia bacteria

Since plants first colonized terrestrial habitats, they have engaged in intricate interactions with a diverse community of microbes known as the phytomicrobiome (Hassani et al.2018). These relationships vary widely, from surface-dwelling phyllosphere and rhizosphere microbes to endophytes nestled within plant tissues, and even extend to the endosymbiosis of microbes within

plant cells, giving rise to mitochondria and chloroplasts (Smith et al. 2015a). While plants having developed their own mechanisms to mitigate various biotic and abiotic stresses in natural environments, they also depend on their microbial partners for growth, survival and protection against microbial invaders (Enebe and Babalola, 2018; Lyu et al. 2020). Indeed, the phytomicrobiome plays crucial roles in plant growth and survival like nutrient acquisition, biotic and abiotic stress tolerance, physiology regulation through molecular dialog, and growth regulation via the production of phytohormones (Hassani et al. 2018; Lyu et al. 2021).

The multicellular host plant and its phytomicrobiome together are referred to as a "holobiont.". Environmental conditions can exert selective pressure on the components of a plant holobiont, influence the composition, diversity, and functions of the microbial community, ultimately impacting the overall fitness and adaptability of the plant to its environment (Vandenkoornhuyse et al. 2015; Lyu et al. 2020; Trivedi et al 2022; Khan, 2023). The relationships between the host plant and its associated phytomicrobiome are bidirectional. When these relationships provide metabolic benefits for both partners, they form a mutualistic association (Smith et al. 2015a; Mesny et al. 2023). The symbiotic association between rhizobia and legumes is a crucial and well-studied example of plant-microbe mutualism (Han et al. 2020). Despite its importance, the establishment of this association is complex and can be influenced by various factors such as plant genotype, compatibility between rhizobial strains, microbial competition, pedoclimatic conditions, and biotic and abiotic stresses.

Sinorhizobium (now *Ensifer*) *meliloti* is a gram-negative soil bacteria that have the ability to form symbiotic associations with its specific host plants, including alfalfa, and fix atmospheric N_2 in a form available to the plant. The success of rhizobia to inhabit ecologically diverse niches is partly due to their complex lifestyles that involve growth and survival in bulk soil, plant rhizospheres, legume infection threads, and mature and senescing legume nodules (Burghardt and diCenzo, 2023). Thus, rhizobia must survive in soil, colonize roots and gain entry to the plant. Rhizobia are attracted towards legume roots by the exudation of chemoattractants metabolites (Poole et al., 2018) and, once they are in close proximity to root hairs, flavonoids are the plant signal molecules responsible for the induction of nodulation *nod* genes in rhizobia (Gifford et al., 2018). Legumes produce many flavonoids but only a few are present in root exudates, and even fewer have been reported to be symbiotically induced (Liu and Murray, 2016). Alfalfa secretes metabolite compounds and flavonoids that attract the bacteria toward its roots and activate the

transcription of *S. meliloti* genes responsible for the production of a lipochitooligosaccharide signal molecule known as Nod factor (Jones et al., 2007; Compton et al., 2020). Nod factors, synthesized by proteins encoded by *nodABC* rhizobial genes, induce an internal signaling cascade in the roots of the legume host and are essential for the infection process in alfalfa (Igolkina et al., 2019). Nod factor initiate the preparation of epidermal root cells to the rhizobial entry and induce the curling of root hairs and the formation of nodules (Buhian and Bensmihen, 2018). Moreover, applied research investigated Nod factor effects on legume growth and stress tolerance responses under controlled conditions and field experiments (Smith et al. 2015b). Formulations of Nod factor produced by rhizobium associated with soybean, *Bradyrhizobium japonicum* applied to seeds have shown to enhances germination of soybean (*Glycine max*), beans (*Phaseolus vulgaris*) and even of other important agronomic nonlegumes crops like sugar beets beets (*Beta vulgaris*), rice (*Oryza sativa*), and corn (*Zea mays*), and cotton (*Gossypium hirsutum*) (Prithiviraj et al., 2003; Smith et al., 2005; Smith et al. 2015b). Nod factor application have also shown beneficial effects on legumes growth under some stressful conditions such as low temperature, acidity, water deficit, soil compaction and salt stress (Duzan et al., 2004; Siczek et al., 2013; Siczek et al., 2014; Prudent et al., 2016; Nandhini et al., 2018; Siczek et al. 2020). However, the host-specificity of Nod factor ensures that the rhizobia can only effectively colonize the roots of compatible host plants that ultimately lead to the formation of nodules. This specificity is crucial for the establishment of successful symbiotic relationships between the bacteria and the plants (Sharma et al. 1993).

Compatible rhizobia induce changes in the flavonoid profile of the host plant. The presence of *S. meliloti* around alfalfa roots alters the composition of root exudates by increasing the amount of *nod* gene-inducing activity (Dakora et al., 1993). Luteolin, a flavone present in seed exudates of alfalfa, induces *nod* genes transcription in its rhizobial partner (Peters et al., 1986; Hartwig et al., 1990). However, Liu and Murray (2016) reported that, methoxychalcone, which is produced by the host-specific enzyme CHALCONE-O-METHYLTRANSFERASE (ChOMT), is the strongest *nod* genes inducer identified in alfalfa root exudates. Gifford et al., (2018) have shown that isoflavonoids, including formononetin, medicarpin as well as coumestrol and prunetin were highly abundant in alfalfa nodules. This “molecular dialog” between legume hosts and their microsymbionts during the initiation of nodule formation and during all the plant life underlines the specificity of the symbiosis and the complexity of the mechanisms involved.

It is well known that the inoculation of alfalfa with *S. meliloti* is highly effective to increase alfalfa yield and it has been shown that the symbiotic efficiency to increase yield depends on the interaction between both the alfalfa genotype and the *S. meliloti* strain (Prévost et al., 1999). As such, the specificity of plant-bacteria interactions seems to be an important parameter for the parallel selection of the partners for an improved symbiotic efficiency (Provorov et al., 1994). In addition, the interaction of these symbionts depends on the pedoclimatic factors and the environmental conditions (Jones and Tisdale, 1921; Bordeleau and Prévost, 1994; Lira et al., 2015). Stresses, including low temperatures not only negatively impacts alfalfa growth and productivity, but also impacts its rhizobial partner and hinders the establishment of the rhizobia-legume interaction as well as its efficiency (Prévost et al., 2003). This is particularly important for rhizobia associated with perennial alfalfa under conditions prevailing in northern environments since a proportion of nodules could be killed during winter, releasing bacteria in the soil that will need to reinitiate the crosstalk with the host plant in the next spring.

2.4 Effects of abiotic stresses on the symbiosis between alfalfa and rizobial strains

Multiple studies have shown that the symbiosis between legumes and rhizobia could modify plant physiology and improve host-legume tolerance to various abiotic stresses such as drought (Staudinger et al., 2016), high temperatures (Khambani et al., 2023), salinity (Bertrand et al., 2020a), alkalinity (Song et al., 2017), acidity (Knežević et al., 2022) and salinity combined to heavy metal (Pacheco-Inausti et al., 2023) thus contributing to their persistence under stressed conditions. While the impact of abiotic stresses on the symbiotic performance have been extensively studied in the last years with emphasis on salinity (22%), heavy metals (18%) and drought (10%) stresses, it was reported that studies focusing of the impact of extreme temperature stresses (cold and heat) on the symbiosis efficiency represented only 2% of the scientific literature (Ramírez and Damo, 2023).

2.4.1 Importance of the legume-rhizobia association in stress tolerance acquisition

The developmental plasticity of belowground plant parts in response to abiotic stresses is of key importance for the long-distance transport of substances to assist plant stress resilience (Li

et al., 2021). As mentioned earlier, legume-rhizobia symbiosis play an important role to improve plants tolerance to various abiotic stresses (Staudinger et al., 2016; Song et al., 2017; Bertrand et al., 2020a; Sindhu et al., 2020) and for low temperatures stresses in particular (Liu et al., 2019; Yuan et al., 2020; Irshad et al., 2021). Legume host modulates resource allocation to nodules depending on resources availability and environmental conditions (Friel and Friesen, 2019) an on the benefit the host is receiving from their symbiotic partners and their efficiency (Regus et al., 2017; Westhoek et al., 2021). On the other hand, the rhizobial partner also exert a control through its sink strength for metabolites to maintain nodule activity (Bertrand et al., 2015; 2016; Marquez-Garcia et al., 2015; Khambani et al., 2023; Pacheco-Inausti et al., 2023). Indeed, the tolerance of some associations between alfalfa host and stress tolerant *S. meliloti* strain were reported (Sanz-Sáez et al., 2012; Bertrand et al., 2015). It has been demonstrated that inoculation with *S. meliloti* strains could modulate the response of alfalfa to high CO_2 concentration and that there is a strain effect on plant physiology (Bertrand et al., 2007b). In studies comparing two alfalfa cultivars differing in their levels of salt tolerance in association with two *S. meliloti* strains with contrasting growth under salt stress, it was shown that the strain exerts a significant effect on the ability of alfalfa to grow and survive under saline conditions and on the total concentration of free amino acids in nodules. Combining both salt-tolerant strain and cultivar was even more efficient to enhance alfalfa salt tolerance (Bertrand et al., 2015; Bertrand et al., 2016). Similar results were obtained with alfalfa populations recurrently-selected for salinity tolerance as they were shown to have higher nodulation index and shoot biomass when combined with a salt-tolerant rhizobial strain, confirming an additive positive effect with a combination of both stress-tolerant partners, resulting in a more efficient symbiosis (Bertrand et al., 2020a). Similar results have been also reported in other legumes-rhizobia associations species exposed to different abiotic stress. When comparing three *Bradyrhizobium* strains in association with soybean growing under different levels of atmospheric CO_2, the most efficient strain had the highest ureides concentration in nodules under elevated CO_2 and the highest nitrogenase activity as well as higher plant yield (Bertrand et al., 2011). This relationship indicated that soybean mobilized energy reserves to increase the activity of nodules colonized with an efficient strain while in return the symbiont synthesized more ureides to support plant growth. Moreover, other recent studies have shown promising results by inoculating drought tolerant rhizobia strains in association with chickpea (Istanbuli et al., 2022), soybean (Igiehon et al., 2019; Aserse et al., 2020), milkvetch (Liu et al.,

2022), pea (Álvarez-Aragón et al., 2023), faba bean (Li et al., 2023), common bean (Del-Canto et al., 2023), and alfalfa (Defez et al., 2017). A robust study based on 4500 alfalfa plants have highlighted the important role that the rhizobial symbiosis exert on low temperature stress tolerance mechanisms (Liu et al., 2019). Authors have shown that more plants with active nodule survived and showed less cell membrane damage than those with inactive nodules or those without nodules, under the same low temperature-stress condition (-6°C). A greater activity of oxidation protective enzymes was reported in the active nodule and inactive nodule groups as compared to plants without nodules, conferring higher tolerance to low temperature in these groups of plants. Nonetheless, while the role of the storage organs roots and crown in the winter survival of perennial plants is well documented, information on the persistence or senescence of the perennial roots nodules during winter is scarce. Altogether, those recent studies demonstrate the importance of the symbiotic relationship between the legume host and its rhizobial partner in stress tolerance acquisition and suggest that nodules could play other important roles in the legumes survival than only provide nitrogen.

2.4.2 Impact of cold temperatures on the symbiosis

2.4.2.1 Nodulation, N fixation and freezing damages

In Canada, low soil temperatures in the spring inhibit rhizobia N fixation and has been shown to be the major cause of the large delay in spring regrowth of perennials. In general, the optimal temperature for nodulation range between 25°C and 30°C while the N fixation activity of legumes is maximum between 15° and 25°C (Trinick, 1982). These temperatures are well above soil temperatures allowing plant regrowth in the spring (Jego et al., 2014). Studies on different legumes crops have shown that cold temperatures can delay nodule formation and the efficiency of the biological N fixation (Jones and Tisdale; 1921, Zhang et al., 1995; Prévost et al., 2003; Lira et al., 2005; Alexandre and Oliveira 2013; Perrone et al., 2020; Thurston et al., 2022). However, in an extensive study conducted with 226 *S. meliloti* strains, Rice et al., (1995) found that nodule formation of alfalfa could occur at temperature as low as 9°C and that strains significantly differ in their abilities to nodulate and produce N-dependant alfalfa growth with root temperatures varying between 10 and 12°C. More recently, a study had tested 60 rhizobial strains to identify the best-performing strains for sainfoin, birdsfoot trefoil, red clover, and alsike clover under low temperature (D'Amours et al., 2023). Authors reported that strains isolated from Canadian soils

were the most efficient in terms of nitrogenase activity when associated with cultivars developed in Canada hinting at a co-evolution of the symbiotic partners under the climatic pressure exerted by harsh winter conditions.

The effects of the overwintering period on the survival or senescence of nodules is not well understood. The overwintering of nodules has previously been reported for perennial legumes of temperate regions (Bergersen et al., 1963; Bal and Khetmalas 1996). The early report of Pate (1961) illustrates precisely the vascular tissue persisting in older portions of the nodule, although all other inner tissues show necrosis. The overwintering of nodules is an important adaptation as it saves the energy-demanding steps of re-establishing a crosstalk between roots and free-living rhizobia in soils. While the studies reported above compared the nodule overwintering in different plant species, it could be inferred that rhizobia strains colonizing nodules could also differ in their freezing tolerance and provide additional protection against freezing to these important organs. In a study looking at the effects of rhizobial strain and CO_2 concentrations on alfalfa freezing tolerance, Bertrand et al., (2007) observed superior freezing tolerance in alfalfa inoculated with strain A2 when compared with strain NRG34. The authors found higher cold-induced expression of three COR genes in roots of the more cold-tolerant alfalfa inoculated with strain A2 than in the less tolerant plants inoculated with strain NRG34 and concluded that rhizobial strain could directly modulate the expression of COR genes coding, among others, for a pathogenesis-related PR-10 protein with antifreeze properties. However, other study have shown that strain NRG34 is cross-adapted to various abiotic stresses such as low temperatures and oxygen deficiency as shown by a greater regrowth and nodulation index of alfalfa inoculated with this strain as compared to two other strains (A2 and Rm1521) under simulated winter conditions (Prévost et al., 2003).

2.4.2.2. Crosstalk between legumes and rhizobia

Soil temperature also influence the interaction between plants and symbionts. The crosstalk between the symbiotic partners is initiated by the exudations of metabolites from plant roots including secondary metabolites such as flavonoids. Abiotic stresses affect the pattern of flavonoids exuded by legumes roots (Dardanelli et al., 2010; Chai and Schachtman, 2022) and induce molecular changes in rhizobia (Janczarek et al., 2015). Low temperatures have been shown to modulates the transcription of genes related to flavonoid and phenylpropanoid biosynthesis (Calzadilla et al., 2016; Xu et al., 2020) causing changes in *nod* genes activity in the symbiont

(Zhang et al., 1996; Zhang et al., 2009). Moreover, cold stress has been shown to decrease lipochitooligosaccharides production by rhizobia while these compounds trigger the symbiosis signaling pathway by being recognized by specific LysM receptor kinase proteins in plant root cells before initiating the formation of nodules (Zhang et al., 1996; Duzan et al., 2006). Thus, cold stress modifies the biochemistry of the plant that could have an impact in the composition of roots exudates and on the ability of rhizobia to grow, colonize the roots systems and survive in nodules. However, little is known on the impact of cold stress on the interaction between the production of flavonoids by legume roots and the molecular responses of its symbiotic partner. As the crosstalk is initiated by the plant, Kapulnik et al., (1987) compared alfalfa populations produced by two cycles of selection for increased N fixation and growth and found that flavonoid extracts from seedling roots contained a 60 % higher concentration of compounds inducing transcription of *nod* genes in *S. meliloti* than comparable extracts of the initial alfalfa population. When comparing recurrently selected populations after 5 and 6 cycles to initial alfalfa Apica TF0 populations, Bertrand and Castonguay (2013) measured higher concentrations of cryoprotective sugars (i.e., sucrose, stachyose, and raffinose) in more freezing-tolerant populations Apica TF5 and TF6 showing that recurrent selection has an impact on the plant biochemistry. Those biochemicals changes could have and indirect impact on the composition of root exudates that also comprise sugars, organic and amino acids that can serve and chemoattractant or be used directly by the rhizobia to sustain their metabolism and thus influence the ability of rhizobia to colonize roots of the legumes host (Bringhurst et al., 2001; Webb et al., 2016). Recent progress has been made in the understanding of the interaction between flavonoids and microbes (Kumar et al., 2024), however, research is still needed to better understand how the flavonoids levels are regulated in roots (Liu and Murray, 2016). For instance, soil N concentration modulates the level of *nod* gene-inducing flavonoids in legumes and influences the nitrogenase activity (Murray et al., 2017). The production of flavonoids in plant tissues may be stimulated by high C content (Solfanelli et al., 2006). As flavonoids are critical for rhizobia infection, the regulation of C and N transfer between host plant and rhizobia appears to be a key mechanism to regulate nodulation (Ailin et al., 2018).

Altogether, selection of cold-adapted rhizobia in association with cold-hardy host plants constitutes a valuable approach to counteract the negative effects of winter stress on legume productivity. Studies of associations between legume and rhizobia partners of contrasted tolerance are necessary to better understand the independent role of each partner in stress tolerance

acquisition. There is a strong interest to investigate the potential effect of low temperatures on the molecular dialog between the legume-host and the rhizobia partner as well as on root exudates composition and its repercussion on the symbiosis with alfalfa under stressful environment.

2.4.3 Competitivity of the strain, the factor not to ignore

There is a lot of diversity in rhizobia genetic and taxonomic characteristics and even a same rhizobia specie show diverse metabolic profiling and strategies to survive as free-living bacteria in soil and to become a symbiont (Masson-Boivin et al., 2009; Lira et al., 2015; Bellabarba et al., 2021; Boivin et al., 2021; Westhoek et al., 2021; Hawkins and Oresnik, 2022; Khambani et al., 2023). Native populations of *Shinorizobium meliloti* are frequent in agricultural soil (Bromfield et al., 1986; Carelli et al., 2000) and they are well adapted to the pedoclimatic conditions. When native strains are not efficient to fix N, they could became parasitic rhizobia for the host plant (Morel Revetria et al., 2023). The ability of a rhizobial strain to compete with native soil rhizobia under various abiotic stress is thus very important in strain selection (Batista et al., 2015; Checcucci et al., 2017). Most of the studies showing high efficiency of a specific rhizobial strain with benefits on legume yield are conducted in sterilized conditions with results varying depending of the substrate that is used (Ji et al., 2017). Moerever, efficient strains are not necessarily competitive and highly-competitive strain can be non-efficient (Mendoza-Suarez et al., 2020, 2021). Indeed, commercial inoculant can be very efficient under specific growth conditions or show high tolerance to specific environmental stresses but be outcompeted by low-efficient native strains under different soil conditions. These low-efficient strains could then occupy a significant portion of the nodule thereby reducing the positive impacts of inoculation and the benefits on host-plants growth (Denison and Kier 2004; Ji et al., 2017; diCenzo et al., 2018; Allito et al., 2020; Mwenda et al., 2023; Rahman et al., 2023).

Competition for nodule occupancy is associated with various rhizobia traits including the ability to communicate with the host plant effectively, the chemotaxis and movement toward seed and root exudates, the production of bacteriocins or other toxins targeting other rhizobia, the ability to catabolize various carbon sources, and the stress tolerance (reviewed in Burghardt and diCenzo 2023). A field-study conducted in Northwestern Canada showed that the root environment has a strong effect on the competitive ability of rhizobia to initiate nodulation at low temperatures (Rice et al., 1995). A comprehensive strain selection scheme should take into account both the efficiency

of the strain in terms of nodulation and N fixation under regional climatic conditions and the competitiveness of the strain in local soils containing natives strains (Irisarri et al., 2019).

Interaction between rhizobium-legume are complex and while competitive ability of a strain is modulated by the environment, it seems to be also driven by the host genotype suggesting a co-evolution in both symbiotic partners or changes in the molecular profiles of the roots exudates resulting of disparities in rhizobia roots colonization (Vargas and Graham, 1989; Boivin et al., 2020; Burghardt et al., 2022). A recent research studying a combination of three *S. meliloti* strains and three alfalfa varieties showed that the strain response to root exudates from the three different cultivars involved hundreds of changes in the transcriptomic response of the *S.meliloti* partner (Fagorzi et al., 2021). Among the differentially expressed genes, 35% were influenced by the strain genotype, 16% were influenced by the plant genotype and 29% were influenced by strain-by-host plant genotype interactions (Fagorzi et al., 2021). Recently, it has been proposed that legumes can select for effective symbionts by making conditional decisions and sanctioning non-effective nodules, resulting in a lower viability of useless rhizobia in their nodules (Westhoek et al., 2021). Plant host could differentially allocate resources to the symbionts according to their efficiency to fix N. Selection of plant genotypes exerting strict sanctions could be beneficial to reduce presence of non-effective natives strains and improve the yield of the legume host (Denison, 2021; Cangioli et al., 2022).The complex legume-rhizobium interaction is further complicated if abiotic stresses or variation in the environments are added. Interestingly, a study comparing acid-tolerant and acid-sensitive symbiotic partners have shown higher colonization by the acid-tolerant strain with the acid-sensitive bean cultivar than when combined with the acid-tolerant cultivar. The acid-tolerant rhizobia strain showed higher nodules occupancy than the acid-sensitive strain but only under low pH (Vargas and Graham, 1989). Thus, roots colonization by rhizobia and successful symbiotic associations are driven by multiple factors and more studies combining different host plant and rhizobia genotypes under different soil conditions are needed to better understand the traits link with the competitive ability of the strain.

A symbiosis that is efficient at low temperature is critical for alfalfa productivity in Canada as cold soil could delay the re-establishment of the symbiotic relationship between rhizobium and alfalfa in the spring, reduce the capacity of N fixation, and decrease the seasonal yield of alfalfa. Climate change and associated predicted warmer temperatures outside of the growing season would possibly aggravate the problem as warmer autumn reduces snow cover and increases winter

rains, thus increasing the risk of winter mortality in alfalfa (Bélanger et al., 2006). To our knowledge the synergistic response of freezing-tolerant alfalfa populations in association with low temperature-tolerant rhizobial strains has never been studied, and little is known about strain-genotype preferential associations under cold stress.

In this thesis the nodulation and growth performance of an initial alfalfa cultivar Apica (A-TF0) compared to those of freezing-tolerant Apica-TF7 (A-TF7) population obtained after seven cycle of recurrent selection, grown in association with various low temperature-tolerant rhizobial strains are assessed. This should allow for the identification of rhizobial strains and strain-population associations with superior symbiotic efficiency under cold-stress conditions. The competitive ability of the selected strains is validated by using different soils with native strains. The characterization of biochemical changes during cold acclimation and deacclimation, associated either to changes due to the plant or the strain or by both partners will allow for a better understanding of the mechanisms involved.

2.5 Connecting Text

The General Introduction and the Literature Review sections of the thesis provided a comprehensive overview of the actual issues and challenges of alfalfa production in northern environments, particularly in the context of current climate change. The overall potential to improve stress tolerance of alfalfa by the association with stress-tolerant *Sinorhizobium meliloti* strains was also thoroughly reviewed with a large emphasis on cold tolerance and on the mechanisms of cold acclimation of perennials. The main purpose of the next chapter is to investigate if the symbiosis with cold-tolerant-rhizobial strain can improve the ability of alfalfa to survive and grow after exposure to sublethal freezing temperatures. A second goal is to identify the most performant strains/alfalfa associations by comparing two alfalfa populations (initial cultivar Apica (A-TF0) and population obtained after seven cycles of recurrent selection for freezing tolerance (A-TF7) in association with five cold-adapted strains and one strain from a commercial inoculum, based on alfalfa regrowth after a simulated freeze-thaw event. An extensive phenotyping study of nodule shape, freezing damages, and distribution on the root system is also carried out to explore the influence of rhizobial strains on those traits and to better understand the link between nodules and regrowth of alfalfa in cold soil.

Chapter 3: Impact of *Sinorhizobium meliloti* strains and plant population on regrowth and nodule regeneration of alfalfa after a freezing event

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D'Amours, E., Bertrand, A., Cloutier, J., Claessens, A., Rocher, S., Seguin, P. (2022). Impact of *Sinorhizobium meliloti* strains and plant population on regrowth and nodule regeneration of alfalfa after a freezing event. *Plant and Soil*. https://doi.org/10.1007/s11104-022-05662-4

Minor modifications were made to conform to the McGill University thesis guidelines.

3.1 Abstract

Purpose

The increase in frequency of freeze-thaw episodes with the diminution of snow cover protection due to climate change compromises the winter survival of alfalfa (*Medicago sativa* L.). Symbiosis with cold-tolerant rhizobial strains can improve the ability of alfalfa to survive and grow under stressful conditions.

Methods

Six strains of *Sinorhizobium (Ensifer) meliloti* were tested in combination with two alfalfa populations bred to differ in their levels of freezing tolerance. Plants of each different combination were grown for eight weeks in a growth chamber before being exposed to temperatures promoting their acclimation to cold. Plants were then exposed to a freezing stress (-11ºC) and regrown for three weeks. Shoot, root and nodule biomass were measured before cold acclimation and three weeks after the freezing stress.

Results

After freezing stress, the alfalfa population A-TF7 had shoot and root biomasses that were respectively 19% and 15% larger than cultivar A-TF0. Alfalfa plants inoculated with strain NRG34 showed both a larger shoot biomass and a higher nodule dry weight than plants inoculated with any other strains. Assessment of freezing damages on nodules showed that plants inoculated with NRG34 had the largest proportion of undamaged nodules or of nodules with a regeneration zone.

Conclusion

This study shows for the first time a relationship between nodule and shoot regrowth after a freezing stress, the latter being linked with the proportion of nodules showing less freezing damage. Our results demonstrated that both the choice of alfalfa populations and *S. meliloti* strains adapted to stress are complementary to increasing alfalfa persistence**.**

3.2 Introduction

Alfalfa (*Medicago sativa* L.), a perennial legume crop, is the most important forage species and the fourth largest crop by area in Canada with 3 million hectares of alfalfa pure stand or alfalfa-based mixtures grown (Statistics Canada 2022). Considered to be the "queen of forages" alfalfa is cultivated worldwide for its high-quality forage (Li and Brummer 2012) and its ability to establish a symbiosis with its nitrogen fixing bacterial partner rhizobia *Sinorhizobium (Ensifer) meliloti.*

The ability to tolerate low freezing temperatures is a major component of winter survival and field persistence of alfalfa in northern latitudes (Bélanger et al., 2006). Predicted climate changes will likely increase the risks of winter injury to alfalfa in eastern Canada due to higher temperatures in autumn causing less favorable hardening conditions and the diminution of snow cover protection during winter (Bélanger et al., 2002). With the reduction of the snow cover and lack of soil insulation, belowground parts of the plant will be more exposed to freezing temperatures causing winter injuries and mortality (Ambroise et al., 2020). Therefore, the improvement of freezing tolerance will continue to be a crucial issue for the persistence of this species under current and future climatic conditions in northern regions (Bertrand et al., 2013; Castonguay et al., 2006). Recurrent selection is a cyclical breeding method involving repeated exposures of a large number of genotypes to a given stress followed by the selection of genotypes showing tolerance to the stress. The large reservoir of genetic diversity in alfalfa allows development of methods using recurrent selection performed indoors to successfully improve the freezing tolerance and field persistence of alfalfa (Castonguay et al., 2009).

Growing evidence show that the symbiosis between legumes and rhizobia could modify plant physiology and improve tolerance to various abiotic stresses such as drought (Staudinger et al., 2016), high temperatures (Alexandre and Oliveira 2011), salinity (Bertrand et al., 2020a), alkalinity (Song et al., 2017) and acidity (Knežević et al., 2022), thus contributing to their persistence under stressed conditions. A recent study based on 4500 alfalfa plants highlighted the important role played by the rhizobial symbiosis in low temperatures stress tolerance. The study reported that more plants with active nodule survived and showed less cell membrane damage than those with inactive nodules or those without nodules, under the same low temperature-stress condition (-6°C) (Liu et al., 2019). Because nodule formation is energy demanding, the host plant exerts a tight control on the number of nodules it forms. Inhibition of nodule formation by host

plant has been reported in the case of non-efficient rhizobia genotypes or when the nitrogen supply in the soil is sufficient (Regus et al., 2015; Zhang et al., 2020). Soil temperature is a critical factor strongly influencing interactions between plant, soil, and the microbiome; cold temperatures can affect every step of the establishment of the interaction between rhizobia and legumes as well as the efficiency of the rhizobia to fix atmospheric N_2 (Alexandre and Oliveira 2013).

The negative effects caused by environmental stresses on the legume-rhizobia symbiosis are well documented and the tolerance of some associations were reported (Sanz-Sáez et al., 2012; Bertrand et al., 2015) but the underlying mechanisms driving these adaptations are not fully characterized (Ferguson et al., 2019). The developmental plasticity of the belowground plant parts in response to abiotic stresses is of key importance for the long-distance transport of substances to assist plant stress resilience (Li et al., 2021). As already mentioned, the host-plant modulates resource allocation to nodules, depending on resources availability and environmental conditions. As a counterpart, the rhizobial partner also exert a control through its sink strength for metabolites to maintain nodule activity (Bertrand et al., 2015, 2016; Marquez-Garcia et al., 2015).

The study of legume-rhizobia symbiosis tolerance to multiple stresses has revealed that biological nitrogen fixation can be improved by the selection of nitrogen-fixing endosymbionts that are well-adapted and tolerant to a broad range of environmental stresses (Atieno and Lesueur 2019). It was shown that rhizobial strains differ in their abilities to nodulate alfalfa at low temperatures (Rice et al., 1995). Furthermore, the level of freezing tolerance and the regrowth of alfalfa after overwintering was influenced by the strains of *S. meliloti*, showing that the selection of adapted strains could improve plant survival after winter (Prévost et al., 2003; Bertrand et al., 2007). In light of these results, there is a high probability that the interaction between freezing-tolerant alfalfa genotypes and cold-adapted *S. meliloti* strains will promote field persistence and counteract the negative effect of low temperatures and freezing stress (Prévost et al., 1999). To our knowledge, no studies have compared the impact of different rhizobia strains on the resource allocation to nodules and their impact on regrowth of alfalfa populations differing in their level of freezing tolerance under simulated fall and winter conditions.

The objective of this study was to compare the regrowth of different combinations of *S. meliloti* strains and alfalfa populations after their exposure to a freezing stress in order to identify the most efficient association strain/population. We hypothesized that alfalfa regrowth after freezing would differ according to the strains used and that better strains could be identified.

Twelve associations were compared (six *S. meliloti* strains with two alfalfa populations) and the detailed phenotyping of aboveground and belowground traits, including nodule damages, were made after the freezing stress to understand the mechanisms of resistance of the associations.

3.3 Materials and Methods

3.3.1 Characterization of plant material

Two populations of alfalfa (*Medicago sativa* L.) were used for this study: the cultivar 'Apica' (A-TF0) adapted for growth in eastern Canada and developed at the Quebec Research and Development Centre (QRDC) of Agriculture and Agri-Food Canada (Michaud et al., 1983), and A-TF7, a population obtained after seven cycles of recurrent selection for improved freezing tolerance from the original cultivar Apica (Castonguay et al., 2009; Bertrand et al., 2014).

To assess their levels of freezing tolerance and determine if the freezing tolerance of A-TF7 was improved as compared to A-TF0, the two alfalfa populations were cold acclimated under natural winter conditions in an unheated greenhouse and, after 16 weeks of overwintering, cold-acclimated potted plants were transferred into a programmable walk-in freezer set at -2°C to assess the lethal temperature for 50% of the plants (LT_{50}). The temperatures tested were -2, -14, -16, -18, -20, -22, -24, -26, and -28°C (6 pots per temperature per populations for a total of 96 pots). After 3 weeks of regrowth, the survival count of the plants at each temperature were recorded and the LT_{50} was calculated (Bertrand et al., 2020b).

3.3.2 *Sinorhizobium meliloti* strains and inoculum production

Sinorhizobium meliloti strains 'A2', 'NRG34', 'I1', 'S27', and 'Rm1521' isolated from different Canadian regions (Table 3.1) were selected from the AAFC collection for this study based on their nodulation performance (nodule number and color) and alfalfa biomass yield after 8 weeks of growth under low temperature (15/10°C, day/night) under controlled conditions (D'Amours et al., in preparation). These strains were also characterized for their genetic diversity using multilocus sequence typing (MLST) analysis based on five *Sinorhizobium (Ensifer) meliloti* housekeeping genes as well as genes related with nodulation capacity and nitrogen fixation, for a total of 18 genes (Rocher et al., in preparation). The choice of strains was also based on previous studies on cold tolerance (A2 and NRG34, Olsen et al., 1994; Rice et al., 1995; Bertrand et al.,

2007) and tolerance to other abiotic factors (Rm1521, Bromfield et al., 1994; Bertrand et al., 2015, 2016, 2020a) of *S. meliloti* strains. The stress tolerant elite strain 'B399' (provided by Instituto de Genética "Edwald Alfredo Favret", INTA, Buenos Aires, Argentina), was also included in the study as a control commercial strain. Strain B399, originally named *Rhizobium meliloti* '102F34' (Nitragin Co., Milwaukee, WI), has been shown to have a high capacity to fix nitrogen under water-deficit field conditions in symbiosis with different alfalfa cultivars (Jozefkowicz et al., 2017) and under various abiotic stresses including cool temperature and elevated CO_2 (Sanz- Sáez et al., 2012). *S. meliloti* strains were grown on yeast mannitol agar plates (Vincent 1970). A unique colony was transferred in yeast mannitol broth (YMB, 8-mL per tube) and then placed in a shaking incubator (120 rpm, Lab-Line Orbit Environ-shaker, Melrose Park, IL) at 28 °C for 24 to 48 h. Then 300 µl of each strain were used to inoculate 125 mL of YMB in a 250 ml Erlenmeyer flask and placed in a shaking incubator (200 rpm, Lab-Line Orbit Environ-shaker, Melrose Park, IL) at 28 °C for 24 to 48 h. For each strain, a viability count was performed to adjust inoculum at 10^8 cells mL^{-1}.

3.3.3 Freezing stress experiment combining contrasting alfalfa populations and cold tolerant *Sinorhizobium meliloti* strains

3.3.3.1 Plant growth conditions

Alfalfa seeds of the two populations were surface-sterilized by immersion in ethanol 95% for 30 sec, and twice in sodium hypochlorite (3.0% v/v) for 2 min, and then washed three times with sterile distilled water and dried. Three seeds were sown in individual Ray Leach Cone-tainersTM (SC-10 Super Cell. Stuewe & Sons Inc, Tangent, OR) previously washed and sterilized and filled with sterilized Turface® MVP®, a heat-treated montmorillonite clay mineral product (Profile Products LLC, Buffalo Grove, IL).

Plants were grown in growth chambers (Conviron model PGW40, Winnipeg, Canada) under a 21/17°C day/night temperature regime, a 16-h photoperiod and a photosynthetic photon flux density of 600-800 µmol photons m^{-2} s^{-1}. Plants were watered once a day during seed germination to avoid Turface dryness. One week after seeding, at the first true trifoliate emergence stage, seedlings were thinned to keep only one plant per cone-tainer and fertilized with 2 mL of sterilized 0.25 N-free Hoagland solution. Each of a total of 384 plants was then inoculated with 1 mL each of the six strains containing 10^8 cells. For each alfalfa populations (A-TF0 and A-TF7),

uninoculated controls were also included to ensure that there was no contamination between tubes but these stunted chlorotic plants were not included in the statistical analysis. For the eight following weeks after inoculation, plants were well-watered daily to keep a constant moisture and 3 mL of sterilized 0.50 N-free Hoagland solution was applied three times a week. During the four weeks following cold acclimation (described below), plants were not fertilized. During the three-week period of regrowth after freezing, plants received 5 mL of sterilized 0.50 N-free Hoagland solution three times a week.

After eight weeks of growth, non-acclimated (NA) plants were collected, and destructive plant measurements were made on 96 plants (8 replicates × 2 alfalfa populations × 6 strains) as described in the next section. Remaining plants were then cold acclimated (CA) for two weeks at 2°C under 150 µmol m^{-2} s^{-1} PPFD and a short photoperiod (8 h) followed by two weeks at -2°C in the dark to stimulate hardening conditions. Following CA, 96 plants were sampled for measurements after thawing one night at 4°C. The remaining plants were transferred into a large programmable walking freezer set at -2°C. Temperature in the freezer was lowered by 3°C during a 30-min period followed by a 90-min plateau at each of the following temperatures: -5°C, -8°C and, finally, -11°C which was the targeted freezing-stress temperature determined by a preliminary experiment as a damaging, non-lethal temperature for the two alfalfa populations under study. At the end of the 90-min plateau at -11°C, plants were removed from the freezer and thawed for 24 h at 4°C in darkness. After thawing, shoots were cut and plants were gradually exposed to the initial optimal regrowth conditions by progressively increasing the air temperature from 4°C to 21/17°C day/night in one hour under a 16 h photoperiod with 600-800 µmol photons m^{-2} s^{-1} PPFD. A third destructive sampling of 96 plants was made two days after the freezing stress (AFS) to collect information on plant damages immediately following the freezing stress. The last sampling of the remaining 96 plants was made after three weeks of regrowth under the initial environmental conditions (Regrowth after freezing; RAF).

The experiment was assigned to a randomized complete block design with eight blocks and a factorial combination of two alfalfa populations inoculated with the six *S. meliloti* strains for a total of twelves combination of treatments. Altogether, 384 individual plants (2 populations × 6 strains × 8 repetitions × 4 sampling events) were grown in individual cone-tainers tube which each represented one experimental unit.

3.3.3.2 Plant measurements

After eight weeks of growth the photosynthetic rate of all non-acclimated-NA plants was measured using a portable photosynthesis system (LI-6400XT, Licor, Inc, Lincoln, NE). Measurements were carried out between 10:00 and 12:00 a.m. and were made on the middle leaflet of the youngest trifoliate fully emerged from the top leaves (one leaflet per plant). Measurements were conducted at the photon flux density of 1000 μmol $m^{-2}s^{-1}$, leaf temperature of 23 ± 2 °C and a relative air humidity of 60%. A second measurement of photosynthetic rate was made on the regrowth of alfalfa three weeks after the freezing stress (RAF).

At each sampling event (NA, CA, AFS, and RAF) plants were carefully removed from their cone-tainers and gently shaken to remove the excess of Turface and then washed three times in distilled water. Excess water was removed by gently pressing roots in absorbent paper towels. Nodules were detached from roots with tweezers and placed in 5-mL tubes kept on ice during sampling. After freezing at -80°C nodules were freezed-dried (Labconco, Model Freezone12, Kansas City, MO) and the dry weight (DW) was recorded. Roots were separated from shoots directly under the crown, and both plant parts were dried separately at 55°C for 72 h. Dry weight (DW) of nodules, roots and shoots were recorded and used to calculate root:shoot and nodules:root ratios. Shoots were only sampled for NA and RAF plants since CA and AFS shoots and leaves were killed by the 2-wks exposure to subfreezing temperature in the dark.

3.3.4 Plant phenotypic traits and nodules characterization after the freezing stress

For a detailed characterization of nodule regeneration, and root and shoot regrowth after the freezing stress (RAF) the phenotyping of the twelve combinations described previously (2 alfalfa population × 6 strains) was made on five replications for a total of 60 plants.

Each plant was gently removed from the Turface and roots were soaked three times in distilled water and quickly pressed with paper towel to absorb the excess water on roots. Shoot height was measured, and plant vigor was assessed visually using the following scale: 1- Plants very chlorotic (yellow), 2- Plants lightly chlorotic (pale green and yellow), 3- Plants green and relatively small, 5- Plants green and vigorous (Risula 2019). The developmental stage of alfalfa was determined based on the classification of Mueller and Fick (1989) 0-Early vegetative, 1-Mid-Vegetative, 2-Late Vegetative, 3-Early bud, 4- Late bud, 5-Early Flower, 6- Late Flower. Roots were separated from shoots under the crown. Root systems were kept on ice during the

characterization of nodulation and nodule sampling. Afterward, shoots and roots were dried at 55°C for 72h to record dry weight and to calculate root:shoot ratio.

Nodule characterization was assessed by examining the following features on each root system (60 plants): 1- nodule abundance; 2- nodule position on the root system; 3- nodule height on the root system. The following traits concerning specifically the nodules of each of the 60 plants were also assessed, 4- nodule shape and 5- nodule freezing-damages.

Nodule abundance was recorded as follows, using the modified scoring index of Knight (2007): 0- zero nodules, 1- less than 10 nodules, 2- between 10 and 30 nodules and 3- over 30 nodules. Nodule position was assessed using the three following classes: 1- mostly lateral nodulation only, 2- mostly crown nodulation only and 3- both crown and lateral nodulation (Risula 2019). Nodule height was assessed by visually scoring the percentage of nodule distribution on the following three root section depths starting just below the crown: from 0 to 2 cm, from 2 to 8 cm, and from 8 to 19 cm depth [modified from Bertrand et al., (2020a)]. Nodule shapes were separated into four categories as described for *Medicago truncatula* (Cai et al., 2018): S; Unbranched and small nodules, E; Unbranched and elongated nodules, B; Bifurcated nodules, PC; Palmate-coralloid nodules. A percentage was attributed for each of these shapes on the 60 plants. For the evaluation of nodule freezing-damages, three classes were used: I; pink nodules presenting no freezing-damages, II; necrotic nodules with pink regeneration zones, III; necrotic nodules. For each plant, the percentage of nodule representing each class was determined visually.

3.3.5 Statistical analysis

To evaluate the freezing tolerance of the two alfalfa populations (A-TF0 and A-TF7), the LT_{50} was computed with the SAS GENMOD procedure using a probit regression model with soil temperature, population, and their interaction considered as independent variables and mean percent survival as the dependent variable. Differences in LT_{50} between populations were established using a chi-square goodness of fit test as described in Castonguay et al., (2009).

Photosynthesis and biomass measurements were analyzed using a two-way analysis of variance (ANOVA) model for a randomized complete block design with the SAS MIXED procedure (SAS® Studio, 2020, Version 3.81, SAS Institute Inc., Cary, NC). The model was used to establish the effects of alfalfa populations with contrasted level of freezing tolerance, *S. meliloti* strains and their interactions, on shoot, root, nodules and total dry weight, root:shoot and

nodule:root ratios, photosynthetic rate, plant development stages and plant height. The ANOVA was performed for each sampling events separately. Residual normality and variance homogeneity were verified using the UNIVARIATE procedure. The Shapiro–Wilk's and Kurtosis's tests were used to verify the normality of the data distribution. Pairwise comparisons of means differences were made using a Fisher's least significance difference test (LSD) at $P \leq 0.05$.

Three weeks after the freezing stress, a visual rating of plant vigor, nodules abundance, position and height on the root system were assessed using the relative frequency of each class or index for each phenotypic trait. The percentage of distribution of nodules within the four shape categories described and of nodule freezing-damages within the three classes described were transformed as reported in Aitchison (1986), as they are not independent. Multivariate analysis of repeated measurements was conducted using the SAS MIXED procedure to establish the effects of alfalfa populations with contrasted level of freezing tolerance, *S. meliloti* strains, and their interactions on the profile of the percent distribution within the four nodules shapes and the three classes of nodule freezing-damages. A maximum likelihood ratio test was performed for model adjustment. Different variances were obtained for each combination and the model presenting the best fit for the data set was used for the comparison based on strains (Supplemental Tables 3.4a and 3.5a). Multiple comparisons between strains were adjusted by Bonferroni's test at $P \leq 0.05$ (Supplemental Tables 3.4b and 3.5b) and back-transformed for the presentation of results in Fig. 3.7

3.4 Results

3.4.1 Freezing tolerance

After seven cycles of recurrent selection (TF7), plant freezing tolerance was significantly improved ($P < 0.0001$) from a lethal temperature for 50% of the plants (LT_{50}) of -20°C in the original cultivar Apica (A-TF0) to -26°C in A-TF7 (Figs. 3.1a and b).

3.4.2 Freezing stress experiment combining contrasting alfalfa populations and cold tolerant *Sinorhizobium meliloti* strains

3.4.2.1 Differential response of alfalfa populations to cold acclimation and freezing stress

After 8 weeks of growth under optimal conditions (NA) we did not observe significant differences in above-ground traits between the two alfalfa populations except for the plant developmental stage (Table 3.2, Supplemental Table 3.1) which differed slightly. The development of A-TF0 was slightly ahead (early flower, stage 5) of that of A-TF7 (late bud, stage 4) (data not shown).

After four weeks of cold acclimation (CA) root DW increased by 16% (average for both alfalfa populations) when compared to the non-acclimated (NA) root DW. After two weeks of cold acclimation (CA) and two days of regrowth after the freezing stress (AFS), the two alfalfa populations differed in their root DW, with an average of 10% larger roots DW for A-TF7 (average of 3.3 g per plant) than A-TF0 (average of 3.0 g per plant) (Table 3.3, Fig. 3.2). The nodule DW and the nodule:root ratio also differed between populations at AFS, with a larger nodule DW (+10%) and nodule:root ratio (+24%) being observed for A-TF0 than A-TF7 (Table 3.3, Fig. 3.3). The largest difference between the two alfalfa populations was observed for the plant regrowth three weeks after the freezing stress (RAF). At that stage, total DW (Fig. 3.4b), as well as shoot DW and root DW (Fig. 3.2) were all significantly larger for A-TF7 than A-TF0 (Tables 3.2 and 3.3). Thus, after freezing, the alfalfa population with highest level of freezing tolerance (A-TF7) averaged 15% more of root DW and 19% more of shoot DW than population A-TF0 (Fig. 3.2) resulting in 17% more total DW (Fig. 3.4b). Significant differences at RAF were also observed with 28% higher nodule:root ratio for A-TF0 than A-TF7 (Table 3.3, Fig. 3.3).

3.4.2.2 Differential responses of plants inoculated with *S. meliloti* strain to cold acclimation and freezing stress

S. meliloti strains did not affect biomass of non-acclimated plants, however, they exerted a significant effect on shoot DW regrowth measured three weeks after the freezing stress (Table 3.2, Supplemental Table 3.1). Shoot DWs of plants inoculated with strain NRG34 isolated from Northwestern Canada was significantly larger than that of plants inoculated with all the other strains except for strain S27. Shoot DW of alfalfa inoculated with strain NRG34 was 19% larger

than shoot DW of plants inoculated with strains A2 and B399 that had the lowest shoot yields (Fig. 3.4c). The root:shoot ratio also differed between strains; B399 had the largest root:shoot ratio (1.62), which was significantly higher than all other strains (average 1.33) except for strains A2 that did not differ significantly (Table 3.2, Fig. 3.4d). We observed a significant interaction between alfalfa populations and *S. meliloti* strains for the photosynthetic rate at RAF (Table 3.2, Supplemental Table 3.1). While there was no difference between strains for A-TF7, the photosynthetic rates of population A-TF0 varied with strains: the average photosynthetic rate with strains NRG34 and S27 (average of 27.3 μmol CO_2 m^{-2} s^{-1}) was larger than with strain B399 (19.4 μmol CO_2 m^{-2} s^{-1}) and Rm1521 (21.1 μmol CO_2 m^{-2} s^{-1}), while it did not differ significantly than with strains A2, and I1 (Fig. 3.4e).

Under optimal growth conditions (NA) the nodule DW and nodule:root ratio did not differ between plants inoculated with the different strains. However, nodule DW and the nodule:root ratio differed significantly between strains at the CA, AFS, and RAF sampling events (Table 3.3, Supplemental Table 3.2). Overall, the cold acclimation process with low temperature and short photoperiod (CA) increased nodule DW (+68%) and the nodule:root ratio (+43%) for all plants as compared to NA plants (Fig. 3.5). Nodule DW was significantly higher on plants inoculated with *S. meliloti* strains A2, Rm1521 and S27 than on plants inoculated with B399 and I1. Nodule DW on plants inoculated with *S. meliloti* NRG34 did not differ from the other strains. The nodule:root ratio was significantly higher on plant inoculated with strain A2 and S27 than with B399 and I1, and intermediate with NRG34 and Rm1521 (Fig. 3.5 upper panel).

A diminution of 22% of nodule DW and of 19% of the nodule:root ratio across all plants compared to CA plant was observed for the sampling event made two days after the freezing stress (AFS) (Fig. 3.5). The strain effect was similar to the results obtained after CA; plants inoculated with strains B399 and I1 showing the lowest nodule DW and nodule:root ratio while plants inoculated with A2 and NRG34 had the highest nodule DW and nodule:root ratio compared to the other strains (Fig. 3.5).

A significant effect of *S. meliloti* strains on nodule DW and nodule:root ratio was observed at the RAF sampling event (Table 3.3). During the three weeks of plant regrowth after freezing stress, the nodule DW of plants inoculated with strain NRG34 increased markedly. Plants inoculated with strain NRG34 had a significant higher nodule DW (0.078 g) than plants inoculated with all other strains (Fig. 3.5). Plants inoculated with strain NRG34 showed a +46% higher nodule

DW than the lowest average values associated with plants inoculated with strains S27, Rm1521, B399 and I1 (average of 0.054 g) and +19% higher DW that plants inoculated with strain A2 (Fig. 3.5). Moreover, plants inoculated with strain NRG34 presented the highest nodule:root ratio (0.025), significantly higher than those inoculated with strains S27, Rm1521, and I1 while there was no difference between plants inoculated with strain A2 (Fig. 3.5).

Inoculation with *S. meliloti* strains significantly affected root DW at the sampling event two days after freezing stress (AFS, Table 3.3). Root DW was significantly higher on plants inoculated with strain B399 (3.4 g per plant) than for plants inoculated with strains S27 and A2 (average of 2.9 g per plant) while the plants inoculated with the other strains had intermediate root DWs (average of 3.1 g/plant for strains I1, NRG34 and Rm1521) (Supplemental Table 3.2).

3.4.3 Plant and nodule phenotyping after freezing stress

A detailed phenotyping of plants was made three weeks after the freezing stress (RAF) to assess if some combinations of alfalfa populations and *S. meliloti* strains had more successful regrowth after freezing. Phenotyping was also conducted to determine which phenotypic traits, particularly regarding nodulation, were associated with more vigorous regrowth.

3.4.3.1 Shoot regrowth

Shoot DW differed significantly between the two alfalfa populations with the freezing-tolerant population A-TF7 showing a significant larger regrowth than A-TF0 (As reported in Fig. 3.2 for RAF plants; Supplemental Table 3.3a). Population A-TF7 presented a larger proportion of 'green and vigorous' shoot regrowth (vigor rate of 4) compared to A-TF0 for which 10% of the plants showed a 'light chlorosis' (vigor rate of 2, Fig. 3.6 left panel). *S. meliloti* strains also exerted a significant effect on shoot DW: plants inoculated with strain NRG34 had a larger shoot DW than plants inoculated with strains A2 and Rm1521 while plants inoculated with strains B399, S27, and I1 had an intermediate shoot regrowth (Supplemental Table 3.3a). The strain effect was also reflected on the vigour of alfalfa regrowth. Plants inoculated with strain NRG34 showed the largest proportion of 'green and vigorous' regrowth (vigor rate 4), followed by I1 and S27, while the regrowth of plants inoculated with B399, A2 and Rm1521 was less vigorous, with a small proportion of 'lightly chlorotic' plants of vigor rate 2 (Fig. 3.6 right panel).

3.4.3.2 Nodulation characteristics

Nodule location on the root system of alfalfa differed according to the different rhizobial strains under study (Figs. 3.7a and b). Plants inoculated with strains NRG34 and S27 presented nodulation on both the crown and lateral roots for 100% of the plants sampled. For plants inoculated with strains Rm1521, I1 and B399, all nodules were located on the crown on 5 to 10% of the plants, while for plants inoculated with A2, nodules were evenly distributed on roots with no crown regrouping (Figs. 3.7a and b).

Percentage of distribution of nodules shapes differed significantly between plants inoculated with each of the strains (Fig. 3.7c outward circle; Supplemental Tables 3.4a and b). Plants inoculated with strain A2 presented the most regular distribution of all nodule shapes with a larger proportion of bifurcated nodules than plants inoculated with the other strains. Plants inoculated with strain NRG34 had the largest proportion of palmate-coralloid nodules than plants inoculated with any other strains while plants inoculated with strains Rm1521, S27, I1 and B399 had intermediate profiles with even proportions of unbranched small nodules, unbranched elongated nodules and palmate-coralloid nodules but low proportion of bifurcated nodules (Fig. 3.7c outward circle).

The proportion of the three classes of freezing damages on nodules differed significantly between plants inoculated with the six different strains (Fig. 3.7c inward circle; Supplemental Tables 3.5a and b).

Plants inoculated with strain NRG34, showed the largest proportion of pink nodules and nodules presenting a regeneration zone (average of 85%) as compared to plants inoculated with all the other strains except for those inoculated with strain S27 (Fig. 3.7c inward circle). Plants inoculated with strain A2 had the largest proportion of necrotic nodules (40%) as compared to plants inoculated with all the other strains (Fig. 3.7c inward circle). The proportion of the three classes of damages were evenly distributed in plants inoculated with strains B399, RM1521, and I1.

3.5 Discussion

Freeze-thaw episodes, which are predicted to occur more often under climate change, can be very damaging to alfalfa crops and compromise their persistence. Better understanding of how

the symbiosis between the host plant and its rhizobial partner are impacted by cold acclimation and freezing stress is required to elaborate strategies to mitigate the impact of cold stress on alfalfa. In this study, we demonstrated that using alfalfa populations obtained by recurrent selection for freezing tolerance and the use of a tolerant *S. meliloti* partner are effective and complementary approaches to enhance the persistence of alfalfa exposed to freezing stress.

The assessment of the LT_{50} of cultivar Apica (A-TF0) and of an alfalfa population obtained after seven cycles of recurrent selection for improved freezing tolerance (A-TF7) revealed a significant increase in freezing tolerance up to -6°C in response to recurrent selection. Moreover, our results showed that cold-acclimated plants of A-TF7 had a 17% larger biomass regrowth than the initial cultivar A-TF0 following a non-lethal freezing stress of -11°C. These results are consistent with previous work on the improvement of the freezing tolerance of alfalfa cultivar Apica (Castonguay et al., 2009; Castonguay et al., 2011) and thus, confirmed the effectiveness of this recurrent selection method performed indoor under control conditions to improve the freezing tolerance of alfalfa and other perennial species (Bertrand et al., 2020b).

One key element of alfalfa persistence is the adequate accumulation of root and crown organic reserves in the form of carbohydrates and amino acids. These reserves are required to sustain the metabolism of overwintering organs and, in the spring, to support plant regrowth (Bélanger et al., 2006). Although most studies have examined the impact of cold-and freezing-stress on the aboveground parts of plants, the role of belowground organs such as roots and nodules in the cold acclimation process, and their impact on freezing tolerance and plant regrowth have largely been overlooked (Nieman et al., 2018; Liu et al., 2019; Ambroise et al., 2020). Here we clearly documented an increase in root DW of alfalfa following four weeks of cold acclimation at low temperature. Although it is well known that roots hardly grow below 5°C (Zhu et al., 2015), perennial plants have been shown to accumulate carbohydrates and amino acids in roots in response to cold acclimation (Dhont et al., 2002, 2003). This transfer of organic reserves from shoot to roots likely contributed to the increase in root biomass that we observed. The observation of a larger increase in root biomass in population A-TF7 as compared to A-TF0 could explain the superior freezing tolerance of the former population since a larger capacity of translocation of cryoprotective sugars and amino acids to the roots is an adaptation strategy that have previously been reported in recurrently-selected populations when compared to the initial genetic background (Castonguay et al., 2011). In a field study, Dhont et al., (2002) reported a marked increase of alfalfa

root biomass with the decline of temperature and photoperiod, concurrently with the accumulation of carbohydrate reserves in belowground organs. The authors also reported a positive correlation between organic reserves in roots and shoot regrowth in the spring in two alfalfa cultivars. Our results show that after three weeks of regrowth after a freezing stress, the difference in root DW was even more pronounced between A-TF7 and A-TF0 than just after cold acclimation showing that the root system is specifically targeted by the method of recurrent selection for improved freezing tolerance that was used. This result also indicates that roots suffered more damages in the less freezing tolerant alfalfa population (Nieman et al., 2018; Ambroise et al., 2020). Future research on plant cold acclimation should include investigations on differential root system architecture and on the impact of root freezing damage on plant regrowth.

The importance of belowground organs in the cold acclimation process of alfalfa is further highlighted by the striking increase in nodule biomass during the four weeks of cold acclimation at low temperature. There is growing evidence that the association between legume-hosts and their rhizobial partners is a key mechanism to increase plant tolerance to various abiotic stresses (Sanz-Sáez et al., 2012; Staudinger et al., 2016; Bertrand et al., 2020a; Song et al., 2017; Sindhu et al., 2020) and to low temperatures stresses in particular (Liu et al., 2019; Yuan et al., 2020; Irshad et al., 2021). Our results showing a large increase of the nodules DW (+68%) and of the nodule:root ratio (+43%) in response to cold acclimation in alfalfa fully support the importance of this mechanism. Legume hosts have the ability to control the investment in number of nodules by the autoregulation of nodulation (AON) pathway (Ferguson et al., 2010) and environmental factors have been shown to affect the resource allocation process from the plant to the nodules (Goh et al., 2016; Friel and Friesen 2019; Zhang et al., 2020). Marquez-Garcia et al. (2015) observed that drought stress induces the senescence of shaded soya leaves before any observation of nodule senescence, suggesting that leaves with a low photosynthetic capacity are sacrificed in favor of nodules for the transfer of nitrogen. Our observation of alfalfa's allocation of resources to nodules during cold acclimation at low temperature by photosynthates translocation agrees with the observation of Gurusamy and al. (2000), who reported that a substantial amount of starch and lipids were transferred to nodules in perennial beach pea before winter.

Our experiment clearly shows that low temperature triggers the allocation of resources into nodules and that the magnitude of this investment is modulated by the strain of the rhizobial partner since there was no significant effect of the *S. meliloti* strains when plants were growing under

optimal conditions (NA) whereas *S. meliloti* strains significantly impacted the nodule DW, the nodule:root ratio, and the regrowth alfalfa after cold acclimation as well as following the freezing stress. It seems that legume-host regulates the resource investment in nodules depending on the benefit that the host will further receive from those symbiotic partners (Regus et al., 2015; Westhoek et al., 2021). Our observation that population A-TF0 invests more into nodules after a freezing stress than the more freezing-tolerant population A-TF7 further support this hypothesis. The less freezing-tolerant population (A-TF0) increased the resource allocation in its symbiotic partner by increasing the nodule:root ratio as the benefit of the symbiosis is likely more important for this population than for a more freezing tolerant population suffering less damages. In our study the nitrogen input was only provided by the rhizobia thus highlighting the importance for alfalfa to invest in nodules to ensure its regrowth after the freezing stress.

In response to freezing, we observed different nodule biomass according to the strain of *S. meliloti* used to inoculate the plants and it could be hypothesized that strains with the largest sink strength are more active after freezing resulting in a larger allocation of cryoprotective compounds such as amino acid, sugars and starch to the nodules, thus increasing their DW. Alfalfa plants under salinity stress conditions have been shown to maintain an active transport from the shoot to the nodules to help maintain nodule activity under stress and it was shown that the sink strength toward nodules was modulated by the level of the salinity tolerance of the strain (Bertrand et al., 2015, 2016). Alfalfa inoculated with strain NRG34, isolated from Northwesthern Canada had larger nodule DW, nodule:root ratio and larger shoot DW than alfalfa inoculated with any other strains. Alfalfa plants inoculated with strain NRG34 also had a better freezing tolerance as showed by their greater shoot weight three weeks following the freezing stress. Plants inoculated with this strain also had a much larger nodule DW than any other strain showing for the first time a strong positive relationship between shoot regrowth and nodule regrowth after a freezing stress. Our results are consistent with previous studies reporting superior adaptation for nodulation and nitrogen fixation at low temperature of strain NRG34 (Rice et al., 1995) and better regrowth potential of overwintering alfalfa inoculated with strain NRG34 (Prévost et al., 2003). However, they are not consistent with the results of Bertrand et al., (2007) who reported superior freezing tolerance of alfalfa cultivar 'AC Caribou' inoculated with strain A2 when compared to plants inoculated with strain NRG34. The use of a different genetic background and experimental conditions could explain these different results.

The only statistically significant interaction between the two alfalfa populations and the *S. meliloti* strains was found for the photosynthetic rate. While there was no difference between strains for the population with the highest level of freezing tolerance (A-TF7), the photosynthetic rate of population A-TF0 varied according to the inoculated strain. Nodules represent a high strength sink and, as such, have been shown to stimulate photosynthetic activity (Kaschuk et al., 2010; Concha et al., 2020; Parvin et al., 2020). The allocation of resources to nodules after freezing resulting in a larger nodule:root ratio for population A-TF0 than A-TF7 may explain the higher rates of photosynthesis for alfalfa population A-TF0 since higher photosynthetic rates are directly linked with strain efficiency (Kaschuk et al., 2010). This suggests that the biomass of active nodules with an efficient nitrogen metabolism is important to support plant regrowth after a freezing stress as they stimulate photosynthesis.

To better understand the link between nodulation and more vigorous plant regrowth, we proceeded with a detailed phenotyping of the above and belowground parts of the plants of the different associations between alfalfa populations and strains three weeks after the freezing stress. We observed that alfalfa shoot regrowth and plant vigor were both superior for plants inoculated with strain NRG34 isolated from Northwestern Canada than with the other strains. We noted distinct profile in the percentage of distribution of nodules shapes among the strains. Higher proportion of small unbranched nodule on the roots of plants inoculated by strain NRG34 than with the other strains suggests a higher potential of regeneration of the nodules infected by that strain as those small nodules were all newly formed. Plants inoculated with strains NRG34 and S27 had higher proportions of palmate-coralloid nodules which is the largest size of nodules observed. It could be supposed that palmate-coralloid nodules stored more resources to ensure the regrowth after the freezing stress and represent a strongest carbon sink to sustain the photosynthetic activity (Cai et al., 2018). We also observed a more even distribution of nodules throughout the root system on crowns and lateral roots for plants inoculated with strains NRG34 and S27 suggesting an additional strategy of those strains to infect and colonize the entire root system.

The freezing damage on nodules differed according to the strain in symbiosis with alfalfa, as shown by the different proportions of undamaged nodules, nodules with regeneration zones, and necrotic nodules. The overwintering of nodules has previously been reported for perennial legumes of temperate regions (Bergersen et al., 1963; Pate 1961; Bal and Khetmalas 1996).

However, to our knowledge, this is the first report of *in vivo* differences in nodule freezing damages and regeneration potential induced in legumes by *S. meliloti* strains. While plants inoculated with strain A2 had the highest nodule DW under optimal growing conditions, they showed the largest proportion of necrotic nodules along with the lowest shoot regrowth three weeks after freezing as compared to the other strains. On the contrary, plants inoculated with strain NRG34, had the largest proportion of active nodules with no damage or with a large regeneration zone after the freezing stress along with the greatest plant vigor and shoot regrowth three weeks after freezing. The positive relationship between less nodule freezing damage and a greater vigorous shoot regrowth reported here opens the door to new strategies to increase legume yield under stress. Taken together, the phenotyping results show that multiple strategies are at play for plants inoculated with strain NRG34 to support a vigorous regrowth after freezing: a capacity to colonize the entire root systems with new active nodules, the presence of large nodules with a strong sink strength, and a higher nodule freezing tolerance.

3.6 Conclusion

Our study shows that both plant recurrent selection and selection of cold and freezing tolerant rhizobial strains are complementary and effective approaches to increase the persistence and regrowth of alfalfa exposed to freeze-thaw episodes. The meaningful allocation of resources to nodules by alfalfa plants exposed to cold acclimation conditions highlights the crucial role of the symbiotic partner to increase alfalfa tolerance to stresses. The differential above and belowground responses modulated by the two alfalfa populations shows that different allocation strategies are at play during cold acclimation depending on the level of freezing tolerance of alfalfa. A detailed plant phenotyping revealed distinct profiles of nodule shapes and different levels of nodules freezing damages depending on the symbiotic *S. meliloti* strain used. These are the first *in vivo* observations of the variability of freezing tolerance among *S. meliloti* strains. These differences also suggest that plant resource allocation to nodules is modulated by their sink strength and metabolic activity after freezing. Alfalfa regrowth after freezing relies in part on nodule tolerance to freezing stress which in turn depend on *S. meliloti* strains.

3.7 Acknowledgments

This work was supported by a grant program of Agriculture and Agri-Food Canada. The first author was financially supported by a scholarship from the Fonds de Recherche Québécois Nature et Technologies. The authors sincerely thank Josée Bourassa, Josée Michaud and Sandra Delaney for their technical assistance. We also thank, Dr Nicolas Ayub from Instituto de Genética "Edwald Alfredo Favret", INTA who kindly provided us the commercial *S. meliloti* strain, B399.

3.8 Statements & Declarations

Funding

This work was supported by a grant program of Agriculture and Agri-Food Canada. The first author was financially supported by scholarship from the Fonds de Recherche Québécois Nature et Technologies.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Emmanuelle D'Amours, Annick Bertrand and Jean Cloutier. The first draft of the manuscript was written by Emmanuelle D'Amours and all authors revised the previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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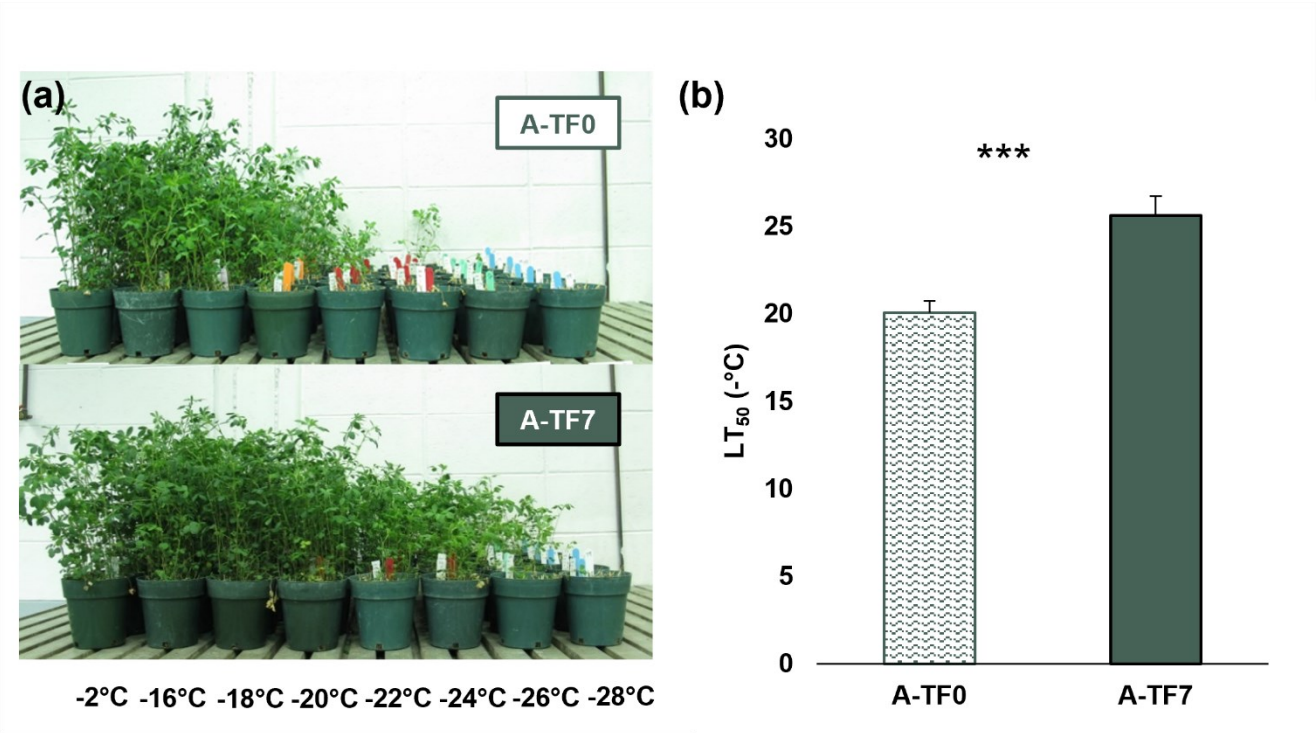

Figure 3.1 (a) Three-weeks regrowth of the two alfalfa populations under study: original cultivar Apica (A-TF0) and A-TF7 obtained after seven cycles of recurrent selection for improved freezing tolerance within cultivar Apica, after being exposed to the freezing temperatures indicated below each row of pots. (b) Freezing tolerance assessed as the freezing lethal temperature for 50% of the plants (LT50 ± SE) of the two alfalfa populations described above (6 pots per temperature per population for a total of 96 pots). Error bars represent the Standard Error of the Means (SEM). *** indicates that $P < 0.001$

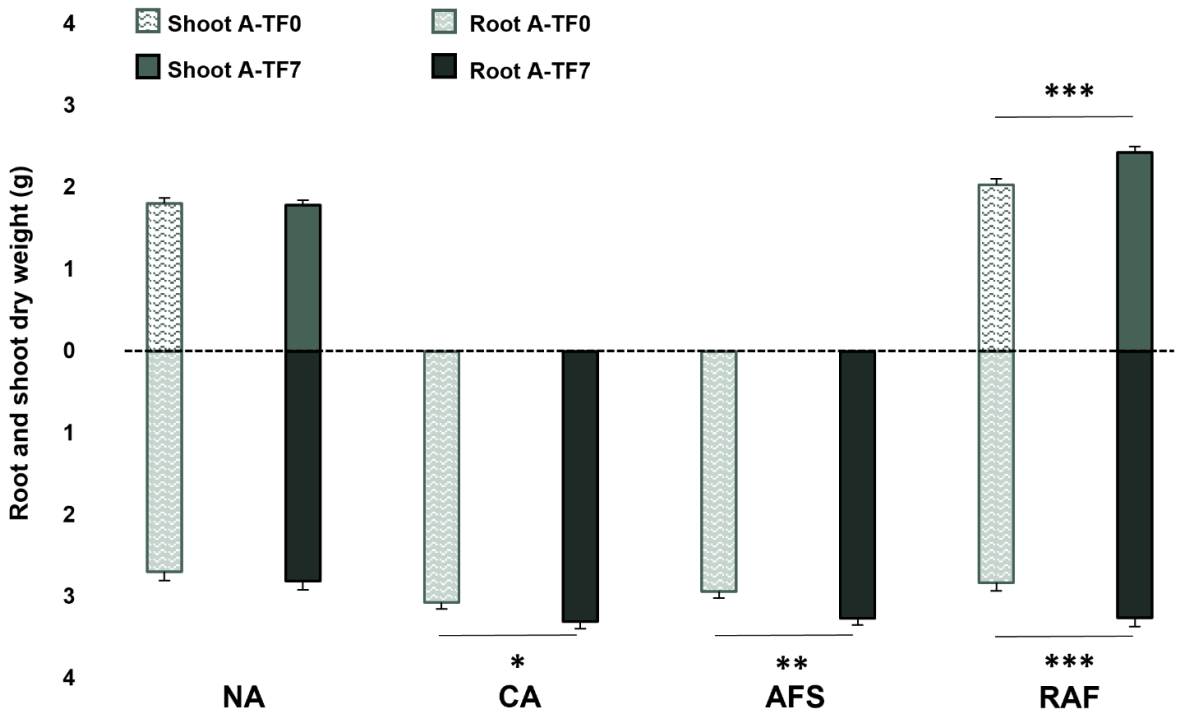

Figure 3.2 Shoot and root dry weight of the two alfalfa populations contrasted in their freezing tolerance levels (A-TF0 and A-TF7). Plants grown under controlled conditions were sampled at four physiological stages (48 plants per population for each sampling event): non-acclimated plants (NA) were grown 8 weeks under a 21/17°C, Day/Night (D/N) temperature regime. Plants were then cold acclimated during 2 weeks at 2°C followed by two weeks at -2°C and sampled again (CA). After their exposure to a freezing stress of -11°C, alfalfa plants were transferred back to optimal regrowth conditions (21/17°C, D/N) and sampled after two days (AFS), and after three weeks (RAF). Shoots were only sampled for NA and RAF plants since CA and AFS shoots and leaves were killed by the 2-wks exposure to subfreezing temperature in the dark while root dry weight was measured at the four stages. Error bars represent the SEM, n = 24. Significant differences between populations at each stage, determined by a t-test, are indicated with the following levels of probability: * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$

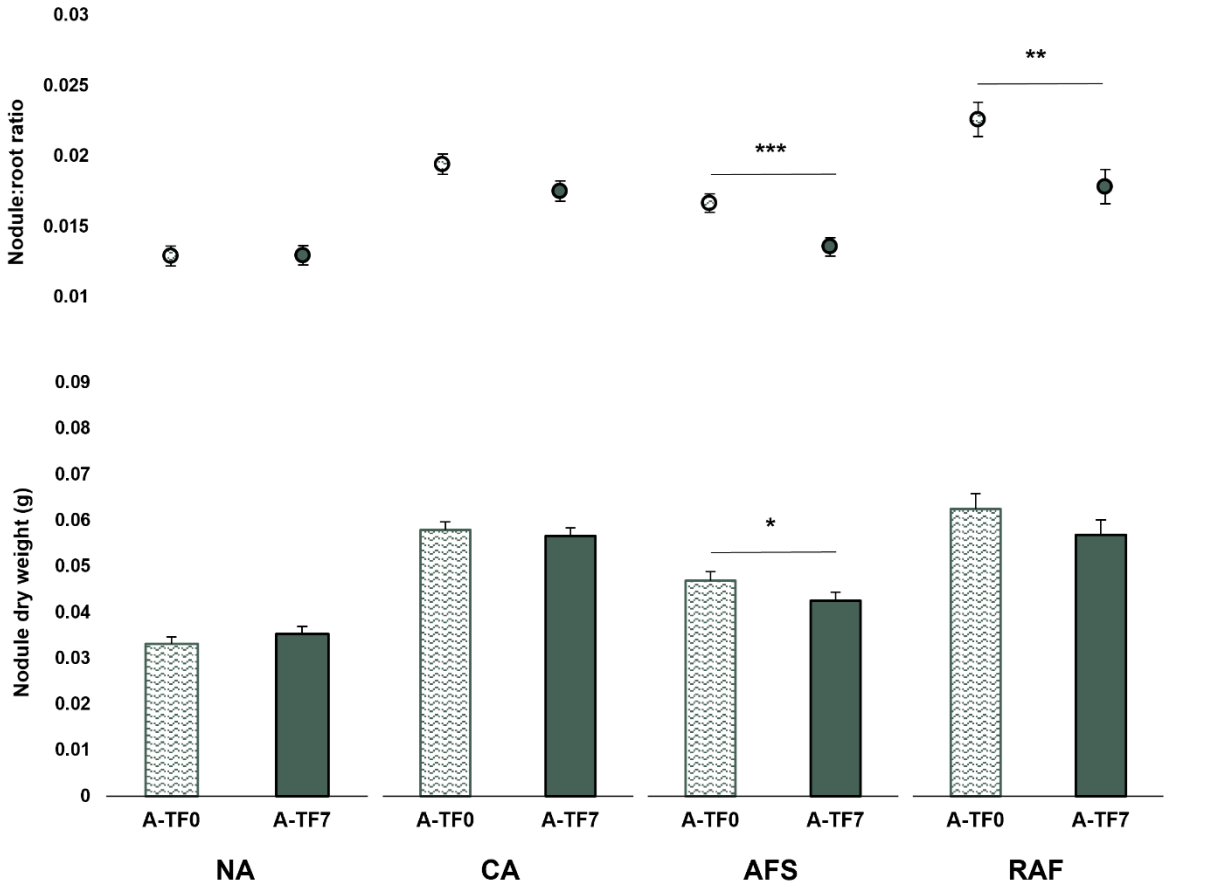

Figure 3.3 Nodule dry weight (DW) and nodule:root DW ratio of the two alfalfa populations with contrasted level of freezing tolerance, A-TF0 and A-TF7 (48 plants per population for each sampling event). Nodules and roots were sampled at the following physiological stages of the plants: non-acclimated (NA), cold-acclimated (CA), two days after the exposure to a freezing stress (AFS) and three weeks regrowth after a freezing stress (RAF). Nodules and roots were freeze-dried, and their dry weight was determined. Error bars represent the SEM, n = 24. Significant differences between populations at each stage are indicated with the following levels of probability: * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$

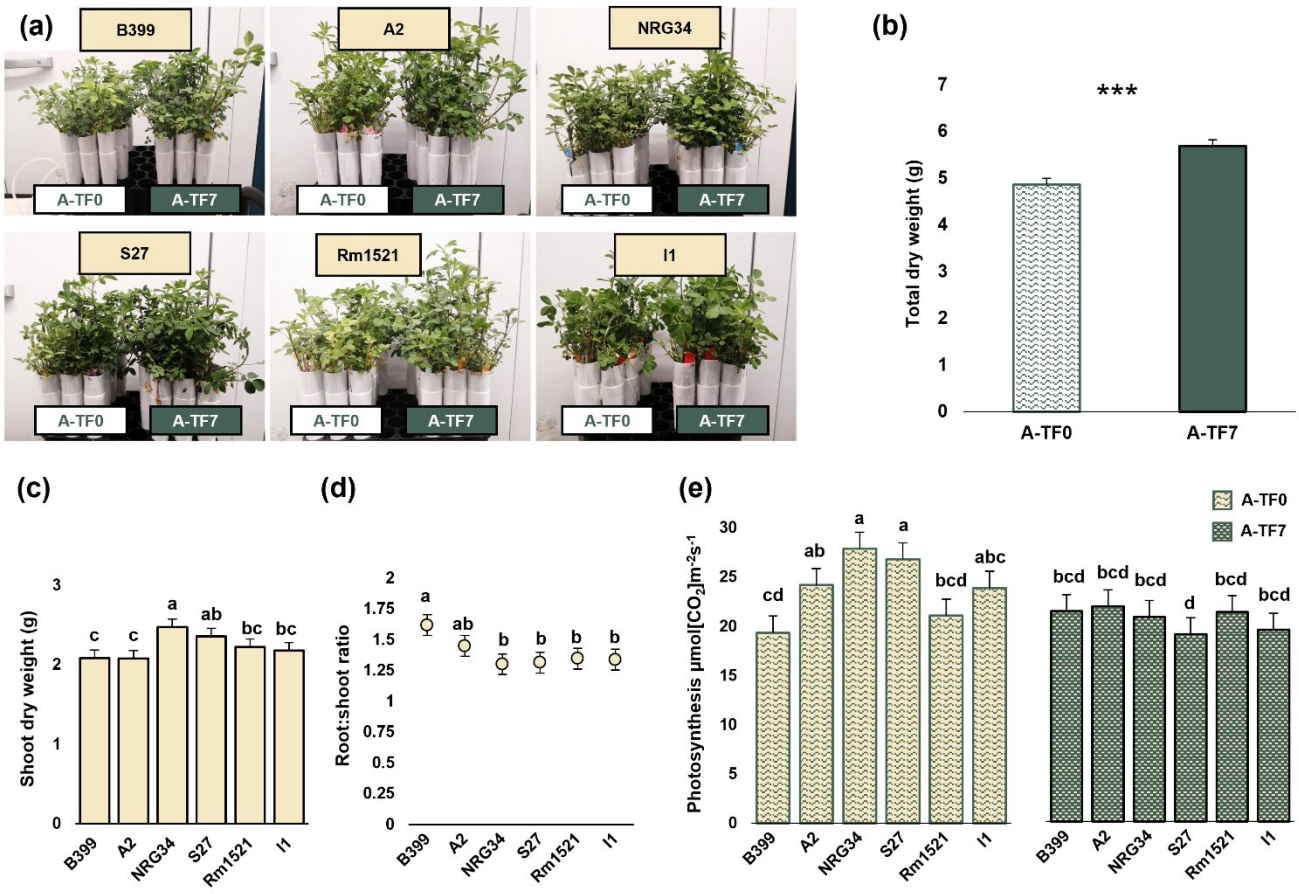

Figure 3.4 (a) Visual representation of the regrowth of each alfalfa population/strain associations 2-wk after a freezing stress of -11°C. The two alfalfa populations are cultivar Apica (A-TF0) and freezing-tolerant A-TF7 inoculated with one of the six *S. meliloti* strains (B399, A2, NRG34, S27, Rm1521 and I1. (b) Total dry weight (DW) of the three weeks regrowth of two alfalfa populations with contrasted level of freezing tolerance (A-TF0 in white, A-TF7 in green) averaged across the 6 strains (48 plants per populations). (c) Average shoot DW of the two alfalfa populations inoculated with each of the six strains (16 plants per strain) measured three weeks after the freezing stress. (d) Average shoot:root ratio of the two alfalfa populations inoculated with each of the six strains (16 plants per strain) measured three weeks after the freezing stress. (e) Photosynthetic rates of the two alfalfa populations (A-TF0 in white, A-TF7 in green), inoculated with the six *S. meliloti* strains (8 plants for each Pop × strain association). Error bars represent the SEM. *** indicates differences in a T-test at $P < 0.001$. Different letters represent significant differences among strains as determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$

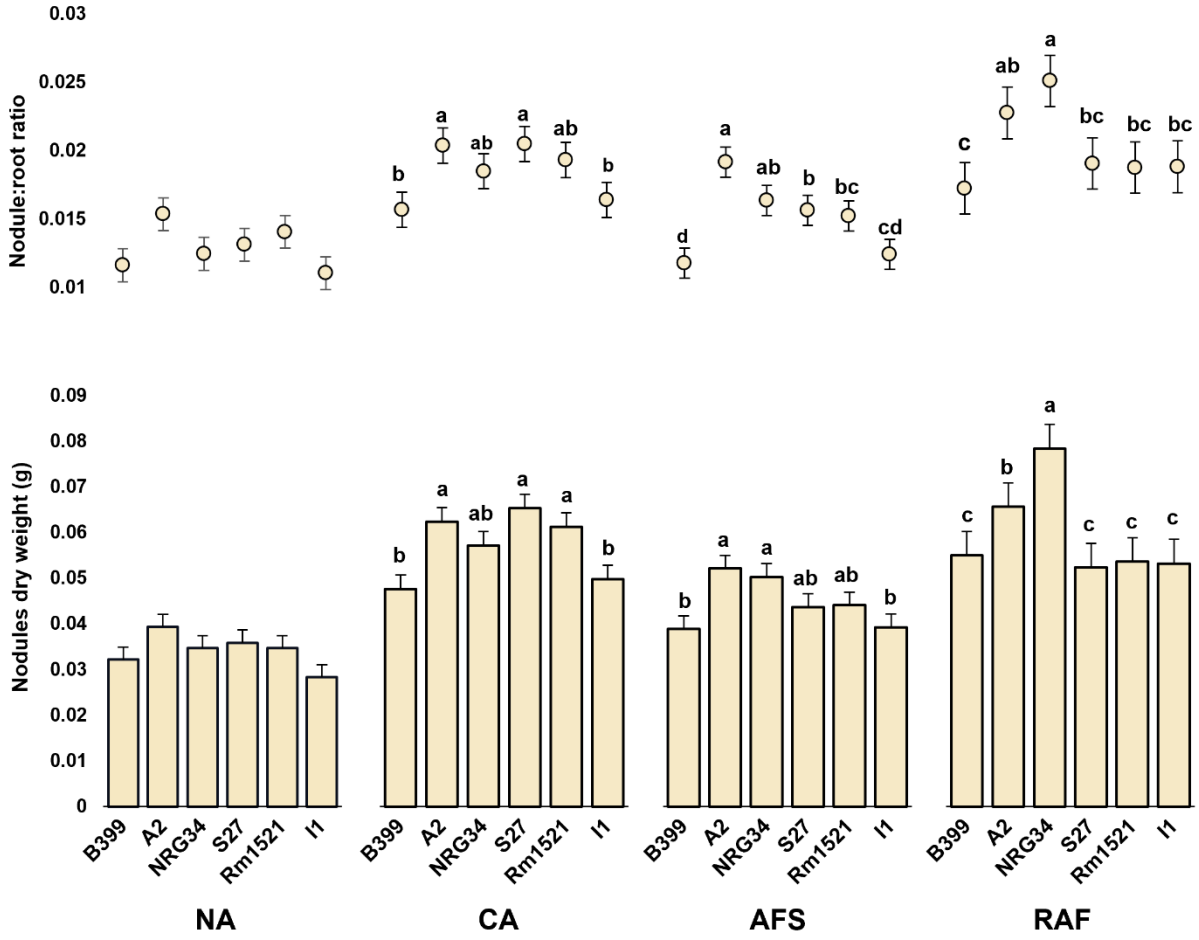

Figure 3.5 Nodule dry weight (DW) and nodule:root DW ratio averaged over the two alfalfa populations in response to inoculation with one of the six different *S. meliloti* strains (B399, A2, NRG34 , S27, Rm1521 and I1). Nodules and roots (16 plants for each strain) were sampled at the following sampling events: non-acclimated (NA), cold-acclimated (CA), two days after the exposure to a freezing stress (AFS) and three weeks regrowth after a freezing stress (RAF). Error bars represent the Standard Error of the Means (SEM). Different letters represent significant differences among strains for each sampling events determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$

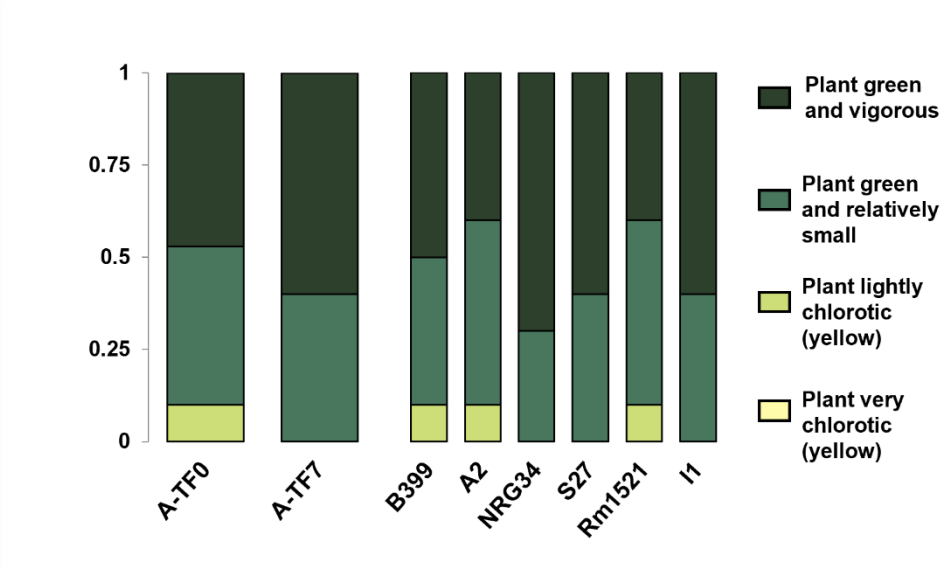

Figure 3.6 Visual rating of plant vigor of the two alfalfa populations (A-TF0 and A-TF7, left panel) and in response to inoculation with the six rhizobia strains (right panel) at the regrowth after freezing (RAF) stage. Each plant was rated according to the following classes: 1- Plants very chlorotic (yellow), 2- Plants lightly chlorotic (pale green and yellow), 3- Plants green and relatively small, 4- Plants green and vigorous. The relative frequency distribution of each class was calculated for the two populations (30 plants per population) and in response to inoculation with each strains by compounding the two populations (10 plants per strain)

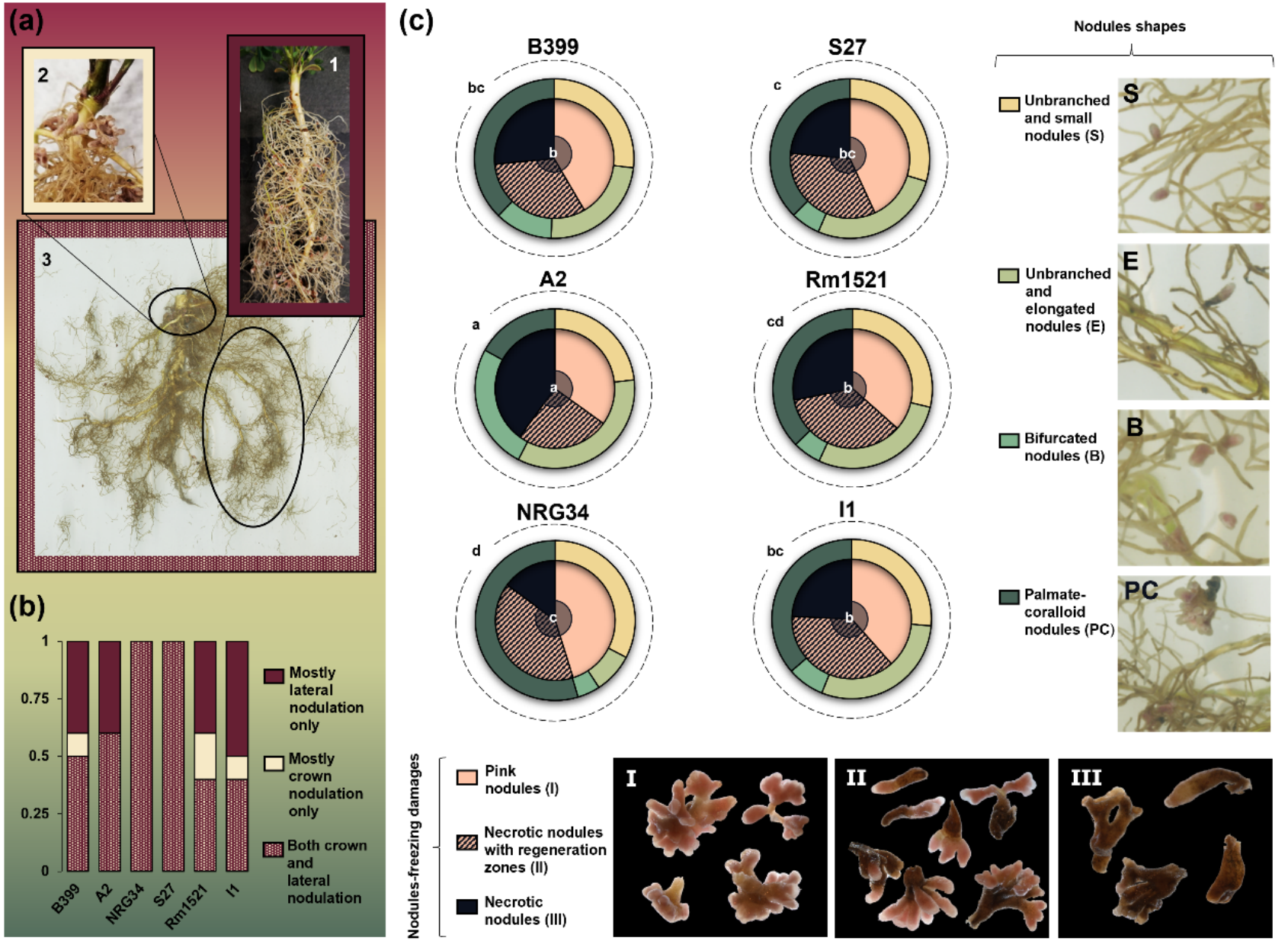
(a)
1
2
3
(b)
1
0.75
0.5
0.25
0
B399
A2
NRG34
S27
Rm1521
I1
Mostly lateral nodulation only
Mostly crown nodulation only
Both crown and lateral nodulation
(c)
B399
bc
b
S27
c
bc
A2
a
a
Rm1521
cd
b
NRG34
d
c
I1
bc
b
Nodules shapes
Unbranched and small nodules (S)
S
Unbranched and elongated nodules (E)
E
Bifurcated nodules (B)
B
Palmate-coralloid nodules (PC)
PC
Nodules-freezing damages
Pink nodules (I)
Necrotic nodules with regeneration zones (II)
Necrotic nodules (III)
I
II
III

Figure 3.7 Nodulation characteristics of alfalfa inoculated with six different *S. meliloti* strains. The following assessments were made on 60 plants (2 alfalfa population x six strains x 5 repetitions) at the regrowth after freezing (RAF) stage. (a) Illustration of the three classes of nodule location on the root system: 1- mostly lateral nodulation only, 2- mostly crown nodulation only and 3- both crown and lateral nodulation. (b) Relative frequency distribution of the three classes of nodules location in response to the six *S. meliloti* strains. (c) Inward circles represent the percentage distribution of visible freezing-damages to the nodules according to the three following classes: I; pink nodules with no damage, II; necrotic nodules with regeneration zones, III; necrotic nodules. Color codes are illustrated in the lower panel. Significant differences for distribution of freezing damages between strains are indicated by different letters at the circle center. Outward circles represent the percentage distribution of different nodule shapes associated to the six *S. meliloti* strains. The four following shape types are illustrated with their color code in the right panel: S; Unbranched and small nodules, E; Unbranched and elongated nodules, B; Bifurcated nodules, PC; Palmate-coralloid nodules. Significant differences in shape distribution between strains are indicated by different letters outside the outward circle. Significant differences were adjusted with Bonferroni's test at $P \leq 0.05$. Since the only significant effect was between strains, results from both alfalfa populations were pooled for n=10

Table 3.1 Identification of *Sinorhizobium meliloti* strains used in this study, the alfalfa cultivars from which strains were isolated, the collection sites, as well as the source and/or a reference presenting the strain

Strain identification	**Alfalfa cultivars**	**Collection Site**	**Source and/or reference**
B399	Unknown	Unknown	Nitragin; Jozefkowicz et al., (2017)
A2	Alpha-69	Lennoxville, QC, Canada	Bordeleau et al., 1977
NRG34	Unknown	Northwestern Canada	Rice et al., 1995
Rm1521	Unknown	Ottawa, ON, Canada	Bromfield et al., 1986
S27	Saranac	Unknown	AAFC
I1	Iroquois	Lennoxville, QC, Canada	Bordeleau et al., 1977

Sources: AAFC, Agriculture and Agri-Food Canada, Québec City, QC, Canada; Nitragin, Milwaukee, WI, USA

Table 3.2 Analysis of variance (*P* values) comparing the effects of two alfalfa populations in symbiosis with six different *S. meliloti* strains and their interactions on total biomass (shoot plus root dry weight), shoot dry weight, root:shoot ratio, photosynthetic rates and plant development stages. Plants grown under controlled conditions were sampled at two physiological stages: non-acclimated plants (NA) were grown 8 weeks under a 21/17°C, Day/Night (D/N) temperature regime. Plants were then cold acclimated for 4 weeks and exposed to a freezing stress of -11°C and transferred back to optimal regrowth conditions (21/17°C, D/N). After three weeks of regrowth (RAF) plants were sampled again. The two alfalfa populations, A-TF0 and A-TF7, were contrasting in their levels of freezing tolerance while the six *S. meliloti* strains were selected based on their nodulation performance at low temperature. Numbers in bold indicate statistically significant effects ($P \leq 0.05$)

	Total dry weight		Shoot dry weight		Root:shoot ratio		Photosynthetic rate		Plant developmental stages	
	Sampling events									
Effects	**NA**	**RAF**	**NA**	**RAF**	**NA**	**RAF**	**NA**	**RAF**	**NA**	**RAF**
Alfalfa Populations (Pop)	0.56	**<0.001**	0.73	**<0.001**	0.40	0.32	0.12	**0.002**	**<0.001**	0.08
***S. meliloti* Strains (Strains)**	0.08	0.13	0.06	**0.01**	0.11	**0.01**	0.09	0.21	0.60	0.07
Pop ×Strains	0.99	0.38	0.79	0.53	0.23	0.72	0.74	**0.03**	0.60	0.52

Table 3.3 Analysis of variance (*P* values) comparing the effects of two alfalfa populations in symbiosis with six different *S. meliloti* strains and their interactions on root dry weight, nodules dry weight and nodules:root ratio. Plants grown under controlled conditions were sampled at four physiological stages: non-acclimated plants (NA) were grown 8 weeks under a 21/17°C, Day/Night (D/N) temperature regime. Plants were then cold acclimated during 2 weeks at 2°C followed by two weeks at -2°C and sampled again (CA). After their exposure to a freezing stress of -11°C, alfalfa plants were transferred back to optimal regrowth conditions (21/17°C, D/N) and sampled after 48h (AFS), and after three weeks (RAF). The two alfalfa populations, A-TF0 and A-TF7, were contrasting in their levels of freezing tolerance while the six *S. meliloti* strains were selected based on their nodulation performance at low temperature. Numbers in bold indicate statistically significant effects ($P \leq 0.05$)

	Root dry weight				**Nodules dry weight**				**Nodules:root ratio**			
	Sampling events											
Effects	**NA**	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**
Alfalfa Populations (Pop)	0.30	**0.02**	**0.001**	**<0.001**	0.33	0.59	**0.05**	0.17	0.96	0.07	**0.001**	**0.002**
***S. meliloti* Strains (Strains)**	0.17	0.90	**0.04**	0.14	0.12	**<0.001**	**0.002**	**0.002**	0.13	**0.04**	**<0.001**	**0.03**
Pop ×Strains	0.77	0.51	0.32	0.37	0.50	0.93	0.68	0.12	0.41	0.69	0.33	0.41

3.10 Connecting Text

Chapter 3 revealed that both the choice of freezing-tolerant alfalfa populations and of cold-adapted *S. meliloti* strains are effective and complementary approaches to improve alfalfa yield and winter persistence. Alfalfa population A-TF7 obtained after seven cycles of selection for improved freezing tolerance and *S. meliloti* stain NRG34 isolated from Northwestern Canadia artic were identified as the symbiotic partners showing the highest freezing tolerance leading to a larger alfalfa yield. The study highlighted the importance of resource allocation made by the host-plant toward its nodules during cold acclimation as shown by a marked increase of nodules dry weight during that short period at near-freezing temperature. Differential allocation to roots or to nodules between alfalfa populations with contrasted levels of freezing tolerance show that distinctive strategies could be used by the plant under stressful events. Phenotyping of alfalfa aboveground and belowground parts after a freezing event revealed that some nodules were necrosed while others showed no damages and/or regeneration zones, depending mainly on the strain used in association with alfalfa. Fewer nodules damages was also shown to be associated with greater shoot regrowth of alfalfa.

While strain-associated freezing tolerance of nodules and their impact on alfalfa yield was for the first time brought to light in chapter 3, the goal of chapter 4 is to further investigate the underlying mechanisms involved in the cold acclimation, freezing stress and cold deacclimation processes of the symbiosis. Four contrasting freezing tolerant alfalfa/rhizobia associations tested in Chapter 3 are compared in Chapter 4. Hence, Chapter 4 focuses on the identification of molecular traits associated with a greater freezing tolerance of alfalfa. Metabolites and gene expression in crown, roots, nodules and root exudates are analyzed during cold acclimation and regrowth of alfalfa to characterize the molecular dialog between each partner of the symbiosis and to monitor the independent effects of each partner of the symbiotic association on metabolites involved in freezing tolerance during cold acclimation and deacclimation.

Chapter 4: Metabolic and genetic responses to simulated overwintering conditions of alfalfa-rhizobia associations contrasted in their freezing tolerance

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Reproduced from:
D'Amours, E., Bertrand, A., Cloutier, J., Claessens, A., Rocher, S., Seguin, P. (2023). Metabolic and genetic responses to simulated overwintering conditions of alfalfa-rhizobia associations contrasted in their freezing tolerance. *Symbiosis, 90*, 321–343. https://doi.org/10.1007/s13199-023-00939-3

Minor modifications were made to conform to the McGill University thesis guidelines.

4.1 Abstract

The study of winter stress tolerance in perennial legumes needs to consider the complete symbiotic system including both plants and bacteria since these two partners are differentially affected by stress conditions. Here, we compared the regrowth after a freezing stress of four different associations of two alfalfa populations differing in freezing tolerance (A-TF0 and A-TF7) inoculated with two *Sinorhizobium (Ensifer) meliloti* strains (B399 and NRG34) of contrasted adaptation to cold. To understand the contribution of each partner to a better regrowth performance of an association after freezing, we identified molecular traits having major roles in cold acclimation, freezing tolerance, and those involved in the crosstalk between alfalfa and its symbiotic partner. Regrowth after exposure to a freezing stress was 35 % larger in the A-TF7 × NRG34 than in the A-TF0 × B399 association. The metabolomic study of roots, crowns and, more specifically, nodules, revealed profound changes in these organs, switching from a sink to support cold acclimation to a source of reserves enabling regrowth after deacclimation. Marked increases in concentrations of stachyose and raffinose, two sugars of the raffinose-family oligosaccharides (RFO), and in the expression level of a gene of the RFO synthetic pathway were observed in response to cold acclimation supporting the importance of a protective role for RFO in alfalfa. Both cold-adapted partners of the symbiotic association contributed to increases in arginine concentration in nodules in response to cold acclimation and deacclimation underscoring the importance of N storage and remobilization for a successful overwintering in alfalfa.

4.2 Introduction

Alfalfa (*Medicago sativa* L.) is the most important forage crop species and the fourth largest crop by area in Canada with 3 million hectares of pure stands or alfalfa-based mixtures (Statistics Canada 2022). Alfalfa ability to establish a symbiosis with the nitrogen (N) fixing bacterial partner *Sinorhizobium (Ensifer) meliloti* reduces the need for N fertilization of this crop as well as for subsequent crops making it a key contributor to the current efforts to reduce agriculture reliance on fossil fuels (Cummings 2005).

In northern latitudes, winter survival, field persistence and yield of alfalfa depend on its ability to tolerate low freezing temperatures (Bélanger et al., 2006; Seppänen et al., 2018). Predicted climate change will increase the risks of winter injury to alfalfa due to higher temperatures in the fall allowing less favorable hardening conditions and to the diminution of insulating snow cover protection during winter due to a higher incidence of freezing rain and thawing events (Bélanger et al., 2002).

Perennial plants such as alfalfa go through the process of cold acclimation that increases their tolerance to freezing temperatures and promotes the accumulation of reserve metabolites to support spring regrowth (Dhont et al., 2002). Cold acclimation elicits several molecular changes in plants (Bertrand et al., 2020a). For instance, low temperature induces starch hydrolysis and modifies the concentration of cryoprotective sugars such as sucrose, and raffinose-family oligosaccharides (RFO) in overwintering roots and crowns of alfalfa (Castonguay et al., 2009). Amino acids like proline, arginine and histidine also accumulate during cold acclimation (Dhont et al., 2006). Proline was shown to stabilize membranes and protect against oxidative stress (Szabados and Savouré 2009). Arginine is known as a precursor of polyamines which are involved in complex signaling networks that regulate stress responses (Alcázar et al., 2011; Pál et al., 2015; Menéndez et al., 2019) and is also an important N reserve to support spring regrowth (Dhont et al., 2006). Cold acclimation elicits major changes in gene expression and several cold regulated (COR) genes have been identified and characterized in alfalfa and other perennial species (Bertrand et al., 2017; Juurakko et al., 2021). While the process of cold acclimation has been extensively studied in alfalfa, there is a knowledge gap regarding the deacclimation process and associated metabolic changes. Deacclimation occurs rapidly upon the return to warm temperatures in the spring. During that period, plants are more susceptible to damage by freeze–thaw cycles (Kalberer et al., 2006). The unpredictable effects of climate change on spring temperatures urge the need to better understand the deacclimation process since perennials will likely be exposed to more frequent and larger temperature fluctuations in springtime.

Alfalfa has been shown to have a large genetic diversity for freezing tolerance which has been successfully leveraged to improve this complex trait using a recurrent selection approach under controlled conditions (Bertrand et al., 2017). A potential relationship between freezing tolerance and changes in gene expression and metabolite composition in cold-acclimated alfalfa is

highlighted by the differential accumulation of COR gene products and of metabolites in alfalfa populations recurrently selected for superior freezing tolerance (Castonguay et al., 2006).

Low temperature stress can not only reduce alfalfa survival and productivity but can also negatively impact its rhizobial partner and hinder the establishment of effective rhizobia-legume interactions (Prévost et al., 2003). The crosstalk between the symbiotic partners is initiated by the secretion of flavonoids by alfalfa (mainly formononetin, medicarpin and coumestrol) which concentrations have been shown to be modified by low temperature (Xu et al., 2021) causing changes in *nod* genes activity in the symbiont (Zhang et al., 1996; Zhang et al., 2009). Moreover, low temperatures has been shown to decrease Nod factors production by rhizobia while these lipo-chitooligosaccharides compounds are essential for the infection process (Zhang et al., 1996; Duzan et al., 2006). D'Amours et al., (2022) recently showed that choice of the rhizobial strain directly affects the level of nodule damage after a freezing stress. Different proportions of undamaged and necrotic nodules were observed according to the rhizobial strain in symbiosis with alfalfa, after exposure to freezing. This observed *in vivo* differences in nodule freezing damages was linked with plant yield: alfalfa inoculated with a freezing-tolerant strain had a larger proportion of active nodules with no damage after the freezing stress along with a greater plant vigor and shoot regrowth as compared to symbiosis with freezing-sensitive strains.

Varying responses of legume-rhizobia associations to environmental stresses were reported (Sanz-Sáez et al., 2012; Bertrand et al., 2020b). The underlying molecular bases of differential tolerance between host-symbiont combinations remain to be elucidated. A feedback regulation between host-plants and nodules has been shown to depend on resources availability and environmental conditions (Marquez-Garcia et al., 2015; Bertrand et al., 2016) as well as on the symbiotic rhizobial strain. For instance, when comparing three *Bradyrhizobium* strains in association with soybean growing under contrasted levels of atmospheric CO_2, the strain associated with the highest yield also induced the highest ureides concentration in nodules under elevated CO_2 along with the highest nitrogenase activity (Bertrand et al., 2011).This relationship indicated a positive-feedback stimulation: soybean mobilized energy reserves to support more nodules, and in return nodules synthesized more ureides to support plant growth.

Strains of *S. meliloti* have been shown to directly affect the level of freezing tolerance of alfalfa (Bertrand et al., 2007) and, recently, alfalfa regrowth after freezing was reported to differ depending on the associated *S. meliloti* strain (D'Amours et al., 2022). To better understand the

mechanisms of tolerance of alfalfa-rhizobia associations, this study focused on metabolites and genes that were previously reported to play major roles in cold acclimation and freezing tolerance of alfalfa and nodules. Flavonoids involved in the crosstalk between alfalfa and rhizobia were also quantified as well as the expression of genes of their biosynthetic pathway.

4.3 Materials and Methods

4.3.1 *Sinorhizobium meliloti* strains and plant material

Based on the results of our previous study on the effect of *S. meliloti* strains on alfalfa yield after a freezing stress (D'Amours et al., 2022), two strains inducing contrasted responses in alfalfa, 'B399' and 'NRG34', were selected for the present study. Plants inoculated with the commercial strain 'B399' (provided by Instituto de Genética "Edwald Alfredo Favret", INTA, Buenos Aires, Argentina) were compared to plants inoculated with strain 'NRG34' isolated from Northwestern Canada (Rice et al., 1995). Both strains were grown in yeast extract mannitol (YEM) broth (Vincent, 1970) at 28 °C for 24 to 48 h and a viability count was performed to adjust inoculum at 10^8 cells mL^{-1}. These strains were used to inoculate two populations of alfalfa contrasted in their levels of freezing tolerance (i.e., A-TF0 and A-TF7). The cultivar 'Apica' (A-TF0) was developed at the Quebec Research and Development Centre (QRDC) of Agriculture and Agri-Food Canada (Michaud et al., 1983) and has a freezing lethal temperature for 50% of the plants (LT50) of -20°C, while population 'A-TF7' was obtained after seven cycles of recurrent selection for improved freezing tolerance from the original cultivar Apica and has a LT50 of -26°C (Bertrand et al., 2020a, D'Amours et al., 2022).

4.3.2 Plant growth conditions

Sterilized seeds of two alfalfa populations were individually seeded in Ray Leach Cone-tainers TM (SC-10 Super Cell. Stuewe & Sons Inc, Tangent, OR) filled with sterilized Turface® (Profile Products LLC, Buffalo Grove, IL). One week after seeding, plants were inoculated with 1 mL of either strain B399 or NRG34 containing 10^8 cells. Uninoculated controls for each alfalfa population (A-TF0 and A-TF7) were included to ensure that there was no uncontrolled sources of

rhizobia contamination. These control plants failed to grow due to the lack of N input from fixation or nutrient solution and were not included in the statistical analysis. Plants were grown and sampled under the experimental conditions illustrated in Figure 4.1 and as described in detail in D'Amours et al., (2022). Briefly, plants were grown in a growth chamber under a temperature regime of 21/17°C day/night and a 16 h-photoperiod and fertilized three times a week with 0.50 N-free Hoagland solution (Hoagland and Arnon 1950). Plants were harvested at the four following sampling events. 1) Non-acclimated (NA) plants were sampled after eight weeks of growth (32 plants: 8 replicates × 2 alfalfa populations × 2 strains). 2) Cold acclimated (CA) plants were sampled after an additional two weeks of growth at 2°C under a 8 h-photoperiod, followed by two weeks at -2°C in the dark (Fig. 4.1). The remaining plants were then exposed to a non-lethal freezing stress in a large programmable freezer in which temperature was gradually reduced from -2°C to -11°C (D'Amours et al., 2022), these frozen plants were then thawed for 24 h at 4°C in darkness and exposed to the initial optimal regrowth conditions by progressively increasing the air temperature from 4°C to 21/17°C day/night. 3) Two days after this freezing stress (AFS), 32 plants were sampled to characterize the symbiosis immediately following the freezing stress. 4) The sampling of the last 32 plants was made after three weeks of regrowth after freezing (RAF) to study the effect of deacclimation.

4.3.3 Root exudates sampling and biochemical analyses

4.3.3.1 Root exudates sampling

In a first step, root exudates were collected at each sampling event (i.e., NA, CA, AFS, and RAF). For this purpose, plants were carefully removed from their cone-tainers and gently shaken to remove the excess of turface and then washed three times in distilled water in order to remove any traces of turface and nutrient solution. Excess water was removed by gently pressing roots in absorbent paper towels. Each plant was then transferred into a 250-mL beaker containing 100 mL of ultrapure water and soaked for 10 minutes to collect root exudates. Plants were then removed from water and the soaking water containing root exudates was filtered using filter papers with 25 µm particle retention (Cytivia, Whatman^{TM} no.4, Marlborough, MA). The remaining filtrate (75 mL) was separated into three equal volumes of 25 mL and transferred in 50-mL screw cap tubes (Sarstedt Inc Nümbrecht, Germany) in order to proceed to the biochemical analysis of three types

of compounds: soluble sugars, amino acids and flavonoids. Tubes were kept on ice during sampling and then covered with perforated parafilm to allow freeze-drying. Root exudates were kept at -40°C until being freeze-dried during 140 hrs to obtain dry-powered exudates (Labconco, Model Freezone12, Kansas City, MO).

4.3.3.2 Soluble sugars and amino acids extraction

A first tube of dry-powered root exudates was diluted in 1 mL of ultrapure water to suspend soluble sugars and free amino acids into an aqueous phase. Tubes were immediately heated 20 min at 65 °C to stop enzymatic activity. Tubes were then cooled in an ice water bath before being vortexed and centrifuged 30 sec at 2,150 × g and the suspension was transferred into a 1.5 mL-microtube and kept frozen at -80°C. Samples were centrifuged for 3 min at 11,350 × g prior to chromatographic analyses for soluble sugars and amino acids as described below.

4.3.3.3 Flavonoids extraction

A second tube of dry-powered root exudates was dissolved in 1 mL of MeOH 80% to extract flavonoids. Tubes were immediately heated 15 min at 65°C then cooled in an ice water bath before being vortexed and centrifuged 30 sec at 2,150 × g. The suspension was transferred to 1.5 mL microtubes and kept frozen at -80°C. Samples were centrifuged for 3 min at 11,350 × g, prior to UPLC analysis.

4.3.4 Plant sampling for extraction of metabolites and RNA

Plants were separated into three major sections: roots systems, crowns and shoots that were kept on ice during sampling. For each plant, nodules were detached from roots using tweezers and transferred into 5-mL tubes. Nodules were freeze-dried prior to biochemical analysis and their total dry weight (DW) was recorded. The crowns, considered as the 2-cm subsections between shoots and roots, were sampled and analyzed separately because of their reported key role in the cold acclimation of alfalfa (Castonguay et al., 2009). Crowns were cut into two longitudinal sub-sections, and their fresh weight was recorded. The first crown sub-section (approx. 0.1 g) was flash frozen and manually ground in liquid nitrogen and kept at −80°C for RNA extraction. The second sub-section of crowns was freeze-dried (Labconco, Model Freezone12), the DW was recorded and samples were ground using an OMNI Bead Ruptor 24 (PerkinElmer, Inc. Kennesaw, GA) before

further biochemical analyses. A 8 cm-long subsample of roots containing a mixture of fine roots and tap roots was weighted (around 0.1 g) and flash frozen in liquid nitrogen for RNA extraction. A second root subsample was weighted (approx. 0.2), freeze-dried, and ground (OMNI Bead Ruptor) prior to biochemical analysis. The remaining part of the root system was dried separately at 55°C for 72 h for the measurement of total root DW (including the calculated DW of all sub sections). Shoots were dried at 55°C for 72 h for the measurement of total shoot dry weight (including the calculated DW of crown sub-sections).

Grinded samples of crowns and roots were analyzed for their soluble sugars and amino acids contents after extraction in methanol-chloroform-water as described in Bertrand et al., (2020b). Grinded samples of nodules were analyzed for their content in soluble sugars, amino acids, and flavonoids. For this purpose, the total dry weight of each nodules samples (between 0.02 and 0.09 g) was extracted in 2 mL of MeOH 80%. Tubes were heated 20 min at 65°C, rapidly cooled on ice and centrifuged (10 min at 1,200 × g at 4 °C). A first subsample of 0.9 mL was evaporated to dryness (Savant Speedvac plus SC210A, Holbrook, NY), solubilized in 0.9 mL of ultrapure water, and kept at -20°C until the analysis of soluble sugars (HPLC, Waters Inc. Milford, MA) and amino acids (UPLC, Acquity, Waters Inc, Milford, MA). A second subsample of 0.9 mL of the supernatant was transferred into 1.5 mL microtubes and frozen at -20°C until flavonoid analysis concentration by UPLC. Subsamples of roots were also extracted in MeOH 80% and treated similarly to analyze their flavonoids concentration. Prior to chromatographic separation by HPLC or UPLC, all samples were centrifuged for 3 min at 11,350 × g.

4.3.5 Metabolites quantification

4.3.5.1 Quantification of carbohydrates and amino acids

Soluble sugars were separated and quantified on a chromatographic analytical system controlled by the Empower II software (Waters, Milford, MA) as described in Bertrand et al., (2020b). Peak identity and quantity were determined for raffinose, stachyose, sucrose, glucose, fructose and pinitol by comparison to standards (Sigma–Aldrich, Oakville, ON, Canada). Starch was quantified in non-soluble residues following the extraction of soluble sugars. Starch in the residues was hydrolyzed into glucose by adding 3 mL of digestion buffer (200 mM sodium acetate, pH 4.5) containing amyloglucosidase (15 U mL^{-1}; Sigma-Aldrich, Oakville, ON, Canada), and

incubation (60 min at 55°C). After centrifugation, the supernatant was collected for quantification of glucose by HPLC, as described above. Starch concentration was calculated by subtracting the amount of soluble glucose from the total amount of glucose measured following digestion with amyloglucosidase. For crowns, roots and nodules, results of carbohydrates determinations were expressed as concentrations on a dry matter (DM) basis (mg g^{-1} DM). For root exudates, results were reported on a root DM basis by taking into account the DM of the root systems that were soaked in ultrapure water to extract exudates. Total soluble sugars (SSTot) is the sum of concentrations of individual sugars raffinose, stachyose, sucrose, pinitol, glucose and fructose and non-structural carbohydrates (NSC) is the sum of SSTot and starch.

Twenty-one amino acids (alanine, arginine, asparagine, aspartate, glutamate, glutamine, glycine, γ-aminobutyric acid (GABA), α- aminobutyric acid (AABA), histidine, proline, methionine, lysine, serine, leucine, isoleucine, ornithine, phenylalanine, threonine, tyrosine and valine) were separated and quantities were determined by comparison to a standard mix containing the 21 amino acids. Each individual amino acid were provided by Sigma–Aldrich (Oakville, ON, Canada) and the standard mix was prepared in our laboratory. For crowns, roots and nodules, results were expressed as concentrations on a DM basis (µmol g^{-1} DM). For root exudates results were reported in µmol on root DM basis by taking into account the DM of the root systems that were soaked in ultrapure water to extract exudates. The total free amino acids (AATot) was the sum of concentrations of the 21 free amino acids.

4.3.5.2 Quantification of flavonoids

Naringenin, luteolin, echinatin, coumestrol, formononetin and medicarpin and their conjugates were separated and quantified using Waters ACQUITY UPLC analytical system controlled by the Empower II software (WATERS, Milford, MA, USA). Flavonoids were separated using a BEH C8 column (2.1 mm × 100 mm, 1.7 µm, Waters), and detected using a Photodiode Array (PDA) Detector set at 287 nm. The chromatographic conditions were as follows: column temperature, 35°C flow rate, 0.35 mL min^{-1}, mobile phase A, 0.2% formic acid in water, mobile phase B: methanol 100%. The gradient was of 65% A at 0.2 mL min^{-1} and a gradual decrease until 2%, before to returning to initial condition of 65% A, for a total run of 8 min. Peak identity and quantity of each flavonoids were determined by comparison to standards. Results for flavonoids determinations in roots were expressed as concentrations on a dry matter (DM) basis

(µg g-^{1}DM). For root exudates, the calculation was as described in the above section. Total flavonoids (FlaTot) are the sum of each individual concentration of the seven flavonoids.

4.3.6 Analysis of gene expression

4.3.6.1 RNA extraction and cDNA synthesis

Total RNA was extracted from 0.1 g of crowns and roots using CTAB-based protocol (Dubé et al., 2013). Total RNA was quantified using NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA). Residual genomic DNA was removed by a treatment with DNaseI (Invitrogen, Burlington, ON, Canada) prior to cDNA synthesis. First strand cDNA was synthesized from 1 µg of total RNA and oligo(dT)18 primers using the Transcriptor First Strand cDNA synthesis Kit (Roche Applied Science, Laval, QC, Canada) following the manufacturer instructions. cDNA synthesis reactions were performed for each sample considered in the subsequent RT-qPCR analyses. Thus, a total of 256 reactions were performed: 8 replicates × 2 alfalfa populations × 2 strains × 4 sampling dates × 2 organs (crowns and roots).

4.3.6.2 RT- qPCR analysis of COR gene expression

The expression of seven genes of interested (GOI) that are cold-regulated (COR) was measured and compared between the different treatments (Table 4.1). The five genes related to cold acclimation and freezing stress tolerance were selected from previous studies on alfalfa (Dubé et al., 2013; Castonguay et al., 2015; Bertrand et al., 2017) and their expressions were analyzed in crown samples. Two genes of interest linked to the phenylpropanoid synthetic pathway leading to flavonoid synthesis were selected based on literature (Gifford et al., 2018) and their expressions were measured in alfalfa root samples. Specific qPCR primers were designed using the methodology described in Castonguay et al., (2020), to amplify these last two genes of interests in *Medicago sativa* samples. Primer design was based on homologous *Medicago tranculata* genes sequences retrieved from a BLASTn search on NCBI database [Medtr4g088190 (IOMT or 2,7,4′-trihydroxyisoflavanone 4′-O-methyltransferase, EC 2.1.1.212) and Medtr5075450 (C4H or cinnamic acid 4-hydroxylase EC 1.14.13.11)] (Gifford et al., 2018). The sequences of all primers selected for RT-qPCR analysis of GOI as well as of the three reference genes described below are listed in Table 4.1. The expression of COR genes was measured in crowns as this tissue plays a

key role in cold acclimation of alfalfa. The expression of genes involved in flavonoid biosynthesis was measured in roots as this tissue is the synthetic site of flavonoids.

The RT-qPCR was carried out in a Mastercycler® ep realplex system (Eppendorf Canada, Mississauga, ON, Canada) using the QuantiTect® SYBR Green PCR kit (QIAGEN, Toronto, ON, Canada) as described by Dubé et al., (2013). The 10-µl reaction mixture contained 3 µl of first-strand cDNA and 0.5 µM of each of the forward and reverse primers. The thermocycler program was set to: 15 min at 95°C for denaturation; 40 cycles of 15 s at 95°C, 15 s annealing at 60°C; 60 s extension at 72°C. Reactions with real-time PCR were carried out with control water samples included as checks for potential contamination with genomic DNA. Efficiency of PCR was calculated from the linear regression of a seven fold dilution of PCR products using the following equation: Efficiency % = $(10^{(-1/slope)} - 1) \times 100$. The threshold cycle (Cq) values at which the PCR product fluorescence rises over the background fluorescence was determined by the instrument software which was set to default parameters. For the normalization of results, reference genes were selected by screening several candidates identified by Castonguay et al., (2015) for the stability of their expression using the geNormPlus M value program included in the qBasePlus software (Biogazelle, Ghent, Belgium). Results were normalized in each tissues with three reference genes: ubiquitin 5 (crown), eukariotic elongation factor 1-alphas (roots) and H-ATPase (crowns and roots). Relative quantification was calculated with the qBase software using the 2-ΔΔCq or comparative Cq method based on the differences in Cq between the target and the reference genes and corrected for PCR efficiency.

4.3.7 Statistical analysis

Statistical analysis of biomass were made using a one-way analysis of variance (ANOVA) for a randomized complete block design with the SAS MIXED procedure (SAS® Studio, 2020, Version 3.81, SAS Institute Inc., Cary, NC). The model was used to compare the effects of the associations between alfalfa populations and *S. meliloti* strains on shoot regrowth and nodules biomass. Concentrations of metabolites and gene expression were analyzed using a two-way analysis of variance (ANOVA) model for a randomized complete block design with the SAS MIXED procedure (SAS® Studio, 2020, Version 3.81, SAS Institute Inc., Cary, NC). The model was used to establish, in a first step, the effects of sampling events. ANOVA were then performed

for each sampling events separately to establish the effects of the two alfalfa populations, the two *S. meliloti* strains, and their interactions on the concentrations of carbohydrates, amino acids, and flavonoids and on genes expression, for each plant organs and root exudates. The Residual normality and variance homogeneity were verified using the UNIVARIATE procedure. The Shapiro–Wilk's and Kurtosis's tests were used to verify the normality of the data distribution. Pairwise comparisons of means differences were made using a Fisher's least significance difference test (LSD) at $P \leq 0.05$. The log 2 fold changes in metabolite concentrations that significantly differed between alfalfa populations and *S. meliloti* strains were calculated in non-acclimated (NA), cold acclimated (CA), 48 hours after freezing stress (AFS) and regrowth after freezing (RAF) plants. The graphical representation of metabolite variations related to either alfalfa populations or *S. meliloti* strains based on this calculation shows the differential contribution of each alfalfa population (A-TF0 vs A-TF7) and each strain (B399 vs NRG34) and to the metabolic changes at each sampling event.

4.4 Results

4.4.1 Comparative assessment of shoot and nodule biomass in alfalfa-rhizobia associations under varying environmental conditions

There was no difference in shoot DW between the non-acclimated (NA) four alfalfa-rhizobia associations tested (Table 4.2). In contrast, shoot regrowth three weeks after exposure to freezing stress (RAF) was significantly different between the populations × strains associations. The shoot DW of the freezing tolerant alfalfa population A-TF7 inoculated with the freezing tolerant strain NRG34 was significantly higher (+35%) than the shoot DW observed with the combination of the less freezing tolerant population and freezing sensitive strain A-TF0 ×B399 (Fig. 4.2a). The shoot DW of A-TF0 × NRG34 was also significantly higher (+17%) than that of A-TF0 × B399. It is noteworthy that the shoot DW of the A-TF7 × B399 was intermediate between those of A-TF0 × B399 and A-TF0 × NRG34 even though it did not significantly differ with from the shoot DW of these two associations. The four alfalfa-rhizobia associations also differed in nodules DW after a freezing stress (Table 4.2, Fig. 4.2b). While no difference in nodules DW was observed in NA and CA plants, the nodules DW of A-TF0 × NRG34 was significantly larger than

both associations with strain B399 two days after the freezing stress (AFS). The significant difference in nodules DW between A-TF0 × NRG34 and the other three alfalfa-rhizobia associations was much larger three weeks after the exposure to a sublethal freezing stress (RAF).

4.4.2 Variations in metabolite concentrations between sampling events

4.4.2.1 Non-structural carbohydrates

Concentrations of starch and soluble sugars in roots, crowns and nodules significantly varied between sampling events (Table 4.3, Fig. 4.3). This observation was also true for root exudates except for glucose and fructose concentrations that did not vary between samplings. Starch concentration under NA conditions was much higher in roots (322 mg g^{-1} DM) and crowns (272 mg g^{-1} DM) than in nodules (1.6 mg g^{-1} DM). After four weeks of CA, starch concentrations were reduced by one half in roots and crowns while its concentration doubled in nodules (Fig. 4.3). Starch concentrations decreased in subsequent AFS and RAF sampling points in nodules, roots and crowns to reach levels that were lower than those measured in NA plants. Concomitantly, a marked increase was observed in total soluble sugars in CA crowns (+222%), roots (+200%) and nodules (+136%) as compared to NA. More specifically, sucrose concentration in nodules, roots and crowns was 2.5 to 4 times higher in CA than in NA plants and then decreased gradually in subsequent AFS and RAF samplings. Concentration of glucose decreased in roots and crowns for CA, AFS and RAF when compared to NA plants while in nodules, a slightly higher glucose concentration was observed in CA as compared to NA. Cold acclimation induced a marked increase of stachyose and raffinose in root exudates, roots, crowns and nodules. While stachyose and raffinose were undetectable in NA nodules, they reached concentrations as high as 9 and 17 mg g^{-1}, respectively, in CA nodules and then decreased progressively to 0 in subsequent sampling events. Generally, the fructose concentration remained stable in nodules, roots, and crowns in response to CA but a significant increase in fructose concentration was observed in nodules of AFS and RAF plants as compared to NA and CA plants. The concentration of pinitol in nodules decreased progressively at each subsequent sampling events in exudates, nodules, roots and crowns as compared to NA.

4.4.2.2 Amino acids

Concentrations of total free amino acids in NA samples were much higher in nodules (523.4 μmol g^{-1} DM) comparatively to roots (143.4 μmol g^{-1} DM), crowns (131.8 μmol g^{-1} DM), and root exudates (2.5 μmol g^{-1} root DM (Fig. 4.3). Total amino acids concentration decreased with CA in root exudates and in nodules while it remained stable between samplings in roots and crowns (Table 4.3, Fig. 4.3). In contrast, individual free amino acids varied in roots, crowns and nodules between samplings except for proline in nodules and crowns, aspartate in nodules, and AABA in roots. Contrarily to the increase that was generally observed in nodules, roots and crowns between NA and the other samplings, individual free amino acids mostly decreased in root exudates at CA and AFS samplings with the exception of methionine and arginine that remained stable. Asparagine was by far the most abundant amino acid, representing 35% of total amino acids in root exudates, 80 % in nodules, 58% in roots and 52% in crowns in NA samples. Cold acclimation induced a decline in asparagine concentration in all samples. In crowns, asparagine concentration re-increased after a 3-wks regrowth (RAF) reaching a level higher than in NA samples. Glutamate concentrations slightly increased in CA and AFS roots and crowns compared to NA. An increase in concentrations of glutamine was observed in CA and AFS nodules while a decrease occurred in roots and crowns at those sampling events. Concentrations of ornithine, arginine and histidine increased significantly in CA roots and crowns (avg. of +82, +77, and +42%, respectively) and even more so in nodules (+917, +798, and +129%, respectively). The concentration of these amino acids remained high in the AFS samples. Concentrations of alanine and glycine increased in CA nodules, remained stable in AFS samples and subsequently decreased to a level lower than that observed in NA nodules in RAF samples. Alanine and glycine concentrations initially decreased in CA roots and crowns and re-increased in AFS samples. Serine concentrations increased with cold acclimation in nodules (+219%), roots (+70%) and crowns (+62%), whereas its concentration decreased in root exudates. An increase in threonine, lysine, isoleucine, leucine, valine, tyrosine and phenylalanine was observed in nodules, roots and crowns 48 hours after a freezing stress (AFS) but their levels remained low. The GABA concentration was at a lower level in nodules and roots at RAF as compared to the other samplings. The AABA concentrations decreased in nodules at CA, AFS and RAF compared to NA, while it increased slightly in response to CA and AFS in crowns.

4.4.2.3 Flavonoids

Total flavonoid concentrations were higher in roots (89 mg g^{-1} DM) than in nodules (52 mg g^{-1} nodules DM) and in root exudates (2.7 mg g^{-1} root DM) of NA plants (Table 4.3, Fig. 4.3). In CA, AFS and RAF plants, concentration of total and individual flavonoids differed between samplings. In general, total flavonoids increased at each subsequent sampling in nodules while it decreased in roots and root exudates (Fig. 4.3). Formononetin was the flavonoid with the highest concentration in all samples, representing slightly more than 75% of all flavonoids in root exudates and nodules, and 95% in roots. Formononetin concentration decreased in response to CA and AFS in nodules and roots while it increased in root exudates in response to CA. Most of individual flavonoids decreased in root exudates with CA and AFS except for formononetin and medicarpin. In roots, concentrations of naringenin and medicarpin were not impacted by the cold acclimation and freezing stress. In nodules, all flavonoids except for fomononetin increased in CA samples and showed an additional increase 48 hours after the freezing stress (AFS) with concentrations of naringenin (+48%), luteolin (+107%), echinatin (+367%), coumestrol (+253%) showing as compared to NA (Fig. 4.3). In roots, only luteolin and echinatin increased with CA and freezing stress, while formononetin and coumestrol decreased when compared to NA.

4.4.3 Variations in metabolite concentrations related to alfalfa populations in cold acclimated (CA) and deacclimated (RAF) plants

Concentrations of various metabolites differed significantly between alfalfa populations in CA and RAF samples (Figs. 4.4-4.5 left panels, Supplemental Tables 4.1-4.4). In CA plants, the freezing-tolerant population A-TF7 had higher concentrations of total soluble sugars and sucrose in roots and crowns while pinitol and glucose were more abundant only in A-TF7 crowns as compared to the less freezing tolerant A-TF0 (Fig. 4.4, left panel). Starch was more abundant in roots of A-TF0 than A-TF7. For amino acids, ornithine was the amino acid showing the most striking difference between the two alfalfa populations with higher level in nodules, roots, and crowns of population A-TF7 when compared to A-TF0. Arginine was the second amino acid with highest level in nodules and in roots of population A-TF7 when compared to A-TF0, followed by histidine in nodules and lysine in both nodules and roots. GABA concentration was higher in nodules and roots of population A-TF0 compared to A-TF7. Methionine concentration was also

higher in crowns of population A-TF0. We also observed higher concentrations of histidine in root exudates and in nodules of population A-TF7. For flavonoids, higher concentration of medicarpin was observed in root exudates and higher concentration of naringenin and luteolin in nodules associated with A-TF0 than those associated with population A-TF7.

Three weeks after the freezing stress (RAF) concentrations of starch in both roots and crowns and raffinose and NSC in crowns were higher in population A-TF7 than in A-TF0 while glucose concentration was higher in A-TF0 roots (Fig. 4.5 left panel, Supplemental Table 4.4). Concentrations of arginine and ornithine were higher in both nodules and roots of A-TF7 than in A-TF0. Histidine concentration was also higher in nodules associated with population A-TF7. Concentrations of glutamine in roots and aspartate and glutamate in both roots and crowns were higher in A-TF7 than in A-TF0. GABA concentration was higher in roots and crowns and alanine and serine in crowns of A-TF0. For flavonoids only a difference in naringenin concentration in root exudates was found between the two alfalfa populations with more than twice the concentration in A-TF0 than in A-TF7 (Fig. 4.5). Variations in metabolite concentrations related to alfalfa populations in NA and AFS plants are presented in supplemental Figures 4.1 and 4.2.

4.4.4 Variations in metabolite concentrations related to *S. meliloti* strains in cold acclimated (CA) and deacclimated (RAF) plants

Differences in the metabolite profiles of alfalfa-rhizobia associations established with freezing-sensitive strain B399 and freezing-tolerant strain NRG34 were observed after cold acclimation (CA). More noticeable variations occurred in nodules than in roots and crowns (Fig. 4 right panel, Supplemental Table 4.2). For non-structural carbohydrates, pinitol concentrations in nodules and crowns as well as glucose and fructose concentrations were higher in nodules of plants inoculated with strain B399 than with NRG34. Differences in cold-induced changes in concentrations of specific free amino acids were also noticeable between strains. Higher concentrations of AABA, aspartate, histidine and threonine in root exudates, AABA and tyrosine in nodules, and GABA and glutamine in roots were detected in the symbiotic association with strain B399. Conversely, higher concentrations of methionine, arginine, and histidine were observed in nodules of alfalfa inoculated with strain NRG34 than in those inoculated with strain

B399. As for flavonoid concentrations, luteolin and naringenin concentrations were higher in nodules of plants inoculated with strain NRG34 than in those associated with strain B399.

After three weeks of regrowth after freezing stress (RAF), higher concentrations of pinitol were still detected in crowns and nodules of alfalfa inoculated with strain B399 (Fig. 4.5 right panel, Supplemental Table 4.4). For amino acids, concentrations of proline, aspartate, and glycine were higher in nodules of alfalfa inoculated with strain B399 than in nodules of alfalfa inoculated with strain NRG34 (Fig. 4.5 right panel). Arginine and phenylalanine were alternatively more abundant in nodules of alfalfa inoculated with strain NRG34 than in nodules of alfalfa inoculated with strain B399. Total flavonoid and formononetin concentrations were higher in roots of alfalfa inoculated with strain NRG34 than in those inoculated with strain B399 as well as concentration of luteolin in nodules (Fig. 4.5). Variations in metabolite concentrations related to *S. meliloti* strains in NA and AFS plants are presented in supplemental Figures 4.1 and 4.2.

Few interactions between the alfalfa population and the *S. meliloti* strain on metabolites concentrations were observed in nodules and crowns in response to CA, AFS and RAF. Higher concentrations of fructose, alanine, AABA and medicarpin for the association A-TF0 × B399 were noticeable cases (Supplemental Tables 4.2-4.4) as well as variations in the concentration of several free amino acids in root exudates in AFS (Supplemental Table 4.3).

4.4.5 Gene expression in crowns and roots in response to sampling event, alfalfa populations and rhizobial strains

The relative expression of all the genes of interest (GOI) measured in crowns was impacted by the sampling events (Table 4.4). When expression profiles were compared to those in NA plants, the most striking modifications of gene expression were observed in response to CA with the up-regulation of galactinol synthase (GaS) and K3-dehydrin, and the down-regulation of sucrose synthase (SuSy) expressions (Table 4.4). The expression levels of these three genes returned to a level comparable to NA plants at the subsequent samplings (AFS and RAF). As observed in AFS crowns, freezing stress induced the down-regulation of SPS gene (Table 4.4). When looking at the effect of alfalfa populations on gene expression, the relative expression of SuSy was higher in A-TF0 than in A-TF7 at the CA and RAF samplings (Figs. 4.6a and 4.6b,

Supplemental Table 4.5). Gene expression of SuSy in crowns did not differ according to strain inoculation.

In roots, the expression of genes coding for enzymes involved in the biosynthetic pathways of flavonoids and secondary metabolites differed according to sampling events (cinnamic acid 4-hydroxylase (C4H; and 2,7,4′-trihydroxyisoflavanone 4′-O-methyltransferase (IOMT; P=0.021). CA and AFS roots show that cold acclimation and freezing stress induced slight but significant up-regulation of C4H gene and down-regulation of the IOMT gene (Table 4.4). The gene expression of both genes in roots were lower in RAF than in NA roots. The relative expression of C4H was higher in A-TF0 than in A-TF7 in the NA and RAF samplings (Figs. 4.7a and 4.7b, Supplemental Table 4.5) while the expression of IOMT was higher in A-TF0 than in A-TF7 in CA plants (Fig. 4.7c, Supplemental Table 4.5). We also observed an interaction between alfalfa population and *S. meliloti* strains on the relative expression of IOMT in AFS sampling (Fig. 4.8d and Supplemental Table 4.5). Relative expression of IOMT was 77% higher for alfalfa population A-TF0 combined with strain B399 when compared to alfalfa population A-TF7 combined with the same strain and both association of alfalfa population inoculated with NRG34 were intermediate and were not different (Fig. 4.7d).

4.5 Discussion

The study of winter stress tolerance in legumes needs to consider the complete symbiotic system including both plants and bacteria since these two partners are differentially affected by stress conditions (Hawkins and Oresnik 2022). Here, we compared the regrowth after a freezing stress of four different associations of alfalfa populations and *S. meliloti* strains and observed up to 35% yield differences between the best (A-TF7 × NRG34) and the worst (A-TF0 × B399) association (Fig. 4.2a). Both partners were shown to contribute to the recovery after exposure to a freezing stress with a 12% difference that was explained by a population effect (A-TF0 vs A-TF7) and 17% that was attributable to a strain effect (B399 vs NRG34). To understand the contribution of each partner to the better regrowth performance of an association after freezing, a thorough investigation of the mechanisms at play was undertaken by monitoring metabolites and genes having major roles in cold acclimation, freezing tolerance, and those involved in the crosstalk

between alfalfa and its symbiotic partner. The metabolomic study of roots, crowns and, more specifically, of nodules, revealed profound changes in these organs, switching from a sink to support cold acclimation to a source of reserves enabling regrowth after deacclimation. Our approach tracing molecular changes separately in alfalfa populations and rhizobial strains provided a novel perspective on their respective contribution to the vigor of spring regrowth of alfalfa. It also allowed the identification of molecular traits potentially conferring superior productivity of alfalfa that was exposed to a sublethal freezing stress.

4.5.1 Effects of cold acclimation, freezing stress and deacclimation on metabolites and gene expression

4.5.1.1 Sugars

In response to cold acclimation we noted an important increase of soluble sugars concentrations in nodules, roots and crowns of alfalfa. Those observations are concordant with similar results reported of the accumulation of soluble sugars in perennial organs of alfalfa in response to low temperature and photoperiod to provide energy for plant overwintering and regrowth (Fig. 4.3, Castonguay et al., 2011; Bertrand et al., 2017). The separate biochemical analysis of nodules allowed us to better understand the pivotal role of these belowground organs. For instance, our results highlight the importance of nodules as large carbon sinks during cold acclimation leading not only to the accumulation of sucrose, stachyose, raffinose, and starch in these organs, but also to a significant increase in their biomass after exposure to cold temperatures (CA) (Fig. 4.2). The allocation of photosynthates into nodules has been linked with the translocation of sugars from other plant organs such as leaves and roots in response to abiotic stress (Bertrand et al., 2016). The large hydrolysis of starch in roots and crowns likely contributed to the new carbohydrates input, including starch, in nodules (Fig. 4.3). An accumulation of starch grains, amyloplast and oleosomes have been observed in perennial beach pea nodules before winter (Gurusamy et al., 2000; Chinnasamy and Bal 2003) and these storage compounds were proposed to serve as energy source for metabolic activities during winter dormancy of nodules that lasts for prolonged periods under arctic/subarctic conditions. In our study, the concentration of total sugars in nodules decreased to levels below those observed in NA nodules upon the return of alfalfa to optimal regrowth conditions (RAF). This observation makes a compelling case for soluble sugars

and starch as key contributors to the nodules survival during overwintering period and as a source of energy to resume N fixation and ultimately support alfalfa regrowth in the spring (D'Amours et al., 2022). An important accumulation of sucrose was observed in nodules, roots and crowns in response to cold acclimation while its concentration decreased in roots exudates with sampling events. Sucrose is a recognized cryoprotectant that can stabilize freeze-dehydrated cells by interacting with cell membrane (Tarkowski and Van den Ende 2015). In alfalfa, a close relationship between sucrose accumulation and superior freezing tolerance has been demonstrated (Castonguay et al., 2011). The decrease in sucrose at regrowth after freezing (RAF) could be partly explained by the quick up-regulation of sucrose synthase (Susy) in nodules after freezing stress to cleave sucrose into fructose and glucose and to provide succinate and malate to the bacteroids, directly feeding into their tricarboxylic acid cycle (TCA) cycle (Geddes and Oresnik, 2014; Liu et al., 2018). Interestingly, fructose concentration increased more than three fold in nodules 48 hours after freezing stress. Fructose has a high capacity for scavenging superoxide and has been shown to be involved in antioxidative protection in pea under chilling stress (Bogdanović et al., 2008). The marked increase in RFO in root exudates, roots, crowns and nodules in response to CA confirm their importance in the freezing tolerance of alfalfa. Concentrations of RFO are intrinsically linked with the level of freezing tolerance of alfalfa due to their cryoprotective actions (Bertrand et al., 2017). Furthermore, RFO are known to support the growth and survival of symbiotic N-fixing bacteria (e.g. *S. meliloti*) in the rhizosphere of germinating seeds and alfalfa seedlings (Bringhurst et al., 2001). The larger concentration of RFO in nodules than in other perennial organs (crowns and roots) highlights the importance of the investment of host plant into nodules protection to maximize their survival and allow for a quick return of N fixation in the spring to support alfalfa regrowth.

4.5.1.2 Amino acids

Amino acids constitute an important source of organic N for spring regrowth and several amino acids possess osmoprotectant and cryoprotectant attributes that help stabilize plant cells under stress (Dhont et al., 2006; Bertrand et al., 2017, 2020a). Cold acclimation has been frequently reported to induce an increase in total amino acids concentration in roots and crowns of alfalfa plants grown under non-limiting nitrogen conditions (Dhont et al., 2006; Castonguay et al., 2011; Bertrand et al., 2020a). In the present study, total amino acids (AATot) did not increase in

nodules, roots and crowns in response to cold acclimation which could be due to the fact that the only source of N was provided by the symbiotic N fixation. The conditions of cold acclimation that were used in this experiment (low temperature and short photoperiod) were far from optimal for symbiotic N fixation which is most effective at 25°C (Alexandre and Oliveira 2013). These conditions likely restricted amino acids synthesis and accumulation, as opposed to what is observed when non-limiting inorganic N source is used with cold acclimated alfalfa plants as in Castonguay et al., (2011). Amino acid concentrations were almost four fold higher in nodules than in roots and crowns, and this was a constant effect at all sampling events, showing the importance of resource investment in nodules by host plants (Fig. 4.3). Asparagine, which is recognized as the primary N fixed compound as well as the principal amino acid transported in xylem in legume species with indeterminate nodules such as alfalfa, was the most abundant amino acid in all samples for non-acclimated plants and represented up to 80% of the pool of free amino acids (Table 4.3, Sulieman et al., 2010; Bertrand et al., 2016). Its progressive decrease in concentration at each sampling event (Fig. 4.3) confirms the slowdown of N fixation under CA as well as the transformation of this major pool of N compounds into other amino acids involved specifically in stress tolerance. For instance, ornithine and arginine as well as their precursor glutamate increased in response to CA and are known to be precursors of polyamines which confer plant resistance to various abiotic stresses (Anwar et al., 2018). Ornithine has also been reported as a signal and regulatory molecule (Majumdar et al., 2016), which could explain why its concentration, while changing significantly in response to CA and freezing, remained low as compared to arginine. Arginine, with its high N to C ratio is considered a storage compound from which N could be readily incorporated into other N-compounds essential for active regrowth, as previously reported in overwintering alfalfa (Dhont et al., 2006; Castonguay et al., 2011). While proline accumulation in responses to low temperatures has previously been observed in alfalfa (Castonguay et al., 2011; Liu et al., 2019; Bertrand et al., 2020a), its direct involvement in the acquisition of freezing tolerance remains unclear. In the present study, proline concentration did not increase in response to decreased temperatures suggesting that proline synthesis is regulated independently of the glutamate-ornithine-arginine pathway (Majumdar et al., 2016). On the other hand, the lack of proline increase could indicate that low temperatures stress increased the degradation of proline to provide a source of carbon and nitrogen to the bacteroid, thereby supporting recent studies suggesting that proline metabolism may play an essential role of energy transfer in the legume-

Rhizobium symbiosis under stress (Sabbioni and Folarni 2022). We also noted an increase of alanine, serine and glycine with cold acclimation in nodules and an increase of those three amino acids in roots and crowns 48 hours after the freezing stress. Alanine accumulation is recognized as an universal first stress signal in a wide variety of organisms including plants (Table 4.3, Ben-Izhak Monselise et al., 2003). Moreover, it has been recently proposed that alanine could play an important role in the symbiosis by sustaining bacteroid metabolism under oxygen limitation (Schulte et al., 2021). Serine/glycine metabolism was shown to be an important key player in biochemical adaptation to environmental stress by regulation of intracellular redox, pH regulation and energy levels (Igamberdiev and Kleczkowski 2018). Another interesting observation is a sharp increase of methionine, tyrosine, phenylalanine, threonine, lysine, isoleucine and leucine concentrations 48 hours after freezing stress (Fig. 4.3, AFS). Methionine synthesis, which is provided by the host plant to the nodules, has been reported to be essential for efficient nodulation by various rhizobia (Barra et al., 2006). Tyrosine and phenylalanine are aromatic amino acid produced by the shikimate pathway which require phosphoenolpyruvate as substrate (Dunn 2014). Tyrosine is essential for the nodule formation and can be used by the bacteroid as source of carbon and nitrogen (Saha et al., 2016). Both tyrosine and phenylalanine can act as substrates for the phenylpropanoids biosynthesis pathway and were found in root exudates (Feduraev et al., 2020). Other protein-bounded amino acids have been reported to increase in response to cold and frost stress in *A. thaliana* and *Camellia sinensis* and, while the authors suggested a role for these amino acids in freezing stress acquisition, the mechanism involved would need further investigations (Hildebrandt 2018; Samarina et al., 2020).

4.5.1.3 Flavonoids

Flavonoids are crucial signaling molecules that play an essential role in the *Rhizobium*-legume symbiosis as chemoattractant and *nod* gene inducers. Flavonoids are excreted by the plant in the rhizosphere to modulate communications with microorganisms, either to ensure protection against pathogens or to attract beneficial microbes (Falcone Ferreyra et al., 2012). Only few and specific flavonoids excreted by the legume-host will activate the expression of a group of bacterial *nod* genes, leading to the synthesis of the Nod factor (lipo-chitooligosaccharides), essential for initiating nodules formation and to maintain symbiotic activity (Reviewed in Liu and Murray, 2016). Following a stress, the crosstalk between the host plants and microorganisms is modified

to face the new conditions and the measurement of flavonoid concentrations in root exudates could give information on the signals exchanged between the partners. An increase of formononetin and medicarpin was observed in root exudates after CA (Fig. 4.3). Formononetin is the most abundant flavonoid in alfalfa while its derivative medicarpin is the second most abundant (Gifford et al., 2018). Formononetin has been reported to be involved in nodules organogenesis (Mathesius 2001) and in the activation of *nod* gene transcription in *Rhizobium meliloti* (Table 4.3, Dakora et al., 1993). Thus, the CA-induced increase of formononetin concentration in roots exudates could be linked to the *de novo* nodules synthesis necessary for the plant to acclimate to cold. On the other hand, formononetin could have been released directly from stressed roots to regulate germination of pathogenic fungi (Tsai and Philipps 1991). Formononetin is also a precursor of medicarpin which was shown to exert an inhibitory effect on incompatible bacterial strains and to repress *nod* gene transcription in alfalfa roots (Hartwig et al., 1990). The increase in medicarpin concentration in root exudates under CA indicates that protection mechanisms against pathogens are also in place. Those mechanisms play an important role in the host range rhizobia specificity of the symbiotic association since pathogenic bacteria can produce similar signaling molecules to facilitate their invasion of the host plant (Wang and Shu 2018). The triggering of protection mechanisms is supported by the observation of large increases in echinatin, coumestrol and medicarpin concomitant with decrease in formononetin in nodules during the experiment. Echinatin is a potent antagonist of Gram+ bacteria with antifungal and antibacterial activity (Dong and Song 2020) while coumestrol has been shown to be induced by fungal infection in alfalfa (Table 4.3, Fields et al., 2018). Increase in medicarpin and decrease in formononetin in CA nodules could have had an inhibitory effect on the *nod* gene activity and the cellular division of *S. meliloti* (Zhang et al., 2009). Flavonoids have also been reported to provide stress protection against UV light, drought, salinity, freezing and to act as scavengers of free radicals such as reactive oxygen species (Schulz et al., 2016; Baskar et al., 2018; Sharma et al., 2019; Laoué et al., 2022). Recently, it has been reported that genes involved in the phenylpropanoids pathways are regulated by low temperatures in alfalfa (Liu et al., 2022). Echinatin could potently increase plant tolerance against several biotic and abiotic stresses (Tripathi et al., 2016; Sharma et al., 2019). Luteolin is essential for the nodulation process by controlling the expression of *nodABC* during the development of the symbiosis between rhizobia and alfalfa and has also been reported as an excellent free-radical scavenger to protect again oxidative stress (Chen et al., 2020), to enhance starch hydrolysis and

soluble sugars accumulation (El-Shafey and AbdElgawad 2012), and to be involved in enhanced salt stress tolerance (Song et al., 2022).

4.5.1.4 COR genes expression

The expression of the following COR genes of carbohydrate synthetic pathways were affected by the exposure to cold temperature in alfalfa crowns: galactinol synthase (GaS), sucrose synthase (Susy), and sucrose phosphatase synthase (SPS) (Table 4.4). For instance, the transcript level of GaS, a key enzyme catalyzing the first step of RFO biosynthesis, increased markedly in response to CA, in accordance with the sharp increase in raffinose and stachyose concentrations that we observed in crowns, and in accordance with previous reports (Cunningham et al., 2003; Bertrand et al., 2016; Liu et al., 2019; Xu et al., 2020). Consistently with previous observations in alfalfa (Bertrand et al., 2017), the gene expression of Susy declined in response to CA and, since Susy is responsible for the cleavage of sucrose into glucose and fructose, its down-regulation along with the up regulation of SPS resulted in sucrose accumulation that we observed in storage organs in response to CA. The Susy progressively returned to the non-acclimated level at the next sampling events which is consistent with the progressive decrease in sucrose concentration in crowns, roots and nodules after the freezing stress. Furthermore, it was suggested that Susy activity might be essential for N fixation in root nodules (Gordon et al., 1999). The quick induction of Susy expression that we observed 48 hours after the freezing stress concurs with an important role for Susy in the deacclimation process as it would positively activate the reprise of biological N fixation in post-freezing nodules (Gordon et al., 1999). A strong up-regulation of K3-dehydrin, coding for an osmotic-stress protein was also observed in response to cold acclimation. The K3-dehydrin is known to be associated with freezing tolerance in cold-acclimated alfalfa trough membrane-stabilizing effect (Dubé et al., 2003; Bertrand et al., 2016; Xu et al., 2020). The transcript levels of K3-dehydrin did not vary between alfalfa-rhizobia associations and, as such, the up-regulation of K3-dehydrin seems to be part of a general protective process of cold acclimation in alfalfa. In general, our results confirmed previous studies showing that COR genes involved in carbohydrate metabolic pathways are crucial for cold acclimation and freezing stress tolerance and that they are also key actors in the deacclimation process. It would be interesting to investigate the level of expression of those genes in nodules and in response to cold/freezing/deacclimation processes to better understand their role in the recovery of the symbiotic association after freezing.

4.5.2 Contribution of each partner to the increased freezing tolerance of the association

4.5.2.1 Sugars and amino acids

We monitored the independent effects of populations and strains on metabolic changes that occurred during cold acclimation (Fig. 4.4) and deacclimation (Fig. 4.5) to better understand their respective contribution to the recovery of the symbiotic association after a freezing stress. In general, metabolites concentration in crowns significantly differed mainly in response to alfalfa populations while strains affected mainly nodules metabolites. For instance, RFOs and sucrose increased in alfalfa crowns in response to CA (Fig. 4.3) but only sucrose was more abundant in freezing-tolerant population A-TF7 as compared to A-TF0, indicating that under conditions of the current study the accumulation of sucrose was more determinant for regrowth after freezing than RFO. Our observations concur with previous reports on cold-induced accumulation of cryoprotective sucrose and RFO in alfalfa populations recurrently selected for superior freezing tolerance (Castonguay et al., 2011). Interestingly, the accumulation of glucose and fructose in CA nodules in response to the inoculation with freeze-sensitive strain B399 seems to indicate that the strain has an effect on the hydrolysis of sucrose by the plant. In response to CA, both symbiotic partners of the most freezing tolerant association (A-TF7 × NRG34) induced an increase in arginine and histidine, confirming the importance of a strategy for N storage during cold acclimation of alfalfa. The response to deacclimation (RAF) also shows a greater remobilisation of N through arginine, ornithine, and histidine in A-TF7 as compared to A-TF0 as well as through arginine for NRG34. During cold acclimation and deacclimation, both symbiotic partners of the most freezing sensitive association (A-TF0 × B399) showed higher accumulation of osmoprotectants and scavenging reacting oxygen species amino acids like GABA, serine, alanine and proline in crowns and proline in nodules (Figs. 4.4 and 4.5). These reactions could indicate that these plants suffered more damage by freezing that triggered reparation mechanisms such as ROS scavenging. On the other hand, higher contents of cryoprotective substances after freezing stress could be beneficial to reduce the risks of damages caused by abrupt freeze-thaw episode in early autumn or spring (Xu et al., 2020).

4.5.2.2 Flavonoids and regulation of C4H and IOMT genes

Production of flavonoids by the phenylpropanoid pathway was influenced by both symbiotic partners at different sampling events. With cold acclimation we found a higher concentration of the nodulation repressor medicarpin in nodules of the less freezing tolerant association (A-TF0 × B399) when compared to the most freezing tolerant association (A-TF7 × NRG34) (Supplemental Table 4.2). The inhibitory and antimicrobial effect of medicarpin could be linked with the higher incidence of freezing damage and necrosis of nodules of those plants as observed in D'Amours et al., (2022). The up-regulation of the cinnamic acid 4-hydroxylase (C4H) gene was observed in roots in response to CA, which is coherent with the observed concentration increases of the flavonoid precursor tyrosine as well as of total flavonoids concentrations in roots and nodules observed at this sampling point. Low temperature has been reported as the main factor responsible for the accumulation of flavonoids with antioxidant properties, either through induced expression of the encoding gene or increase enzymatic activity of C4H (He et al., 2022). However, as a major rate-limiting enzyme in the phenylpropanoid biosynthesis that separates the pinocembrin pathway from the lignin/monolignol synthesis pathway, the up-regulation of C4H could also be linked to the accumulation of other secondary metabolites including lignin (Gifford et al., 2018). In alfalfa, isoflavone O-methyltransferase (IOMT) catalyses the reaction leading to 7-O-methyl daidzein, the precursor of formononetin and, further down the pathway to medicarpin (He et al., 1998). The IOMT was down-regulated with CA and its expression was lower in freezing-tolerant population A-TF7 than in A-TF0. This is consistent with the decrease of formononetin observed in both roots and nodules at CA sampling and with the higher concentration of medicarpin in root exudates of A-TF0. A lesser repression of expression of the gene involved in synthesis of formononetin and medicarpin by cold and freezing stress could be associated with less freezing tolerance. Upon return to conditions allowing regrowth, at RAF, the expression of IOMT further increased. A larger formononetin concentration in roots of plants inoculated with NRG34 at RAF suggests that strain-induced signals are more active during regrowth after freezing in the specific A-TF7 × NRG34 association. However further investigations are necessary to better understand the role of flavonoids in plant stress tolerance (He et al., 2022)

4.6 Conclusion

This study highlights the importance to consider the rhizobial symbiosis in strategies aimed at improving stress tolerance in legumes. Rhizobia and plants play complementary roles to ensure enhanced regrowth after freezing. It was shown that root nodules, while accumulating a large pool of free amino acids and carbohydrates during cold acclimation, turned into a source of reserves that enable regrowth in spring after deacclimation. This work also identified metabolic and genetic traits that confer superior yields to alfalfa populations exposed to a sublethal freezing stress such as the accumulation and remobilization of N storage amino acids during cold acclimation and deacclimation, respectively. Choosing the right partners when applying rhizobial inoculants may contribute in improving the stress tolerance of alfalfa.

4.7 Acknowledgments

This work was supported by a grant program of Agriculture and Agri-Food Canada. The first author was financially support-ed by a scholarship from the Fonds de Recherche Québécois Nature et Technologies. The authors sincerely thank Josée Bourassa, Josée Michaud, Sandra Delaney and David Gagné for their technical assistance and Dr Yves Castonguay for his thorough revision of the manuscript. We also thank, Dr Nicolas Ayub from Instituto de Genética “Edwald Alfredo Favret”, INTA who kindly provided us the commercial *S. meliloti* strain, B399.

4.8 Statements & Declarations

Funding

This work was supported by a grant program of Agriculture and Agri-Food Canada. The first author was financially supported by scholarship from the Fonds de Recherche Québécois Nature et Technologies.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Emmanuelle D'Amours, Annick Bertrand and Jean Cloutier. The first draft of the manuscript was written by Emmanuelle D'Amours and all authors revised the previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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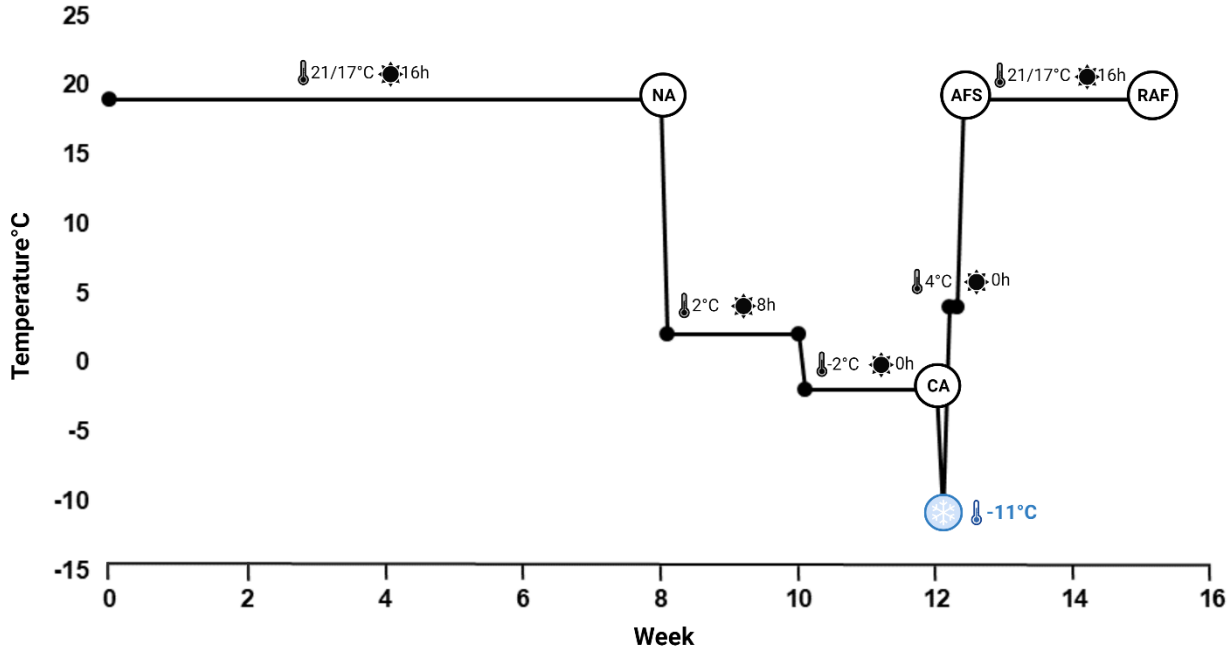

Figure 4.1 Plants growth conditions and sampling events. Plants grown under controlled conditions were harvested at four sampling events: non-acclimated plants (NA) were sampled after being grown for 8 weeks under a 21/17°C, Day/Night (D/N) temperature regime. Plants were then cold acclimated for 4 weeks (CA) and sampled, and then exposed to a freezing stress of -11°C and transferred back to optimal regrowth conditions (21/17°C, D/N). Two days after freezing stress (AFS) and three weeks of regrowth after freezing (RAF) plants were sampled again

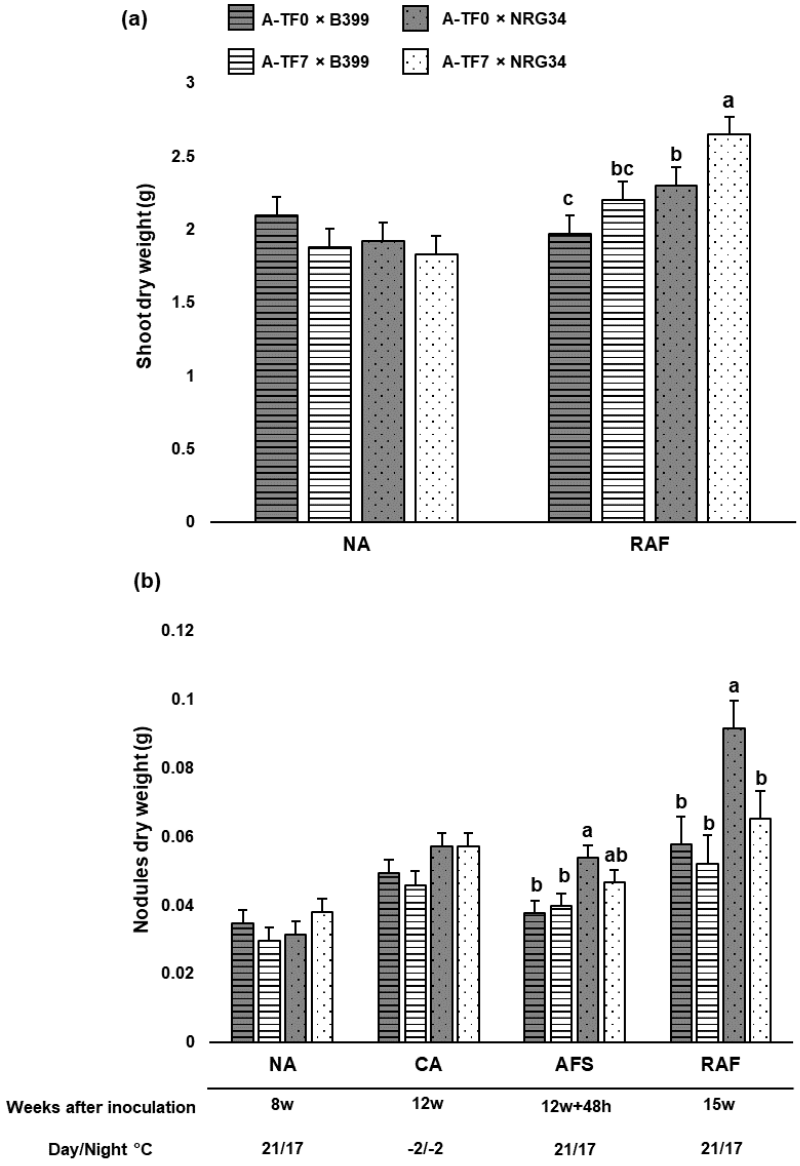

Figure 4.2 Shoot dry weight (DW) (a), and nodules DW (b) of four associations combining two alfalfa populations A-TF0 (in grey) and A-TF7 (in white) inoculated with two *S. meliloti* strains B399 (lined pattern) and NRG34 (dotted pattern). Alfalfa plants were grown under controlled conditions and sampled at four sampling events: non-acclimated plants (NA) were grown 8 weeks under a 21/17°C, Day/Night (D/N) temperature regime. Plants were then cold acclimated during 2 weeks at 2°C followed by two weeks at -2°C and sampled again (CA). After their exposure to a freezing stress of -11°C, alfalfa plants were transferred back to optimal regrowth conditions (21/17°C, D/N) and sampled after two days (AFS), and after three weeks (RAF). Shoots were only

sampled for NA and RAF plants since CA and AFS shoots and leaves were killed by the 2weeks exposure to subfreezing temperature in the dark while nodule dry weight was measured at the four sampling events. Error bars represent the Standard Error of the Mean (SEM), n = 8. No letter means that there is no significant differences between treatments for that event sampling while different letters represent significant differences as determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$

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

Figure 4.3 Average concentration of metabolites measured in exudates, nodules, roots and crowns of the four alfalfa × strain associations at each sampling event(n = 32). Metabolite concentrations of sugars (mg/g DM), amino acids (µmol/g DM) and flavonoids (µg/g DM) were measured in non-acclimated plants (NA), cold-acclimated (CA) plants, plants exposed to a freezing stress and transferred back to optimal regrowth conditions for 48 h (AFS), and after a 3-weeks regrowth (RAF). Colors represent the log 2 fold change of either a decrease (red) or an increase (blue) of metabolite concentrations compared to NA plants. Color code is shown at the bottom of the last column of the Figure 4.3. Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Formo, formononetin, FlaTot, total flavonoids; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, leucine; Lys, lysine; Met, methionine; NSC, nonstructural carbohydrates; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine; Orn, Ornithine; AATot, Total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid

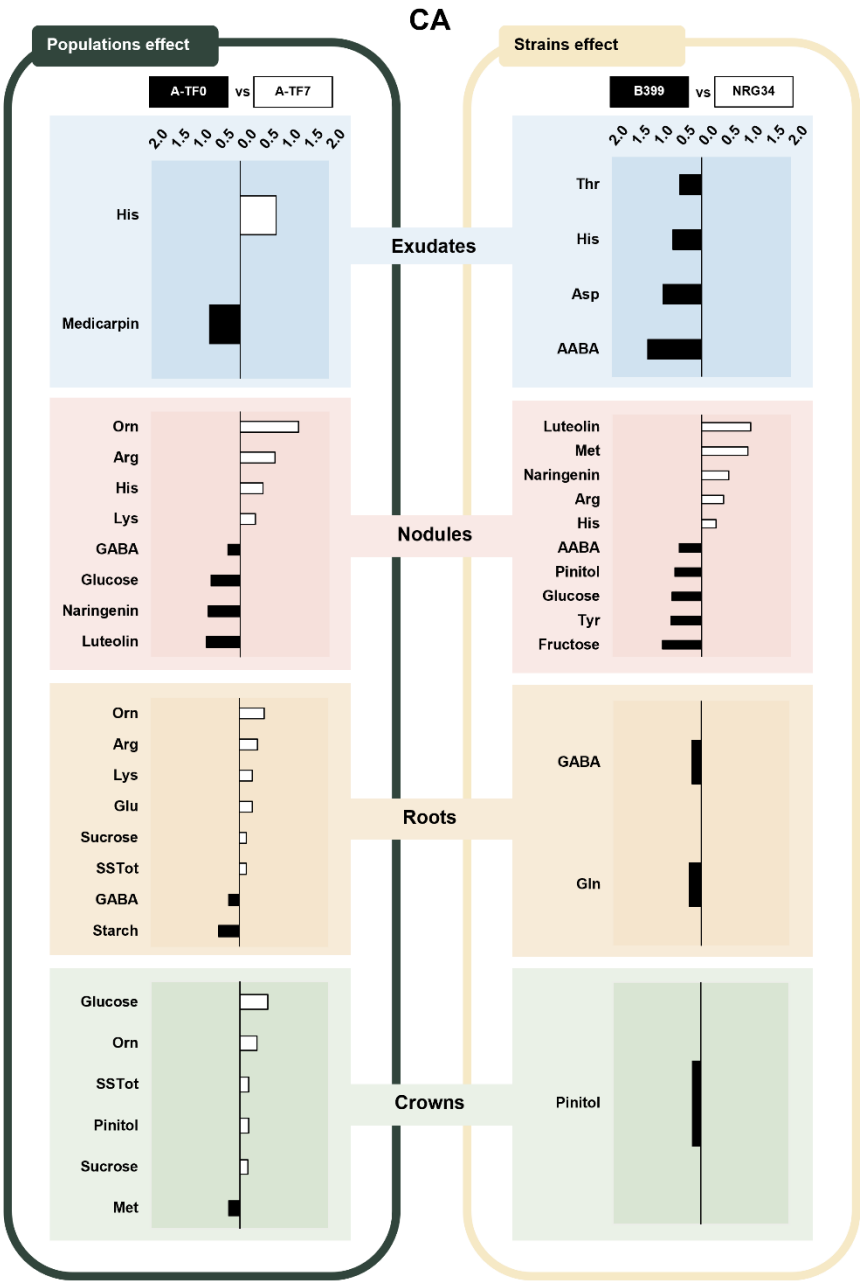

Figure 4.4 Graphic representation of log 2 fold changes in metabolites concentration showing the differential contribution of each alfalfa population (A-TF0 vs A-TF7) and each *S. meliloti* strain (B399 vs NRG34) to the metabolic changes in CA root exudates, nodules, roots and crowns. The left panel compares the two alfalfa populations with significant higher concentration in A-TF7 on the right to zero line (white) and significant higher concentration in A-TF0 on the left (black). The right panel compares the two rhizobial strains with significant higher concentration in response to inoculation with NRG34 on the right to zero line (white) and significant higher concentration in

response to B399 on the left (black). Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Formo, formononetin, FlaTot, total flavonoids, Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, leucine; Lys, lysine; Met, methionine; NSC, nonstructural carbohydrates; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine; Orn, ornithine, AATot, Total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid

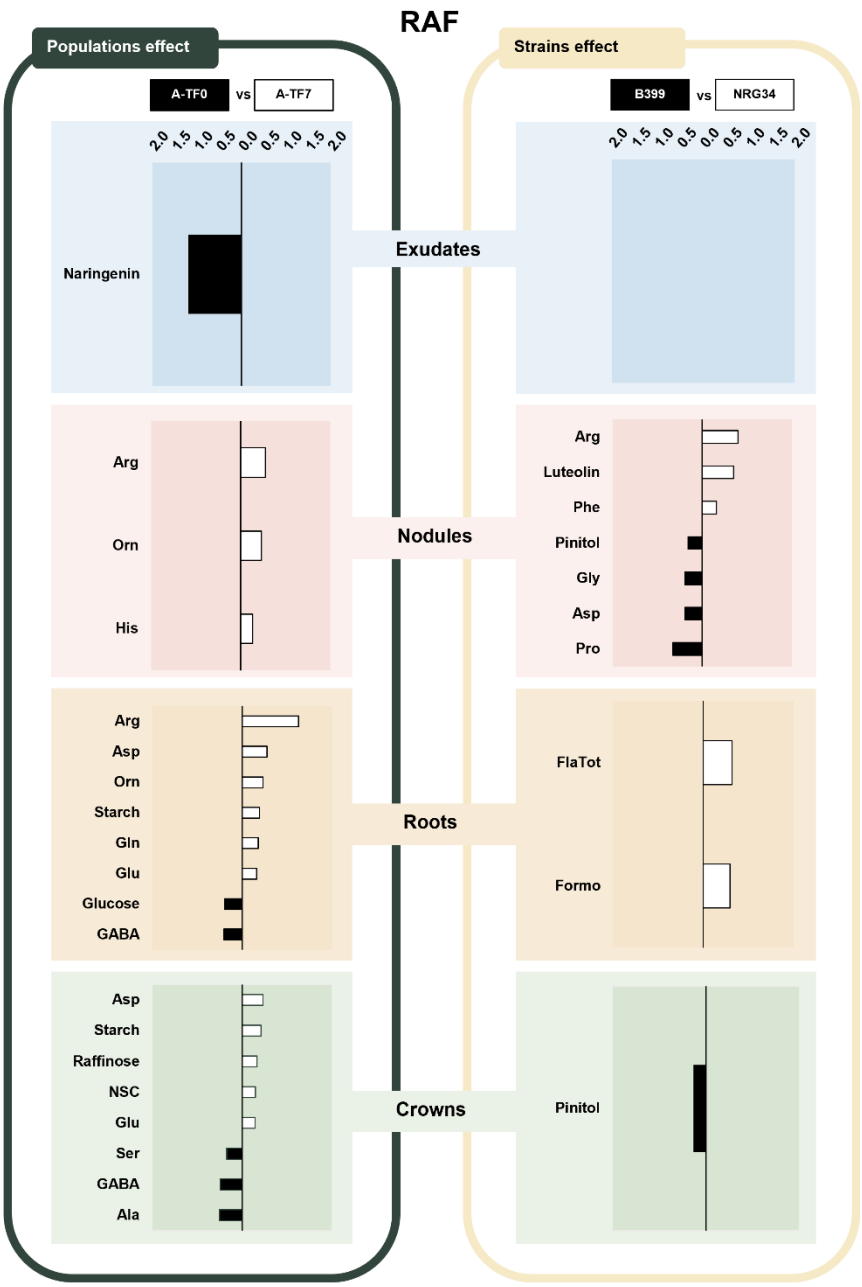

Figure 4.5 Graphic representation of log 2 fold changes in metabolites concentration showing the differential contribution of each alfalfa population (A-TF0 vs A-TF7) and each *S. meliloti* strain (B399 vs NRG34) to the metabolic changes in RAF root exudates, nodules, roots and crowns. The left panel compares the two alfalfa populations with significant higher concentration in ATF-7 on the right to zero line (white) and significant higher concentration in ATF-0 on the left (black). The right panel compares the two rhizobial strains with significant higher concentration in response to

inoculation with NRG34 on the right to zero line (white) and significant higher concentration in response to B399 on the left (black). Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Formo, formononetin, FlaTot, total flavonoids, Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, leucine; Lys, lysine; Met, methionine; NSC, nonstructural carbohydrates; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine; Orn, ornithine, AATot, Total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid

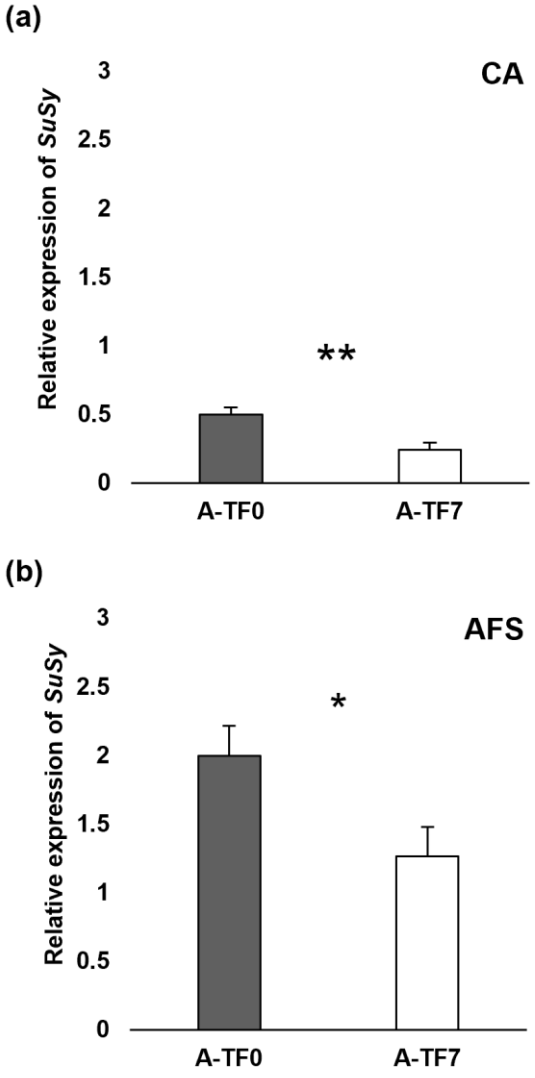

Figure 4.6 Relative expression in alfalfa crowns of sucrose synthase (SuSy) in (a) cold-acclimated (CA) and (b) 48 hours after freezing stress in two alfalfa populations contrasted in their freezing tolerance levels (A-TF0 and A-TF7) analyzed by RT-qPCR. Error bars represent the SEM, n = 16. Significant differences between populations at each event, determined by a t-test, are indicated with the following levels of probability: * $P \leq 0.05$, ** $P < 0.01$ ***

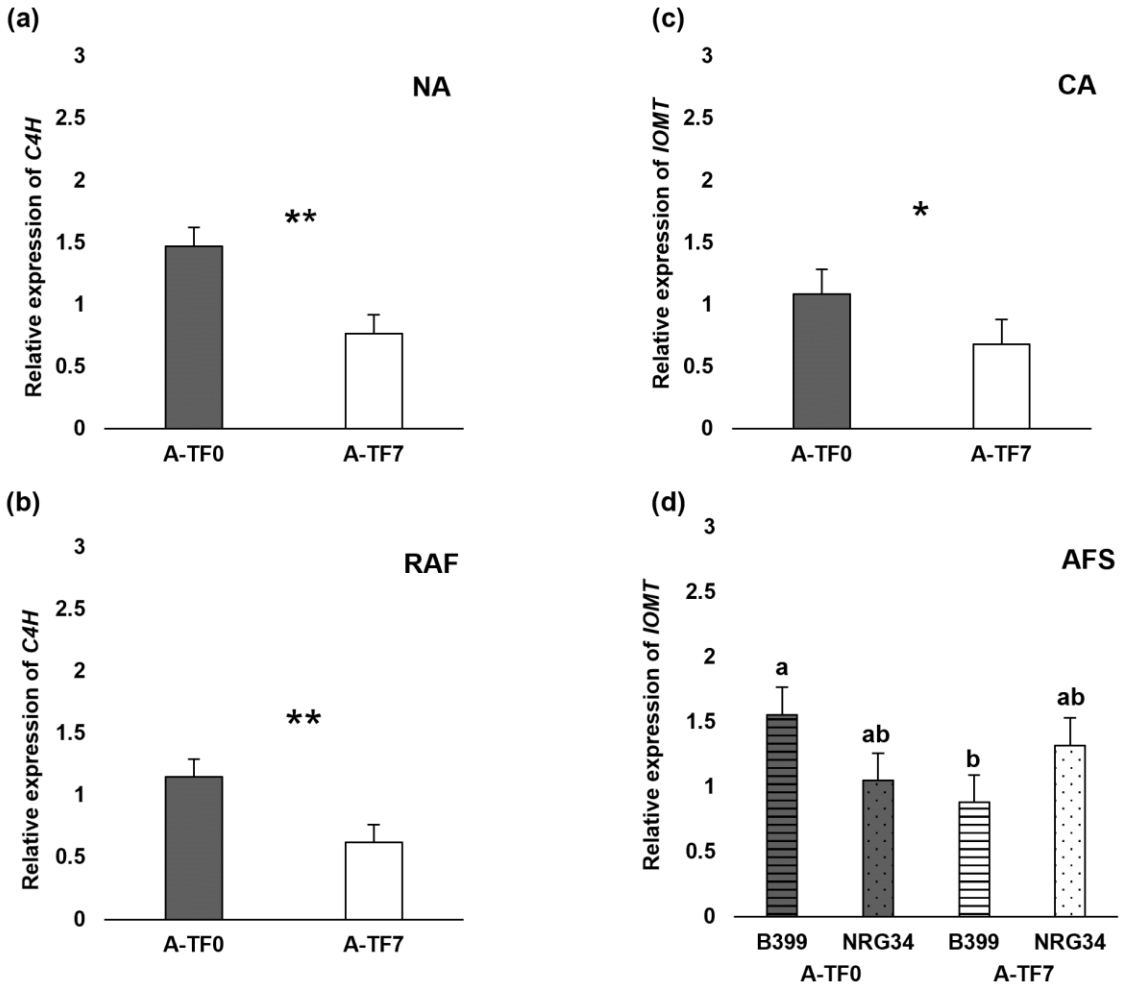

Figure 4.7 Relative expression in roots of cinnamic acid 4-hydroxylase (C4H) in non-acclimated NA alfalfa (a) and in RAF alfalfa regrowth (b). Relative expression of isoflavone O-methyltransferase (IOMT) (c) *i*n two CA alfalfa populations contrasted in their freezing tolerance levels (A-TF0 and A-TF7) and of four associations combining two alfalfa populations (grey scale) A-TF0 (in grey) and A-TF7 (in white) inoculated two *S. meliloti* strains (patterns), B399 (lined) and NRG34 (dotted), sampled 48 h after freezing (AFS) (d) Error bars represent the SEM for n = 16. Significant differences between populations at each event, determined by a t-test, are indicated with the following levels of probability: * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$. Different letters indicate significant differences in relative expression of IOMT due to pop × strains interactions at $P \leq 0.05$ (n=8)

Table 4.1 List of genes of interest (GOI) and reference genes (Ref) selected for expression analysis in alfalfa crowns and/or roots, sequence homology based on *Medicago trunculata* genes retrieved using a BLAST search in the NCBI database, sequences of PCR primers, and PCR fragment sizes are also presented

Plant Sample	Gene	Gene annotation	Type	Primer sequence (5'-3')	Primer reference	Amplicon size (bp)
Crown	GaS	Galacticnol synthase	GOI	ACCCTCCTGAGAACCAAACC GGCCTCACAGAAACAGTCCA	Bertrand et al., 2017	197
Crown	SPS	Sucrose phosphate synthase	GOI	TCCCAAGCCCTCAGATACC CTGCTTCCGACTCCCTTCA	Castonguay et al., 2015	146
Crown	Susy	Sucrose synthase	GOI	CCGATTGACATCCTTCTACCC GTCCTTTGACTCCTTCCTCCT	Castonguay et al., 2015	235
Crown	K3-dehydrin	K3-dehydrin	GOI	GGAGCTGTGGACAAGATCAAGG GTGTCCTTGTCCATGTCCAGTACA	Dubé et al., 2013	345
Crown / Roots	Aptase	Vacuolar H+- ATPase A subunit	Ref	CTACGAACGTGCTGGGAAAG GAGGGTTGCAGATGTCACG	Castonguay et al., 2015	124
Crown	UBQ2	Ubiquitin 2	Ref	GGACTCAAGGTGGCCAAAC GAGGGTTGCAGATGTCACG	Castonguay et al., 2015	197
Roots	C4H	Cinamic acid 4-hydoxylase	GOI	CCCCGGAATCATTCTTGCCT TTTGATTGTCCGGGTGGAGG	Designed for this study	93
Roots	IOMT	Isoflavone-O-methytransferase	GOI	CGCACAACGGATTCTTCGAG ACGAACCCGAAAGAGTTGGA	Designed for this study	144
Roots	eEF-1α	Eukariotic elongation factor 1-alphas	Ref	GAGCCAAAGAGACCCACAGAC TCAGTGAGAGCCTCGTGGT	Castonguay et al., 2015	192

Table 4.2 *P* values of the analysis of variance comparing the effects of four alfalfa populations × rhizobia strains associations (A-TF0 × B399, A-TF0 × NRG34, A-TF7 × B399 and A-TF7 × NRG34) on shoot dry weight (g) and nodules dry weight (g) of alfalfa. Measurements were made at four sampling events: in non-acclimated plants (NA), cold-acclimated (CA) plants, plants exposed to a freezing stress and transferred back to optimal regrowth conditions for 48 h (AFS), and after a 3-weeks regrowth (RAF). Shoot DW could not be measured in CA and AFS plants as the shoots were damaged by these treatments (not assessed, n.a.). Numbers in bold represent significant differences at $P \leq 0.05$

	Event sampling			
	NA	**CA**	**AFS**	**RAF**
Shoot dry weight	0.480	n.a	n.a	**<0.001**
Nodules dry weight	0.430	0.120	**0.016**	**0.011**

Table 4.3 Analysis of variance *(P values*) comparing the effect of sampling events on the concentration of different metabolites (sugars, amino acids, and flavonoids) in root exudates, nodules, roots and crowns of four alfalfa populations-rhizobial strains associations. Alfalfa plants grown under controlled conditions were sampled at four sampling events: non-acclimated (NA) plants were sampled after eight weeks of growth; Cold acclimated (CA) plants (were sampled after four weeks of cold-acclimation 48 h after freezing stress (AFS), AFS plants were sampled and plants were sampled after three weeks of regrowth after freezing (RAF). Reported metabolic functions of sugars and amino acids related to cold acclimation and freezing stress tolerance in plants are presented as well as the reported functions of flavonoids in the rhizosphere with their corresponding references

Metabolites	Metabolic functions	References	Event sampling			
			Alfalfa organs			
			Exudates	Nodules	Roots	Crowns
			P values			
Sugars						
Sucrose	Osmotic adjustment	Dhont et al., 2002	<0.001	<0.001	<0.001	<0.001
Glucose	Osmotic adjustment	Dhont et al., 2002	0.0903	0.0049	0.0133	<0.001
Stachyose	Cryoprotectant	Castonguay et al., 2011	<0.001	<0.001	<0.001	<0.001
Raffinose	Cryoprotectant	Castonguay et al., 2011	<0.001	<0.001	<0.001	<0.001
Fructose	TCA cycle/ antioxidative protection under cold stress	Bogdanović et al., 2008	0.1533	<0.001	<0.001	<0.001
Pinitol	Osmoprotectant	Castonguay et al., 2011	0.0068	<0.001	<0.001	<0.001
SSTot				<0.001	<0.001	<0.001
Starch	Carbon storage	Gurusamy et al., 2000; Dhont et al., 2002;		0.015	<0.001	<0.001
NSC				<0.001	<0.001	<0.001
Amino Acids						
Glu	Nitrogen assimilation and transport	Liu et al., 2018	0.508	0.00	<0.001	<0.001
Gln	Nitrogen assimilation and transport	Liu et al., 2018	<0.001	0.00	<0.001	<0.001

Pro	Osmoprotectant	Castonguay et al., 2011	<0.001	0.36	<0.001	0.48
Orn	Polyamine synthesis, Arg pathway	Majumdar et al., 2016	<0.001	<0.001	<0.001	<0.001
Arg	Cryoprotectant, chelator of nitrate to prevent damages to membranes and ice formation	Alcázar et al., 2011	0.761	<0.001	<0.001	<0.001
His	Nitrogen storage	Dhont et al., 2006	<0.001	<0.001	<0.001	<0.001
Asp	Carbon skeleton, precursor leading to the biosynthesis of multiple biomolecules required for plant growth and defense	Galili 2011	0.024	0.24	<0.001	<0.001
Asn	Nitrogen assimilation and transport	Sulieman et al., 2010	<0.001	<0.001	0.02	<0.001
Ala	First stress signal, accumulation induced by radical formation	Ben-Izhak Monselise et al., 2003; Schulte et al., 2021	<0.001	<0.001	<0.001	<0.001
Thr	Conjugated with metabolism of branched-chain amino acids Val and Leu	Hildebrandt 2018	<0.001	<0.001	<0.001	<0.001
Lys	Degradation to Val, Leu, Ile, alternative respiratory substrate under a stress	Hildebrandt 2018	0.036	<0.001	<0.001	<0.001
Met	Conjugated with metabolism of branched-chain amino acids/essential for efficient nodulation by rhizobia	Hildebrandt 2018; Barra et al., 2006	<0.001	<0.001	<0.001	<0.001
Ile	Involved in fatty acid branching pattern for membrane adaptation to low temperatures	Klein et al., 1999	<0.001	<0.001	<0.001	<0.001
Leu	Conjugated with metabolism of branched-chain amino acids	Hildebrandt 2018	<0.001	<0.001	<0.001	<0.001
Val	Conjugated with metabolism of branched-chain amino acids	Hildebrandt 2018	<0.001	<0.001	<0.001	<0.001
Ser	Carbon skeleton, regulation of intracellular redox	Igamberdiev and Kleczkowski 2018	<0.001	<0.001	<0.001	<0.001
Gly	Osmotic adjustment	Igamberdiev and Kleczkowski 2018	<0.001	0.01	<0.001	<0.001
GABA	ROS scavenging	Abd Elbar et al., 2021	<0.001	<0.001	<0.001	0.05
AABA	Isomere of GABA not well studied	Bertrand et al., 2016	<0.001	<0.001	0.09	<0.001

Tyr	Precursor of phenolic compounds, act as antioxidants with mechanisms involving both free radical scavenging and metal chelation	Feduraev et al., 2020	<0.001	<0.001	<0.001	<0.001
Phe	Precursor of phenolic compounds, act as antioxidants with mechanisms involving both free radical scavenging and metal chelation	Feduraev et al., 2020	0.001	<0.001	<0.001	<0.001
AATot			<0.001	<0.001	0.07	0.11
Flavonoids						
Naringenin	*nod* gene inducer	Peters et al., 1986	<0.001	0.01	0.46	
Luteolin	*nod* gene inducer, ROS scavenging	Peters et al., 1986; Chen et al., 2020	<0.001	<0.001	<0.001	
Echinatin	*nod* gene inducer	Hartwig et al., 1990	0.000	<0.001	<0.001	
Coumestrol	Phytoestrogen, plant defense response stress, *nod* gene repressing activity on *S. meliloti*	Fields et al., 2018/ Zuanazzi et al., 1998	0.005	<0.001	0.00	
Formononetin	*nod* gene inducer	Dakora et al., 1993	0.018	0.00	<0.001	
Medicarpin	Repress *nod* gene induction in alfalfa roots	Zuanazzi et al., 1998	<0.001	<0.001	0.31	
FlaTot			0.054	<0.001	<0.001	

Table 4.4 Relative expression levels of seven gene of interest (GOI) in crowns (five GOI) and roots (two GOI) of four associations combining two alfalfa populations A-TF0 and A-TF7 inoculated with two *S. meliloti* strains, B399 and NRG34. Alfalfa plants grown under controlled conditions were sampled at four sampling events: before cold-acclimated (NA) , after cold-acclimation (CA), after freezing stress (AFS) and after three weeks of regrowth after freezing (RAF). Relative expression levels and analysis of variance (p-value for n = 32) of the effects of sampling event are presented

Alfalfa organs	Gene	Gene annotation	Relative expression levels by sampling event				
Crowns			NA	CA	AFS	RAF	P value
	GaS	Galactinol synthase	0.34	46.93	2.13	0.81	<0.001
	SPS	Sucrose phosphate synthase	1.23	1.60	0.64	0.99	<0.001
	Susy	Sucrose synthase	1.93	0.37	1.63	1.52	<0.001
	K3-dehydrin	K3-dehydrin	0.20	33.79	0.97	0.87	<0.001
Roots							
	C4H	Cinamic acid 4-hydoxylase	1.10	1.36	1.42	0.88	0.018
	IOMT	Isoflavone-O-methytransferase	1.92	0.88	1.20	0.93	0.021

4.10 Connecting Text

The aim of chapter 4 was to better understand the underlying mechanisms leading to the improved freezing tolerance of a symbiosis by investigating the molecular contributions of each partner, either the plant population or the rhizobial strain, in the process of cold acclimation, resistance to freezing stress and deaclimation and on the crosstalk between alfalfa and rhizobia. The pivotal role of root nodules switching from an energy sink during cold acclimation to a source of energy to promote shoot regrowth of alfalfa was clearly demonstrated in Chapter 4. The effect of the recurrent alfalfa selection on metabolites concentration was most significant in crowns while the effect of the *S. meliloti* strains was exerted mainly in nodules. Hence, Chapter 4 has shown that symbiotic association between freezing tolerant alfalfa population A-TF7 and cold-adapted rhizobia strain NRG34 can improve the regrowth of alfalfa after a freezing stress by both playing a role on storage and remobilization of amino acids in nodules. Strain NRG34 was shown to improve alfalfa freezing tolerance in previous chapters and additional major characteristics are investigated in Chapter 5 to determine its potential as inoculant.

In Chapter 5, the efficiency and competitive ability under low temperature simulating spring soils conditions of the freezing tolerant strain identified NRG34 are assessed and compared to other cold-adapted strains. Efficiency of *S. meliloti* strain to early nodulate and fix N under low temperatures should improve spring growth of alfalfa and ensure a successful root colonization under cold conditions. Hence, in Chapter 5 the efficiency of four *S. meliloti* strains to nodulate and fix N is firstly tested. Thereafter, the competitive ability of cold-adapted selected strains over native rhizobia population in two soils from southern and northern Quebec is realized by tagging the selected strains thus allowing for the measurement of their nodule occupancy when in competition with native strain. The competitive ability of a strain to colonize root nodules is one major criteria to ensure a transfer from laboratory strain selection to a successful field application.

Chapter 5: Selection of effective and competitive *Sinorhizobium meliloti* strains that nodulate alfalfa under low temperature

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Reproduced from:
D'Amours, E., Bertrand, A., Cloutier, J., Claessens, A., Rocher, S., Seguin, P. (2024). Selection of effective and competitive *Sinorhizobium meliloti* strains that nodulate alfalfa under low temperature. *Rhizosphere, 29*:100860. https://doi.org/10.1016/j.rhisph.2024.100860.

Minor modifications were made to conform to the McGill University thesis guidelines.

5.1 Abstract

Rhizobia strain selection is mostly based on nodulation efficiency with host-plant. Selected strains also require a high capacity to compete with indigenous soil rhizobia under various abiotic stresses to prevent nodule occupation by competitive but low-efficient native strains. In this experiment, three highly-efficient strains were selected out of six genetically-divergent strains, based on nodulation speed, nitrogenase activity, and alfalfa biomass accumulation under spring conditions. Strain competitiveness was assessed by determining the percent of alfalfa nodules occupancy by selected tagged strains. Two alfalfa populations, A-TF0 and A-TF7, differing in their tolerance to freezing as well as two soils originating from south and north of Quebec containing indigenous rhizobia were tested. More nodules were recorded on roots of freezing-tolerant alfalfa in southern soil. Highly-efficient strain NRG34, isolated from northern Canada, proved to be very competitive as shown by a greater nodule occupancy percentage than any other strain in cold soil.

Selection of rhizobial strains that are efficient in soils under low temperature is important for alfalfa (*Medicago sativa* L.) to benefit from an extended growing season in spring and fall. Cold soil temperatures can affect every steps of interactions between rhizobia and legumes, as well as the efficiency of rhizobia to fix atmospheric nitrogen (N) (Alexandre and Oliveira, 2013). The optimum soil temperature for symbiosis is between 25-30°C and lower temperatures are known to delay root hair infection and nodule formation (Junior et al., 2005). *Sinorhizobium meliloti* strains have been shown to largely differ in their ability to nodulate and produce N-dependent alfalfa growth at low root temperatures (Rice et al., 1995) and to survive to freezing stress (D'Amours et al., 2022).

The ability of a rhizobial strain to adapt to soil conditions strongly influences not only its nodulation and N fixation efficiency but also its competitiveness with other strains, each trait having to be assessed separately since efficient strains are not necessarily competitive and vice versa (Mendoza-Suarez et al., 2021). Thus, the capacity of a strain to compete with indigenous soil rhizobia under various abiotic stress is an important selection criterion (Checcucci et al., 2017), particularly in soils containing competitive but low-efficient native strains. Under adverse soil conditions, highly competitive native strains could outcompete commercial inoculant-strains and

occupy a significant portion of the nodule thereby reducing the positive impacts of inoculation on host-plants (diCenzo al. 2018). A comprehensive strain selection scheme should take into account both the efficiency of the strain in terms of nodulation and N fixation under regional climatic conditions, and the competitiveness of the strain in local soils containing indigenous strains (Irisarri et al., 2019).

Abiotic stresses affect alfalfa carbohydrate synthesis and translocation to the bacteroid thus having an impact on N fixation since this process depends on plant carbohydrates supply to nodules (D'Amours et al., 2023a, Friel and Fersen 2019). Abiotic stress also modifies the competitive ability of rhizobia to nodulate the host plant and of alfalfa to recruit rhizobia strains by its impact on root exudate composition (D'Amours et al., 2023a). It has recently been reported that stress-tolerant soybean genotypes could modify the recruitment of rhizosphere microbiome in favor of stress-tolerant microorganisms having the ability to enhance host-plant growth (Liu et al., 2019; Plett et al., 2021).

The goal of this study was, first, to select under low temperature, the three most efficient *S.meliloti* strains out of a group of six genetically divergent strains, in association with two alfalfa populations differing for their freezing tolerance. Then, the competitive ability of the selected three *gus*A-tagged-strains was assessed by measuring the percent occupancy of alfalfa nodules by each strain in soils originating from two different locations and thus colonized with different native strains.

To select the most performant strains in association with alfalfa, five *Sinorhizobium meliloti* strains that were shown to be genetically divergent using a multilocus sequence typing (MLST) analysis (A2, S27, Rm1521, I1, NRG34), and a commercial control strain (B399) were grown in sterilized Yeast Mannitol Broth (YMB) with a 10:1 (w:w) Mannitol:Yeast extract ratio, and a pH of 6.8. Alfalfa seeds of initial cultivar Apica (A-TF0) and freezing tolerant population A-TF7 obtained after seven cycles of recurrent selection for freezing tolerance were surface-sterilized. Germinated seedlings were aseptically transferred into individual cone-tainers (SC-10 Super Cell. Stuewe & Sons Inc, Tangent, OR) filled with Turface®MVP®, sterilized twice during 45 minutes at 15 PSI and 121°C. Plants were grown in growth chambers (Conviron model PGW40, Winnipeg, Canada) under a 15/10°C day/night temperature regime, a 16-h photoperiod and a photosynthetic photon flux density of 500 µmol photons m^{-2} s^{-1}, measured 5cm above the canopy, and provided by a mixture of fluorescent and incandescent lamps. The cone-tainers of each

population (A-TF0 and A-TF7) were inoculated with 10^8 cells of each selected strain, plus a non-inoculated control, for a total of 336 individual plants (4 replicates × 2 plant populations × 6 strains × (6 sampling dates + 1 pot for nitrogenase activity measurement at the last sampling). Plants were sampled each week, between the second weeks and seventh weeks after inoculation, , and nodules number, shoots and roots dry weight (DW) were measured each week. Nitrogenase activity was measured on week 7 using the acetylene reduction test as described in D'Amours et al., (2023b). The selection of the three most performant strains under low temperatures was based on: i) nodules number, ii) nitrogenase activity, and iii) alfalfa shoot biomass.

For the competitivity experiment, the two alfalfa populations were grown individually in cone-tainers filled with two soils originating from southern and northern sites in Quebec, Canada (Sainte-Anne-de-Bellevue and Normandin; soil characterization reported in Table 5.1) to compare nodule occupancy of the three most-efficient selected strains identified in the first experiment: NRG34, Rm1521 and S27. Each strain was tagged with a stable plasmid vector pJBA21Km carrying a constitutively expressed *gusA* gene (Wielbo and Skorupska 2001) and a kanamycin resistance gene. The pJBA21Km plasmid was introduced into the selected *S. meliloti* strains by electroporation as described in Wielbo and al. (2007). *S. meliloti* strains that successfully incorporated the plasmid were selected on Yeast Mannitol Agar (YMA) medium obtained by adding 15 g of agar to YMB medium, supplemented with 500 μg ml^{-1} of kanamycin and 50 μg ml^{-1} of X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide), the β-glucuronidase substrate.
One-week old seedlings were inoculated with 300 μl (10^8 cells mL^{-1}) of each of the three strains previously tagged. Plants were grown under low temperature conditions previously described during six weeks. At week 6, biomass was harvested, dried at 55°C during 72h, and weighted. Alfalfa roots were stained to reveal Gus activity as described in Wilson et al., (1995) (see Supplemental Fig. 5.1 for more details). For each strain, each alfalfa population, and each soil type, the number of blue nodules (tagged strains) and white nodules (colonized by indigenous strains of each soil) were counted (Marek-Kozaczuk et al., 2014). Nodules number, biomass measurement, nitrogenase activity and nodules occupancy were analyzed using a two-way ANOVA model for a randomized complete block design with the SAS MIXED procedure (SAS® Studio, 2020) after verification of residual normality and variance homogeneity and the normality of data distribution. Pairwise comparisons of means differences were made using Fisher's least significant difference (LSD) test at $P \leq 0.05$.

In the first experiment, all inoculated alfalfa plants grown at 15/10°C had visible nodules after two weeks (Table 5.2). The appearance of nodules after only two weeks of growth showed that alfalfa is an early nodulating species in cold soils as compared to other perennial legume species such as birdsfoot trefoil, sainfoin, red clover and alsike clover for which the first nodules are visible after four weeks under similar conditions (D'Amours et al., 2023b). Overall, at week 7, the average number nodules was higher for alfalfa inoculated with strain S27, A2, RM1521 and NRG34 (average of 40.4) than for alfalfa inoculated with strain B399 and I1 (average of 29.4) (Table 5.2). After 7 weeks of growth nitrogenase activity significantly differed in response to the rhizobial strains (Figure 5.1A). Alfalfa inoculated with strains Rm1521 and S27 had the highest C_2H_4 increase rate (average of 5.60 μmole $C_2H_4.hour^{-1}.plant^{-1}$) that was significantly higher than for plants inoculated with strains A2 and I1 but similar to plants inoculated with strains B399 and NRG34. The overall shoot DW of alfalfa from week 2 to 7 also differed in response to the rhizobial strains and, although shoot growth was null for control plants and was not included in the statistical analysis, the differences observed between strains followed a tendency similar to nitrogenase activity, with superior shoot DW for alfalfa inoculated with strains RM1521 and S27, intermediates for B399 and NRG34 and inferior for A2 and I1 (Figure 5.1B), confirming the strong link between nitrogenase activity and yield of various perennial legume species (D'Amours et al., 2023b). A strong link between N fixation efficiency and plant biomass was also reported in a study comparing nodule occupancy of *Trifolium repens* by native and commercial inoculants (Irisarri et al., 2019), and on the effect of these inoculants on the biomass yield of *Trifolium pratense* (Batista et al., 2015).

For the second experiment on strain competitivity, total nodule number was much smaller for alfalfa grown in the northern-soil from Normandin as compared to the southern-soil from Sainte-Anne-de-Bellevue (Table 5.3). This difference could partly be explained by soil chemical properties since the southern soil had higher concentration of $N\text{-}NH_4$, $N\text{-}NO_3$, P and K than the northern soil while the number of indigenous rhizobia assessed by the Most Probable Number (MPN, Somasegaran and Hoben 1994) was in the same order of magnitude for the two soils (Table 5.1). The restriction in nodule formation in the northern soil could also be due to a greater compaction that likely also caused a reduction of plant DW (Table 5.3). While there was no difference between the two alfalfa populations in term of total nodules number and shoot dry weight in the northern-soil, the freezing-tolerant alfalfa population A-TF7 had a 42% larger

nodules number and 20% larger shoot dry weight than population A-TF0 in the southern-soil (Table 5.3). The larger number of nodules for A-TF7 could be partly explained by a greater rhizobia recruitment by this population due to higher concentration of flavonoids and stachyose in root exudates as reported by D'Amours et al., (2023a). Flavonoids and sugars are involved in the first steps of the crosstalk between alfalfa and its symbiotic partner and higher concentrations of these signaling molecules could have led to the synthesis of nod factors that are essential for the initiation of nodule formation (Liu and Murray 2016). Population A-TF7 could also have a higher efficiency than A-TF0 to supply carbon to nodules in cold soils thus favoring N fixation by providing energy to the bacteriods (Liu et al., 2019; Plett et al., 2021).

For both soils, alfalfa plants inoculated with selected tagged strains produced significantly more nodule and larger shoot dry weight than non-inoculated control (Table 5.3), confirming that the selected strains are more efficient that native strains to form nodules resulting in an improved alfalfa yield. Plants inoculated with the freezing tolerant strain NRG34 (D'Amours et al., 2022) had the largest number of nodules and that strain had the highest percentage of occupancy in soils from both locations, confirming the efficiency and the broad spectrum of competitivity of this strain under low temperature with different native rhizobia indigenous from a northern and a southern soil of Quebec.

Our study shows that cold tolerant *S. meliloti* strains can improve alfalfa growth under low temperature in soils from contrasted environmental conditions. Strain NRG34 showed a higher competitive ability to nodulate alfalfa in two soils with different physicochemical and biochemical properties, showing a greater percentage nodule occupancy than any other strains. Strain NRG34 seems to be cross-adapted to various abiotic stresses such as low temperatures, oxygen deficiency and freezing stress as shown by a greater alfalfa regrowth, nodulation index and nodules biomass and less nodules necrosis as compared to other strains under simulated winter conditions (Prévost et al., 2003, D'Amours et al., 2022). Selection for freezing tolerance in alfalfa populations was also shown to be effective to increase the number of nodules in soils under low temperature, which translated into larger plant biomass, likely through a better recruitment of rhizobia by root exudates.

5.2 Acknowledgments

This work was supported by a grant program of Agriculture and Agri-Food Canada. The first author was financially supported by a scholarship from the Fonds de Recherche Québécois Nature et Technologies. The authors sincerely thank Josée Bourassa, Josée Michaud, Sandra Delaney and Normand Bertrand for their technical assistance. We also thank, Dr Nicolas Ayub from Instituto de Genética "Edwald Alfredo Favret", INTA who kindly provided the commercial *S. meliloti* strain, B399 and Dr Jerzy Wielbo who provided the plasmid used in this study.

5.3 Statements & Declaration

Funding

This work was supported by a grant program of Agriculture and Agri-Food Canada. The first author was financially supported by scholarship from the Fonds de Recherche Québécois Nature et Technologies.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Emmanuelle D'Amours, Annick Bertrand and Jean Cloutier. Annie Claessens provided the alfalfa populations. Solen Rocher and Jean Cloutier performed the strain genetic diversity analysis. The first draft of the manuscript was written by Emmanuelle D'Amours and all authors revised the previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request

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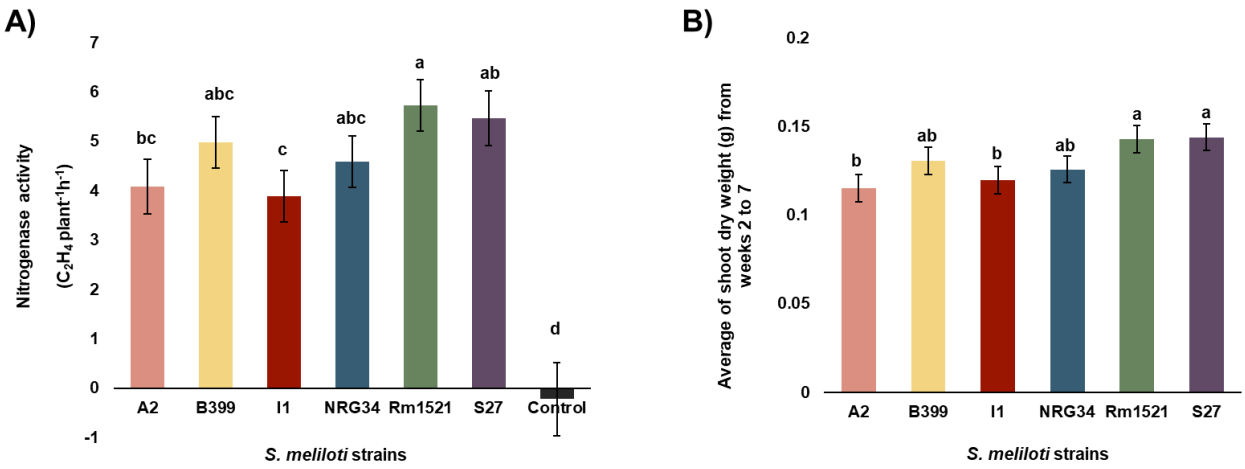

Figure 5.1 (a) Rates of nitrogenase activity assessed 7 weeks after inoculation by the reduction of acetylene (C_2H_2) in ethylene (C_2H_4) by the enzyme nitrogenase as described in D'Amours et al., (2023b) measured after 0.5, 1, and 2 hrs in gas-tight bags enclosing individual alfalfa plant (*Medicago sativa* L.) inoculated with six different strains of *Sinorhizobium meliloti* or uninoculated (Control). Air in bags was sampled after 0.5, 1, and 2 hrs. Results represent the increase in concentration of C_2H_4 (µmol) per hour per alfalfa plant. Different letters represent significant differences among strains as determined by the Fisher's least significant difference (LSD) test at $P < 0.05$. Error bars represent the Standard Error of the Means (SEM). (b) Average shoot dry weight per alfalfa plant (g) from week-two to week-seven after inoculation with six different strains of *Sinorhizobium meliloti*

Table 5.1 Description of two contrasted soils (originating from Normandin, northern site and Sainte-Anne-de-Bellevue, southern site) used in an evaluation of alfalfa rhizobial strain competitivity study

Location	Years since establishment of alfalfa field	Textural class[1]	Soil type[1]	pH [1]	C/N[1]	$N\text{-}NH_4$[1]	$N\text{-}NO_3$[1]	P[1]	K[1]	MPN[2]
						mg kg^{-1}		kg ha^{-1}		g^{-1} soil
Northern Normandin, Qc, Canada	2	Sand – Clay-Loam	G2	6.3	12.6	<5	16	50	103	2.4×10^4
Southern Saint-Anne-de-Bellevue, Qc, Canada	2	Loam	G2	6.5	10.9	12	14	239	387	1×10^4

[1] Soil texture and chemical properties were assessed by standardized soil analytical methods; [2]The most probable number (MPN) was assessed by the method of Somasegaran and Hoben (1994)

Table 5.2 Number of nodules per root system at weeks 2 and 7 of alfalfa plants (*Medicago sativa* L.) inoculated with one of six rhizobial strains. Each value is the average number of nodules of eight plants. Different letters in a column represent significant difference at $P < 0.05$ as determined by the Fisher's least significant difference (LSD) test

Strain	Week 2	Week 7
A2	18.6^{a}	43.1^{a}
B399	18.1^{a}	29.3^{b}
I1	13.5^{a}	29.4^{b}
NRG34	19.1^{a}	39.0^{a}
Rm1521	16.9^{a}	38.6^{a}
S27	18.5^{a}	40.9^{a}

Table 5.3 Total number of nodules, number of tagged nodules, percentage of nodule occupancy, and shoot dry weight per plant of two alfalfa (*Medicago sativa* L.) populations inoculated with one of three *Sinorhizobium meliloti* strain or with no inoculation (Control treatment containing native strains of each soil) in two soils of contrasted locations. Plants were grown under controlled conditions with cold temperature 15/10°C. Day/Night . The two soils originated from two contrasted sites (Normandin, northern site and Sainte-Anne-de-Bellevue, southern site) of the province of Quebec, Canada. The two alfalfa populations A-TF0 and A-TF7 and three *S. meliloti* strains contrasted in their levels of freezing tolerance and the controls were compared. Different letters represent significant difference at P < 0.05. as determined by the Fisher's least significant difference (LSD) test for each effect and each variable

	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**						
Control	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}

*Within the population effect, for each variable, small letters indicate significant differences in nodule numbers at P<0.05 between populations in the two soils with a decreasing quantity from a to c. The absence of letters indicates that there is no significant differences between populations

** Within the strain effect, for each variable, small letters indicate differences at P<0.05 between strains in the two soils with a decreasing quantity from a to f

***nd: the number of tagged nodules could not be precisely determined due to the instability of the plasmid within strain S27 throughout the 6-weeks of experiment and this treatment was removed from the statistical

Chapter 6: General discussion

Agriculture is the main source of nitrogen (N) pollution including one of the main source of nitrous oxide (N_2O) emission, a powerful greenhouse gas (GHG) contributing to global climate change, which, in turn, affects negatively croplands (Rochette et al., 2008). This vicious cycle reinforces the urgent need to move towards sustainable agriculture. A recent publication has shown that if innovative policies with improved management practices were globally adopted in agriculture, N pollution could be reduced of 26 million tons annually (-32%), while an additional 17 million tons (+20%) of crop-N could be produced with 22 tons less N fertilizer needed (-21%), representing $476 billion US dollars in societal benefits for food supply, human health, ecosystems and climate (Gu et al., 2023). One of the eleven keys proposed by authors was to incorporate legumes crops in rotation with other crops. The cost of these integrated best management practices would be balanced by an agricultural N credit system that would acknowledges the responsibilities and limitations of the multiple parties along the food chain, including farmers, suppliers, processors, retailers, consumers, and governments (Gu et al., 2021).

As a perennial plant requiring very little management and as the most broadly adapted perennial legume species across Canada and worldwide, alfalfa is the legume species of choice to be incorporated in crop rotations to increase soil nitrogen reserves and improve soil health. On the other hand, impact of climate changes with warmers fall, less snow accumulation in winter and freeze-thaw episodes can be damaging to alfalfa and could compromise its persistence. Thus there is a need to implement new approaches to extend the persistence of alfalfa over many years in the field to fully exert the leverage effect of this species on the ecosystems. Among the tools that are available to increase the persistence of alfalfa, the two that really stand out, are the genetic improvement of alfalfa and the selection of rhizobial strains that are adapted to northern environments. These two approaches are at the core of this thesis in which it was clearly demonstrated that the association between alfalfa populations obtained by recurrent selection for freezing tolerance and inoculation with a cold-tolerant *S. meliloti* partner are effective and complementary approaches to enhance overwintering persistence of alfalfa. Moreover, it was shown that alfalfa-rhizobia symbiosis could improve the freezing tolerance of alfalfa and that

nodules play a pivotal role to increase alfalfa persistence by changing over between fall and spring, from sink to promote plant cold acclimation to an energy source for the benefit of spring regrowth.

Regarding the genetic improvement of alfalfa, the results presented in this thesis further confirmed the effectiveness of the recurrent selection method performed indoor under controlled conditions to improve the freezing tolerance of alfalfa. The lethal temperature of alfalfa population A-TF7, obtained after seven cycles of recurrent selection, was shown to be significantly improved by -6°C (Fig.3.1) and the shoot regrowth after freezing as well as shoot dry weight under simulated spring conditions in soil from southern Quebec of A-TF7 were respectively 19 and 20% larger than for the initial cultivar Apica, A-TF0 (Fig 3.2 and Table 5.3). Chapter 3 highlighted the importance of alfalfa to allocate cryoprotective and N storage compounds to belowground organs. This effective adaptation strategy was revealed by the observation that both the root systems and nodules biomass markedly increased during cold acclimation and by the direct link observed between larger belowground organs and successful alfalfa regrowth after a freezing stress. As for the selection of rhizobial strain, several strains isolated from different Canadian regions were tested under low temperature to identify the ones that were better adapted to cold. For the first time, differences in nodule freezing tolerance according to the inoculated strain in association with alfalfa was brought to light by assessing nodule freezing damages. The synergy between both partners to improve the freezing tolerance of alfalfa was also demonstrated and the underlying mechanisms were investigated in this thesis.

Chapter 3 shows that the two alfalfa populations under study share common strategies to cold acclimate and resume regrowth after a freezing stress. For instance both populations invest in their belowground biomass thus reinforcing the need to investigate the role of belowground parts in freezing tolerance by studying root architecture and damages by freezing stress to roots and nodules. It was observed that the less freezing tolerant population A-TF0 invested more into nodules biomass after a freezing stress than the freezing-tolerant population A-TF7 suggesting that it is more important for a less freezing-tolerant population (A-TF0) to allocate resources in its symbiotic partner by increasing the nodule:root ratio as the benefit of the symbiosis than for a more freezing tolerant population suffering less damages (Fig 3.3). However, in this study, the only source of N provided was through the biological N-fixation of the rhizobium and the substrate was sterilized. Thus, additional research including a treatment with N fertilization could be interesting to elucidate if investments to nodules and on metabolites profiles would differ in

response to environmental factors when N is not a limiting factor. It would be particularly interesting in the view of the results of the experiment that was made in agricultural soil reported in chapter 5. Under simulated spring conditions the freezing-tolerant alfalfa population A-TF7 had a 42% larger nodules number than population A-TF0 (Table 5.3). While the number of nodules number is not necessary linked with nodule dry weight, these results seems to indicate that plant response to environmental factors differs when using a sterilized inert substrate compared to using an agricultural soil including its complex microbiome and fertility levels. These results reinforce the complexity of interaction between alfalfa and rhizobia driven by environmental factors and the soil microbiome, since root and nodules can be colonized by various bacteria species other than rhizobia that could also improve stress tolerance (Schaedel and Grossman, 2021; Fan et al., 2023). Considering recurrent selection exerts changes in plant chemistry it could be interesting to further investigate if those biochemical changes could influence the roots and nodules microbial ecology.

In this thesis, twelve *Sinorhizobium meliloti* strains isolated from different Canadian regions and selected for their genetic diversity were tested through a series of experiments under controlled conditions simulating seasonal variations in cold soil typical of northern environments and were compared to a commercial strain, 'B399'. Strain B399, originally named *Rhizobium meliloti* strain '102F34' has been one of the most widely used inoculants in alfalfa production in the last 50 years and was shown to have a high capacity to fix N under various abiotic stresses and with different alfalfa cultivars (Sanz-Sáez et al., 2012; Jozefkowicz et al., 2017; Brambilla et al., 2018). Selected strains and the commercial strain in symbiosis with alfalfa population differing in their level of freezing tolerance were exposed to cold stress through a series of experiments conducted in controlled conditions. Through this thesis, results have confirmed our hypothesis showing that strain selection plays an important role in improving freezing stress tolerance and alfalfa growth under low temperature. In Chapters 3 and 4, *Sinorhizobium meliloti* strain NRG34 isolated from Northwestern Canada was shown to induce a better freezing tolerance as observed by higher shoot regrowth of the plant after freezing stress, larger nodule dry weight and fewer nodules damages caused by freezing stress than any other strain (Figs 3.4c, 3.5, 3.7c and 4.2). These chapters report for a first time a strong positive relationship between shoot regrowth and nodule regrowth after a freezing stress highlighting moreover that nodule freezing tolerance differs according to the strain in symbiosis with alfalfa, and that necrotic nodules have a capacity of regeneration after freezing. The experimental conditions used in Chapter 5 were selected to

represent soil temperatures in the spring in eastern Canada. Early nodulation of alfalfa under low temperature compared to other perennial legumes was surprising and could explain in part the high yield and quick establishment of alfalfa compared to other perennial legumes (Table 5.2). Although, in sterilized turface the strain NRG34 did not really stand out compared with other cold-adapted strains in term of nodulation speed, yield biomass and nitrogenase activity, the superiority of that strain was clearly demonstrated by its higher competitive ability to nodulate alfalfa in two soils with different physicochemical and biological properties (Fig. 5.1; Tables 5.2 and 5.3). In non-sterilized agricultural soils containing natives rhizobia, strain NRG34 had a greater percentage of nodule occupancy than any other strains (Table 5.3). Furthermore, the root system of alfalfa inoculated with that strain had more nodules and biomass yield was greater. Altogether, strain NRG34 seems to be cross-adapted to various abiotic stresses such as low temperatures, oxygen deficiency and freezing stress as shown by a greater alfalfa regrowth, nodulation index and nodules biomass and less nodules necrosis as compared to other strains under simulated winter conditions (Prévost et al., 2003).

Inoculation with the selected *S. meliloti* strain NRG34 has shown promising results under controlled conditions and laboratory studies to enhance alfalfa overwintering persistence and to nodulate alfalfa under spring conditions but it is important to emphasize that results in the field could differ due to the contrasting pedoclimatic conditions influencing both symbiotic partners and to the interactions with the soil microbiome. Multiple field experiments across Canada would be necessary to validate the efficiency of strain NRG34 under real field conditions and in association with various alfalfa cultivars before considering the commercialization of that strain. As the nature of inoculum carriers can be a key element affecting the viability and persistence of inoculum in soils and that new alternatives to peat like biochar (Hardy and Knight, 2021; Bolan et al., 2023) and manure compost-based products (Li et al., 2023) have been proposed, new studies should be conducted to test these factors. Moreover, long-term field trials are necessary to validate the persistence and efficiency of the inoculum in years following the establishment. The whole genome of strain NRG34 has been sequenced but results were not presented in this thesis. Detailed analysis of the genome sequences could make possible the evaluation of alfalfa root colonization by NRG34 in field trials by a simple DNA analysis of crushed nodules while also providing a deepened understanding of the functional properties and specificity of this high-efficient cold tolerant strain.

The results of this thesis have shown that both plant recurrent selection and selection of cold and freezing tolerant rhizobial strains are complementary and effective approaches to increase the persistence and regrowth of alfalfa exposed to freeze–thaw episodes. In Chapter 4, we identified molecular traits having major roles in cold acclimation, freezing tolerance, and those involved in the crosstalk between alfalfa and its symbiotic partner to better understand the contribution of each partner to a better regrowth performance of an association after freezing. We compared the regrowth after a freezing stress of four different associations of two alfalfa populations differing in freezing tolerance (A-TF0 and A-TF7), inoculated with two *S. meliloti* strains (B399 and NRG34) of contrasted adaptation to cold. Regrowth after exposure to a freezing stress was 35% larger in the freezing tolerant association A-TF7 × NRG34 than in the freezing sensitive association, A-TF0 × B399, showing the synergetic benefit of combining two tolerant partners on alfalfa freezing tolerance (Fig. 4.2). The experimental approach used in this study allowed to monitor the independent effects of alfalfa populations and rhizobial strains on metabolic changes occurring during alfalfa cold acclimation and deacclimation to understand their respective contribution to the recovery of the symbiotic association after a freezing stress. While the significant differences of metabolite concentrations in crowns were mainly due to an effect of alfalfa populations, the differences of metabolites observed in nodules were mainly an effect of *S. meliloti* strains (Figs 4.4 and 4.5; Supplemental Tables 4.2 and 4.4). The studies of metabolomic and gene expression changes all concorded in showing the importance of carbon and nitrogen storage in crowns, nodules and roots and in their remobilization to support spring regrowth of alfalfa. For instance, the expression of cold-regulated genes coding for galactynol synthase (GaS), a key enzyme catalyzing the first step of raffinose-family oligosaccharides (RFO) biosynthesis, increased markedly in response to cold acclimation concomitantly with a sharp increase in raffinose and stachyose concentration in alfalfa crowns (Fig 4.3; Table 4.4). These two sugars are likely involved in the general process of cold acclimation in alfalfa as no significant differences were observed in RFO concentrations between the two alfalfa populations with contrasted level of freezing tolerance. Sucrose, however, was more abundant in freezing-tolerant population A-TF7 as compared to A-TF0, indicating that the accumulation of this sugar could in part explain the higher level of freezing tolerance of A-TF7 hinting at a central role for this sugar in the acquisition of freezing tolerance in alfalfa (Fig 4.4, left panel, Supplemental Table 4.2). Moreover, the quick induction of genes coding for sucrose synthase (SuSy) observed only 48 h upon the return to

regrowth conditions after the freezing stress indicates that sucrose could be involved not only in the acquisition of freezing tolerance but also in the deacclimation process by procuring a source of succinate and malate to the bacteroids following its cleavage into fructose and glucose by Susy (Table 4.4). As such, it would positively activate the reprise of biological N fixation in post-freezing nodules. Both cold-adapted partners of the symbiotic association contributed to increases in arginine concentration in nodules in response to cold acclimation and deacclimation underscoring the importance of N storage and remobilization for a successful overwintering in alfalfa (Figs 4.4 and 4.5; Supplemental Tables 4.2 and 4.4). For its part, the freezing sensitive association showed higher accumulation of osmoprotectants and scavenging reacting oxygen species (ROS) amino acids like GABA, serine, alanine and proline in crowns and proline in nodules suggesting that these plants suffered more damages by freezing and that it triggered reparation mechanisms such as ROS scavenging (Figs 4.4 and 4.5; Supplemental Tables 4.2 and 4.4). Thorough analyses of metabolic changes occurring during deacclimation of perennial plants, as was done in this thesis, are rarely reported in the literature although understanding the mechanisms of deacclimation seems to be more and more urgent in the light of the unpredictable effects of climate change on spring temperatures that will likely expose perennials to more frequent and larger temperature fluctuations in springtime. Here it was shown that many compounds linked with freezing tolerance remained at higher concentration in the freezing-tolerant association for a long period following a freezing stress. Since there is a metabolic cost for any biological system to synthesize or retain metabolites as was shown in crowns and nodules during alfalfa deacclimation, it could be inferred that these compounds are beneficial to reduce the risks of damages caused by abrupt freeze–thaw episode in early autumn or spring and that there is a need for further research on deacclimation.

The measurement of metabolites in root exudates including the concentration of flavonoids of the phenylpropanoid pathway showed that they were influenced by both symbiotic partners at the different sampling events, confirming our hypothesis that cold stress actively impact the cross-talk between the symbiotic partners and that the metabolic response differs between the most performant and the less performant host/strains associations. In response to cold acclimation and freezing stress, most of the individual sugars, amino acids and flavonoids decreased in root exudates contrarily to the increase that was generally observed in nodules, roots and crowns (Fig 4.3; Supplemental Tables 4.2 and 4.3). Stachyose and raffinose were among the very few

metabolites that increased in root exudates during cold acclimation. These RFO-galactosides have been shown to be present in non-sterile soil around the roots of legumes and alfalfa seedlings and to be able to support the growth and survival of symbiotic N-fixing bacteria (Bringhurst et al., 2001) and, as such, could be actively involved in cold-adapted rhizobia recruitment. The flavonoids formononetin and medicarpin also increased in concentration during cold acclimation and both flavonoids exert their action on *nod* genes expression showing the intricated retroactive link between the plant and its symbiotic partner in cold soil. While the heart of this thesis was to better understand the mechanisms of tolerance of alfalfa-rhizobia by measuring targeted metabolites and genes previously reported to play major roles in cold acclimation and freezing tolerance of alfalfa, the results of the analysis of root exudates have set the table to further explore the molecular dialog between symbiotic partners and the impact of abiotic stresses on these interactions. Further research could also explore other important metabolites involved in the crosstalk as well in cold acclimation and deacclimation. For instance, oleosomes and their high-energy phospholipid contents as well as amyloplasts have been proposed to play an important role in the protection of perennial beach pea nodule cells from cold stress and to be used as energy to fuel N fixation in spring (Chinnasamy et al., 2003). Lipids have also been reported to play roles in cold acclimation and deacclimation of alfalfa (Xu et al., 2020; Li et al., 2022), thus, specific metabolites involved in the lipolytic activity could be interesting to investigate.

Altogether, one of the most important feature that this thesis brought to light is the importance to consider the rhizobial partner in strategies aimed at improving stress tolerance in legumes with emphasis to study the nodules metabolism. In Chapter 3, a striking increase in the nodules dry weight of all associations in response to cold acclimation highlighted the importance of belowground organs in the overwintering performance of alfalfa. Information on the overwintering of nodules of perennial legumes in temperate regions is scarce (Pate, 1961; Bergersen et al., 1963; Bal and Khetmalas 1996) and it was generally taken for granted that in northern environments, nodules of perennial legumes such as alfalfa were decaying in the fall and that new nodules were formed in the spring for the next growing season (Thurston et al., 1930). Here, the detailed phenotyping of aboveground and belowground traits after a freezing stress in Chapter 3 showed clearly that a proportion of necrotic nodules damaged by freezing could generate regeneration zone and that this was linked with the intrinsic freezing tolerance of the rhizobial strain with repercussions on the shoot regrowth of alfalfa. Furthermore, the separate biochemical

analysis of crown, roots, nodules and roots exudates under simulated overwintering conditions presented in Chapter 4 have shown the importance of resource allocation made by the plant toward the nodules in response of cold acclimation by the important increase of starch, sucrose and important cryoprotective sugars observed. The decreased concentration of total sugars and amino acids in nodules to support alfalfa regrowth demonstrated that nodules play a pivotal role as large carbon sinks during cold acclimation and as a source of energy to resume N fixation and ultimately support alfalfa regrowth in the spring. While the importance of nodules was clearly demonstrated in this thesis, those results raise important questions regarding the overwintering survival of nodules and on their persistence on years following the field establishment of alfalfa. Indeed, a majority of studies have focused on the early nodulation process with short laboratory experiment or on the first year after legumes establishment. Li et al., (2012) reported that nodule development on the root system of perennial alfalfa changed dramatically with the age of the stand. The authors have shown that in 1 yr-old alfalfa stands, nodules were almost all located in the topsoil (0-15 cm) while nodule densities were generally increasing from first-year production to the third year in the lower topsoil (15–30 cm) and subsoil (30-80 cm). The authors also added that nodules of alfalfa in the first year of establishment appeared to be more reddish in color, whereas nodules under second and third years of production tended to be more yellowish or brownish which possibly indicated differences in nodule health and activity. These observations reinforce the importance to characterize differences in nodule phenotype as was reported in this thesis. When comparing the freezing tolerance of young established alfalfa plants to that of older plants, it was observed that the level of freezing tolerance of young plants (-25 to -30 °C at the crown) was significantly above the threshold of -15 °C generally recognized as critical temperature for alfalfa winter survival while older plants maintained freezing tolerance levels of -15 to -18°C which is much closer to the critical threshold (Bélanger et al., 2006). In the view of these observations and of those reported in this thesis on nodule phenotyping, it could be supposed that the observed decrease in winter survival of older alfalfa stands could be due to a larger proportion of senescent or necrotic nodules in older alfalfa plants. Therefore, further researches following persistence of nodulation in subsequent years of alfalfa and other perennial legumes could provide information on the evolution of the symbiosis and to validate if there is a link between the nodules health, the strain used for inoculation and the decrease of winter survival of older alfalfa stands.

Chapter 7: Conclusions and Future directions

7.1 Conclusions

The work presented in this thesis highlights the need for including the symbiotic association with tolerant rhizobia strains to improve stress tolerance of legumes. In this thesis, six *S. meliloti* strains including five strains isolated from different regions of Canada and one commercial strain as control were tested in association with two alfalfa populations bred to differ in their level of freezing tolerance. The twelve alfalfa-rhizobia associations were exposed to overwintering simulated conditions and result have shown that both plant recurrent selection and selection of cold and freezing tolerant rhizobial strains are complementary and effective approaches to increase overwintering persistence and regrowth of alfalfa exposed to freeze–thaw episodes in northern environments. Indeed, alfalfa population with higher level of freezing tolerance, A-TF7 and plants inoculated with strain NRG34 isolated from Northwestern Canada have shown larger aboveground yield after freezing than the original cultivar Apica and than plants inoculated with any others strain. The meaningful allocation of resources to nodules by alfalfa plants exposed to cold acclimation conditions revealed the importance of this belowground organs to improve stress tolerance of alfalfa. The differential above and belowground responses modulated by the two alfalfa populations shows that different allocation strategies are at play during cold acclimation depending on the level of freezing tolerance of alfalfa. Moreover, the detailed plant phenotyping after freezing stress has shown different levels of nodules freezing damages depending on the symbiotic *S. meliloti* strain used. To our knowledge, it is the first time that variability of freezing tolerance among *S. meliloti* strains are reported *in vivo* showing that alfalfa regrowth after freezing relies in part on nodule tolerance to freezing stress which in turn depends on *S. meliloti* strains.

The underlying mechanisms involved in symbiotic associations were studied through the measurement of targeted metabolites and of cold regulated genes in crowns, roots, nodules and root exudates of the four most contrasted alfalfa-rhizobia associations in response to cold acclimation, freezing stress and deacclimation. The importance of root nodules in the overwintering process of alfalfa was highlighted by the accumulation of carbohydrates and a large pool of free amino acids during cold acclimation which switched into a source of reserves that

enabled alfalfa regrowth in the spring after deacclimation. By monitoring the independent effects on metabolites concentrations of the alfalfa population and the *S. meliloti* strains, the results revealed that both partners of the alfalfa-rhizobia symbiosis play complementary roles to improve regrowth after a freezing event. The metabolomic study has identified biochemical and genetic traits that confer superior regrowth yields after a sublethal freezing stress of alfalfa such as the accumulation and remobilization of sucrose and N storage amino acids during cold acclimation and deacclimation, respectively. This study has greatly improved our knowledge in the molecular dialog between host legumes plant and its rhizobia partner under stressful environment.

Results of the thesis revealed that cold-tolerant *S. meliloti* strains can improve alfalfa growth under low temperature in soils from contrasted environmental conditions suggesting that inoculation with a cold-adapted strain could result in early nodulation and larger spring growth. Competitive trial in two soils from northern and southern Quebec has revealed that strain NRG34 presents a high competitive ability to nodulate alfalfa by showing a greater percentage of nodule occupancy than any other strains. Moreover, alfalfa population selected for higher freezing tolerance were shown to effectively increase the number of nodules in soils under low temperature, which translated into larger plant biomass. The analysis of root exudates indicates that the freezing-tolerant alfalfa population likely exert a better recruitment of effective strains. Overall, the results presented in this thesis shows a promising commercialization potential of the alfalfa population A-TF7 and of the strain NRG34 as inoculant to improve yield performance and persistence of alfalfa fields in northern environments.

7.2 Future directions

Alfalfa is the most important forage crop species in Canada and its productivity is essential to the economic profitability of the dairy and beef sectors. Sustainable practices must be developed not only to increase the productivity and mitigate the detrimental effects of environmental stressors but also to reduce the negative impacts of agriculture on climate changes. This work has laid the foundation to study the overwintering of nodules and their persistence through several subsequent years of alfalfa production. Studies to characterize root exudates metabolites involved in the crosstalk between alfalfa and rhizobia in response to abiotic stress are necessary to better

understand the underlying mechanisms of the symbiotic legume-rhizobia partnership. The role of flavonoids as *nod* gene inducers is touched upon in this thesis but would warrant a more thorough investigation to understand their role when the symbiosis has to resume following stress-induced damages to the nodules. One of the future research direction suggested as a follow-up of this study would be an in-depth investigation of the link that was demonstrated in this thesis between nodules winter survival and spring regrowth of alfalfa. Validation of the positive effects of the freezing-tolerant alfalfa population in association of *S. meliloti* strain NRG34 under field conditions to improve overwintering persistence and yield production under various environmental conditions and winter tests in the field are necessary to validate the breakthrough findings reported in this thesis. The efficiency of alfalfa-rhizobia associations with higher level of freezing tolerance and of the NRG34 strain in association with other alfalfa cultivars would need to be determined by multiple field trials across Canada and long-term trials to assess the persistence and the yield of alfalfa over many production years. The yield performance of the freezing-tolerant alfalfa population in summer growth season needs to be tested to ensure the seasonal performance of the symbiosis. To implement applied results based on this research, the NRG34 strain should be developed as an inoculum and various carriers should be tested to ensure the success of colonization and the persistence of that strain through multiple growing seasons. Success at all these steps would ultimately lead to the commercialization of a new alfalfa cultivar using the ATF7-population as parental germplasm and of the NRG34 *S. meliloti* strain to support alfalfa cultivation and adaptation in Canadian northern environments.

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Appendices

Appendix 1 Supplementary information for Chapter 3

Supplemental Table 3.1 Means of total biomass (shoot plus root dry weight), shoot dry weight, root:shoot ratio and photosynthetic rates comparing the effects of two alfalfa populations in symbiosis with six different *S. meliloti* strains, and their interactions, at different sampling events. Plants grown under controlled conditions were sampled at two sampling events: non-acclimated plants (NA) were grown 8 weeks under a 21/17°C, Day/Night (D/N) temperature regime. Plants were then cold acclimated for 4 weeks and exposed to a freezing stress of -11°C and transferred back to optimal regrowth conditions (21/17°C, D/N). After three weeks of regrowth (RAF) plants were sampled again. The two alfalfa populations, A-TF0 and A-TF7, were contrasting in their levels of freezing tolerance while the six *S. meliloti* strains were selected based on their nodulation performance at low temperature. Numbers in bold and letters in superscript indicate statistically significant effects ($P \leq 0.05$) and different letters represent significant differences determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$

	Total dry weight		**Shoot dry weight**		**Root:shoot ratio**		**Photosynthetic rate**		**Plant development stages**	
	g DW		**g DW**				**µmol[CO_2]$m^{-2}s^{-1}$**			
	Sampling events									
	NA	**RAF**	**NA**	**RAF**	**NA**	**RAF**	**NA**	**RAF**	**NA**	**RAF**
Alfalfa Populations (Pop)										
A-TF0	4.50	**4.86**	1.81	**2.04**	1.54	1.42	21.9	23.9	**5.2**	2.6
A-TF7	4.59	**5.69**	1.79	**2.43**	1.59	1.37	20.2	20.8	**4.0**	2.2

***S. meliloti* Strains (Strains)**										
B399	4.87	5.42	1.99	**2.09^{c}**	1.48	**1.62^{a}**	18.4	20.4	5.1	2.1
A2	4.40	5.05	1.77	**2.0^{c}**	1.50	**1.45^{ab}**	20.7	23.1	4.4	3.3
NRG34	4.85	5.64	1.87	**2.48^{a}**	1.63	**1.30^{b}**	21.2	24.4	4.4	2.7
S27	4.43	5.39	1.64	**2.36^{ab}**	1.76	**1.31^{b}**	24.3	23.0	4.4	2.2
Rm1521	4.19	5.14	1.68	**2.23^{bc}**	1.52	**1.35^{b}**	21.2	21.3	4.8	2.1
I1	4.54	5.02	1.83	**2.18^{bc}**	1.51	**1.34^{b}**	20.4	21.7	4.7	2.2
Pop × Strains										
A-TF0 × B399	4.74	5.04	2.10	1.97	1.30	1.59	18.6	**19.4^{cd}**	5.9	2.3
A-TF0 × A2	4.31	4.30	1.78	1.75	1.44	1.50	21.5	**24.2^{ab}**	4.9	3.3
A-TF0 × NRG34	4.88	5.46	1.92	2.30	1.58	1.38	20.6	**27.9^{a}**	5.3	3.1
A-TF0 × S27	4.37	4.90	1.57	2.16	1.84	1.32	25.9	**26.8^{a}**	4.6	2.4
A-TF0 × Rm1521	4.18	4.80	1.67	2.00	1.55	1.43	22.2	**21.1^{bcd}**	5.6	2.0
A-TF0 × I1	4.53	4.67	1.83	2.05	1.53	1.31	22.6	**23.9^{abc}**	5.0	2.9
A-TF7 × B399	5.01	5.80	1.88	2.20	1.67	1.64	18.2	**21.5^{bcd}**	4.3	1.9
A-TF7 × A2	4.49	5.81	1.77	2.41	1.56	1.40	19.8	**22.0^{bcd}**	4.0	3.3
A-TF7 × NRG34	4.82	5.82	1.83	2.65	1.67	1.22	21.8	**20.9^{bcd}**	3.5	2.3
A-TF7 × S27	4.49	5.87	1.71	2.56	1.68	1.31	22.6	**19.2^{d}**	4.1	2.0
A-TF7 × Rm1521	4.21	5.49	1.69	2.45	1.50	1.26	20.3	**21.4^{bcd}**	4.0	2.3
A-TF7 × I1	4.55	5.36	1.84	2.31	1.48	1.37	18.2	**19.6^{bcd}**	4.4	1.5

Supplemental Table 3.2 Means of root dry weight, nodules dry weight and nodules:root ratio comparing the effects of two alfalfa populations in symbiosis with six different *S. meliloti* strains and their interactions, at different sampling events. Plants grown under controlled conditions were sampled at four sampling events: non-acclimated plants (NA) were grown 8 weeks under a 21/17°C, Day/Night (D/N) temperature regime. Plants were then cold acclimated during 2 weeks at 2°C followed by two weeks at -2°C and sampled again (CA). After their exposure to a freezing stress of -11°C, alfalfa plants were transferred back to optimal regrowth conditions (21/17°C, D/N) and sampled after 48h (AFS), and after three weeks (RAF). The two alfalfa populations, A-TF0 and A-TF7, were contrasting in their levels of freezing tolerance while the six *S. meliloti* strains were selected based on their nodulation performance at low temperature. Numbers in bold and letters in superscript indicate statistically significant effects ($P \leq 0.05$) and different letters represent significant differences determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$

	Root dry weight				Nodule dry weight				Nodules:root ratio			
	g DW				g DW							
	Sampling events											
	NA	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**
Alfalfa Populations (Pop)												
A-TF0	2.69	**3.07**	**2.94**	**2.83**	0.033	0.058	**0.047**	0.063	0.013	0.019	**0.017**	**0.023**
A-TF7	2.81	**3.31**	**3.26**	**3.26**	0.035	0.057	**0.043**	0.057	0.013	0.017	**0.013**	**0.018**
***S. meliloti* Strains (Strains)**												
B399	2.89	3.20	**3.36**^{a}	3.33	0.032	**0.048**^{b}	**0.039**^{b}	**0.055**^{c}	0.012	**0.016**^{b}	**0.012**^{d}	**0.017**^{c}
A2	2.63	3.10	**2.88**^{b}	2.98	0.039	**0.062**^{a}	**0.052**^{a}	**0.066**^{b}	0.015	**0.020**^{a}	**0.019**^{a}	**0.023**^{ab}
NRG34	2.98	3.26	**3.14**^{ab}	3.16	0.035	**0.057**^{ab}	**0.050**^{a}	**0.078**^{a}	0.012	**0.018**^{ab}	**0.016**^{ab}	**0.025**^{a}
S27	2.80	3.25	**2.93**^{b}	3.03	0.036	**0.065**^{a}	**0.044**^{ab}	**0.052**^{c}	0.013	**0.020**^{a}	**0.016**^{b}	**0.019**^{bc}
Rm1521	2.52	3.19	**3.05**^{ab}	2.92	0.035	**0.061**^{a}	**0.044**^{ab}	**0.054**^{c}	0.014	**0.019**^{ab}	**0.015**^{bc}	**0.019**^{bc}
I1	2.71	3.12	**3.24**^{ab}	2.84	0.028	**0.050**^{b}	**0.039**^{b}	**0.053**^{c}	0.011	**0.016**^{b}	**0.012**^{cd}	**0.019**^{bc}
Pop ×Strains												
A-TF0 × B399	2.65	2.99	3.37	3.07	0.035	0.049	0.038	0.058	0.013	0.017	0.011	0.019
A-TF0 × A2	2.53	3.12	2.70	2.56	0.038	0.062	0.055	0.061	0.015	0.020	0.021	0.024
A-TF0 × NRG34	2.96	3.04	3.09	3.16	0.031	0.057	0.054	0.092	0.012	0.020	0.017	0.029
A-TF0 × S27	2.80	3.13	2.59	2.75	0.036	0.069	0.046	0.056	0.013	0.022	0.018	0.022
A-TF0 × Rm1521	2.51	3.20	2.80	2.80	0.030	0.060	0.045	0.049	0.012	0.019	0.017	0.018
A-TF0 × I1	2.70	2.91	3.08	2.62	0.029	0.050	0.044	0.060	0.011	0.018	0.014	0.022
A-TF7 × B399	3.13	3.40	3.35	3.60	0.030	0.046	0.040	0.052	0.010	0.014	0.012	0.015
A-TF7 × A2	2.72	3.07	3.07	3.39	0.041	0.063	0.049	0.071	0.015	0.021	0.017	0.021
A-TF7 × NRG34	2.99	3.49	3.18	3.17	0.038	0.057	0.047	0.065	0.013	0.017	0.015	0.021
A-TF7 × S27	2.79	3.37	3.27	3.31	0.036	0.062	0.041	0.049	0.013	0.019	0.013	0.016
A-TF7 × Rm1521	2.52	3.18	3.30	3.04	0.039	0.062	0.044	0.058	0.016	0.020	0.014	0.019
A-TF7 × I1	2.71	3.33	3.41	3.05	0.028	0.050	0.034	0.046	0.011	0.015	0.010	0.015

Supplemental Table 3.3a Means of shoot dry weight, root dry weight, root:shoot ratio on the 60 plants used for the phenotyping (2 alfalfa population x six strains x 5 repetitions) three weeks regrowth after exposed to a freezing stress (RAF). *P* values of the analysis of variance comparing the effects of two alfalfa populations in symbiosis with six different *S. meliloti* strains and their interactions are also presented. Bold indicates statistically significant effects ($P \leq 0.05$) and different letters represent significant differences determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$

	Shoot (g DW)	Root (g DW)	Root:shoot ratio
Alfalfa Populations (Pop) *P* values	**0.003**	0.190	0.202
A-TF0	**2.56**	2.20	0.87
A-TF7	**2.98**	2.36	0.81
***S. meliloti* strains (Strains)** *P* values	**0.036**	0.282	0.743
B399	**2.80^{ab}**	2.26	0.83
A2	**2.51^{b}**	2.13	0.85
NRG34	**3.13^{a}**	2.40	0.79
S27	**2.79^{ab}**	2.48	0.91
Rm1521	**2.48^{b}**	2.15	0.87
I1	**2.88^{ab}**	2.24	0.81
Pop ×Strains *P* values	0.407	0.440	0.207
A-TF0 x× B399	2.56	1.96	0.78
A-TF0 × A2	2.35	2.01	0.86
A-TF0 × NRG34	2.79	2.45	0.89
A-TF0 × S27	2.52	2.45	1.00
A-TF0 × Rm1521	2.57	2.05	0.80
A-TF0 × I1	2.56	2.28	0.90
A-TF7 × B399	3.04	2.57	0.87
A-TF7 x A2	2.67	2.25	0.85
A-TF7 x× NRG34	3.47	2.35	0.68
A-TF7 × S27	3.07	2.51	0.83
A-TF7 × Rm1521	2.40	2.26	0.93
A-TF7 × I1	3.2	2.19	0.71

Supplemental Table 3.3b Means of the percentage of distribution of nodules freezing damages within each of the three classes and means of the percentage of distribution of nodules shapes within each of the four categories, observed on 60 plants used for the phenotyping (2 alfalfa population x six strains x 5 repetitions) three weeks regrowth after being exposed to a freezing stress (RAF)

	Visible Nodules-Freezing damages			Nodules shapes			
	(%)			(%)			
	Classes			Categories			
	I	II	III	S	E	B	PC
Alfalfa Populations (Pop)							
A-TF0	41	32	27	29	26	7	39
A-TF7	40	38	23	28	24	9	38
***S. meliloti* strains (Strains)**							
B399	41	33	26	26	23	11	40
A2	36	32	32	24	30	22	25
NRG34	47	38	15	34	12	1	53
S27	41	34	25	29	27	3	40
Rm1521	36	36	28	32	29	3	36
I1	39	36	24	26	30	7	37
Pop × Strain							
A-TF0 × B399	42	28	30	28	28	11	33
A-TF0 × A2	41	19	40	26	34	16	25
A-TF0 × NRG34	49	38	13	36	7	0	58
A-TF0 × S27	35	37	28	27	28	2	43
A-TF0 × Rm1521	36	36	28	29	29	2	39
A-TF0 × I1	41	34	25	27	31	9	33
A-TF7 × B399	41	37	22	25	17	12	46
A-TF7 × A2	32	45	23	22	26	28	24
A-TF7 × NRG34	44	39	17	32	17	3	49
A-TF7 × S27	47	31	21	32	27	5	37
A-TF7 × Rm1521	37	36	27	34	28	4	33
A-TF7 × I1	38	38	24	26	29	4	41

I; no damage, II; necrotic nodules with regeneration zone, III; necrotic nodules
S; Unbranched and small nodules, E; Unbranched and elongated nodules, B; Bifurcates nodules, PC; Palmate-coralloid nodules

Supplemental Table 3.4a Comparison of the models using a maximum likelihood ratio test on the transformed additive logratio datas comparing the effects of two alfalfa populations in symbiosis with six different *S. meliloti* strains and their interactions on the different profiles of percentages of distribution for the four nodules shapes categories. The line in bold indicates the statistical model selected (p>0.05) presenting the best fit for the data set

Models	**Level**	**Num parameters**	**-2LogL**	**Q Statistics**	**V (DF)**	**p-Value**
Complete with UN	1	72+36=108	NA			
Complete with ANTE(1)	1a*	60+36=96	253.2			
Pooled variance Common profile	2	6+3	418.9	165.8	87	0.0000
Pooled variance Profile by Pop	2a	6+6	417.2	164.0	84	0.0000
Pooled variance Profile by Strain	2b	6+18	366.0	112.9	72	0.0015
Pooled variance Profile by Pop*Strain	2c	6+36	347.0	93.8	54	0.0006
Separate variance Pop Common profile	3	12+3	410.7	157.5	81	0.0000
Separate variance Pop Profile by Pop	3a	12+6	408.9	155.8	78	0.0000
Separate variance Pop Profile by Strain	3b	12+18	353.8	100.7	66	0.0038
Separate variance Pop Profile by Pop*Strain	3c	12+36	335.0	81.8	48	0.0017
Separate variance Strain Common profile	4	36+3	345.0	91.9	57	0.0023
Separate variance Strain Profile by Pop	4a	36+6	342.6	89.4	54	0.0017

Separate variance Strain Profile by Strain	4b	36+18	307.6	54.4	42	0.0943
Separate variance Strain Profile by Pop*Strain	4c	36+36	289.3	36.2	24	0.0527
Separate variance Combin Common profile	5	60+3	321.6	68.5	33	0.0003
Separate variance Combin Profile by Pop	5a	60+6	318.9	65.7	30	0.0002
Separate variance **Combin** **Profile by Strain**	**5b**	**60+18**	**269.4**	**16.2**	**18**	**0.5763**

*The basic complete model 1a with 96 parameters was used for the comparison.

Supplemental Table 3.4b Multiple comparisons of the six *S. meliloti* strains with Bonferroni's correction for the different profiles of percentages of distribution of the four nodules shapes categories. Probability is significant only at p values < 0.05/15 = 0.003 while values of p < 0.10/15 = 0.006 indicates a trend. Different letters representing significant differences adjusted with Bonferroni's test at $P \leq 0.05$ are indicated in the lower panel

Label	NumDF	DenDF	ChiSq	FValue	FProb
A2 vs B399	1	102	14.78	14.78	0.0002
A2 vs I1	1	102	35.09	35.09	<.0001
A2 vs NRG34	1	102	185.89	185.89	<.0001
A2 vs Rm1521	1	102	95.13	95.13	<.0001
A2 vs S27	1	102	111.44	111.44	<.0001
B399 vs I1	1	102	2.42	2.42	0.1231
B399 vs NRG34	1	102	28.53	28.53	<.0001
B399 vs Rm1521	1	102	11.57	11.57	0.0010
B399 vs S27	1	102	12.28	12.28	0.0007
I1vs NRG34	1	102	12.97	12.97	0.0005
I1vs Rm1521	1	102	2.76	2.76	0.0998
I1 vs S27	1	102	2.84	2.84	0.0952
NRG34 vs Rm1521	1	102	6.92	6.92	0.0098
NRG34 vs S27	1	102	10.39	10.39	0.0017
Rm1521 vs S27	1	102	0.01	0.01	0.9186

A2	B399	I1	S27	Rm1521	NRG34
a	bc	bc	c	cd	d

Supplemental Table 3.5a Comparison of the models using a maximum likelihood ratio test on the transformed additive logratio datas comparing the effects of two alfalfa populations in symbiosis with six different *S. meliloti* strains and their interactions on the different profiles of percentages of distribution for the three nodules freezing-damages classes. The line in bold indicates the statistical model selected (p>0.05) presenting the best fit for the data set.

Models	**Level**	**Num parameters**	**-2LogL**	**Q Statistics**	**V (DF)**	**p-Value**
Complete	1*	24+36 = 60	87.5			
Pooled variance Common profile	2	3+2=5	225.8	138.2	55	0.0000
Pooled variance Profile by Pop	2a	3+4=7	222.4	134.9	53	0.0000
Pooled variance Profile by Strain	2b	3+12=15	206.7	119.2	45	0.0000
Pooled variance Profil par Pop*Strain	2c	3+24=27	187.3	99.8	33	0.0000
Separate variance Pop Common profile	3	6+2=8	221.7	134.2	52	0.0000
Separate variance Pop Profile by Pop	3a	6+4=10	218.3	130.9	50	0.0000
Separate variance Pop Profile by Strain	3b	6+12=18	202.1	114.7	42	0.0000
Separate variance Pop Profile by Pop*Strain	3c	6+24=30	182.9	95.5	30	0.0000
Separate variance Strain Common profile	4	18+2=20	173.5	86.1	40	0.0000
Separate variance Strain Profile by Pop	4a	18+4=22	173.1	85.6	38	0.0000

Separate variance Strain Profile by Strain	4b	18+12=30	153.6	66.2	30	0.0002
Separate variance Strain Profile by Pop*Strain	4c	18+24=40	132.5	45.0	18	0.0004
Separate variance Combin Common profile	5	36+2=38	137.5	50.1	22	0.0006
Separate variance Combin Profile by Pop	5a	36+4=40	136.7	49.3	20	0.0003
Separate variance Combin Profile by Strain	**5b**	**36+12=48**	**108.3**	**20.8**	**12**	**0.0535**

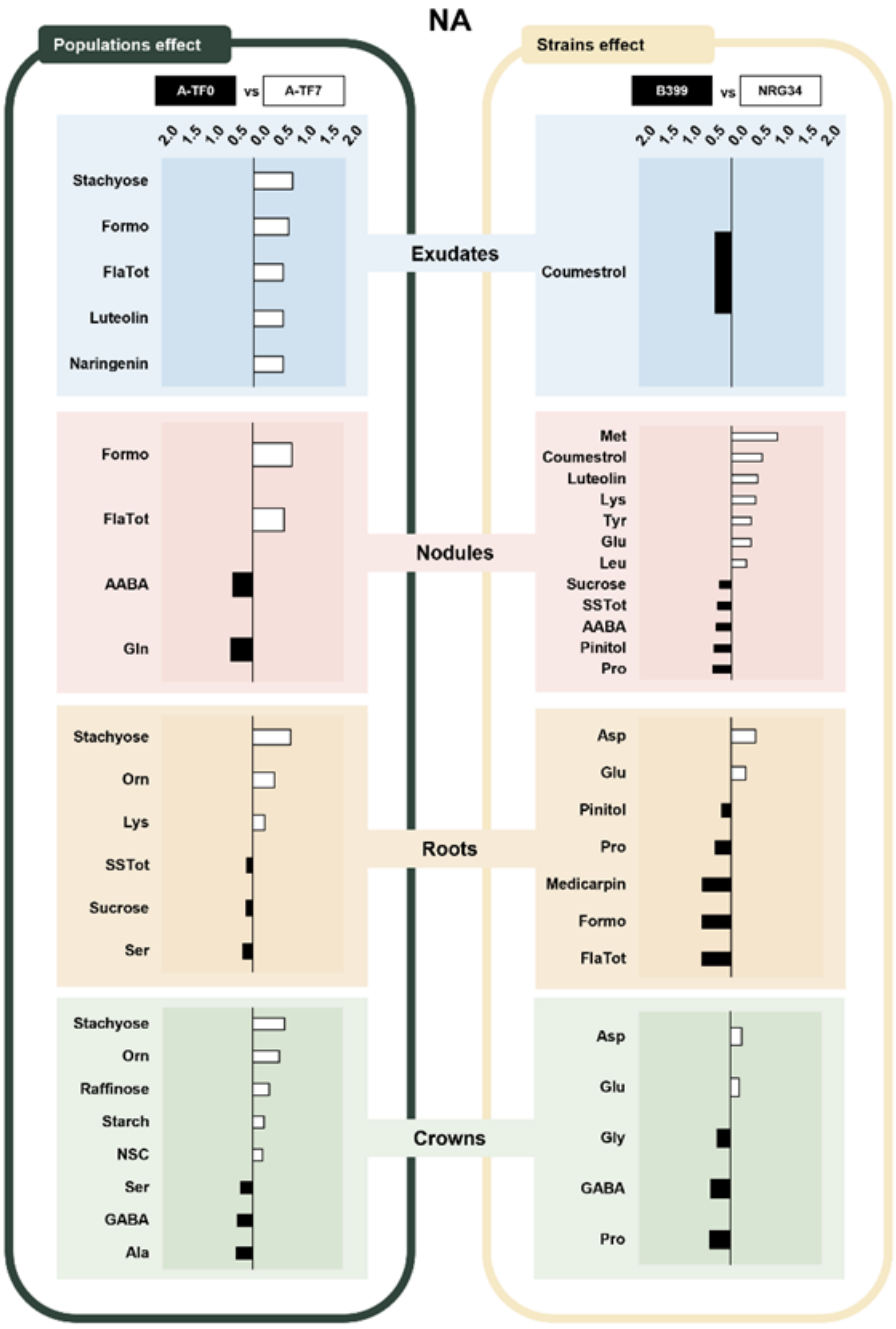
* The basic complete model 1 with 60 parameters was used for the comparison

Supplemental Table 3.5b Multiple comparisons of the six *S. meliloti* strains with Bonferroni's correction on the different profiles of percentages of distribution for the three nodules freezing-damages classes. Probability is significant only at p values < 0.05/15 = 0.003 while values of p < 0.10/15 = 0.006 indicates a trend. Different letters representing significant differences adjusted with Bonferroni's test at $P \leq 0.05$ are indicated in the lower panel

Label	NumDF	DenDF	ChiSq	FValue	ProbChiSq	FProb
A2 vs B399	1	48	10.81	10.81	0.0010	0.0019
A2 vs I1	1	48	38.96	38.96	<.0001	<.0001
A2 vs NRG34	1	48	43.09	43.09	<.0001	<.0001
A2 vs Rm1521	1	48	13.50	13.50	0.0002	0.0006
A2 vs S27	1	48	9.18	9.18	0.0025	0.0039
B399 vs I1	1	48	0.00	0.00	0.9604	0.9606
B399 vs NRG34	1	48	8.15	8.15	0.0043	0.0063
B399 vs Rm1521	1	48	1.17	1.17	0.2787	0.2841
B399 vs S27	1	48	0.31	0.31	0.5799	0.5824
I1 vs NRG34	1	48	13.72	13.72	0.0002	0.0005
I1 vs Rm1521	1	48	5.48	5.48	0.0192	0.0234
I1 vs S27	1	48	0.39	0.39	0.5329	0.5358
NRG34 vs Rm1521	1	48	21.19	21.19	<.0001	<.0001
NRG34 vs S27	1	48	3.17	3.17	0.0752	0.0815
Rm1521 vs S27	1	48	2.01	2.01	0.1558	0.1623

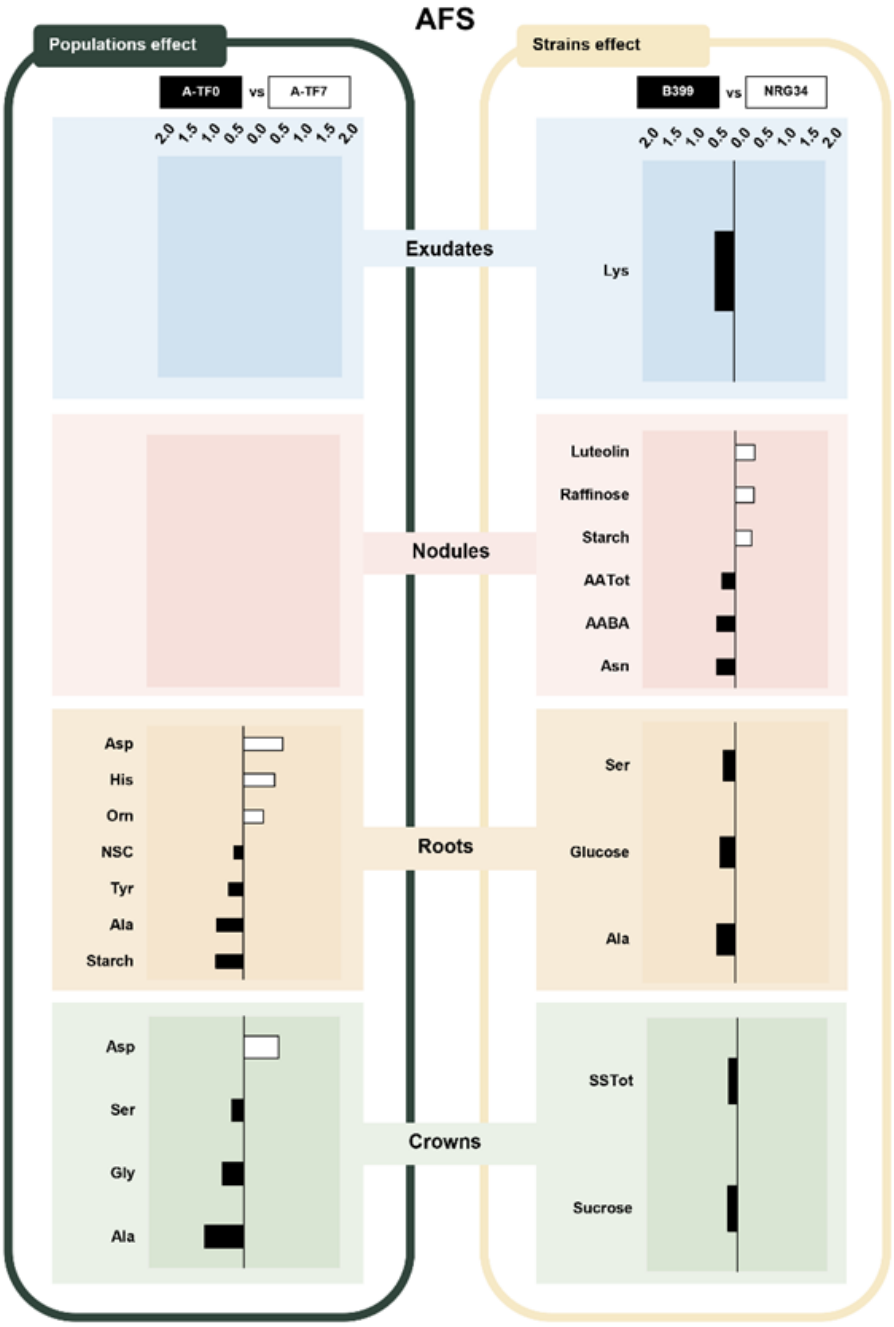
A2	B399	I1	Rm1521	S27	NRG34
a	b	b	b	bc	c

Appendix 2 Supplementary information for Chapter 4



Supplemental Figure 4.1 Graphic representation of log 2 fold changes in metabolites concentration showing the differential contribution of each alfalfa population (A-TF0 vs A-TF7) and each *S. meliloti* strain (B399 vs NRG34) to the metabolic changes in NA root exudates, nodules, roots and crowns. The left panel compares the two alfalfa populations with significant higher concentration in ATF-7 on the right to zero line (white) and significant higher concentration

in ATF-0 on the left (black). The right panel compares the two rhizobial strains with significant higher concentration in response to inoculation with NRG34 on the right to zero line (white) and significant higher concentration in response to B399 on the left (black). Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Formo, formononetin, FlaTot, total flavonoids, Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, leucine; Lys, lysine; Met, methionine; NSC, non structural carbohydrates; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine; Orn, ornithine, AA-Tot, Total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid



Supplemental Figure 4.2 Graphic representation of log 2 fold changes in metabolites concentration showing the differential contribution of each alfalfa population (A-TF0 vs A-TF7) and each *S. meliloti* strain (B399 vs NRG34) to the metabolic changes in AFS root exudates, nodules, roots and crowns. The left panel compares the two alfalfa populations with significant higher concentration in ATF-7 on the right to zero line (white) and significant higher concentration

in ATF-0 on the left (black). The right panel compares the two rhizobial strains with significant higher concentration in response to inoculation with NRG34 on the right to zero line (white) and significant higher concentration in response to B399 on the left (black). Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Formo, formononetin, FlaTot, total flavonoids, Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, leucine; Lys, lysine; Met, methionine; NSC, nonstructural carbohydrates; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine; Orn, ornithine, AA-Tot, Total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid

Supplemental Table 4.1 Metabolites concentrations of two alfalfa populations (A-TF0 vs A-TF7) in symbiosis with two *S. meliloti* strains (B399 and NRG34), and their interactions in non-acclimated (NA) root exudates, nodules, roots and crowns. Numbers in bold indicate statistically significant effects ($P \leq 0.05$). Different letters represent significant populations × strain interactions as determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$. Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Coum, coumestrol; Formo, formononetin, FlaTot, total flavonoids; Gln, glutamine; Glu, glutamic acid; Gluc, glucose; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lut, luteolin, Lys, leucine; Lys, lysine; Medic, medicarpin; Met, methionine; Nar, naringenin; NSC, nonstructural carbohydrates; Phe, phenylalanine; Pin, pinitol; Pro, proline; Raff, raffinose; Ser, serine; Suc, Sucrose; Stach, Stachyose, Thr, threonine; Tyr, tyrosine; Val, valine; Orn, ornithine; AATot, total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid;

	Metabolites																																						
	Sugars									Amino Acids																								Flavonoids						
Exudates	Suc	Gluc	Stach	Raff	Fruc	Pin	SS Tot	Starch	NSC	Glu	Gln	Pro	Orn	Arg	His	Asp	Asn	Ala	Thr	Lys	Met	Ile	Leu	Val	Ser	Gly	GABA	AABA	Tyr	Phe	AA Tot	Nar	Lut	Echi	Coum	Formo	Medic	FlaTot
A-TF0	0.559	0.330	**0.008**	0.002	0.345	0.244	1.488			0.179	0.037	0.719	0.016	0.007	0.010	0.181	0.797	0.077	0.038	0.008	0.004	0.009	0.011	0.018	0.091	0.025	0.157	0.001	0.004	0.009	2.40	**0.0295**	**0.0351**	0.224	0.115	**1.51**	0.151	2.071
A-TF7	0.469	0.393	**0.014**	0.003	0.458	0.294	1.631			0.184	0.040	0.725	0.018	0.012	0.012	0.191	0.938	0.079	0.040	0.009	0.004	0.009	0.012	0.019	0.082	0.024	0.193	0.001	0.004	0.009	2.60	**0.0459**	**0.0548**	0.248	0.114	**2.58**	0.189	3.231
P-values	0.529	0.403	**0.046**	0.094	0.167	0.310	0.600			0.834	0.616	0.962	0.555	0.215	0.392	0.713	0.483	0.882	0.747	0.688	0.965	0.811	0.840	0.812	0.508	0.679	0.241	0.343	0.587	0.877	0.637	**0.013**	**0.049**	0.622	0.934	**0.040**	0.256	0.053
B399	0.594	0.398	0.011	0.002	0.400	0.301	1.705			0.177	0.040	0.816	0.019	0.011	0.012	0.183	0.891	0.080	0.040	0.009	0.004	0.010	0.012	0.020	0.091	0.025	0.177	0.001	0.004	0.009	2.63	0.0366	0.0480	0.246	**0.128**	2.38	0.193	3.034
NRG34	0.434	0.325	0.012	0.003	0.404	0.237	1.414			0.185	0.037	0.627	0.015	0.008	0.010	0.189	0.844	0.075	0.038	0.008	0.004	0.009	0.011	0.018	0.083	0.024	0.173	0.001	0.004	0.008	2.37	0.0388	0.0419	0.226	**0.101**	1.71	0.147	2.268
P-values	0.270	0.342	0.867	0.333	0.959	0.192	0.291	nd	nd	0.748	0.768	0.156	0.254	0.292	0.555	0.824	0.812	0.709	0.703	0.702	0.645	0.634	0.628	0.516	0.546	0.630	0.909	0.884	0.813	0.676	0.556	0.721	0.521	0.679	**0.035**	0.185	0.172	0.190
A-TF0 × B399	0.735	0.320	0.007	0.002	0.297	0.269	1.630			0.175	0.038	0.833	0.018	0.009	0.010	0.176	0.871	0.080	0.037	0.009	0.004	0.009	0.012	0.019	0.094	0.027	0.169	0.001	0.004	0.009	2.60	0.0307	0.0393	0.272	0.137	1.89	0.188	2.564
A-TF0 ×NRG34	0.382	0.340	0.009	0.002	0.394	0.220	1.346			0.182	0.035	0.605	0.014	0.006	0.009	0.186	0.724	0.073	0.039	0.008	0.004	0.009	0.011	0.018	0.089	0.023	0.144	0.001	0.004	0.008	2.19	0.0282	0.0310	0.177	0.094	1.13	0.113	1.578
A-TF7 × B399	0.452	0.476	0.015	0.002	0.503	0.334	1.781			0.179	0.041	0.800	0.020	0.014	0.013	0.190	0.912	0.080	0.043	0.009	0.004	0.010	0.012	0.020	0.088	0.024	0.185	0.001	0.004	0.009	2.66	0.0425	0.0568	0.220	0.120	2.87	0.198	3.505
A-TF7 ×NRG34	0.485	0.311	0.014	0.003	0.414	0.254	1.482			0.188	0.040	0.650	0.016	0.009	0.011	0.192	0.964	0.078	0.037	0.009	0.004	0.009	0.011	0.018	0.077	0.024	0.202	0.001	0.004	0.009	2.55	0.0493	0.0528	0.275	0.109	2.29	0.180	2.958
P-values	0.187	0.227	0.843	0.054	0.253	0.757	0.978			0.961	0.913	0.765	0.909	0.794	0.645	0.866	0.619	0.830	0.468	0.872	0.322	0.997	0.803	0.994	0.828	0.624	0.500	0.951	0.983	0.688	0.727	0.452	0.818	0.123	0.194	0.859	0.396	0.702
Nodules																																							
A-TF0	62.0	2.83	0.00	0.00	0.74	28.0	93.5	1.31	88.7	15.8	**2.85**	27.1	0.19	2.19	3.58	4.97	435	13.7	1.72	0.75	0.078	0.779	0.819	1.65	3.50	2.41	27.1	**0.453**	0.454	0.455	546	1.11	1.18	6.57	3.07	**28.1**	2.18	**39.6**
A-TF7	54.5	2.75	0.00	0.00	0.83	30.9	89.0	1.94	90.9	16.3	**2.06**	22.7	0.29	2.15	3.56	5.01	397	12.1	1.63	0.88	0.065	0.658	0.760	1.38	3.44	2.23	27.0	**0.331**	0.516	0.429	501	1.35	0.99	5.73	2.20	**51.0**	2.78	**64.1**
P-values	0.075	0.734	1.000	1.000	0.607	0.060	0.284	0.347	0.763	0.803	**0.045**	0.173	0.214	0.727	0.971	0.916	0.305	0.747	0.338	0.092	0.510	0.250	0.379	0.088	0.844	0.393	0.942	**0.003**	0.320	0.695	0.287	0.262	0.262	0.486	0.058	**0.005**	0.281	**0.009**
B399	**63.3**	2.98	0.00	0.00	0.98	**33.2**	**100.5**	1.89	96.2	**13.7**	2.24	**28.2**	0.24	2.09	3.22	4.84	394	13.7	1.63	**0.66**	**0.048**	0.628	**0.705**	1.45	3.20	2.30	27.8	**0.437**	**0.411**	0.402	502	1.04	**0.88**	2.55	**4.75**	43.5	2.46	52.7
NRG34	**53.2**	2.59	0.00	0.00	0.59	**25.7**	**82.1**	1.36	83.4	**18.4**	2.67	**21.6**	0.23	2.26	3.91	5.14	439	12.1	1.72	**0.97**	**0.095**	0.809	**0.875**	1.59	3.74	2.35	26.3	**0.346**	**0.559**	0.482	545	1.42	**1.29**	2.72	**7.55**	35.6	2.51	51.0
P-values	**0.019**	0.099	1.000	1.000	0.028	**<0.001**	**<0.001**	0.417	0.097	**0.024**	0.252	**0.047**	0.939	0.941	0.164	0.510	0.231	0.206	0.319	**0.001**	**0.024**	0.093	**0.018**	0.383	0.110	0.840	0.426	**0.021**	**0.023**	0.224	0.304	0.092	**0.018**	0.699	**0.025**	0.285	0.922	0.852
A-TF0 × B399	65.6	3.01	0.00	0.00	**0.76 ab**	31.7	101.0	1.47	90.2	13.4	2.59	29.3	0.20	1.95	2.96	4.77	389	13.7	1.67	0.61	0.055	0.622	0.710	1.53	3.07	2.60	27.7	0.506	0.368	0.374	498	1.08	1.00	3.00	4.89	30.3	2.03	37.0
A-TF0 ×NRG34	58.4	2.64	0.00	0.00	**0.72 b**	24.3	86.1	1.15	87.2	18.2	3.10	24.8	0.17	2.22	4.19	5.16	482	12.5	1.77	0.89	0.101	0.936	0.929	1.78	3.93	2.23	26.5	0.399	0.540	0.535	593	1.13	1.35	3.13	8.24	26.0	2.34	42.2
A-TF7 × B399	61.0	2.95	0.00	0.00	**1.19 a**	34.7	99.9	2.32	102.2	14.0	1.89	27.0	0.28	2.43	3.49	4.90	399	13.8	1.59	0.72	0.041	0.634	0.699	1.37	3.33	2.01	27.9	0.367	0.453	0.430	506	1.01	0.76	2.09	4.60	56.9	2.89	68.3
A-TF7 ×NRG34	47.9	2.55	0.00	0.00	**0.46 b**	27.2	78.1	1.56	79.6	18.6	2.24	18.4	0.30	2.08	3.63	5.13	396	11.6	1.68	1.04	0.090	0.681	0.821	1.39	3.54	2.46	26.0	0.294	0.578	0.429	497	1.70	1.22	2.31	6.86	45.1	2.68	59.9
P-values	0.466	0.955	1.000	1.000	**0.046**	0.962	0.414	0.739	0.195	0.960	0.829	0.521	0.756	0.525	0.270	0.861	0.202	0.698	0.975	0.793	0.932	0.206	0.471	0.479	0.329	0.066	0.834	0.654	0.698	0.217	0.212	0.144	0.735	0.912	0.648	0.600	0.637	0.432
Roots																																							
A-TF0	**52.3**	2.41	**0.16**	0.103	3.96	9.63	**68.6**	308	376	5.76	1.37	28.7	**0.230**	5.33	1.79	1.15	74.5	3.74	1.11	**0.077**	0.028	0.154	0.170	0.377	**2.81**	0.380	5.74	0.048	0.109	0.268	134	0.00	0.00	0.087	0.025	80.4	4.33	84.9
A-TF7	**47.3**	1.92	**0.29**	0.109	3.40	9.52	**62.5**	336	399	6.10	1.37	28.9	**0.321**	7.12	2.19	1.29	91.5	3.66	1.06	**0.093**	0.029	0.152	0.168	0.365	**2.42**	0.399	5.37	0.039	0.116	0.274	153	0.00	0.00	0.012	0.000	87.9	4.81	92.7
P-values	**0.021**	0.078	**0.001**	0.527	0.152	0.815	**0.023**	0.082	0.15	0.285	0.985	0.906	**0.006**	0.108	0.257	0.236	0.074	0.772	0.470	**0.034**	0.704	0.834	0.872	0.647	**0.013**	0.475	0.426	0.267	0.315	0.793	0.100	1.000	1.000	0.220	0.326	0.643	0.609	0.649
B399	50.5	2.09	0.22	0.108	3.22	**10.26**	66.4	313	380	**5.28**	1.29	**32.2**	0.288	6.65	1.85	**1.00**	82.2	3.88	1.07	0.084	0.030	0.148	0.168	0.380	2.60	0.412	6.00	0.043	0.116	0.275	146	0.000	0.00	0.025	0.087	**102.2**	**5.54**	**108.0**
NRG34	49.1	2.24	0.23	0.104	4.13	**8.90**	64.7	330	395	**6.57**	1.44	**25.4**	0.263	5.80	2.13	**1.45**	83.8	3.51	1.11	0.085	0.027	0.158	0.171	0.363	2.63	0.367	5.12	0.044	0.109	0.267	141	0.000	0.00	0.000	0.012	**66.0**	**3.60**	**69.7**
P-values	0.494	0.568	0.658	0.577	0.371	**0.010**	0.500	0.288	0.31	**<0.001**	0.242	**0.001**	0.405	0.434	0.429	**0.001**	0.863	0.186	0.597	0.882	0.076	0.409	0.831	0.516	0.853	0.092	0.065	0.868	0.353	0.689	0.642	1.000	1.000	0.326	0.220	**0.0**	**0.050**	**0.033**
A-TF0 × B399	53.2	2.27	0.14	0.105	3.26	10.04	69.0	294	363	5.19	1.33	30.7	0.234	5.91	1.79	0.92	74.6	3.99	1.07	0.079	0.030	0.149	0.172	0.389	2.71	0.411	6.20	0.044	0.112	0.272	136	0.00	0.00	0.051	0.173	99.2	5.65	105.3
A-TF0 ×NRG34	51.5	2.55	0.18	0.101	4.66	9.23	68.2	322	390	6.33	1.40	26.7	0.226	4.75	1.79	1.39	74.4	3.49	1.15	0.075	0.027	0.159	0.169	0.365	2.91	0.350	5.28	0.051	0.106	0.264	131	0.00	0.00	0.000	0.000	61.5	3.01	64.6
A-TF7 × B399	47.8	1.91	0.29	0.111	3.18	10.48	63.8	333	397	5.38	1.25	33.7	0.342	7.39	1.91	1.08	89.8	3.78	1.06	0.090	0.031	0.147	0.164	0.370	2.49	0.412	5.80	0.042	0.121	0.278	156	0.00	0.00	0.000	0.000	105.3	5.43	110.7
A-TF7 ×NRG34	46.7	1.93	0.28	0.106	3.61	8.56	61.2	339	400	6.81	1.48	24.1	0.300	6.85	2.46	1.50	93.2	3.53	1.06	0.095	0.027	0.157	0.173	0.360	2.34	0.385	4.95	0.037	0.112	0.270	150	0.00	0.00	0.000	0.024	70.5	4.19	74.7
P-values	0.890	0.633	0.536	0.943	0.440	0.266	0.721	0.493	0.435	0.631	0.524	0.131	0.576	0.773	0.432	0.863	0.849	0.655	0.556	0.518	0.646	1.000	0.655	0.776	0.236	0.503	0.945	0.435	0.840	0.999	0.980	1.000	1.000	0.326	0.111	0.929	0.461	0.889
Crown																																							
A-TF0	43.5	4.67	**0.350**	**0.104**	4.15	10.6	63.4	**249**	**312**	7.07	1.89	27.1	**0.27**	7.45	2.49	3.47	67.8	**2.97**	0.948	0.105	0.096	0.160	0.168	0.337	**2.96**	0.420	**4.74**	0.096	0.111	0.274	131							
A-TF7	43.9	5.78	**0.573**	**0.135**	4.87	11.2	66.4	**296**	**362**	7.92	1.65	24.8	**0.40**	9.19	3.17	4.01	70.5	**2.31**	0.826	0.120	0.107	0.155	0.155	0.316	**2.46**	0.411	**3.75**	0.087	0.102	0.323	133							
P-values	0.880	0.075	**0.019**	**0.013**	0.256	0.453	0.297	**0.018**	**0.016**	0.077	0.074	0.475	**0.001**	0.193	0.126	0.110	0.776	**0.033**	0.083	0.215	0.439	0.785	0.499	0.374	**0.019**	0.785	**0.008**	0.514	0.357	0.175	0.887							
B399	45.7	4.99	0.439	0.120	4.28	11.3	66.8	277	344	**7.01**	1.68	**29.9**	0.37	9.45	2.79	**3.41**	70.9	2.89	0.877	0.115	0.103	0.152	0.163	0.337	2.75	**0.457**	**4.87**	0.088	0.104	0.315	139							
NRG34	41.7	5.46	0.484	0.119	4.74	10.5	63.0	268	331	**7.98**	1.85	**22.0**	0.30	7.19	2.87	**4.07**	67.4	2.39	0.897	0.109	0.101	0.163	0.159	0.316	2.68	**0.374**	**3.63**	0.095	0.109	0.282	125				nd			
P-values	0.103	0.431	0.606	0.924	0.469	0.295	0.191	0.617	0.50	**0.046**	0.190	**0.019**	0.112	0.093	0.853	**0.052**	0.723	0.102	0.769	0.600	0.935	0.446	0.810	0.390	0.740	**0.028**	**0.002**	0.625	0.583	0.366	0.315							
A-TF0 × B399	45.5	4.30	0.317	0.107	3.73	10.8	64.8	251	316	6.43	1.75	29.8	0.29	8.55	2.59	2.89	66.9	3.27	0.902	0.106	0.092	0.147	0.169	0.330	2.81	0.452	5.47	0.085	0.109	0.277	133							
A-TF0 ×NRG34	41.5	5.04	0.384	0.102	4.57	10.4	62.0	246	308	7.72	2.03	24.3	0.25	6.36	2.39	4.06	68.6	2.66	0.993	0.103	0.100	0.173	0.166	0.344	3.12	0.388	4.02	0.108	0.113	0.271	128							
A-TF7 × B399	45.8	5.67	0.561	0.133	4.83	11.9	68.9	303	372	7.60	1.62	29.9	0.45	10.35	2.99	3.93	74.8	2.50	0.852	0.125	0.113	0.157	0.157	0.344	2.69	0.462	4.27	0.092	0.099	0.353	144							
A-TF7 ×NRG34	41.9	5.89	0.584	0.137	4.91	10.5	64.0	289	353	8.24	1.68	19.7	0.36	8.02	3.34	4.08	66.3	2.12	0.800	0.115	0.102	0.154	0.152	0.288	2.24	0.359	3.23	0.083	0.105	0.293	122							
P-values	0.981	0.662	0.800	0.687	0.551	0.553	0.101	0.831	0.795	0.485	0.386	0.461	0.540	0.959	0.522	0.126	0.599	0.701	0.298	0.745	0.524	0.336	0.970	0.157	0.073	0.598	0.558	0.257	0.900	0.444	0.532							

Supplemental Table 4.2 Metabolites concentrations of two alfalfa populations (A-TF0 vs A-TF7) in symbiosis with two *S. meliloti* strains (B399 and NRG34), and their interactions in cold-acclimated (CA) root exudates, nodules, roots and crowns. Numbers in bold indicate statistically significant effects ($P \leq 0.05$). Different letters represent significant populations × strain interactions as determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$. Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Coum, coumestrol; Formo, formononetin, FlaTot, total flavonoids; Gln, glutamine; Glu, glutamic acid; Gluc, glucose; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lut, luteolin, Lys, leucine; Lys, lysine; Medic, medicarpin; Met, methionine; Nar, naringenin; NSC, non structural carbohydrates; Phe, phenylalanine; Pin, pinitol; Pro, proline; Raff, raffinose; Ser, serine; Suc, Sucrose; Stach, Stachyose, Thr, threonine; Tyr, tyrosine; Val, valine; Orn, ornithine; AATot, total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid

	Metabolites																																							
	Sugars									Amino Acids																								Flavonoids						
Exudates	Suc	Gluc	Stach	Raff	Fruc	Pin	SS Tot	Starch	NSC	Glu	Gln	Pro	Orn	Arg	His	Asp	Asn	Ala	Thr	Lys	Met	Ile	Leu	Val	Ser	Gly	GABA	AABA	Tyr	Phe	AA Tot	Nar	Lut	Echi	Coum	Formo	Medic	FlaTot
A-TF0	0.453	0.362	0.066	0.078	0.362	0.110	1.43			0.150	0.003	0.150	0.005	0.008	**0.002**	0.142	0.024	0.041	0.013	0.006	0.005	0.005	0.008	0.012	0.038	0.010	0.071	0.0002	0.002	0.005	0.673	0.025	0.031	0.097	0.081	2.11	**0.623**	2.97
A-TF7	0.606	0.429	0.071	0.087	0.434	0.136	1.76			0.190	0.006	0.118	0.010	0.014	**0.004**	0.205	0.062	0.042	0.017	0.007	0.004	0.007	0.010	0.012	0.035	0.012	0.070	0.0003	0.003	0.006	0.863	0.025	0.021	0.086	0.073	2.41	**0.385**	3.00
P-values	0.107	0.517	0.604	0.359	0.531	0.283	0.144			0.078	0.345	0.348	0.103	0.103	**0.014**	0.177	0.141	0.875	0.104	0.165	0.068	0.094	0.144	0.978	0.697	0.221	0.945	0.315	0.329	0.478	0.201	0.941	0.211	0.565	0.708	0.599	**0.030**	0.955
B399	0.508	0.459	0.068	0.080	0.468	0.143	1.73			0.178	0.006	0.121	0.009	0.013	**0.004**	**0.224**	0.055	0.045	**0.017**	0.007	0.005	0.007	0.009	0.012	0.040	0.012	0.081	**0.0003**	0.003	0.006	0.885	0.025	0.023	0.099	0.090	2.31	0.548	3.10
NRG34	0.551	0.332	0.069	0.085	0.328	0.103	1.47			0.162	0.003	0.147	0.006	0.009	**0.003**	**0.123**	0.031	0.039	**0.012**	0.006	0.005	0.006	0.008	0.012	0.033	0.010	0.060	**0.0001**	0.002	0.005	0.651	0.025	0.029	0.083	0.064	2.21	0.460	2.87
P-values	0.640	0.223	0.859	0.615	0.226	0.114	0.253	nd	nd	0.454	0.153	0.241	0.156	0.242	**0.045**	**0.038**	0.334	0.238	**0.042**	0.225	0.722	0.325	0.214	0.991	0.206	0.256	0.086	**0.017**	0.115	0.399	0.119	0.932	0.431	0.425	0.240	0.862	0.396	0.738
A-TF0 × B399	0.337	0.439	0.066	0.074	0.439	0.120	1.47			0.144	0.004	0.126	0.007	0.010	0.003	0.180	0.031	0.041	0.014	0.006	0.005	0.006	0.009	0.011	0.039	0.011	0.075	0.0003	0.003	0.006	0.732	0.026	0.024	0.103	0.093	2.394	0.674	3.31
A-TF0 ×NRG34	0.568	0.286	0.066	0.082	0.286	0.099	1.39			0.156	0.003	0.116	0.004	0.007	0.002	0.104	0.017	0.042	0.011	0.005	0.005	0.005	0.006	0.013	0.036	0.008	0.068	0.0001	0.002	0.005	0.614	0.024	0.037	0.092	0.069	1.824	0.572	2.62
A-TF7 × B399	0.679	0.480	0.069	0.086	0.498	0.165	1.98			0.212	0.009	0.174	0.012	0.017	0.005	0.268	0.078	0.049	0.020	0.008	0.004	0.008	0.010	0.013	0.042	0.012	0.088	0.0004	0.003	0.006	1.038	0.023	0.022	0.096	0.086	2.230	0.423	2.88
A-TF7 ×NRG34	0.534	0.379	0.073	0.088	0.370	0.107	1.55			0.168	0.003	0.119	0.007	0.012	0.003	0.143	0.045	0.036	0.013	0.007	0.004	0.007	0.009	0.011	0.029	0.011	0.053	0.0002	0.003	0.006	0.687	0.026	0.021	0.075	0.060	2.598	0.347	3.13
P-values	0.053	0.802	0.846	0.750	0.912	0.456	0.448			0.209	0.449	0.414	0.778	0.726	0.589	0.597	0.698	0.217	0.432	0.705	0.791	0.946	0.677	0.190	0.393	0.730	0.250	0.496	0.810	0.930	0.427	0.505	0.367	0.788	0.972	0.421	0.899	0.483
Nodules																																								
A-TF0	162	**4.56**	8.12	16.7	0.807	26.5	218	6.02	224	17.6	3.05	26.3	**1.40**	**14.2**	**5.79**	4.63	311	20.1	1.46	**0.740**	0.085	0.888	0.719	1.87	12.0	3.04	**27.8**	0.301	0.947	0.309	454	**1.55**	**1.79**	5.86	20.17	24.2	9.58	63.2
A-TF7	156	**2.89**	10.11	16.7	0.729	26.4	213	3.27	216	18.7	2.95	23.1	**3.48**	**24.4**	**8.26**	5.32	409	20.4	1.44	**0.939**	0.070	0.942	0.795	1.86	10.2	3.64	**23.0**	0.280	1.062	0.378	560	**0.94**	**1.06**	5.86	15.18	20.7	6.62	50.3
P-values	0.727	**0.015**	0.162	0.994	0.639	0.951	0.719	0.135	0.61	0.551	0.908	0.578	**0.003**	**0.002**	**0.003**	0.228	0.072	0.894	0.746	**0.036**	0.488	0.642	0.531	0.983	0.310	0.669	**0.020**	0.389	0.387	0.144	0.100	**0.010**	**0.025**	0.993	0.127	0.571	0.126	0.112
B399	165	**4.57**	8.15	16.2	**0.996**	**31.9**	227	5.31	232	16.4	3.07	26.3	2.88	**16.1**	**6.25**	4.84	354	20.1	1.44	0.758	**0.051**	0.904	0.738	1.74	10.0	3.55	26.3	**0.341**	**1.240**	0.349	498	**0.99**	**0.91**	5.70	20.82	18.4	**10.10**	56.9
NRG34	153	**2.88**	10.08	17.2	**0.540**	**21.0**	205	3.98	209	19.9	2.94	23.2	2.00	**22.5**	**7.80**	5.11	366	20.4	1.45	0.921	**0.104**	0.925	0.777	1.99	12.2	3.12	24.4	**0.240**	**0.769**	0.338	517	**1.50**	**1.95**	6.02	14.53	26.5	**6.10**	56.7
P-values	0.482	**0.014**	0.172	0.669	**0.010**	**<0.001**	0.181	0.465	0.17	0.065	0.884	0.592	0.163	**0.029**	**0.051**	0.634	0.831	0.923	0.851	0.083	**0.021**	0.856	0.746	0.340	0.225	0.762	0.334	**<0.001**	**0.001**	0.817	0.761	**0.013**	**0.002**	0.746	0.058	0.195	**0.043**	0.980
A-TF0 × B399	157	5.70	7.12	15.2	1.158	31.9	218	7.15	225	15.2	2.63	27.7	1.84	13.7	5.22	4.05	323	18.4	1.43	0.677	0.047	0.908	0.704	1.75	10.9	2.18	27.6	0.355	1.129	0.332	460	1.28	1.08	4.99	26.12	19.5	**13.64a**	66.6
A-TF0 ×NRG34	167	3.41	9.12	18.3	0.456	21.2	219	4.90	224	20.0	3.48	24.9	0.96	14.7	6.37	5.20	298	21.8	1.49	0.803	0.123	0.867	0.735	1.99	13.1	3.89	28.0	0.246	0.764	0.285	448	1.82	2.51	6.73	14.23	28.9	**5.52b**	59.8
A-TF7 × B399	173	3.43	9.17	17.2	0.833	31.9	235	3.47	239	17.5	3.51	24.8	3.92	18.5	7.28	5.62	385	21.8	1.46	0.840	0.055	0.900	0.772	1.73	9.0	4.93	25.1	0.326	1.352	0.365	535	0.70	0.73	6.40	15.52	17.2	**6.55b**	47.1
A-TF7 ×NRG34	139	2.34	11.04	16.2	0.625	20.9	190	3.07	193	19.9	2.39	21.5	3.04	30.3	9.23	5.01	433	19.0	1.42	1.039	0.085	0.983	0.818	2.00	11.3	2.36	20.8	0.234	0.773	0.391	586	1.18	1.38	5.32	14.83	24.2	**6.68b**	53.6
P-values	0.200	0.348	0.964	0.405	0.143	0.943	0.156	0.608	0.1846	0.525	0.282	0.958	0.995	0.062	0.600	0.126	0.487	0.218	0.478	0.687	0.293	0.595	0.949	0.959	0.964	0.141	0.235	0.731	0.423	0.438	0.614	0.882	0.222	0.174	0.089	0.845	**0.037**	0.399
Roots																																							
A-TF0	**165**	2.14	3.55	4.03	3.23	8.18	**186**	**132.3**	318	**7.09**	0.914	17.5	**0.394**	**10.0**	2.75	2.56	57.6	3.33	0.781	**0.121**	0.032	0.253	0.225	0.710	4.79	0.276	**8.13**	0.055	0.324	0.284	118	0.684	0.201	1.948	0.00	75.1	5.86	83.8
A-TF7	**184**	2.12	3.98	4.10	3.66	8.95	**206**	**95.8**	302	**8.66**	0.943	20.8	**0.579**	**13.2**	2.62	3.13	68.8	2.95	0.861	**0.148**	0.025	0.216	0.206	0.420	4.10	0.307	**6.88**	0.059	0.301	0.252	135	0.450	0.253	0.824	0.00	72.9	16.06	90.5
P-values	**0.001**	0.940	0.240	0.896	0.529	0.126	**0.000**	**0.015**	0.297	**0.001**	0.705	0.296	**0.007**	**0.035**	0.820	0.073	0.263	0.195	0.206	**0.016**	0.256	0.305	0.427	0.343	0.115	0.293	**0.017**	0.754	0.646	0.249	0.189	0.758	0.769	0.264	ns	0.903	0.363	0.746
B399	175	2.05	3.64	4.12	3.17	8.81	197	118.9	316	7.52	**1.016**	20.2	0.481	12.0	2.81	2.89	67.3	3.24	0.836	0.128	0.029	0.208	0.203	0.399	4.46	0.297	**8.03**	0.066	0.340	0.247	133	1.097	0.235	1.788	0.00	58.7	15.65	77.4
NRG34	174	2.21	3.90	4.01	3.71	8.31	196	109.2	305	8.23	**0.841**	18.1	0.492	11.2	2.56	2.80	59.1	3.04	0.805	0.140	0.027	0.261	0.227	0.731	4.42	0.285	**6.98**	0.047	0.285	0.289	121	0.036	0.218	0.984	0.00	89.4	6.27	96.9
P-values	0.766	0.542	0.466	0.856	0.432	0.321	0.808	0.499	0.49	0.101	**0.035**	0.497	0.858	0.615	0.649	0.764	0.405	0.500	0.622	0.255	0.724	0.146	0.322	0.278	0.926	0.677	**0.040**	0.132	0.260	0.135	0.364	0.177	0.924	0.421	ns	0.100	0.402	0.348
A-TF0 × B399	165	1.98	3.51	4.20	2.81	8.02	185	137.5	323	6.52	0.954	17.7	0.385	10.2	2.66	2.52	60.2	3.20	0.739	0.111	0.034	0.202	0.205	0.385	4.43	0.283	8.22	0.058	0.359	0.250	120	0.684	0.201	1.948	0.00	75.1	5.86	71.6
A-TF0 ×NRG34	165	2.30	3.60	3.85	3.65	8.33	187	127.1	314	7.65	0.873	17.3	0.402	9.8	2.83	2.59	55.0	3.47	0.822	0.130	0.030	0.304	0.246	1.035	5.16	0.269	8.05	0.052	0.288	0.318	117	1.367	0.175	2.660	0.00	62.2	5.19	96.0
A-TF7 × B399	185	2.12	3.77	4.03	3.54	9.59	208	100.2	308	8.51	1.079	22.7	0.576	13.7	2.95	3.26	74.4	3.28	0.933	0.145	0.025	0.215	0.202	0.413	4.50	0.312	7.84	0.075	0.321	0.245	146	0.827	0.296	0.915	0.00	55.1	26.11	83.3
A-TF7 ×NRG34	182	2.12	4.20	4.17	3.78	8.30	205	91.3	296	8.82	0.808	18.9	0.582	12.7	2.29	3.00	63.1	2.61	0.788	0.150	0.025	0.218	0.209	0.428	3.69	0.301	5.91	0.043	0.281	0.259	125	0.072	0.210	0.732	0.00	90.8	6.00	97.8
P-values	0.754	0.552	0.635	0.670	0.661	0.114	0.598	0.959	0.9138	0.337	0.236	0.589	0.924	0.817	0.445	0.594	0.756	0.116	0.076	0.481	0.721	0.165	0.496	0.299	0.083	0.962	0.081	0.292	0.755	0.318	0.497	0.688	0.697	0.534	ns	0.787	0.338	0.810
Crown																																							
A-TF0	**172**	**3.39**	4.11	3.04	3.17	**8.52**	**194**	107	301	10.8	0.808	21.6	**0.56**	13.0	4.77	6.23	39.5	1.70	0.654	0.116	**0.101**	0.22	0.16	0.75	4.61	0.30	3.22	0.12	0.18	0.19	110							
A-TF7	**195**	**5.25**	4.83	3.06	5.85	**9.79**	**223**	84	307	10.8	0.923	24.1	**0.73**	14.9	3.63	7.02	50.2	1.57	0.651	0.127	**0.085**	0.18	0.14	0.30	4.15	0.28	3.46	0.14	0.15	0.19	124							
P-values	**0.005**	**0.013**	0.155	0.977	0.008	**0.028**	**0.000**	0.105	0.7549	0.987	0.389	0.398	**0.031**	0.397	0.254	0.268	0.349	0.409	0.951	0.428	**0.044**	0.516	0.486	0.345	0.270	0.614	0.532	0.171	0.534	0.813	0.389							
B399	188	4.05	4.21	3.18	3.91	**9.83**	213	102	315	10.6	0.909	23.5	0.65	13.3	3.99	6.53	45.6	1.56	0.661	0.120	0.092	0.16	0.14	0.29	4.26	0.30	3.50	0.14	0.20	0.19	117							
NRG34	178	4.59	4.73	2.91	5.11	**8.48**	204	89	293	11.1	0.823	22.2	0.64	14.5	4.41	6.72	44.0	1.71	0.645	0.124	0.093	0.24	0.16	0.76	4.50	0.27	3.18	0.13	0.14	0.19	117				nd			
P-values	0.207	0.432	0.311	0.688	0.210	**0.020**	0.225	0.360	0.2033	0.509	0.520	0.662	0.955	0.577	0.669	0.791	0.889	0.377	0.771	0.784	0.861	0.226	0.450	0.325	0.577	0.343	0.409	0.364	0.161	0.741	0.996							
A-TF0 × B399	175	3.79	4.12	3.38	**3.59 b**	9.12	199	110	309	10.1	0.786	21.1	0.55	11.1	3.81	5.76	35.1	1.57	0.628	0.115	0.098	0.18	0.17	0.31	4.19	0.32	3.60	0.12	0.23	0.19	100							
A-TF0 ×NRG34	169	2.99	4.10	2.69	**2.76 b**	7.93	189	105	294	11.5	0.830	22.1	0.57	14.9	5.74	6.70	43.8	1.84	0.681	0.117	0.103	0.27	0.14	1.18	5.04	0.27	2.84	0.12	0.13	0.19	119							
A-TF7 × B399	201	4.31	4.31	2.98	**4.24 b**	10.54	227	95	322	11.0	1.031	25.9	0.75	15.5	4.17	7.30	56.1	1.56	0.693	0.124	0.086	0.15	0.10	0.27	4.34	0.28	3.39	0.15	0.16	0.19	133							
A-TF7 ×NRG34	188	6.19	5.36	3.13	**7.46 a**	9.04	219	72	292	10.7	0.816	22.3	0.71	14.2	3.09	6.74	44.3	1.58	0.609	0.131	0.083	0.22	0.18	0.33	3.95	0.28	3.52	0.13	0.14	0.19	114							
P-values	0.665	0.061	0.291	0.536	**0.039**	0.771	0.902	0.532	0.6377	0.309	0.333	0.443	0.697	0.260	0.139	0.297	0.369	0.445	0.210	0.872	0.577	0.904	0.033	0.384	0.144	0.415	0.243	0.297	0.291	0.890	0.250							

Supplemental Table 4.3 Metabolites concentrations of two alfalfa populations (A-TF0 vs A-TF7) in symbiosis with two *S. meliloti* strains (B399 and NRG34), and their interactions in 48 hours after a freezing stress (AFS) root exudates, nodules, roots and crowns. Numbers in bold indicate statistically significant effects ($P \leq 0.05$). Different letters represent significant populations × strain interactions as determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$. Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Coum, coumestrol; Formo, formononetin, FlaTot, total flavonoids; Gln, glutamine; Glu, glutamic acid; Gluc, glucose; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lut, luteolin, Lys, leucine; Lys, lysine; Medic, medicarpin; Met, methionine; Nar, naringenin; NSC, non structural carbohydrates; Phe, phenylalanine; Pin, pinitol; Pro, proline; Raff, raffinose; Ser, serine; Suc, Sucrose; Stach, Stachyose, Thr, threonine; Tyr, tyrosine; Val, valine; Orn, ornithine; AATot, total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid

	Metabolites																																									
	Sugars									Amino Acids																								Flavonoids							
Exudates	Suc	Gluc	Stach	Raff	Fruc	Pin	SS Tot	Starch	NSC	Glu	Gln	Pro	Orn	Arg	His	Asp	Asn	Ala	Thr	Lys	Met	Ile	Leu	Val	Ser	Gly	GABA	AABA	Tyr	Phe	AA Tot	Nar	Lut	Echi	Coum	Formo	Medic	FlaTot
A-TF0	0.196	0.345	0.037	0.009	0.367	0.112	1.066			0.150	0.002	0.118	0.004	0.009	0.004	0.158	0.004	0.069	0.021	0.007	0.005	0.012	0.016	0.020	0.042	0.017	0.094	0.000	0.004	0.007	0.764	0.015	0.025	0.086	0.080	1.58	0.190	1.97
A-TF7	0.249	0.419	0.038	0.008	0.434	0.122	1.270			0.180	0.001	0.139	0.005	0.010	0.005	0.147	0.005	0.060	0.025	0.008	0.005	0.013	0.018	0.021	0.043	0.017	0.094	0.000	0.004	0.007	0.809	0.012	0.028	0.054	0.042	1.87	0.165	2.17
P-values	0.396	0.278	0.819	0.494	0.336	0.556	0.193			0.143	0.540	0.258	0.309	0.326	0.265	0.595	0.635	0.326	0.059	0.222	0.419	0.376	0.392	0.522	0.816	0.739	0.982	0.346	0.965	0.661	0.584	0.682	0.457	0.094	0.387	0.306	0.465	0.752
B399	0.243	0.398	0.037	0.008	0.424	0.122	1.231			0.168	0.001	0.133	0.005	0.009	0.005	0.155	0.003	0.061	0.023	**0.009**	0.005	0.013	0.017	0.021	0.043	0.017	0.092	0.000	0.004	0.006	0.791	0.008	0.028	0.066	0.080	1.68	0.158	2.02
NRG34	0.202	0.366	0.039	0.009	0.378	0.111	1.105			0.162	0.002	0.124	0.005	0.009	0.005	0.151	0.006	0.068	0.024	**0.007**	0.005	0.013	0.017	0.021	0.042	0.017	0.096	0.000	0.003	0.007	0.783	0.019	0.025	0.073	0.042	1.77	0.197	2.12
P-values	0.510	0.634	0.655	0.763	0.505	0.539	0.415	nd	nd	0.768	0.196	0.625	0.953	0.787	0.586	0.839	0.214	0.429	0.698	**0.050**	0.433	0.889	0.733	0.998	0.838	0.926	0.806	0.768	0.803	0.352	0.923	0.071	0.598	0.717	0.377	0.751	0.257	0.560
A-TF0 × B399	0.206	**0.289 b**	0.033	0.009	0.325	**0.097 b**	**0.959 b**			**0.129 b**	0.001	**0.094 b**	**0.004 b**	**0.007 b**	0.004	0.144	0.003	**0.054 b**	0.019	**0.007 b**	0.005	**0.010 b**	**0.014 b**	**0.017 b**	**0.037 b**	**0.015 b**	**0.075 a**	0.000	**0.003 a**	**0.005 b**	**0.647 b**	0.006	0.025	0.069	0.126	**1.19 b**	0.137	**1.55 b**
A-TF0 ×NRG34	0.186	**0.402 ab**	0.041	0.009	0.410	**0.126 ab**	**1.174 ab**			**0.172 ab**	0.003	**0.143 ab**	**0.006 ab**	**0.010 ab**	0.005	0.173	0.004	**0.084 a**	0.024	**0.007 b**	0.005	**0.014 ab**	**0.018 ab**	**0.023 ab**	**0.048 a**	**0.019 a**	**0.011 b**	0.000	**0.004 a**	**0.008 a**	**0.881 ab**	0.023	0.025	0.103	0.033	**1.97 ab**	0.243	**2.39 ab**
A-TF7 × B399	0.280	**0.507 a**	0.040	0.008	0.522	**0.147 a**	**1.504 a**			**0.207 a**	0.001	**0.172 a**	**0.006 ab**	**0.012 a**	0.006	0.166	0.003	**0.068 ab**	0.027	**0.002 a**	0.005	**0.015 a**	**0.020 a**	**0.024 a**	**0.05 a**	**0.020 a**	**0.011 b**	0.000	**0.004 a**	**0.007 a**	**0.934 a**	0.009	0.031	0.064	0.034	**2.17 a**	0.179	**2.48 ab**
A-TF7 ×NRG34	0.218	**0.330 ab**	0.037	0.008	0.346	**0.097 b**	**1.037 b**			**0.153 ab**	0.001	**0.105 b**	**0.005 a**	**0.008 b**	0.004	0.128	0.007	**0.052 b**	0.024	**0.006 b**	0.005	**0.012 ab**	**0.015 ab**	**0.018 ab**	**0.037 b**	**0.01 b**	**0.079 a**	0.000	**0.003 b**	**0.006 b**	**0.684 b**	0.015	0.026	0.043	0.050	**1.57 b**	0.151	**1.85 b**
P-values	0.740	**0.040**	0.364	0.855	0.067	**0.027**	**0.034**			**0.024**	0.265	**0.003**	**0.050**	**0.014**	0.075	0.112	0.468	**0.022**	0.077	**0.041**	0.792	**0.037**	**0.024**	**0.018**	**0.028**	**0.048**	**0.018**	0.092	**0.023**	**0.039**	**0.007**	0.337	0.557	0.148	0.214	**0.020**	0.056	**0.035**
Nodules																																									
A-TF0	66.2	3.41	2.45	2.90	2.70	12.3	90.0	1.45	91.5	12.8	3.92	20.3	0.99	21.0	8.57	4.78	257	15.0	5.36	3.03	0.222	6.19	8.75	9.58	9.27	6.30	22.67	0.112	1.66	1.68	419	1.80	2.37	13.9	24.5	20.8	5.64	69.1
A-TF7	69.1	3.54	2.62	3.47	2.44	13.5	94.7	1.50	96.2	13.1	3.20	21.9	1.06	23.9	9.66	4.41	240	16.6	5.30	3.09	0.256	5.94	8.73	9.19	9.16	6.39	24.35	0.075	1.77	1.78	410	1.85	2.11	10.7	18.9	24.5	5.02	63.2
P-values	0.679	0.700	0.768	0.143	0.437	0.315	0.568	0.748	0.568	0.827	0.238	0.593	0.798	0.492	0.124	0.531	0.590	0.304	0.890	0.805	0.529	0.717	0.982	0.715	0.905	0.962	0.514	0.039	0.681	0.603	0.782	0.869	0.268	0.162	0.250	0.414	0.591	0.557
B399	66.5	3.57	2.07	**2.73**	2.77	14.1	91.7	**1.29**	93.0	12.8	3.84	22.0	1.21	21.8	9.43	5.01	**283**	16.8	5.57	3.14	0.210	6.18	8.99	9.75	9.09	7.96	24.72	0.106	1.66	1.57	**455**	1.78	**1.92**	10.9	25.2	22.2	5.29	67.6
NRG34	68.8	3.38	3.00	**3.63**	2.37	11.8	93.0	**1.66**	94.6	13.1	3.28	20.2	0.84	23.1	8.80	4.17	**214**	14.7	5.09	2.98	0.269	5.96	8.49	9.03	9.33	4.73	22.31	0.081	1.77	1.90	**374**	1.87	**2.56**	13.7	18.1	23.0	5.37	64.8
P-values	0.737	0.573	0.131	**0.025**	0.259	0.058	0.878	**0.017**	0.844	0.796	0.350	0.563	0.151	0.770	0.365	0.161	**0.041**	0.179	0.309	0.479	0.278	0.755	0.614	0.493	0.793	0.114	0.353	0.137	0.683	0.090	**0.029**	0.762	**0.012**	0.216	0.143	0.851	0.941	0.782
A-TF0 × B399	66.6	3.65	2.19	2.63	3.01	13.8	91.9	1.30	93.2	13.1	3.89	21.5	1.02	19.7	9.45	5.50	280	16.5	5.97	3.08	0.203	6.68	9.46	10.37	9.91	7.43	25.58	**0.144 a**	1.69	1.57	453	1.92	2.20	14.0	32.5	22.1	6.30	79.4
A-TF0 ×NRG34	65.9	3.17	2.71	3.17	2.39	10.9	88.2	1.61	89.8	12.5	3.94	19.0	0.97	22.3	7.69	4.06	234	13.4	4.75	2.99	0.242	5.70	8.04	8.78	8.63	5.17	19.75	**0.079 b**	1.62	1.80	385	1.68	2.55	13.8	16.5	19.4	4.97	58.9
A-TF7 × B399	66.4	3.49	1.94	2.84	2.52	14.4	91.6	1.29	92.9	12.4	3.79	22.5	1.41	23.9	9.41	4.53	286	17.1	5.16	3.20	0.217	5.67	8.52	9.12	8.28	8.50	23.85	**0.068 b**	1.62	1.56	457	1.63	1.64	7.8	18.0	22.3	4.27	55.8
A-TF7 ×NRG34	71.7	3.59	3.30	4.10	2.35	12.7	97.7	1.70	99.5	13.8	2.61	21.3	0.71	23.9	9.91	4.29	194	16.0	5.43	2.97	0.296	6.21	8.94	9.27	10.04	4.29	24.86	**0.082 b**	1.91	2.00	362	2.07	2.57	13.7	19.8	26.7	5.77	70.7
P-values	0.658	0.387	0.486	0.349	0.507	0.612	0.545	0.736	0.544	0.483	0.311	0.833	0.201	0.757	0.112	0.314	0.478	0.506	0.119	0.739	0.710	0.281	0.354	0.411	0.110	0.623	0.191	**0.025**	0.523	0.583	0.703	0.285	0.231	0.183	0.070	0.432	0.224	0.089
Roots																																								
A-TF0	141	1.69	2.03	1.19	2.86	8.10	157	**142**	299	9.50	0.778	21.0	**0.318**	9.8	**2.42**	**1.66**	59.1	**7.74**	1.71	0.426	0.149	1.17	1.47	1.47	6.26	0.636	7.49	0.0518	**0.605**	0.659	134	0.031	0.725	0.750	0.00	61.2	7.30	70.0
A-TF7	146	1.75	2.06	1.06	2.93	9.18	163	**96**	260	9.69	0.777	22.0	**0.420**	13.1	**3.78**	**2.91**	65.2	**5.28**	1.87	0.427	0.166	1.18	1.45	1.38	5.61	0.547	6.33	0.0562	**0.491**	0.683	143	0.033	0.536	0.770	0.00	58.8	9.35	69.4
P-values	0.273	0.686	0.918	0.348	0.868	0.076	0.209	**0.004**	0.009	0.720	0.994	0.563	**0.038**	0.108	**0.009**	**0.003**	0.648	**0.005**	0.192	0.970	0.339	0.905	0.909	0.580	0.066	0.060	0.064	0.444	**0.028**	0.691	0.573	0.953	0.469	0.885	ns	0.859	0.752	0.965
B399	147	**1.92**	1.92	1.11	3.06	8.88	164	117	281	9.29	0.821	21.8	0.346	12.2	2.90	2.30	63.8	**7.41**	1.80	0.434	0.167	1.19	1.51	1.42	**6.46**	0.631	7.49	0.0571	0.575	0.658	143	0.033	0.553	0.813	0.00	61.3	13.40	76.1
NRG34	140	**1.52**	2.17	1.13	2.74	8.40	156	122	278	9.90	0.735	21.2	0.392	10.8	3.29	2.27	60.6	**5.61**	1.78	0.419	0.148	1.16	1.41	1.43	**5.41**	0.552	6.33	0.0509	0.521	0.683	135	0.031	0.708	0.710	0.00	58.6	3.25	63.4
P-values	0.109	**0.015**	0.363	0.897	0.456	0.410	0.090	0.692	0.83	0.273	0.164	0.722	0.335	0.493	0.416	0.945	0.813	**0.035**	0.842	0.688	0.283	0.770	0.483	0.899	**0.005**	0.091	0.063	0.282	0.282	0.673	0.594	0.953	0.552	0.506	ns	0.847	0.126	0.345
A-TF0 × B399	142	2.00	1.86	1.17	3.39	8.41	159	137	297	9.07	0.841	20.4	0.283	10.0	2.11	1.94	65.3	8.18	1.80	0.428	0.168	1.20	1.53	1.46	6.71	0.652	7.51	0.0578	0.605	0.672	141	0.000	0.766	0.851	0.00	57.9	11.14	70.7
A-TF0 ×NRG34	140	1.39	2.20	1.20	2.34	7.79	155	147	302	9.93	0.715	21.6	0.353	9.6	2.73	1.38	52.9	7.31	1.63	0.425	0.130	1.14	1.41	1.48	5.80	0.619	7.46	0.0458	0.605	0.646	128	0.061	0.684	0.650	0.00	64.5	3.46	69.4
A-TF7 × B399	153	1.85	1.97	1.05	2.73	9.36	169	96	265	9.52	0.801	23.3	0.409	14.4	3.70	2.66	62.2	6.64	1.81	0.441	0.167	1.18	1.49	1.37	6.21	0.610	7.47	0.0563	0.544	0.644	146	0.066	0.340	0.775	0.00	64.7	15.66	81.6
A-TF7 ×NRG34	140	1.66	2.14	1.06	3.13	9.00	157	97	254	9.87	0.754	20.8	0.432	11.9	3.86	3.16	68.3	3.92	1.93	0.414	0.166	1.18	1.42	1.39	5.02	0.484	5.19	0.0560	0.437	0.721	141	0.000	0.732	0.770	0.00	52.8	3.05	57.3
P-values	0.304	0.179	0.758	0.967	0.105	0.820	0.434	0.760	0.555	0.642	0.512	0.316	0.618	0.602	0.625	0.181	0.492	0.265	0.249	0.746	0.295	0.778	0.854	1.000	0.678	0.317	0.074	0.304	0.280	0.393	0.786	0.090	0.366	0.530	ns	0.507	0.705	0.395
Crown																																								
A-TF0	121	2.44	2.19	0.81	2.48	9.59	138	113	251	13.8	1.006	27.3	0.505	14.0	4.20	**2.63**	44.2	**8.05**	1.56	0.360	0.056	0.872	1.08	1.25	**6.18**	**0.703**	4.22	0.155	0.446	0.584	133								
A-TF7	129	2.59	2.13	0.77	3.04	10.10	148	89	237	12.7	0.739	22.9	0.621	13.7	5.17	**4.33**	36.9	**4.54**	1.47	0.378	0.063	0.877	1.09	1.05	**5.18**	**0.517**	2.92	0.158	0.405	0.618	116								
P-values	0.203	0.711	0.775	0.713	0.197	0.565	0.171	0.089	0.33	0.104	0.013	0.093	0.056	0.897	0.217	**0.003**	0.558	**0.015**	0.374	0.621	0.399	0.953	0.982	0.148	**0.008**	**0.028**	0.061	0.838	0.418	0.452	0.291								
B399	**133**	2.76	2.15	0.77	3.08	9.99	**152**	103	256	13.1	0.868	24.7	0.526	13.1	3.93	3.35	36.6	7.17	1.50	0.364	0.054	0.864	1.08	1.10	6.00	0.670	4.04	0.156	0.459	0.602	120								
NRG34	**117**	2.27	2.17	0.81	2.44	9.71	**134**	99	233	13.4	0.877	25.5	0.600	14.6	5.44	3.61	44.4	5.41	1.53	0.374	0.066	0.886	1.09	1.21	5.35	0.549	3.09	0.158	0.392	0.600	129				nd				
P-values	**0.014**	0.223	0.939	0.679	0.143	0.752	**0.012**	0.716	0.12	0.743	0.927	0.781	0.215	0.551	0.062	0.625	0.533	0.204	0.806	0.760	0.110	0.788	0.938	0.430	0.074	0.145	0.167	0.919	0.198	0.963	0.583								
A-TF0 × B399	125	3.02	2.22	0.79	3.08	9.82	144	118	309	13.1	1.023	24.9	0.464	13.0	3.49	2.89	39.7	8.39	1.52	0.347	0.054	0.846	1.07	1.13	6.29	0.735	4.51	0.163	0.456	0.602	125								
A-TF0 ×NRG34	116	1.85	2.16	0.82	1.88	9.37	132	108	294	14.5	0.989	29.7	0.547	15.0	4.90	2.37	48.7	7.70	1.59	0.373	0.059	0.899	1.10	1.38	6.06	0.670	3.93	0.147	0.436	0.566	142								
A-TF7 × B399	141	2.50	2.08	0.75	3.08	10.16	160	89	322	13.1	0.713	24.6	0.588	13.2	4.37	3.81	33.6	5.95	1.49	0.380	0.053	0.882	1.09	1.07	5.72	0.605	3.58	0.149	0.461	0.602	116								
A-TF7 ×NRG34	117	2.68	2.17	0.80	3.01	10.05	136	89	292	12.2	0.765	21.2	0.653	14.1	5.98	4.85	40.1	3.12	1.46	0.375	0.072	0.872	1.08	1.03	4.64	0.428	2.26	0.168	0.349	0.634	116								
P-values	0.246	0.101	0.727	0.936	0.192	0.846	0.373	0.704	0.638	0.103	0.664	0.123	0.878	0.810	0.897	0.149	0.920	0.434	0.563	0.661	0.354	0.696	0.842	0.295	0.238	0.492	0.580	0.223	0.367	0.445	0.602								

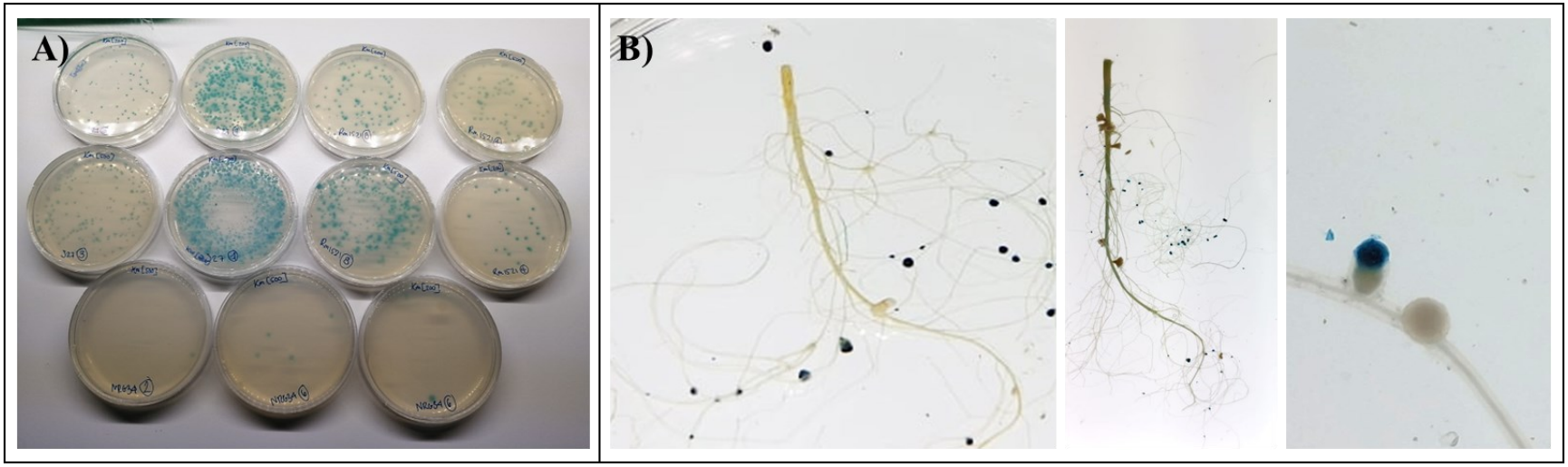
Supplemental Table 4.4 Metabolites concentrations of two alfalfa populations (A-TF0 vs A-TF7) in symbiosis with two *S. meliloti* strains (B399 and NRG34), and their interactions in three weeks regrowth after freezing stress (RAF) root exudates, nodules, roots and crowns. Numbers in bold indicate statistically significant effects ($P \leq 0.05$ Different letters represent significant populations × strain interactions as determined by the Fisher's least significant difference (LSD) test at $P \leq 0.05$. Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Coum, coumestrol; Formo, formononetin, FlaTot, total flavonoids; Gln, glutamine; Glu, glutamic acid; Gluc, glucose; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lut, luteolin, Lys, leucine; Lys, lysine; Medic, medicarpin; Met, methionine; Nar, naringenin; NSC, non structural carbohydrates; Phe, phenylalanine; Pin, pinitol; Pro, proline; Raff, raffinose; Ser, serine; Suc, Sucrose; Stach, Stachyose, Thr, threonine; Tyr, tyrosine; Val, valine; Orn, ornithine; AATot, total amino acid; SSTot, total soluble sugars; AABA, α-aminobutyric acid; GABA, γ-aminobutyric acid

	Metabolites																																						
	Sugars									Amino Acids																						Flavonoids							
Exudates	Suc	Gluc	Stach	Raff	Fruc	Pin	SS Tot	Starch	NSC	Glu	Gln	Pro	Orn	Arg	His	Asp	Asn	Ala	Thr	Lys	Met	Ile	Leu	Val	Ser	Gly	GABA	AABA	Tyr	Phe	AA Tot	Nar	Lut	Echi	Coum	Formo	Medic	FlaTot
A-TF0	0.085	0.674	0.037	0.012	0.768	0.172	1.72			0.192	0.045	0.661	0.020	0.010	0.010	0.201	0.651	0.077	0.039	0.012	0.006	0.014	0.016	0.023	0.094	0.031	0.138	0.001	0.006	0.010	2.257	**0.027**	0.136	0.458	0.145	1.24	0.158	2.16
A-TF7	0.172	0.707	0.038	0.005	0.740	0.232	1.87			0.194	0.043	0.620	0.018	0.010	0.010	0.242	0.605	0.066	0.033	0.009	0.005	0.011	0.013	0.019	0.070	0.026	0.137	0.001	0.005	0.009	2.147	**0.012**	0.052	0.268	0.088	1.24	0.111	1.77
P-values	0.444	0.856	0.819	0.144	0.897	0.298	0.76			0.960	0.802	0.817	0.608	0.781	0.711	0.235	0.746	0.371	0.480	0.347	0.069	0.307	0.376	0.381	0.169	0.338	0.975	0.470	0.211	0.497	0.802	**0.045**	0.373	0.127	0.289	0.999	0.224	0.520
B399	0.223	0.685	0.037	0.009	0.728	0.210	1.87			0.193	0.041	0.642	0.020	0.010	0.010	0.215	0.519	0.067	0.035	0.010	0.006	0.013	0.015	0.021	0.077	0.028	0.123	0.001	0.006	0.010	2.062	0.020	0.149	0.427	0.137	1.04	0.131	1.90
NRG34	0.034	0.696	0.039	0.008	0.780	0.193	1.72			0.193	0.047	0.639	0.017	0.010	0.010	0.228	0.738	0.075	0.037	0.011	0.005	0.013	0.014	0.021	0.087	0.029	0.152	0.001	0.005	0.010	2.342	0.019	0.039	0.299	0.096	1.43	0.137	2.03
P-values	0.110	0.956	0.655	0.871	0.810	0.768	0.76	nd	nd	0.991	0.369	0.987	0.506	0.917	0.813	0.689	0.137	0.498	0.835	0.823	0.787	0.982	0.770	0.990	0.523	0.948	0.082	0.927	0.761	0.917	0.523	0.806	0.245	0.302	0.446	0.412	0.882	0.843
A-TF0 × B399	0.129	0.703	0.033	0.012	0.766	0.151	1.77			0.195	0.044	0.697	0.021	0.010	0.010	0.199	0.516	0.074	0.037	0.012	0.006	0.014	0.016	0.022	0.085	0.030	0.130	0.001	0.006	0.010	2.134	0.027	0.217	0.583	0.185	0.84	0.149	2.00
A-TF0 ×NRG34	0.042	0.645	0.041	0.012	0.770	0.192	1.67			0.190	0.046	0.625	0.018	0.009	0.011	0.203	0.787	0.080	0.041	0.012	0.006	0.015	0.016	0.024	0.103	0.031	0.146	0.001	0.006	0.011	2.380	0.027	0.055	0.334	0.105	1.63	0.168	2.32
A-TF7 × B399	0.317	0.668	0.040	0.006	0.690	0.270	1.97			0.191	0.039	0.588	0.019	0.010	0.010	0.230	0.522	0.061	0.034	0.009	0.005	0.012	0.014	0.019	0.069	0.026	0.117	0.001	0.005	0.009	1.989	0.014	0.081	0.270	0.089	1.24	0.114	1.81
A-TF7 ×NRG34	0.027	0.746	0.037	0.004	0.790	0.195	1.78			0.197	0.048	0.653	0.016	0.010	0.010	0.254	0.689	0.071	0.033	0.010	0.005	0.011	0.012	0.018	0.072	0.026	0.157	0.001	0.004	0.009	2.305	0.010	0.023	0.265	0.087	1.24	0.107	1.73
P-values	0.374	0.711	0.364	0.913	0.825	0.316	0.92			0.833	0.547	0.698	0.936	0.979	0.828	0.774	0.718	0.863	0.793	0.909	0.557	0.792	0.836	0.798	0.645	0.896	0.442	0.925	0.623	0.702	0.935	0.818	0.579	0.322	0.469	0.412	0.732	0.747
Nodules																																							
A-TF0	47.6	3.27	0.000	0.000	2.32	12.3	65.4	1.35	66.7	13.5	3.94	23.9	**0.306**	**1.85**	2.84	4.09	274	10.46	5.36	0.799	0.137	0.972	0.771	2.11	4.03	2.07	16.5	0.090	0.308	0.665	365	1.77	2.71	11.23	28.7	39.4	9.10	93
A-TF7	49.7	2.93	0.000	0.000	2.17	13.9	68.7	1.57	70.3	16.4	3.53	21.1	**0.422**	**3.23**	3.41	4.48	283	9.28	5.30	0.974	0.171	1.164	0.932	2.31	3.95	2.11	15.7	0.102	0.292	0.690	375	1.82	2.59	7.44	28.3	33.5	8.01	82
P-values	0.769	0.147	ns	ns	0.566	0.145	0.680	0.058	0.664	0.175	0.523	0.488	**0.014**	**0.013**	**0.037**	0.506	0.702	0.285	0.890	0.186	0.212	0.191	0.115	0.444	0.810	0.822	0.612	0.252	0.734	0.733	0.746	0.905	0.807	0.077	0.961	0.474	0.562	0.449
B399	54.8	3.28	0.000	0.000	2.42	**14.5**	75.0	1.50	76.5	16.1	3.83	**27.5**	0.390	**2.06**	3.12	**4.85**	281	11.00	5.57	0.839	0.128	1.021	0.829	2.12	4.19	**2.37**	18.4	0.105	0.276	**0.603**	382	1.68	**2.02**	9.78	28.7	30.2	9.48	83
NRG34	42.4	2.92	0.000	0.000	2.07	**11.7**	59.1	1.42	60.5	13.8	3.63	**17.5**	0.337	**3.02**	3.14	**3.71**	276	8.74	5.09	0.934	0.180	1.114	0.873	2.30	3.79	**1.81**	13.7	0.087	0.324	**0.751**	358	1.91	**3.28**	8.89	28.2	42.7	7.63	93
P-values	0.100	0.130	ns	ns	0.163	**0.016**	0.059	0.480	0.060	0.283	0.753	**0.023**	0.245	**0.001**	0.956	**0.054**	0.857	0.046	0.309	0.468	0.059	0.522	0.662	0.486	0.202	**0.017**	0.006	0.081	0.318	**0.051**	0.449	0.594	**0.019**	0.668	0.943	0.143	0.329	0.482
A-TF0 × B399	53.4	3.62	0.000	0.000	**2.80 a**	14.0	73.8	1.39	75.2	15.8	4.23	30.3	0.352	1.59	2.96	4.96	285	**12.76 a**	5.97	0.854	0.118	0.954	0.789	2.15	4.45	2.43	**20.4 a**	0.101	0.280	0.590	394	1.58	2.25	12.72	28.5	32.7	11.01	89
A-TF0 ×NRG34	41.7	2.92	0.000	0.000	**1.83 b**	10.5	57.0	1.30	58.3	11.2	3.64	17.5	0.259	2.53	2.72	3.22	262	**8.17 b**	4.75	0.743	0.156	0.989	0.752	2.07	3.60	1.70	**12.5 b**	0.079	0.336	0.740	337	1.95	3.17	9.75	28.8	46.1	7.19	97
A-TF7 × B399	56.2	2.93	0.000	0.000	**2.04 b**	15.0	76.2	1.60	77.8	16.4	3.44	24.7	0.428	2.12	3.28	4.75	276	**9.25 b**	5.16	0.824	0.138	1.089	0.870	2.09	3.93	2.30	**16.4 ab**	0.109	0.271	0.617	371	1.77	1.79	6.85	28.9	27.7	7.95	76
A-TF7 ×NRG34	43.2	2.93	0.000	0.000	**2.31 ab**	12.8	61.2	1.53	62.8	16.4	3.62	17.4	0.416	3.93	3.55	4.20	291	**9.32 b**	5.43	1.124	0.204	1.239	0.994	2.53	3.97	1.93	**15.0 b**	0.094	0.312	0.763	380	1.88	3.38	8.03	27.7	39.2	8.06	89
P-values	0.933	0.134	ns	ns	**0.020**	0.595	0.908	0.915	0.908	0.274	0.549	0.504	0.371	0.231	0.342	0.302	0.468	**0.040**	0.119	0.123	0.601	0.689	0.419	0.324	0.169	0.402	**0.047**	0.742	0.879	0.980	0.314	0.770	0.515	0.324	0.918	0.909	0.302	0.867
Roots																																							
A-TF0	76.4	**1.95**	0.265	0.165	2.03	7.06	87.8	**182**	269	**4.83**	**1.39**	21.7	**0.150**	**2.18**	0.84	**1.21**	68.2	3.14	0.933	0.082	0.032	0.129	0.126	0.336	2.06	0.390	**4.47**	0.059	0.221	0.194	113	1.11	0.421	0.87	0.386	21.2	4.68	28.7
A-TF7	70.2	**1.49**	0.274	0.162	1.68	7.54	81.3	**237**	318	**6.01**	**1.77**	23.5	**0.207**	**5.19**	1.13	**1.77**	78.9	2.78	0.837	0.087	0.033	0.118	0.112	0.312	1.80	0.356	**3.37**	0.048	0.175	0.192	129	0.04	0.464	1.26	0.542	28.7	1.41	32.4
P-values	0.120	**0.042**	0.838	0.830	0.286	0.299	0.123	**0.009**	0.010	**<0.001**	**0.031**	0.545	**0.017**	**<0.001**	0.162	**0.003**	0.350	0.201	0.293	0.457	0.948	0.395	0.325	0.456	0.138	0.271	**0.045**	0.091	0.234	0.856	0.271	0.329	0.781	0.672	0.135	0.072	0.283	0.384
B399	75.6	1.86	0.297	0.175	1.92	7.69	87.6	209	296	5.43	1.57	23.2	0.190	3.41	0.99	1.52	68.3	3.12	0.941	0.082	0.031	0.125	0.125	0.331	1.87	0.390	3.93	0.054	0.191	0.189	116	1.12	0.404	1.12	0.314	**19.9**	1.02	**23.9**
NRG34	70.9	1.59	0.242	0.152	1.80	6.90	81.6	210	291	5.40	1.58	22.1	0.167	3.96	0.98	1.46	78.8	2.80	0.830	0.087	0.034	0.122	0.113	0.317	1.99	0.356	3.91	0.052	0.205	0.198	125	0.03	0.481	1.01	0.614	**30.0**	5.07	**37.2**
P-values	0.227	0.219	0.185	0.192	0.696	0.091	0.154	0.962	0.772	0.410	0.953	0.707	0.309	0.459	0.935	0.740	0.361	0.260	0.226	0.513	0.629	0.822	0.364	0.676	0.493	0.266	0.964	0.723	0.720	0.424	0.513	0.317	0.614	0.668	0.417	**0.018**	0.186	**0.004**
A-TF0 × B399	82.6	2.19	0.314	**0.193**	2.21	7.50	95.0	172	267	4.94	1.38	22.6	0.160	1.46	0.77	0.92	57.1	3.47	1.020	0.083	0.029	0.134	0.139	0.349	2.06	0.422	4.62	0.066	0.223	0.192	102	2.21	0.357	0.89	0.290	16.8	1.07	21.6
A-TF0 ×NRG34	70.2	1.72	0.217	**0.137**	1.86	6.62	80.7	191	272	4.72	1.40	20.8	0.141	2.90	0.91	1.39	79.2	2.81	0.847	0.081	0.035	0.124	0.113	0.322	2.07	0.358	4.33	0.052	0.218	0.197	123	0.00	0.485	0.85	0.482	25.7	8.28	35.8
A-TF7 × B399	68.7	1.52	0.279	**0.156**	1.63	7.88	80.2	245	326	5.93	1.77	23.7	0.220	5.37	1.22	1.08	79.5	2.77	0.861	0.081	0.033	0.116	0.112	0.312	1.68	0.359	3.24	0.043	0.159	0.185	130	0.03	0.450	1.35	0.338	23.1	0.96	26.2
A-TF7 ×NRG34	71.6	1.46	0.268	**0.168**	1.74	7.19	82.4	229	311	6.09	1.77	23.3	0.193	5.01	1.05	1.50	78.4	2.78	0.812	0.093	0.032	0.120	0.113	0.312	1.92	0.353	3.49	0.053	0.191	0.199	128	0.06	0.477	1.17	0.745	34.3	1.86	38.6
P-values	0.061	0.339	0.296	**0.050**	0.480	0.838	0.057	0.373	0.600	0.410	0.929	0.806	0.864	0.225	0.443	0.863	0.315	0.236	0.495	0.281	0.424	0.560	0.323	0.665	0.500	0.342	0.610	0.064	0.626	0.678	0.449	0.303	0.742	0.780	0.768	0.779	0.300	0.826
Crown																																							
A-TF0	64.6	3.86	0.472	**0.132**	4.30	7.20	80.6	**130**	**210**	**7.16**	2.51	25.2	0.383	3.79	1.65	**3.56**	83.2	**2.96**	0.992	0.090	0.047	0.148	0.135	0.373	**3.00**	0.498	**4.21**	0.114	0.215	0.217	141								
A-TF7	67.5	3.58	0.540	**0.165**	4.23	8.08	84.1	**173**	**257**	**8.74**	2.37	26.1	0.456	5.64	1.71	**4.90**	83.6	**2.10**	0.948	0.087	0.041	0.137	0.121	0.347	**2.37**	0.420	**3.01**	0.097	0.210	0.239	144								
P-values	0.525	0.563	0.310	**0.012**	0.911	0.173	0.507	**0.014**	**0.012**	**0.001**	0.618	0.784	0.293	0.073	0.839	**0.001**	0.969	**0.006**	0.654	0.791	0.060	0.412	0.333	0.363	**0.017**	0.076	**0.003**	0.158	0.886	0.158	0.827								
B399	69.3	4.03	0.553	0.152	4.62	**8.33**	87.0	156	243	8.17	2.25	25.5	0.447	4.17	1.67	4.32	73.4	2.44	0.995	0.085	0.043	0.144	0.131	0.362	2.53	0.454	3.52	0.109	0.213	0.225	131								
NRG34	62.8	3.41	0.459	0.145	3.90	**6.95**	77.7	147	224	7.73	2.63	25.9	0.391	5.27	1.69	4.14	93.4	2.62	0.945	0.092	0.046	0.141	0.125	0.358	2.83	0.464	3.71	0.103	0.212	0.231	153								
P-values	0.162	0.205	0.162	0.554	0.253	**0.036**	0.088	0.575	0.28	0.308	0.191	0.890	0.415	0.276	0.946	0.602	0.076	0.545	0.605	0.387	0.252	0.840	0.651	0.898	0.240	0.810	0.593	0.589	0.976	0.704	0.143				nd			
A-TF0 × B399	67.5	4.21	0.533	0.137	4.85	7.36	84.6	127	211	7.38	2.14	24.1	0.389	2.50	1.48	3.42	62.9	2.90	0.976	0.084	0.044	0.140	0.135	0.357	2.75	0.482	3.96	0.126	0.204	0.208	117								
A-TF0 ×NRG34	61.7	3.50	0.412	0.126	3.74	7.04	76.5	132	209	6.94	2.88	26.4	0.376	5.08	1.81	3.70	103.5	3.02	1.007	0.096	0.050	0.156	0.136	0.390	3.25	0.515	4.46	0.102	0.226	0.225	164								
A-TF7 × B399	71.2	3.84	0.574	0.167	4.39	9.31	89.5	185	274	8.96	2.36	26.8	0.505	5.83	1.85	5.22	84.0	1.98	1.014	0.086	0.041	0.147	0.128	0.367	2.32	0.427	3.07	0.091	0.222	0.241	146								
A-TF7 ×NRG34	63.9	3.31	0.506	0.163	4.06	6.85	78.8	161	240	8.53	2.38	25.4	0.406	5.45	1.56	4.58	83.3	2.21	0.883	0.089	0.042	0.127	0.114	0.327	2.42	0.414	2.96	0.103	0.199	0.237	142								
P-values	0.872	0.849	0.689	0.811	0.528	0.101	0.810	0.367	0.348	0.993	0.212	0.549	0.532	0.147	0.295	0.189	0.068	0.842	0.407	0.574	0.276	0.177	0.614	0.215	0.435	0.595	0.413	0.146	0.492	0.488	0.087								

Supplemental Table 4.5 Analysis of variance (*P* values) comparing the effects of two alfalfa populations (A-TF0 vs A-TF7) in symbiosis with two *S. meliloti* strains (B399 and NRG34), and their interactions on the expression of genes coding galactinol synthase (GaS), sucrose phosphate synthase (SPS), sucrose synthase (SuSy), K3-dehydrin and msa CID in alfalfa crowns and genes coding cinnamic acid 4-hydroxylase (C4H) and ,7,4′-trihydroxyisoflavanone 4′-O-methyltransferase (IOMT) in alfalfa roots. Plants grown under controlled conditions were sampled at four physiological stages: non-acclimated plants (NA) were grown 8 weeks under a 21/17°C, Day/Night (D/N) temperature regime. Plants were then cold acclimated during 2 weeks at 2°C followed by two weeks at -2°C and sampled again (CA). After their exposure to a freezing stress of -11°C, alfalfa plants were transferred back to optimal regrowth conditions (21/17°C, D/N) and sampled after 48h (AFS), and after three weeks (RAF). The two alfalfa populations, A-TF0 and A-TF7, and the two *S. meliloti* strains, B399 and NRG34 were contrasting in their levels of freezing tolerance Numbers in bold indicate statistically significant effects ($P \leq 0.05$)

	GaS				SPS				SuSy				K3-dehydrin				C4H				IOMT			
	Sampling events																							
Effects	**NA**	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**	**NA**	**CA**	**AFS**	**RAF**
Alfalfa Populations (Pop)	0.104	0.415	0.060	0.285	0.143	0.150	0.753	0.872	0.974	**0.002**	**0.022**	0.064	0.980	0.747	0.168	0.068	**0.003**	0.078	0.661	**0.006**	0.352	**0.031**	0.350	0.939
***S. meliloti* Strains (Strains)**	0.06	0.830	0.659	0.907	0.539	0.682	0.557	0.683	0.357	0.624	0.149	0.228	0.329	0.577	0.199	0.457	0.761	0.946	0.417	0.324	0.876	0.685	0.875	0.578
Pop × Strains	0.798	0.703	0.437	0.772	0.273	0.618	0.785	0.655	0.636	0.745	0.116	0.426	0.215	0.282	0.611	0.901	0.997	0.379	0.233	0.931	0.384	0.331	**0.034**	0.395

Appendix 3 Supplementary information for Chapter 5



Supplemental Figure 5.1 A) The pJBA21Km plasmid was introduced into selected *S. meliloti* strains by electroporation with the following conditions (2.5 kV, 200Ω, 25μF) modified from Wielbo and al. 2007 and *S. meliloti* were selected YMA agar medium supplemented with 500μg ml–1 kanamycin and 50 μg ml–1 X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucuronide) substrate for β-glucuronidase. B) Alfalfa roots were stained for β-glucuronidase (Gus) activity in 50 mM sodium phosphate buffer (pH 7.2) with 50 μg ml–1 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-GlcA), 0.1 mM EDTA, 0.38 mM K3Fe(CN)6 and 0.38 mM K4Fe(CN)6 for about 8 h at 37°C. Following staining, roots were cleared using 25% (v/v) household bleach (1.25 % final concentration hypochlorite) for15 min, followed by extensive washing with distillated water, modified form (Wilson et al., 1995)