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Contribution des légumineuses, des champignons endophytes et mycorhiziens dans la nutrition azotée des prairies indigènes semi-arides

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Contribution des légumineuses, des champignons endophytes et mycorhiziens dans la nutrition azotée des prairies indigènes semi-arides

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Résumé

Les prairies indigènes présentent une source importante d'alimentation pour le pâturage du bétail dans les prairies Canadiennes semi-arides. L'addition de légumineuses fixatrices d'azote et de phosphore dans les prairies indigènes peut améliorer la productivité et la valeur nutritive de fourrage. Ces pratiques peuvent induire des modifications de la structure et de la diversité des communautés fongiques du sol, ce qui peut en retour avoir un impact sur la production et le contenu nutritionnel du fourrage.

L'objectif de cette étude était de développer un système de pâturage à bas niveau d'intrants, productif, autonome et durable. À court terme, nous voulions 1) déterminer l'effet des légumineuses (*Medicago sativa,* une légumineuse cultivée ou *Dalea purpurea,* une légumineuse indigène) et la fertilité en phosphore du sol sur la productivité et la valeur nutritive des graminées indigènes, comparées avec celles de la graminée introduite *Bromus biebersteinii* en mélange avec le *M. sativa*, 2) identifier l'effet de ces pratiques sur la diversité et la structure des communautés des champignons mycorhiziens à arbuscules (CMA) et des champignons totaux, 3) identifier l'effet des légumineuses et des CMA sur les interactions compétitives entre les graminées de saison fraîche et les graminées de saison chaude.

Les expériences menées au champ ont montré que *M. sativa* améliorait les teneurs en azote et en phosphore des graminées indigènes au début de l'été, ainsi que la teneur en azote de la graminée de saison chaude *Bouteloua gracilis* à la fin de l'été de l'année sèche 2009. Par contre, la fertilité en phosphore du sol n'ait pas affecté la productivité des plantes. D'autre part, l'inclusion des légumineuses augmentait la diversité des CMA dans le mélange de graminées indigènes. Cette modification présentait des corrélations positives avec la productivité et la quantité totale d'azote chez le *M. sativa* et avec la teneur en phosphore des graminées indigènes, au début de l'été. La structure des communautés de champignons totaux était influencée par l'interaction entre le mélange des espèces et la fertilité en phosphore du sol

seulement en 2008 (année humide). Cet effet pourrait être lié en partie avec la productivité des plantes et l'humidité du sol.

Les expériences menées en chambre de culture ont montré que les CMA peuvent favoriser la productivité des graminées de saison chaude au détriment des graminées de saison fraîche. En effet, *Glomus cubense* augmentait la productivité de la graminée de saison chaude *B. gracilis*, en présence de *M. sativa*. Cet effet pourrait être associé à l'effet négatif du *G. cubense* sur la fixation de l'azote par le *M. sativa* et à la diminution de l'efficacité d'utilisation de l'azote de certaines graminées de saison fraîche résultant en une augmentation de la disponibilité de l'azote pour *B. gracilis*. Par contre, le *Glomus* sp. augmentait la biomasse de *Schizachyrium scoparium*, autre graminée de saison chaude, en absence de légumineuse. Ce phénomène pourrait être attribuable à une amélioration de l'efficacité d'utilisation du P de cette graminée.

En conclusion, mes travaux de recherche ont montré que la légumineuse cultivée *M. sativa* peut améliorer la valeur nutritive des graminées indigènes au début de l'été ainsi que celle de la graminée de saison chaude *B. gracilis*, dans des conditions de sécheresse sévère de la fin de l'été. De plus, l'addition de *M. sativa* dans le mélange de graminées indigènes peut contribuer à augmenter le nombre des espèces bénéfiques des CMA pour la production et la nutrition du fourrage au début de l'été.

Mots clés: prairies semi-arides, graminées indigènes, légumineuses, champignons totaux, champignons mycorhiziens à arbuscule, productivité, nutriments, fourrage.

Abstract

The native grasslands are considered as the main feed source for livestock grazing, in semi-arid regions of the Canadian prairies. The addition of N fixing legumes and phosphorus to semi-arid native grasslands may increase the productivity and nutritive value of forage. However, these practices may also shape the structure and diversity of soil fungal communities which in turn may impact forage production and nutritive value.

The global objective of this research was to design productive, self-sustaining, permanent and with low inputs pastures. The specific objectives were 1) to demonstrate the effect of N-fixing legumes (the cultivated legume *Medicago sativa* or the native legume *Dalea purpurea*) and soil P fertility on the productivity and nutritive value of native grasses mixes in comparison to the mixture of the introduced grass *Bromus biebersteinii* and *M. sativa,* 2) identify the effect of these practices on the diversity and community structure of arbuscular mycorrhizal (AM) fungi and total fungi, and 3) identify the effect of legumes and AM fungi on competitive interactions between native cool-season grasses and native warm-season grasses.

The field experiment showed that *M. sativa* improved the nitrogen and phosphorus concentrations of native grasses mixes early in the summer, as well as the N concentration of the warm-season grass *B. gracilis*, in late summer of the driest year 2009. In contrast, the soil phosphorus fertility had no effect on plant productivity. On the other hand, the inclusion of legumes to the mix of native grasses generally increased AM fungal diversity. This shift was positively correlated with the productivity and nitrogen uptake by *M. sativa* and with the phosphorus concentration of native grasses mixes in early summer. The structure of the total fungal community was affected by the interaction between species mixtures and soil P fertility only in the wet year (2008), suggesting that this effect was likely driven in part by plant productivity and soil moisture.

The growth chamber experiment showed that the AM fungi may favoured the growth of warm-season grasses under competition with cool-season grasses. However, *Glomus cubense* increased the productivity of warm-season grass *B. gracilis* when growing with *M. sativa*. This effect might be related to a negative impact of *G. cubense* on the nitrogen-fixing activity of *M. sativa* and to a lower N-use efficiency of certain cool-season grasses, which resulted in increased soil N availability for *B. gracilis*. In contrast, *Glomus* sp. enhanced the growth of *S. scoparium*, another warm-season grass in the absence of legumes, and this may be related to improved P-use efficiency in this grass.

We concluded that the cultivated legume *M. sativa* can improve the nutritive value of native grasses mixes early in the summer and also of warm season grass under severe drought conditions in late summer. In addition, the inclusion of *M. sativa* within native grass mixes may contribute to promote beneficial AM fungi taxa that were involved in forage production and nutrition early in the summer.

Keywords: semi-arid grassland, native grasses, legumes, total fungi, AM fungi, productivity, nutrients, forage

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Liste des sigles et abréviations

AMF: arbuscular mycorrhizal fungi

ANOVA: analysis of variance

ATP: Adénosine triphosphate

bp: base pairs

C: carbone

cm: centimètre

CMA: champignon mycorhizien à arbuscules

ddH_2O: eau bi-distillée

DNA: deoxyribonucleic acid

DSE: dark Septate endophyte

EC: electrical conductivity

Fig: figure

g: gramme

h: heure

ITS: Internal transcribed spacer

K: potassium

kg ha^{-1}: Kilogramme par hectare

kg: kilogramme

L: litre

m: mètre

MANOVA: multivariate analysis of variance

mg: milligramme

min: minute

ml: millilitre

mm: millimètre

Mol: mole

ms: millisiemens

N: nitrogen (azote)

N_2: azote atmosphérique

NCBI: National center for biotechnology information

NH^{4+}: ammonium

NO_3: nitrate

NSERC: Natural Science and Engineering Research Council of Canada

OUT: operational taxonomic unit

P: phosphore

P_2O_5: anhydride de phosphate

pb: pair of bases

PCoA: Principal coordinate analysis

PCR: polymerase chain reaction

PERMANOVA: permutational multivariate analysis of variance

pmol: pico mole

PO_4: phosphate

rDNA: ribosomal DNA

s: seconde

sp.: species (espèce)

UPGMA: Unweighted Pair Group Method with Arithmetic

Zn: zinc

$\delta^{15}N$: nitrogen isotopic ratio

µg: micro gramme

µL: micro Litre

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Chapitre 1: Introduction générale

Une large portion des prairies canadiennes a été couverte par des prairies indigènes dont la superficie à l'origine s'élevait à 61.5 millions d'hectares, mais ces prairies sont actuellement beaucoup plus réduites, elles occupent uniquement 11.4 millions hectares. Ces pertes sont essentiellement attribuées à l'usage élevé des espèces introduites ou des céréales qui ont remplacé les espèces indigènes afin d'avoir une production intensive de fourrage ou de produits agricoles (Jefferson et al., 2005; Iwaasa et al., 2012). Au cours de la dernière décennie, les politiques et les organismes privés ou publics ont encouragé les producteurs à revégétaliser les zones perturbées avec des espèces indigènes qu'avec des espèces introduites afin de protéger et conserver les prairies indigènes qui sont en voie de disparition (Jefferson et al., 2005). Les prairies indigènes présentent un grand intérêt écologique. Elles offrent un fourrage de bonne qualité au bétail et contribuent à la biodiversité, la lutte contre l'érosion et la séquestration du carbone dans le sol (Jefferson et al., 2004; Jefferson et al., 2005; Bailey et al., 2010; Muir et al., 2011). Les espèces indigènes sont plus tolérantes à la sècheresse, plus durables mais elles produisent moins de biomasse et de graines que les espèces introduites (Kilcher and Looman, 1983; Lawrence and Ratzlaff, 1989). La productivité élevée des espèces introduites pourrait s'expliquer par leurs taux élevé d'établissement, la fertilisation et la période d'échantillonnage. La faible productivité observée chez les espèces indigènes est expliquée en partie par le fait que ces espèces sont plus difficiles à installer et qu'elles demandent plus de temps pour s'établir dans les prairies que les espèces introduites (Kilcher and Looman, 1983). L'addition de la fertilisation phosphatée peut faciliter l'installation des espèces indigènes, puisque le phosphore est considéré comme un élément important pour la croissance des racines des plantes (Brady and Weil, 2001).

Un système de pâturage ensemencé avec des espèces indigènes peut avoir une bonne productivité tout au long de la saison, suite à une gestion appropriée. En effet, les espèces indigènes ensemencées en mélange plutôt qu'en monocultures peuvent fournir une productivité élevée (Schellenberg, 2008). Un mélange formé de graminées de saison fraîche et de graminées de saison chaude peut assurer une productivité de fourrage de qualité élevée du début à la fin de l'été (Schellenberg et al., 2012), puisque ces espèces de graminées sont caractérisées par une production saisonnière différente. En effet, les espèces de saison fraîche sont des plantes dominantes et productives au printemps mais qui ont une croissance réduite en été, alors que les espèces de saison chaude ont une croissance active pendant l'été (Abougendia, 1995). En fait, les communautés de plantes plus diversifiées peuvent acquérir plus de nutriments et devenir plus productives que les communautés de plantes moins diversifiées, dans des conditions où les ressources sont limitées (Houper et al., 2005; Mommer et al., 2010). Ceci peut résulter de la complémentarité d'utilisation par les plantes de différentes ressources ou des mêmes ressources à des moments différents, ce qui conduit à une réduction de la compétition entre les espèces et à une meilleure exploitation des ressources (eau, énergie solaire, nutriments) (Vandermeer, 1992; Houper et al., 2005).

L'intégration des légumineuses dans un système de pâturage peut améliorer et maintenir le rendement fourrager, ainsi que le contenu protéique des mélanges graminées-légumineuses tout au long de la saison (Schultz and Stubbendieck, 1982; Frame and Harkess, 1987; Fagerberg, 1988; Bullock et al., 2007; Temperton et al., 2007; Nyfeler et al., 2009). Les légumineuses présentent des intérêts économiques et écologiques très importants puisqu'elles réduisent la dépendance aux fertilisants azotés et les impacts négatifs de ceux-ci sur l'environnement. En effet, ces légumineuses permettent de réduire le coût des intrants, de diminuer la consommation d'énergie fossile, la pollution des nappes d'eau en nitrates et les émissions de gaz à effet de serre (GES) (Ledgard et al., 2009; Schils et al., 2013; Schmeer et al., 2014).

En plus, elles fournissent souvent un apport très important d'azote au sol grâce aux bactéries fixatrices d'azote de l'ordre des Rhizobiales (Mosier, 2002). Toutefois, la capacité fixatrice d'azote peut être affectée par la disponibilité des nutriments dans le sol. En effet, une forte disponibilité d'azote ainsi que le stress hydrique peuvent induire une diminution de l'activité de la nitrogénase et la biomasse des nodules et par la suite, une réduction de l'activité fixatrice d'azote (Ledgard and Steele, 1992; Soussana and Tallec, 2010; Nyfeler et al., 2011). Par contre, la disponibilité du phosphore dans le sol est essentielle pour la croissance des nodules et l'activité de la nitrogénase chez les légumineuses, puisque l'activité fixatrice d'azote est un processus énergétique qui nécessite 16 mol d'ATP pour 1 mol d'azote fixé (Crews, 1993; Allahdadi et al., 2007; Kurppa et al., 2010; Soussana and Tallec, 2010). Une partie de l'azote libéré par les légumineuses dans le sol peut être transférée aux graminées associées à travers la décomposition et la minéralisation des résidus de légumineuses (Ledgard and Steele, 1992; Sierra and Desfontaines, 2009) ou directement par les exsudats racinaires (Lory et al., 1992; Govindarajulu et al., 2005; Jalonen et al., 2009). En effet, Zanetti et al. (1997) a montré que l'augmentation du rendement en azote chez la graminée *Lolium perenne* est due principalement à l'azote fixé provenant du *Trifolium repens*. Un autre processus permet d'augmenter le rendement et l'efficacité d'utilisation de l'azote disponible pour les graminées et les légumineuses, lorsqu'elles sont en association. Les légumineuses fixent plus l'azote atmosphérique lorsqu'elles sont en présence de graminées qu'en monoculture et par conséquent, les graminées accèdent à plus que leur part de l'azote disponible du sol, un phénomène qu'on appelle en anglais: 'N sparing' (Temperton et al., 2007).

Dans les prairies canadiennes, la légumineuse cultivée *Medicago sativa* L. est très connue pour sa longévité, sa grande productivité et sa contribution à l'azote des fourrages. *Dalea purpurea* Vent. est aussi considérée comme une espèce fourragère de qualité nutritive et d'appétence élevées dans les prairies canadiennes (Schellenberg and Banerjee, 2002). Cette légumineuse est une plante indigène de saison

chaude qui pousse plus tardivement que *M. sativa* et qui augmente la valeur nutritive des pâturages en fin de saison. Toutefois, un pâturage basé sur l'utilisation des légumineuses seules peut induire des pertes de bétails à cause du météorisme en plus d'être moins productif. Il est préférable d'utiliser des mélanges de graminées et de légumineuses (Popp et al., 2000). Les légumineuses améliorent la valeur nutritive des graminées indigènes associées, surtout à la fin de leur période de croissance, quand les graminées de saison fraîche s'assèchent et que leurs valeurs nutritives diminuent (Skerman et al., 1988).

Plusieurs études ont révélé que les champignons du sol jouent un rôle crucial dans la détermination de la productivité, la diversité et la structure des communautés végétales (Bever et al., 1997; van der Heijden et al., 1998; van der Heijden et al., 2008; Rillig et al., 2014). La composition des communautés de plantes peut avoir en retour des effets marqués sur la diversité et la composition des populations fongiques du sol (Scheublin et al., 2004; Bainard et al., 2014). Dans la prairie Nord Américaine, la plupart des racines des plantes et le sol environnant sont colonisés par des champignons endophytes et des champignons mycorhiziens à arbuscules (Jumpponen and Trappe, 1998; Mandyam and Jumpponen, 2008; Khidir et al., 2010; Porras-Alfaro et al., 2011).

Les CMA sont souvent considérés comme un bon indicateur de la fertilité biologique du sol puisqu'ils sont capables d'assurer une production végétale durable par l'amélioration de la croissance des plantes et l'absorption des éléments nutritifs, particulièrement dans les sols où la disponibilité des nutriments est faible. Les CMA transfèrent le phosphore et d'autres éléments nutritifs (N, K, Zn, etc.) à la plante en échange de glucides (Smith and Read, 2008). Grâce aux hyphes fongiques, les CMA augmentent le volume et la surface de contact avec le sol prospecté par les racines, favorisant la nutrition minérale de la plante hôte (Smith and Read, 2008). De plus, les CMA réduisent les maladies chez les plantes (St-Arnaud and Vujanovic, 2007) et augmentent leur tolérance aux stress hydriques (Subramanian and Charest, 1998; Jeffries, 2003) et aux métaux lourds (Audet and Charest, 2006). Ils peuvent aussi augmenter la fixation

azotée chez les légumineuses (Barea et al., 2002) puisque les CMA peuvent fournir des quantités supplémentaires de phosphore nécessaires pour l'activité fixatrice d'azote (Azcón et al., 1991; Scheublin et al., 2004).

Les CMA sont aussi considérés comme un facteur déterminant de la coexistence des plantes au sein des communautés végétales (Hart et al., 2003; Stein et al., 2009) puisqu'ils permettent de modifier la compétition entre les espèces (Scheublin et al., 2007; Wagg et al., 2011) et la distribution des nutriments entre les espèces voisines (Scheublin et al., 2007). En effet, la structure de la communauté végétale peut dépendre du degré de dépendance mycorhizienne des plantes. Par exemple, la performance des graminées de saison chaude est plus dépendante aux CMA que celle des graminées de saison fraîche (Hetrick et al., 1988; Wilson and Hartnett, 1998; Hoeksema et al., 2010). Dans les associations graminées-légumineuses, on a montré que les CMA favorisaient plus la croissance des légumineuses que des graminées (Scheublin et al., 2007; Wagg et al., 2011), alors que d'autres études ont montré inversement que les CMA augmentaient plus la capacité compétitive de la graminée que celle de la légumineuse, grâce à l'azote fixé libéré par la légumineuse et transféré aux graminées (Hamel et al., 1991).

L'influence des CMA sur la performance des plantes en compétition est caractérisée par une variété de réponses qui peuvent être négatives, neutres, ou positives (Johnson et al., 1997; Hart et al., 2003; Klironomos, 2003; Smith et al., 2011) selon le degré de compatibilité physiologique des CMA avec la plante hôte (van der Heijden et al., 1998), la disponibilité des nutriments (Johnson, 2010) et les interactions compétitives entre les espèces (Mariotte et al., 2013). Cela pourrait être expliqué par l'équilibre entre le coût de la symbiose pour la plante hôte (carbone) et le gain (nutriments) qu'elle enregistre (Johnson et al., 1997; Hart et al., 2003; Klironomos, 2003; Johnson, 2010; Smith et al., 2011). Par exemple, une forte colonisation par les champignons peut réduire la compétitivité des plantes colonisées si le carbone demandé par les CMA dépasse les bénéfices reçus par ces plantes (Hart et al.,

2003). D'autres études ont montré que la réduction de la croissance des plantes ayant un faible taux de colonisation par les CMA peut être due à une diminution de l'absorption du phosphore par les racines en réponse à la mycorhization qui n'est pas compensée par la contribution des champignons à l'acquisition de cet élément nutritif par la plante (Grace et al., 2009).

Une grande diversité d'espèces de CMA contribue à favoriser la diversité et la coexistence des plantes (et vice versa) (van der Heijden et al., 1998; van der Heijden, 2003), parce que la capacité des plantes à sélectionner le CMA le plus efficace et le plus compatible est plus élevée (Hart et al., 2003; Hodge et al., 2010). D'ailleurs, il a été montré que les plantes préfèrent allouer plus de carbone aux espèces de CMA qui leur sont bénéfiques (Kiers et al., 2011; Chagnon et al., 2012). Les différentes fonctions des champignons mycorhiziens associés aux plantes peuvent réduire le chevauchement des niches entre les espèces voisines et augmenter la complémentarité entre les plantes, résultant en une augmentation de la productivité totale des communautés végétales (Wagg et al., 2011). De plus, la diversité des CMA peut maintenir la productivité des plantes dans des environnements hétérogènes et aussi dans des conditions environnementales changeantes (Wagg et al., 2011). La disponibilité des nutriments du sol peut aussi affecter les populations fongiques et en particulier les CMA (Johnson, 2010). En effet, une forte disponibilité des nutriments dans le sol réduit en général l'effet des CMA dans l'amélioration de la nutrition des plantes et en retour la plante réduit l'allocation des ressources carbonées aux CMA, ceci entrainant une diminution de l'abondance de ces champignons (Kiers et al., 2011; Smith and Smith, 2012).

Des champignons endophytes sont trouvés très fréquemment dans les racines des plantes et dans le sol environnant dans l'écozone des prairies, qui se caractérisent par le déficit hydrique (Porras-Alfaro et al., 2008; Khidir et al., 2010; Porras-Alfaro et al., 2011). Ces champignons endophytes peuvent améliorer la tolérance des plantes à la sécheresse et au stress hydrique. Ils peuvent aussi conférer à la plante une

meilleure résistance aux organismes pathogènes et aux herbivores par la production de métabolites inhibiteurs (Mandyam and Jumpponen, 2005). De plus, les endophytes sont impliqués dans la transformation des formes organiques de nutriments en formes disponibles à la plante grâce à la sécrétion d'enzymes hydrolytiques dans la rhizosphère (Caldwell et al., 2000; Mandyam and Jumpponen, 2005; Upson et al., 2009; Mandyam et al., 2010; Newsham, 2011). Usuki et Narisawa (2007) ont montré que quelques espèces endophytes ont des effets bénéfiques sur la plante en améliorant leur nutrition azotée. Similairement aux CMA, les endophytes peuvent également influencer la diversité et la productivité des communautés de plantes. En effet, ils peuvent former des associations mutuelles, pathogènes ou commensales (Schulz and Boyle, 2005) selon la plante hôte (Kageyama et al., 2008) et selon le statut nutritionnel de l'environnement (Bethlenfalvay et al., 1983). Le type d'interaction entre la plante et les endophytes dépend de la physiologie et du génotype de la plante, du génotype et de la virulence des champignons et des conditions environnementales (Donoso et al., 2008). Récemment, il a été démontré que les interactions entre les CMA et les endophytes peuvent entrainer une modification de la structure des communautés végétales par le changement de l'abondance des espèces et par conséquence, la productivité des plantes (Rillig et al., 2014).

L'inclusion des légumineuses et l'addition du phosphore dans un système de pâturage de graminées indigènes de saison fraîche et de graminées de saison chaude dans la prairie semi-aride pourraient augmenter la productivité et la valeur nutritive de fourrage tout au long de la saison de pâturage. Ces pratiques pourraient aussi modifier la structure et la diversité des communautés fongiques du sol, ce qui pourrait en retour influencer leurs interactions avec la plante et leur impact sur la dynamique, la productivité et la nutrition des plantes fourragères. Les champignons mycorhiziens à arbuscules jouent un rôle important dans la compétition, la diversité et la productivité des communautés

végétales (van der Heijden et al., 1998; Hart et al., 2003; Stein et al., 2009; Chagnon et al., 2012). De plus, une grande proportion des champignons endophytes a été trouvée dans le sol et dans le système racinaire des plantes dans l'écozone des prairies semi-arides. Les rôles de ces champignons restent ambigus. Donc, il est très intéressant d'éclaircir leur relation avec les communautés de plantes et la fertilité du sol.

Dans ce projet de thèse, l'objectif global était d'avoir à long terme un système de pâturage productif, autonome et permanent par l'utilisation des légumineuses fixatrice d'azote et d'une modification initiale de la fertilité en phosphore du sol.

Les objectifs spécifiques étaient: 1) de déterminer la contribution des légumineuses fixatrices d'azote (*M. sativa*, une légumineuse cultivée, ou *D. purpurea*, une légumineuse indigène) et l'ajout de phosphore dans l'amélioration de la productivité et de la nutrition des graminées indigènes, en comparaison avec celles d'un mélange de la graminée introduite *Bromus biebersteinii* cultivée et de *M. sativa*, ce mélange qui est couramment utilisé dans la région semi-aride du Canada; 2) d'analyser l'effet de l'ajout de phosphore inorganique et de divers mélanges de plantes (sept graminées indigènes, sept graminées indigènes + *M. sativa*, sept graminées indigènes + *D. purpurea* ou *B. biebersteinii* + *M. sativa*) sur la structure et la diversité des champignons mycorhiziens à arbuscules et d'autres champignons naturellement présent dans le sol des prairies; 3) d'examiner l'effet interactif de la présence d'une légumineuses (*M. sativa* ou *D. purpurea*) et d'un CMA (*Glomus cubense* ou *Glomus* sp.) sur la relation de compétition existant entre trois espèces de graminées indigènes de saison fraîche (*Elymus canadensis*, *Elymus trachycaulus* ssp. *subsecundus* et *Elymus lanceolatus* ssp. *lanceolatus*) et deux espèces de graminées indigènes de saison chaude (*Schizachyrium scoparium* et *Bouteloua gracilis*) en conditions contrôlées (chambre de culture).

Nos hypothèses de départ étaient:

1) L'addition de légumineuse fixatrice d'azote et l'ajout de P inorganique augmente la productivité et la nutrition azotée du fourrage et en particulier celles des graminées indigènes au début et à la fin de l'été.

2) L'addition des légumineuses et l'augmentation de la fertilité en phosphore du sol influence différemment la diversité et la structure des communautés des champignons mycorhiziens à arbuscules et des champignons totaux dans le sol des prairies.

3) L'effet combiné des champignons mycorhiziens à arbuscules et des légumineuses change la relation de compétition qui existe entre les espèces de graminées indigènes de saison fraîche et de saison chaude.

Chapitre 2: Contribution of legumes to promote the nutritive value of forage in semi-arid grassland pastures

Ce chapitre est actuellement soumis dans une revue scientifique internationale:

Klabi R, TH Bell, C Hamel, MP Schellenberg, A Iwaasa, A Raies, M St-Arnaud. Contribution of legumes to the production of high quality forage in semi-arid grassland pastures. PloS One, soumis 2015-07-16 (PONE-D-15-31346).

Les résultats de ce chapitre ont été présentés lors des conférences suivantes:

*Rim Klabi, Terrence H. Bell, Chantal Hamel, Alan Iwaasa, Michael P. Schellenberg, Aly Raies et Marc St-Arnaud. Contribution of legumes to the production of sustainable forage and plant nitrogen and to increase the soil AM fungi diversity in grassland communities. Conférence internationale sur Mycorhizes ICOM8, Flagstaff, Arizona, USA, 3-7 Août 2015.

*Rim Klabi, Terrence H. Bell, Chantal Hamel, Alan Iwaasa, Michael P. Schellenberg, Aly Raies et Marc St-Arnaud. Effet de la légumineuse *Medicago sativa* sur la diversité des champignons mycorhiziens à arbuscules, les rendement fourragers et azotés dans les prairies semi-arides. Colloque Mycorhizes 2015, Ottawa, 8 May 2015.

*Rim Klabi, Chantal Hamel, Alan Iwaasa et Aly Raies. Nitrogen fixation and isotopic composition of native and tame forage plants. In 16th Soil and Crops conference, Saskatoon, Saskatchewan 24 Février 2010.

2.1 Préface

Pour déterminer l'importance de l'addition des légumineuses et l'ajout de phosphore sur l'amélioration de la production et la valeur nutritive du fourrage au début et à la fin de la période de croissance dans les prairies indigènes des régions semi-arides, nous avons testé, dans ce chapitre, l'effet de l'inclusion des légumineuses (*Medicago sativa*, une légumineuse cultivée, *Dalea purpurea*, une légumineuse indigène) et de la fertilité en phosphore du sol (addition de 0, 50 ou 200 P_2O_5 kg ha^{-1} à l'ensemencement) sur la productivité et la nutrition azotée des graminées indigènes comparativement avec celles de la graminée introduite *Bromus biebersteinii.* Cette dernière est considérée comme une espèce productive dans les zones semi-arides des prairies canadiennes. Nous avons également testé la contribution des champignons mycorhiziens à arbuscules dans la productivité et la nutrition du fourrage.

2.2 Abstract

The inclusion of legumes in grasslands may promote the productivity and nutritive value of forage over longer grazing periods. This study was designed to assess the effect of legumes (*Medicago sativa* or *Dalea purpurea*) and soil P fertility (addition of 0, 50, or 200 P_2O_5 kg ha^{-1} at seeding) on the dry matter and nutrient content of a mix of native grasses, as compared to the commonly used cultivated forage grass *Bromus biebersteinii* grown with *M. sativa.* Plant harvests were performed in September 2008, July and September 2009. Plants were analyzed for their nutrient content, $\delta^{15}N$ value and dry matter. Results show that the inclusion of *M. sativa* increased the nutrient concentration (N and P) of native grass mixes by 19% and 25% respectively, early in the growing season, as well as the N concentration in *Bouteloua gracilis* by 21% in late summer of the driest year, 2009. However, the higher N concentration observed in native grasses without reducing the $\delta^{15}N$ value indicated that the N nutrition of those grasses was essentially dependent on higher soil N sparing, which is the N not used by legumes. In contrast, the soil P

fertility did not affect plant productivity. In addition, AM fungi diversity that was higher in the mixture of native grasses and *M. sativa* was positively correlated with the dry matter and nitrogen uptake of *M. sativa* and also with the phosphorus concentration of native grasses, early in the summer of 2009. Overall, this study show that *M. sativa* can enhance the nutritive value of seeded native grasses early in the summer, but also of the warm-season grass *B. gracilis* under severe drought conditions, which are typical of late summer in the semi-arid prairies and suggests that this legume may contribute to promote functionally beneficial AM fungi taxa for forage production and nutrition in native grassland, early in the growing season.

Keywords Forage dry matter, introduced grass; legumes; native grass mix; nutrients.

2.3 Introduction

Native grasslands have many important functional roles in semi-arid regions. These include providing high quality forage for livestock grazing, reducing soil erosion, enhancing soil organic carbon sequestration, limiting the effects of drought on biomass production, and contributing to biodiversity, while maintaining habitat space for native wildlife (Richards et al., 1998; Jefferson et al., 2004; Weigelt et al., 2009; Bailey et al., 2010; Muir et al., 2011). In general, native grass species yield less biomass and fewer seeds relative to introduced grass species that have been introduced for the purpose of producing high-yield forage (Kilcher and Looman, 1983; Lawrence and Ratzlaff, 1989). However, some studies have shown that monocultures of native grasses in semi-arid regions can provide higher forage yield than monocultures of introduced grasses (Jefferson et al., 1999; Willms et al., 2005). It appears that the generally higher forage yield of introduced species may depend on soil fertility and harvesting time (Jefferson et al., 2005). For example, the productivity of native species was higher than that of introduced

species in an unfertilized grassland, while introduced species produced more biomass when fertilizer was added (Johnston et al., 1968; Knowles, 1987; Jefferson et al., 2005). Additionally, some native species have been shown to be more productive than introduced species in dry periods of the summer (Knowles, 1987).

In contrast, the invasion of native grasslands by introduced species may impair ecosystem stability, nutrient cycling, and local biodiversity. As a result, both private and public organizations that are responsible for the management and preservation of native grasslands in the Canadian Prairies have supported the re-seeding of disturbed areas with native species (Asay et al., 2001; Jefferson et al., 2005). A number of producers have shown that the production of native grasses is sustainable over time, and that these grasses encroach less upon neighbouring native species than do introduced species (Jefferson et al., 2004). Recently, several studies have indicated that seeding native species in well-managed systems can produce large amounts of high quality forage for livestock grazing, both early and late in the growing season (Jefferson et al., 2002; Schellenberg et al., 2012), as opposed to introduced species which tend to become dormant under the dry conditions of mid-summer (Knowles, 1987). In addition, forage grasses tend to be more productive in mixture than monocultures (Tilman et al., 2001; Schellenberg, 2008), especially when they contain both warm- and cool-season grasses. Warm- and cool-season grasses reach maturity at different times in the growing season, which may contribute to more sustainable pastures that support longer grazing periods (Schellenberg et al., 2012). Cool-season grasses are generally seeded in forage pastures. Their productivity is high in spring, but decreases over the summer months. In contrast, warm-season grasses reach maturity in summer (Abougendia, 1995) and have the potential to increase the feeding value of native forage mixtures for late-season grazing (Schellenberg et al., 2012).

Integrating N-fixing legumes into native grass mixtures may help to sustain and even increase herbage production in unfertilized pastures (Temperton et al., 2007; Nyfeler et al., 2009). This practice

can also increase the total protein content of grass-legume mixtures, as well as the rate of digestion by grazing animals (Schultz and Stubbendieck, 1982; Frame and Harkess, 1987; Fagerberg, 1988; Suter et al., 2015). Grasses growing with neighbouring legumes can receive large amounts of fixed N, which often improves their N nutrition and vigour (Boller and Nösberger, 1987; Høgh-Jensen and Schjoerring, 1997; Gubsch et al., 2011; Nyfeler et al., 2011). Legumes grown alongside non–leguminous plants fix more N_2 than legumes grown in monoculture (Carlsson and Huss-Danell, 2003; Corre-Hellou et al., 2006; Fustec et al., 2010), and consequently, the N nutrition of grasses is closely related to the high availability of soil N that was not consumed by legumes (Temperton et al., 2007). In dry prairie soils, where nutrients are often limited, the addition of phosphorus to mixtures containing legumes and grasses may help to promote N fixation by legumes (Israel, 1987; Hayat et al., 2008; Divito and Sadras, 2014) and facilitate plant establishment, since native species may establish more slowly than introduced species.

In Southwest Saskatchewan, the cultivated N-fixing legume *Medicago sativa* L. is commonly included in forage systems, due to its high nutritive value and rapid growth in semi-arid regions. *Dalea purpurea* Vent. is also considered to be an important forage legume. This legume is native to the Canadian Prairies, and has a higher protein content than *M. sativa*, as well as increased palatability for grazing livestock (Schellenberg and Banerjee, 2002). *Dalea purpurea* is a warm-season plant, maturing later in the season than *M. sativa*, and potentially providing more nutritious feed for cattle late in the growing season. Incorporating legumes in low-input forage systems reduces the need for expensive and environmentally-damaging industrial N-fertilizers (Foley et al., 2005; Deutsch et al., 2006).

In semi-arid grasslands, the plant roots and surrounding soils are colonized by a high diversity of fungi including arbuscular mycorrhizal fungi (AM fungi) (Porras-Alfaro et al., 2011; Klabi et al., 2015). The AM fungi may play an important role for plant growth by enhancing nutrients acquisition (eg. P, N, Zn etc.), while in turn they receive carbon and energy from plants (Smith and Read, 2008; Smith and

Smith, 2012). We recently found that soil arbuscular mycorrhizal (AM) fungi diversity was increased by the addition of legumes to semi-arid native grasslands (Klabi et al., 2015), which in turn may impact forage production. Moreover, it has been shown that high AM fungi diversity have a positive influence on plant diversity and productivity (van der Heijden et al., 1998), which can result from the fact that plants may be able to select the most beneficial AM fungi, while these fungi may prefer to associate with plants that provide high carbon allocation (Kiers et al., 2011). Consequently, legumes may prefer to interact with AM fungi that are more efficient in acquisition of P or of others nutrients that are important for nitrogen fixation and hence for legumes growth (Scheublin et al., 2004; Klabi et al., 2015). In addition, AM fungi can improve plant drought tolerance (Jeffries, 2003; Gupta and Kumar, 2000). which may also be an important benefit in semi-arid grasslands.

We conducted a field experiment in the Canadian Prairies to determine the effect of phosphorus (P) addition and legume introduction (either *M. sativa* or *D. purpurea*) on the dry matter and nutrient content of a native grass mix in comparison to a monoculture of the introduced grass *Bromus biebersteinii* Roem. & Schult., which is a commonly used cultivated forage species in semi-arid regions of Canada. The inclusion of legumes in grass mixtures is an interesting low-cost option for sustaining the productivity of unfertilized pastures from year to year. We hypothesized that including legumes within the native grass mix, or along with the introduced *B. biebersteinii*, would increase forage production and nutritional content. Specifically, we tested: 1) whether N-fixing legumes contribute to increased dry matter, N concentration, and total N uptake by the total species mixture and in particular by the mix of native grasses, and 2) whether legumes increase the N concentration in the tissues of different species of native grass at the end of growing season. Plant growth parameters were estimated at the end of the growing season over two consecutive years (September), as well as during the vegetative growth stage (July) of the second year of the experiment.

2.4 Materials and methods

2.4.1 Site description

The field experiment was established in 2006 on a loamy Brown Chernozem soil located at the South Farm of the Semiarid Prairie Agricultural Research Centre in Swift Current, Saskatchewan, Canada (50°16`N 107°44` W). The soil contained 9.23 mg kg^{-1} of NO_3^--N, 26.63 mg kg^{-1} of PO_4^--P, and 282.85 mg kg^{-1} of K on average at plant establishment. The soil NO_3^--N, PO_4^--P and exchangeable potassium (K) were determined by extraction in 0.001 M $CaCl_2$ (Hamm et al. 1970). This soil have adequate level of K and PO_4^--P, but the concentration of P is unlikely to increase plant production.

The experimental site was seeded with barley (*Hordeum vulgare* L.) in 2005. Total annual precipitation at the site was 431.8 mm in 2008, which was higher than normal (the 1981-2010 average of 392.5 mm), and 266.5 mm in 2009, which was lower than normal, and the mean annual temperature was 3.8 °C in 2008 and 3.3 °C in 2009. Precipitation during the growing season (1 April to 30 September) of the study years was abovenormal (358.8 mm vs the 1981-2010 average of 269.4 mm) in 2008 and belownormal (182.5) in 2009. The mean temperature was closed to normal (12.7 °C vs the 1981-2010 average of 13.1 °C) in 2008 and 13.3 °C in 2009 according to a weather station located within 1000 m of the study site (Klabi et al., 2015).

2.4.2 Experimental design

The experimental treatments were the factorial combination of four species mixtures (described below) and three concentrations of soil P addition. Treatments were arranged in four complete randomized blocks. In total, there were forty-eight 5.48×7.32 m plots, separated by 1.83 m in all directions. Species mixtures were (1) seven native grasses, (2) the same native grasses + the cultivated legume *Medicago sativa* L. (alfalfa), (3) the same native grasses + the native legume *Dalea purpurea*

Vent. (purple prairie clover), and (4) the introduced forage grass *Bromus biebersteinii* Roem. & Schult. (meadow brome) + *M. sativa*. The native grass mix contained five cool-season species, which were *Elymus canadensis* L. (Canada wildrye), *E. lanceolatus* (Scribn. & J.G. Sm.) Gould ssp. *lanceolatus* (northern wheatgrass), *E. trachycaulus* (Link) Gould ex Shinners ssp. *subsecundus* (Link) (awned wheatgrass), *Nassella viridula* (Trin.) Barkworth (green needlegrass) and *Pascopyrum smithii* (Rydb.) Barkworth & D.R. Dewey (western wheatgrass), and two warm-season species, which were *Bouteloua gracilis* (Kunth.) Lag. ex Griffiths (blue grama) and *Schizachyrium scoparium* (Michx.) Nash (little bluestem). The grass and legume seeds were developed by Agriculture and Agri-Food Canada for Ducks Unlimited Canada Inc., and provided by Ducks Unlimited Canada Inc. (Saskatchewan Provincial Office). The total seeding rate was 11.1 kg ha^{-1} for the mixture of seven native grasses growing alone, 9.2 kg ha^{-1} for the mixtures of seven native grasses growing with legumes and 16.1 kg ha^{-1} for the mixtures of *B. biebersteinii* and *M. sativa.* The seeding rate for each species in the mixture is provided in Table 1. Row spacing was 30.48 cm and the seeding depth was~0.5 cm. The seeds were sown in May 2006 using a self-propelled hydrostatic plot seeder (Semiarid Prairie Agriculture Research Center, Design lab, Swift current, SK). At the time of seeding (i.e. two years prior to initial sampling), species mixtures received 0, 50, or 200 kg/ha of P_2O_5 as triple super-phosphate, which was raked manually through the top 10 cm of soil. The phosphorus fertilizers was applied to establish three different soil P concentrations. On July 2006, Roundup Renew (glyphosate) was wick applied above the young crop plants for weed control.

2.4.3 Plant sampling

Plants were sampled at the end of each growing season (i.e. the last week of September in 2008 and 2009) and in the first week of July 2009, when the most plants (cool season species) peak in production, in semi-arid regions. Dry matter was determined by clipping the plants at ground level from

one 0.25 m^2 quadrat that was placed randomly within each species mixture. In 2008, the total dry matter of species mixes was measured, while in 2009, the dry matter of legumes and grasses was determined independently. The plant material taken within these quadrats was dried at 40°C for three days and weighed. Plant dry matter is expressed as kg ha^{-1}. In addition, each individual native grass, each legume species, and *B. biebersteinii* were collected separately from each plot and dried at the end of the growing season over two consecutive years, except for *E. lanceolatus* ssp. *lanceolatus* and *P. smithii*, which were collected together due to their similar morphology preventing separation. Dry plant materials were ground, and subsamples of ground plant material were digested with H_2SO_4/Se/Na_2SO_4 (Varley, 1966). The concentration of N (Noel and Hambleton, 1976) and P in plant tissues (Milbury et al., 1970) was determined using a segmented flow auto analyzer (Technicon, AAII System, Tarrytown, NY). Total P and N uptake by total species mixes was determined by multiplying plant dry matter by the concentration of each nutrient in plant tissues. A second subsample of ground plant material was finely ground to powder in a bead miller and a 0.006-g subsample was placed in tin capsules for isotopic $^{15}N/^{14}N$ analyses using a Carlo Erba 17 NA1500 elemental CN analyzer coupled to an Optima isotope ratio mass spectrometer, in order to evaluate the contribution of biological N_2 fixation to herbage N concentration and N uptake.

2.4.4 Soil sampling

Soil samples were collected in August 2009 to determine soil nutrient availability. Samples were taken from two different soil depths (0-15 and 30-45 cm) using a soil corer (5 cm Ø × 15 cm length). Two core samples were randomly taken from each of the two soil layers in each plot and pooled to produce a single composite sample for each depth and plot. Plant available PO_4-P was determined by extracting 2.5 g soil with $NaHCO_3$ (Olsen et al., 1954) and plant available NO_3-N was determined by KCl extraction

(Maynard and Kalra, 1993). Soil P concentrations increased with soil P addition in the 0-15 cm soil layer but was not affected by species mixtures, in 2009 (Table S1). This indicated that the soil P addition had successfully create three different soil P concentrations (low-, middle-, high soil P) that persisted over the experimental period, but this effect diminished with soil depth, and was not observed at 30-45 cm. In contrast, soil N availability was not affected by species mixtures (Table S1).

Table 1 Seeding rate of species seeded in different species mixtures

Species mixtures	Species name	Seeding rate (Kg ha^{-1})
Native grasses	*E. canadensis*	0.2
	E. lanceolatus ssp. *lanceolatus*	2.0
	E. trachycaulus ssp. *subsecundus*	0.2
	N. viridula	2.5
	P. smithii	2.2
	B. gracilis	0.8
	S. scoparium	3.2
Native grasses + *M. sativa*	*E. canadensis*	0.2
	E. lanceolatus ssp. *lanceolatus*	1.2
	E. trachycaulus ssp. *subsecundus*	0.2
	N. viridula	2.5
	P. smithii	1.5
	B. gracilis	0.8
	S. scoparium	2.2
	M. sativa	0.2
Native grasses + *D. purpurea*	*E. canadensis*	0.2
	E. lanceolatus ssp. *lanceolatus*	1.3
	E. trachycaulus ssp. *subsecundus*	0.2
	N. viridula	2.5
	P. smithii	1.5
	B. gracilis	0.8
	S. scoparium	2.2
	D. purpurea	0.5
B. biebersteinii + *M. sativa*	*B. biebersteinii*	15.3
	M. sativa	0.8

2.4.5 Determining the nitrogen isotopic ratio

The natural abundance of ^{15}N in plant tissues can be expressed as a $\delta^{15}N$ value, which allows us to estimate the sources of nitrogen flux in the aboveground and belowground portions of different species mixtures (Hogberg, 1997). The $\delta^{15}N$ value denotes the deviation in ‰ of the sample from the ratio of $^{15}N/^{14}N$ in atmospheric N_2, and is calculated with the equation (1) (Robinson, 2001):

$$\delta^{15}N = \left((R_{sample} - R_{atm})/R_{atm}\right) \times 1000 \qquad (1)$$

Where R_{sample} is the $^{15}N/^{14}N$ isotope ratio of the sample and R_{atm} is the $^{15}N/^{14}N$ ratio of atmospheric N_2 ($R_{atm} = 0.00366$). The $\delta^{15}N$ of atmospheric N_2 by definition is 0 ‰.

The $\delta^{15}N$ of a particular plant species can vary depending on the surrounding species mixture. The $\delta^{15}N$ of N_2-fixing legumes is usually much lower than that of other plant species, as it is close to the atmospheric $\delta^{15}N$ (around 0‰), since most of their N is derived from N fixation (Hogberg, 1997). The $\delta^{15}N$ of grasses growing without legumes is usually higher, reflecting the higher $\delta^{15}N$ of the soil (Shearer and Kohl, 1986). Thus, if the $\delta^{15}N$ of plants that do not fix N_2 (such as grasses) is lower when they are grown alongside N_2-fixing plants (such as legumes) than when they are grown alone, it indicates a transfer of fixed N_2 (Temperton et al., 2007).

2.4.6 Soil AM fungi diversity

The effect of plant assemblage composition and soil P concentrations on communities of arbuscular mycorrhizal fungi was analyzed from soil cores harvested from the same experimental design, and the results are reported in Klabi et al. (2015). The AM fungal diversity data from this paper were used in the present work to test for relationships between this variable and either plant dry matter and nutrient content. Briefly, total DNA was extracted from 0.26 g soil cores taken from the 0-15 cm soil depth of all plots except those fertilized with 50 kg/ha of P_2O_5. A 18S rRNA gene fragment was amplified using the

primer set AMV4.5NF and AMDGR (Sato et al. 2005) and then sequenced using one half sequencing plate on the 454 GS FLX Titanium sequencing platform (Roche, Branford, CT, USA). Quality processing of AM fungal 18S rRNA gene was performed in Mothur (v.1.31.2) (Schloss et al. 2011). The sequence data generated were deposited in the NCBI Sequence Read Archive and are available under the SRA project number SRP046836. Shannon diversity was calculated in order to compare AM fungi diversity between treatments. More details are available in Klabi et al. (2015).

2.4.7 Statistical analysis

Statistical analyses were conducted using JMP 9 software (SAS Institute Inc.). Treatment effects were analyzed separately at each time point. The normality of the data was tested using the Shapiro-Wilk test, and non-normal data were log-transformed prior to analysis. The effect of species mixture, soil P concentrations, and their interaction on total dry matter, nutrient uptake and nutrient concentration for the whole plant biomass at each sampling time were tested by 2-way ANOVA. Blocks were included as a random effect. ANOVAs were also performed separately for grass and legume dry matter in 2009. One-way ANOVA was used to compare the $\delta^{15}N$ and N concentration in the tissues of legume species to those in native grass mix tissues grown without legumes at each time of sampling in order to show if the legumes were actively fixing nitrogen. The effect of treatments on the nutrient concentration and $\delta^{15}N$ of individual species of native grasses, *B. biebersteinii,* and legumes and on the $\delta^{15}N$ of combined native grasses, was also tested by 2-way ANOVA. Overall plant dry matter was compared between different sampling times using one-way ANOVA. The significance of differences between means was tested with the Tukey's range test. However, $\delta^{15}N$ and the nutrient concentration of the introduced grass *B. biebersteinii* relative to the native grasses growing alone were measured in September 2008 only and were compared using the Student's t-test. Regression analysis was conducted to determine the relationship

between plant dry matter or nutrient content in different species mixtures with AM fungal diversity data published in Klabi et al. (2015) at each sampling time.

2.5 Results

2.5.1 Plant dry matter

Total dry matter was highest in July 2009 (4965 ± 443 kg ha^{-1}), and was higher at the end of the growing season in 2008 (2980 ± 132 kg ha^{-1}) than in 2009 (1887 ± 160 kg ha^{-1}) (Figure S1). Plant dry matter also varied by species mixture, but only in 2009 (Figure 1A). At the end of the growing season in 2008, a much wetter year, the dry matter of species mixtures with legumes was similar to the dry matter of the native grass mix grown alone. In 2009, which was the dry year, the mixtures containing *M. sativa* had the highest overall plant dry matter in July. However, by the end of the growing season, the dry matter of the native grass mix + *M. sativa* was similar to native grass mixes growing without legumes, while the dry matter of *B. biebersteinii* + *M. sativa* was higher.

When the dry matter of grasses was measured alone, the dry matter of the native grass mix was not affected by legumes in either July or September of 2009, and was not significantly different from the dry matter of *B. biebersteinii* (Figure 1B). The growth of *M. sativa* was always high, and significantly higher than that of *D. purpurea* (Figure 1B). There was no effect of soil P concentrations on forage dry matter.

2.5.2 Evidence of nitrogen fixation

The legume species growing in mixture had a higher N concentration and lower $\delta^{15}N$ (around 0‰) value than native grasses tissues growing without legumes (Table S2), indicating that the legumes were clearly different from grasses, and confirming active N fixation by legumes (Hogberg, 1997; Temperton et al., 2007). Additionally, soil P concentrations differentially affected the $\delta^{15}N$ value of the two legume

species in July 2009 (Table S3). High soil P reduced the $\delta^{15}N$ of *D. purpurea* compared to that of *M. sativa.* In contrast, the $\delta^{15}N$ of *D. purpurea* was higher than that of *M. sativa* grown with *B. biebersteinii* at low soil P concentrations.

2.5.3 N uptake, N concentration, and isotopic composition of $\delta^{15}N$

N concentration and N uptake by the total plant dry matter were influenced by species mixture in both years and were higher in the native grasses grown with *M. sativa* than with *D. purpurea* (Table 2). In 2009, the tissue N concentration of *M. sativa* was higher than that of *D. purpurea* at each sampling time, leading to the highest N uptake by forage plants over time. However, mixing grasses with a legume did not generally increase the total N uptake by grasses, although *M. sativa* did contribute to increased N concentration in combined native grass tissues by 19%, in July 2009. The $\delta^{15}N$ in native grass tissues was not reduced by the presence of this legume. In contrast the introduced grass tissues grown with *M. sativa* had lower $\delta^{15}N$ values compared to native grasses growing alone, but only in September 2008 and July 2009 (Table S4).

At the end of the growing season, most native grass species did not benefit from the presence of legumes, except for the cool-season grass *E. canadensis* and the warm-season grass *B. gracilis*, although this varied with year, soil P concentrations, and legume species (Table S5). In 2008 and at low soil P, the concentration of N in *E. canadensis* tissue was enhanced by 35% in the presence of *D. purpurea*, compared to that of this grass grown without legumes. This effect was not seen at the higher P levels or in 2009. In contrast, *M. sativa* enhanced the N concentration of *B. gracilis* tissues by 21%, irrespective of soil P fertility, in 2009 but not in 2008. The $\delta^{15}N$ estimated for different species of native grasses was not decreased by the presence of legumes at the end of growing season (Table S6).

2.5.4 P uptake and P concentration

In September 2008, total phosphorus uptake was influenced by the interaction between soil P concentrations and species mixture (Table 3). High soil P promoted total P uptake by species mixtures that contained *M. sativa* compared to that of native grasses mixes growing without legumes, but the concentration of P estimated in the tissues of those mixtures was similar among treatments. In July 2009, the highest P uptake and concentration were observed in mixtures containing *M. sativa,* irrespective of soil P concentrations, but this effect disappeared by the end of the growing season in the same year.

Among legume species, the P uptake by *M. sativa* was always higher than that of *D. purpurea* (Table 3). In September 2008, the P concentration in legumes growing with native grasses was higher than that of *M. sativa* grown with *B. biebersteinii,* and the high soil P fertility increased the P concentration in legume tissues. In contrast, the concentration of P in legume tissues was affected by the interaction of soil P fertility and species mixture in July 2009, but not in September of the same year. High soil P increased the concentration of P in *M. sativa* when grown with the native grass mix, while the concentration of P in *D. purpurea* was promoted when P was added at 50 kg/ha. In September 2009, the concentration of P in *D. purpurea* tissues grown alongside the native grass mix was higher than that of *M. sativa* grown alongside *B. biebersteinii* (Table 3).

In July 2009, *M. sativa* enhanced the concentration of P in the native grass mix (Table 4). In contrast, at the end of growing season, the P concentration of different native grass species was not affected by species mixture or soil P fertility, except for that of *B. gracilis* in 2009 (Table S4). In the tissues of this grass species, high soil P improved the concentration of P, irrespective of legume treatment. The concentration of P in *B. biebersteinii* grown with *M. sativa* was greater than that of the native grass mix grown alone in September 2008 and in July 2009, but not in September 2009 (Table 4).

2.5.5 Relationships between plant dry matter, nutrient content, and AM fungal diversity

Since AM fungal diversity was previously found to be higher in the mixture of legumes with the native grasses (Klabi et al., 2015), we examined the correlation between this variable and either plant dry matter and nutrient content. A positive relationship was found between AM fungal diversity and the dry matter and nitrogen uptake by the entire species mixture, particularly by the legume *M. sativa* in July 2009. AM fungal diversity was also positively correlated with the P concentration in native grass tissues when grown with *M. sativa* during the same period (Figure S2), but those effects were not observed in the mixture of native grasses and *D. purpurea,* or at the end of growing season in 2009.

Table 2 Nitrogen uptake and concentration in total dry matter, grass and legume dry matter, as influenced by species mixtures over the two years of sampling.

	Total dry matter		Grass dry matter		Legume dry matter	
September 2008[¥]	N uptake[±] (Kg ha^{-1})	N concentration (g kg^{-1})[±]	N uptake[±] (Kg ha^{-1})	N concentration[±] (g kg^{-1})	N uptake[±] (Kg ha^{-1})	N concentration[±] (g kg^{-1})
Native grasses	17.3 a	6.3 a	-	6.3 a	-	-
Native grasses + *M. sativa*	30.5 b	9.0 b	-	-	-	20.4 c
Native grasses + *D. purpurea*	17.4 a	6.8 a	-	-	-	9.5 a
B. biebersteinii + *M. sativa*	26.1 b	8.1 b	-	6.5 a	-	17.9 b
P value	**<0.001**	**<0.001**		0.376		**<0.001**
July 2009						
Native grasses	25.6 a	8.4 a	25.6 a	8.4 a		
Native grasses + *M. sativa*	100.4 b	15.8b	26.8 a	10.0 b	79.8 b	21.7 b
Native grasses + *D. purpurea*	24.1 a	8.5 a	23.7 a	8.4 a	0.3 a	17.5 a
B. biebersteinii + *M. sativa*	111.4 b	14.6 b	28.8 a	8.6 a	89.1 b	20.8 b
P value	**<0.001**	**<0.001**	0.231	**<0.001**	**<0.001**	**0.001**
September 2009						
Native grasses	9.2 a	6.6 a	9.2 a	6.6 a	-	-
Native grasses + *M. sativa*	22.5 b	10.0 b	7.7 a	7.2 a	15.6 b	13.2 b
Native grasses + *D. purpurea*	8.4 a	7.0 a	8.2 a	6.9 a	0.08 a	10.5 a
B. biebersteinii + *M. sativa*	25.6 b	9.3 b	8.4 a	6.4 a	18.5 b	12.9 b
P value	**<0.001**	**<0.001**	0.731	0.229	**<0.001**	**0.022**

[±]Within a column and a sampling time, means are not significantly different ($p \leq 0.05$, ANOVA; n = 12) when followed by the same letter (a, b, c) according to Tukey's range tests or Student's t-tests (N concentration for *B. biebersteinii* vs native grasses in 2008). Bolded values are significant**.**

[¥]In 2008, the total plant dry matter was estimated so the dry matter of grasses or legumes alone are not available, except for the nitrogen concentration in *B. biebersteinii* and legume species that was also determined separately.

Table 3 Phosphorus uptake and concentration in total dry matter and legumes dry matter as influenced by the interaction between species mixtures and soil P concentrations at different times of sampling.

		Total P uptake (kg ha^{-1})[±]				Total P concentration [±] (g kg^{-1})			
	Species mixtures	Low soil P	Middle soil P	High soil P	Mean	Low soil P	Middle soil P	High soil P	Mean
Sept. 2008	Native grasses	2.1 ax	3.6 ay	2.5 ax	2.7	1.0	1.0	1.2	1.1 a
	Native grasses + *M. sativa*	2.9 ax	3.6 ax	4.0 bx	3.5	0.9	1.1	1.1	1.0 a
	Native grasses + *D. purpurea*	3.1 ax	2.3 ax	2.7 abx	2.7	1.1	1.0	1.0	1.0 a
	B. biebersteinii + *M. sativa*	3.3 ax	2.9 ax	4.0 bx	3.4	1.1	1.0	1.2	1.1 a
	Mean	2.9	3.1	3.3		1.0 x	1.0 x	1.1 x	
July 2009	Native grasses	3.8	3.7	3.8	3.7 a	1.2	1.3	1.2	1.3 a
	Native grasses + *M. sativa*	6.4	10.9	9.2	8.9 b	1.3	1.3	1.6	1.4 b
	Native grasses + *D. purpurea*	3.9	3.1	3.4	3.4 a	1.2	1.2	1.3	1.2 a
	B. biebersteinii + *M. sativa*	10.9	8.3	11.9	10.4 b	1.3	1.3	1.5	1.4 b
	Mean	6.2 x	6.5 x	7.1 x		1.3 x	1.3 x	1.4 x	
Sept. 2009	Native grasses	1.5	1.6	1.5	1.6 ab	1.1	1.2	1.1	1.1 b
	Native grasses + *M. sativa*	1.8	2.0	1.7	1.8 ab	0.8	0.8	1.0	0.9 ab
	Native grasses + *D. purpurea*	1.3	1.3	1.1	1.3 a	1.0	1.0	1.2	1.1 b
	B. biebersteinii + *M. sativa*	2.3	2.0	1.8	2.0 b	0.8	0.1	0.8	0.8 a
	Mean	1.7 x	1.8 x	1.5 x		0.9 x	0.1 x	1.0 x	
		P uptake by legumes (kg ha^{-1})[±¥]				P concentration in legumes (g kg^{-1})[±¥]			
Sept. 2008	Native grasses + *M. sativa*	-	-	-		1.1	1.2	1.3	1.2 b
	Native grasses + *D. purpurea*	-	-	-	-	1.1	1.2	1.8	1.3 b
	B. biebersteinii + *M. sativa*	-	-	-	-	0.9	0.8	1.0	0.9 a
	Mean	-	-	-	-	1.0 x	1.0 x	1.4 y	
July 2009	Native grasses + *M. sativa*	2.9	6.9	4.3	4.7 b	1.2 ax	1.3 abxy	1.6 ay	1.4
	Native grasses + *D. purpurea*	0.02	0.02	0.03	0.02 a	1.3 ax	1.7 by	1.2 ax	1.4
	B. biebersteinii + *M. sativa*	5.5	2.8	6.7	5.0 b	1.1 ax	1.1 ax	1.3 ax	1.2
	Mean	2.8 x	3.3 x	3.7 x		1.2	1.4	1.4	
Sept. 2009	Native grasses + *M. sativa*	0.8	0.8	0.5	0.7 b	0.6	0.6	0.7	0.6 ab
	Native grasses + *D. purpurea*	0.003	0.005	0.009	0.006 a	0.7	0.6	0.9	0.7 b
	B. biebersteinii + *M. sativa*	0.8	0.5	0.7	0.6 b	0.5	0.4	0.6	0.5 a
	Mean	0.5 x	0.4 x	0.4 x		0.6 x	0.5 x	0.7 x	

[±]Means are not significantly different ($p \leq 0.05$, ANOVA, n = 4) when followed by the same letter within a column (a, b) or within a row (x, y), according to the Tukey's range tests. Sept.: September.

[¥]In 2008, the total plant dry matter was estimated so the dry matter of grasses or legumes alone are not available, except for the P concentration in legumes species that was determined separately.

Table 4 Phosphorus uptake and phosphorus concentration in total grass dry matter as influenced by species mixtures at different times of sampling.

	P concentration in grasses (g kg^{-1}) [±]			P uptake by grasses (kg ha^{-1})[±]		
Species mixtures	September 2008[¥]	July 2009	September 2009	September 2008	July 2009	September 2009
Native grasses	1.0 a	1.2 a	1.1 a	-	3.7 a	1.5 a
Native grasses + *M. sativa*	-	1.5 b	1.1 a	-	4.1 ab	1.2 a
Native grasses + *D. purpurea*	-	1.2 a	1.0 a	-	3.4 a	1.2 a
B. biebersteinii + *M. sativa*	1.2 b	1.6 b	1.1 a	-	5.4 b	1.4 a
P value	**0.022**	**<0.001**	0.876	-	**<0.001**	0.360

[±]Within each column, means are not significantly different ($p \leq 0.05$, ANOVA; n = 12) when followed by the same letter (a, b) according to Tukey's range tests or with the Student's t-test (*B. biebersteinii* vs native grasses in 2008). Bolded values are significant.

[¥]The P concentration in *B. biebersteinii* tissues was determined separately in 2008.

Table S1 Soil nutrients concentrations (PO_4-P, NO_3-N) measured at two soil depths (from 0 to15 cm and from 30-45 cm) as influenced by species mixtures and by soil P addtion in 2009.

Soil depths	0-15 cm		30-45 cm	
Soil nutrients	PO_4-P (mg kg^{-1})$^{\pm}$	NO3-N (mg kg^{-1})$^{\pm}$	PO_4-P (mg kg^{-1})$^{\pm}$	NO3-N (mg kg^{-1})$^{\pm}$
Species mixtures				
Native grasses	22.15 a	0.51 a	1.96 a	0.23 a
Native grasses + *M. sativa*	22.22 a	0.62 a	2.54 a	0.30 a
Native grasses + *D. purpurea*	24.36 a	0.61 a	2.36 a	0.30 a
B. biebersteinii + *M. sativa*	18.83 a	0.46 a	2.46 a	0.35 a
P value	0.129	0.312	0.398	0.098
Soil P concentration				
Low soil P	16.47 a	0.50 a	1.91 a	0.33 a
Middle soil P	20.52 b	0.60 a	2.76 a	0.31 a
High soil P	28.69 c	0.56 a	2.31 a	0.25 a
P value	**<0.001**	0.405	0.067	0.487

$^{\pm}$Within each column, means are not significantly different ($p \leq 0.05$, ANOVA; n = 12) when followed by the same letter according to Tukey's range tests. Bolded values are significant.

Table S2 Comparison of nitrogen isotopic ratio ($\delta^{15}N$) and N concentration in legumes species and those in native grasses mixes, when grown alone at different times of sampling.

		$\delta^{15}N^{\pm}$			N concentration (g kg^{-1}) $^{\pm}$		
Plant mixtures	Plant tissues	September 2008	July 2009	September 2009	September 2008	July 2009	September 2009
Native grasses	Grasses	5.6 b	11.7 b	2.3 b	6.3 a	8.4 a	6.6 a
Native grasses + *M. sativa*	*M. sativa*	-1.5 a	-1.7 a	-3.6 a	20.4 c	21.7 c	13.2 c
Native grasses + *D. purpurea*	*D. purpurea*	-0.3 a	-3.0 a	-4.8 a	9.5 b	17.5 b	10.5 b
B. biebersteinii + *M. sativa*	*M. sativa*	-1.2 a	-2.1 a	-2.5 a	17.9 c	20.8 c	12.9 c
P value		**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

$^{\pm}$Within each column, means are not significantly different ($p \leq 0.05$, ANOVA; n = 12) when followed by the same letter (a, b, c) according to Tukey's range tests. Bolded values are significant.

Table S3 Nitrogen isotopic ratio ($\delta^{15}N$) in legumes species as influenced by the interaction between species mixtures and soil P concentrations in July 2009 (n = 4; P = 0.002).

	$\delta^{15}N$[±]			
	P concentrations in soil (mg kg^{-1})			
Plant mixtures	Low soil P	Middle soil P	High soil P	Mean
Native grasses + *M. sativa*	-1.3 abx	-1.8 ax	-2.0 bx	-1.7
Native grasses + *D. purpurea*	-0.6 by	-3.7 axy	-4.6 ax	-3.0
B. biebersteinii + *M. sativa*	-2.4 axy	-1.7 ay	-2.5 bx	-2.2
Mean	-1.4	-2.4	-3.0	

[±]Means are not significantly different ($p \leq 0.05$, ANOVA) when followed by the same letter within a column (a, b) or within a row (x, y), according to the Tukey's range test.

Table S4 Nitrogen isotopic ratio ($\delta^{15}N$) in native grasses or *B. biebersteinii* dry matter as influenced by species mixtures at different times of sampling.

	Grasses dry matter		
	$\delta^{15}N$[±]		
	September 2008[¥]	July 2009	September 2009
Native grasses	5.6 b	11.7 b	2.3 a
Native grasses + *M. sativa*	-	16.9 b	4.7 a
Native grasses + *D. purpurea*	-	22.7 b	7.7 a
B. biebersteinii + *M. sativa*	3.2 a	2.3 a	3.4 a
P value	**0.008**	**<0.001**	0.140

[±]within each column, means are not significantly different ($p \leq 0.05$, ANOVA; n = 12) when followed by the same letter (a, b) according to Tukey's range tests or with the Student's t-test (*B. biebersteinii* vs native grasses in 2008). Bolded values are significant.

[¥]In 2008, the total plant dry matter was estimated so the dry matter biomass of grasses or legumes alone are not available, except for the $\delta^{15}N$ in *B. biebersteinii* tissues that was determined separately.

Table S5 Nutrients concentration in the tissues of *E. canadensis* and *B. gracilis* as influenced by the presence of legumes and soil P concentrations over two years of sampling.

September 2008	N concentration in *E. canadensis*[±] (g kg^{-1})			
	Low soil P	Middle soil P	High soil P	Mean
No legumes	4.9 ax	6.8 ay	5.5 axy	5.7
M. sativa	5.0 abx	5.4 ax	5.7 ax	5.4
D. purpurea	6.6 bxy	4.6 ax	7.1 ay	6.1
Mean	5.5	5.6	6.1	
September 2009	N concentratio in *B. gracilis*[±] (g kg^{-1})			
	Low soil P	Middle soil P	High soil P	Mean
No legumes	5.1	5.9	6.3	5.8 a
M. sativa	7.6	6.8	6.5	7.0 b
D. purpurea	6.1	5.7	6.9	6.2 ab
Mean	6.3 x	6.1 x	6.6 x	
September 2009	P concentration in *B. gracilis*[±] (g kg^{-1})			
	Low soil P	Middle soil P	High soil P	Mean
No legumes	0.8	1.1	1.1	1.0 a
M. sativa	1.2	1.2	1.2	1.2 a
D. purpurea	0.9	0.9	1.5	1.1 a
Mean	0.9 x	1.0 xy	1.3 y	

[±]Means are not significantly different ($p \leq 0.05$, ANOVA) when followed by the same letter within a column (a, b) or within a row (x, y), according to the Tukey's range test.

Table S6 Nitrogen isotopic ratio ($\delta^{15}N$) of individual native grass species, as influenced by the presence of legumes at different times of sampling.

	$\delta^{15}N^{\pm}$					
September 2008	EL+PS[¥]	ET	NV	EC	SS	BG
No legumes	29.5 a	29.5 a	15.5 a	14.2 a	32.3 a	28.1 a
M. sativa	16.5 a	25.9 a	18.3 a	20.2 a	23.5 a	35.7 a
D. purpurea	23.9 a	16.3 a	22.0 a	25.6 a	12.4 a	27.5 a
P value	0.236	0.387	0.571	0.575	0.613	0.304
September 2009	EL+PS[¥]	ET	NV	EC	SS	BG
No legume	5.9 a	6.3 a	5.2 a	6.9 a	6.8 a	7.0 a
M. sativa	11.8 a	5.0 a	5.1 a	6.5 a	19.2 b	8.1 a
D. purpurea	6.2 a	7.7 a	5.2 a	7.9 a	7.4 a	11.2 a
P value	0.914	0.863	0.661	0.479	**0.017**	0.760

[±]Within each column means are not significantly different ($p \leq 0.05$, ANOVA; n = 12) when followed by the same letter (a, b) according to Tukey's range tests.

[¥]EL+PS=*E. lanceolatus* ssp. *lanceolatus* + *P. smithii*; ET=*E. trachycaulus* ssp. *subsecundus*; NV=*N. viridula*, EC=*E. canadensis*; SS=*S. scoparium*; BG=*B. gracilis.* Bolded values are significant

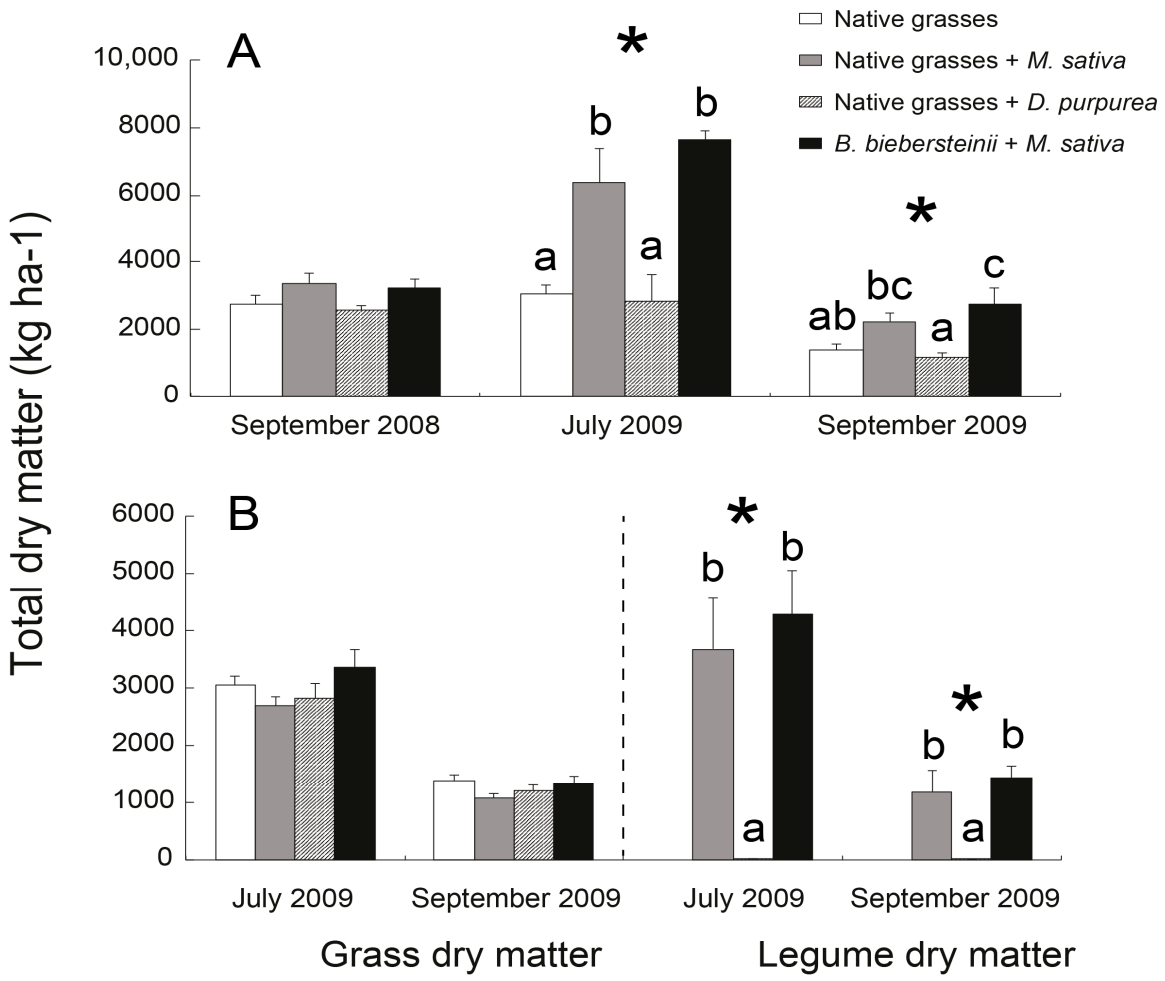

Figure 1 Total dry matter (A) measured in September in 2008 and 2009, and once in July 2009, and (B) the total dry matter of grasses or legumes measured separately in 2009, as influenced by species mixtures at each time of sampling. Significance was tested by ANOVA. Values are means ± SE, n=12. Significant differences ($p \leq 0.05$) between species mixtures at each time of sampling (*) are indicated by different letters over columns.

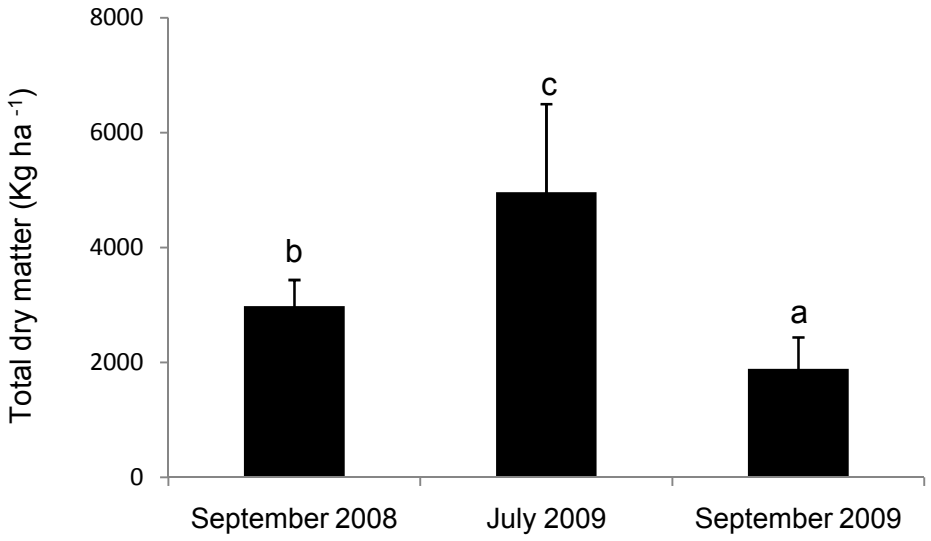

Figure S1 Total dry matter as influenced by different times of sampling ($P<0.001$). Significance was tested by ANOVA. Values are means ± SE, n = 48. Different letters over columns indicate significant differences according to a Tukey's range tests.

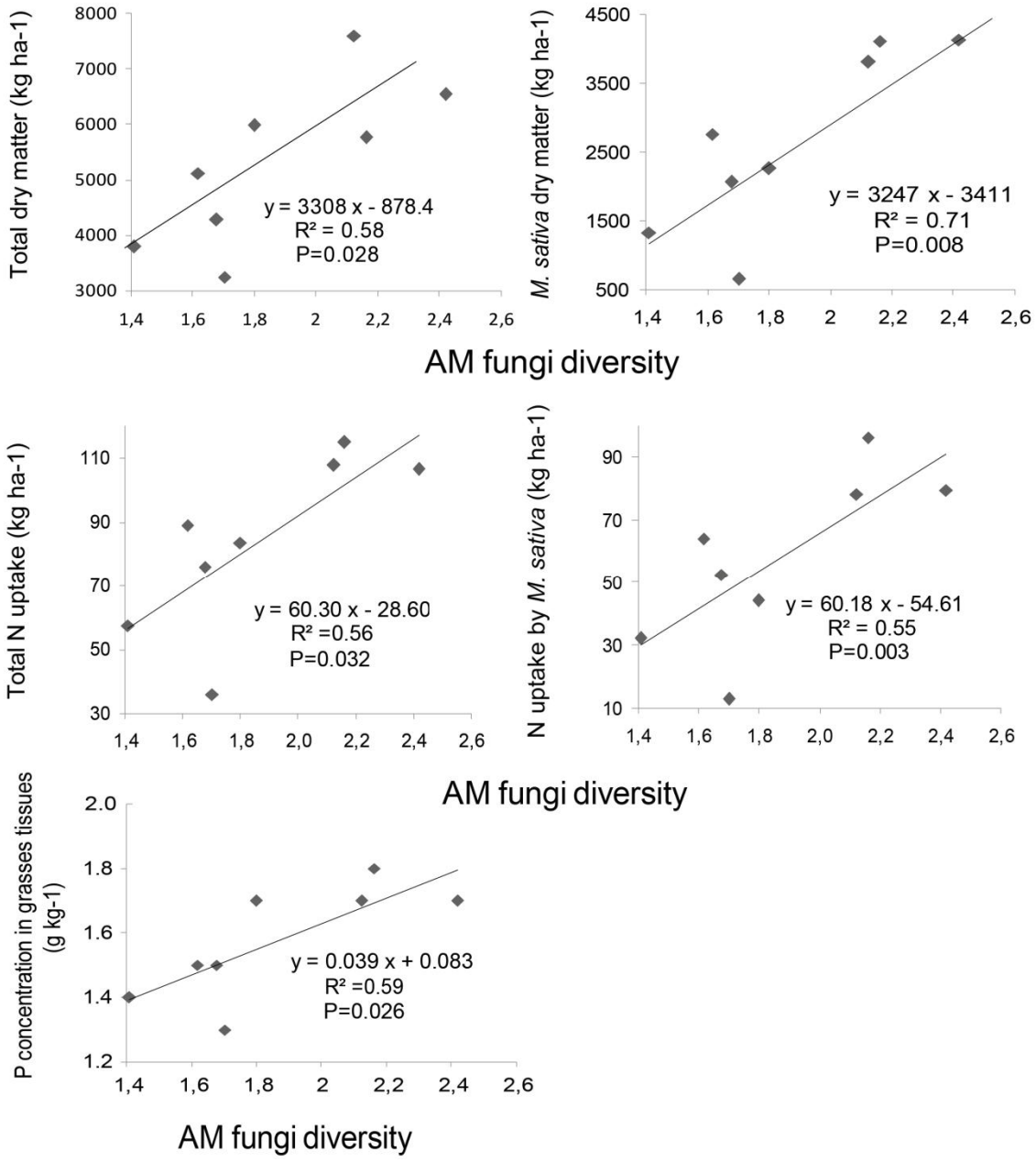

Figure S2 Significant relationships between total dry matter, legume dry matter, total N uptake, N uptake by *M. sativa* and P concentration in native grasses tissues, and AM fungi diversity in the mixture containing *M. sativa* and native grasses mixes in July 2009; points represent two concentrations of soil P (low soil P and high soil P).

2.6 Discussion

Our results show that the inclusion of *M. sativa* enhanced nitrogen and phosphorus concentrations in the tissues of the native grasses (all grasses measured together), early in the growing season of 2009. This indicates that *M. sativa* improved the nutritive value of combined native grasses early in the summer. Additionally, this legume appears to have the ability to improve the N concentration in the warm season grass *B. gracilis* tissues late in the summer of the drought year, 2009. Our results confirm those of others studies, which showed that the inclusion of legumes to native pastures may enhance the nutritive value of forage (Posler et al., 1993; Schellenberg and Banerjee, 2002). The native legume *D. purpurea* also increased N concentrations in the tissues of the cool season grass *E. canadensis* at the end of growing season, in the wet year of 2008, but only at low soil P. This effect was likely related to plant competitive interactions and soil moisture condition. The nitrogen ratio isotopic analysis showed that the N nutrition of native grasses grown with *M. sativa* was mostly dependent on high soil N sparing that was not consumed by legumes.

2.6.1 Legumes enhance overall plant dry matter, but not that of grasses

The inclusion of the cultivated legume *M. sativa* to mixtures containing the native grass mix or monocultures of the introduced grass *B. biebersteinii* led to dry matter that were similar to, or higher than, grasses grown in the absence of the legume. At the end of 2008, the dry matter of native grasses grown alongside *M. sativa* and without legumes was similar. In 2009, mixtures containing *M. sativa* were most productive early in the growing season, while the mixture of native grasses and *M. sativa* produced similar dry matter to native grasses grown without legumes and the dry matter of the *B. biebersteinii* and *M. sativa* was higher, at the end of the growing season. Our findings concur with previous findings showing that a mixture of *M. sativa* and either a native forage shrub (winterfat) or another shrub species (Gardner' s saltbush) yielded a forage

biomass that was higher than, or similar to, those shrub species grown alone or to *M. sativa* grown alone (Schellenberg and Banerjee, 2002). The high dry matter observed in species mixtures containing *M. sativa* appear to be mainly attributed to the vigorous growth of this cultivated legume, since native grasses dry matter was similar in the presence or absence of *M. sativa* in 2009, and the dry matter of the introduced grass *B. biebersteinii* was similar to that of native grasses mixes growing with or without legumes, when grasses and legumes were measured separately.

Total plant dry matter was higher at the end of the growing season in 2008 than in 2009. In 2008, precipitation and soil moisture levels were higher, which likely benefitted plant growth. In addition, plant dry matter was greater early in the growing season of 2009 than at the end of the growing season. In general, the native cool-season grasses and introduced grasses that are also cool-season species initiate their growth in April and their maximum production occurs early by July, but their productivity is reduced or they may have entered into dormancy in late summer, when soil moisture is not available for their growth. In contrast, the warm season species are more productive during the summer months and the peak production occurred by September because they are tolerant to drought conditions of semi-arid prairies (Abougendia, 1995; Cherney and Kallenbach 2007; Schellenberg et al., 2012). In the present study, the warm-season grasses (and the warm-season legume *D. purpurea*) did not help to sustain forage productivity at the end of the growing season three years after establishment, obviously due to the low abundance and establishment of some warm season species in the mixture (Appendix 1 and 2). In addition, the warm season species are charecterized by slow growth rate, witch lead to produce less biomass compared to cool season grasses.

2.6.2 Legume effects on nutrient uptake and concentration

The species mixtures that contained *M. sativa* had the highest N uptake and N concentration over time. This was mostly related to the higher N concentration within this legume, which may benefit it's growth, compared to *D. purpurea*. In addition, a positive relationship was found between AM fungal diversity and the dry matter of *M. sativa* and N uptake by this legume in early summer, and this correlation was higher in the native grass mix grown alongside *M. sativa* (Klabi et al., 2015). This suggests that high AM fungal diversity may have favoured soil N uptake by *M. sativa*, which could benefit the productivity of this legume when in competition with native grasses; alternatively this may be explained by enhanced legume P nutrition or increased availability of other soil nutrients that are important for nodule growth and nitrogen fixation activity, which could also be a product of AM fungi diversity (Barea et al., 1987; Azcón et al., 1991; Olivera et al., 2004; Scheublin et al., 2004; Divito and Sadras, 2014). Larimer et al. (2014) showed that inoculation with AM fungi or rhizobia independently improved the nitrogen content of the native legume *Amorpha canescens*, whereas the interactive effect of the two symbionts was even more effective. In addition, Wagg et al. (2011) showed that following manipulation of AM fungal diversity, high AM fungal diversity (four species) enhanced the biomass of the legume *Trifolium pratense* when in competition with the grass *Lolium multiflorum.* AM fungi may help to increase the biomass of *M. sativa* by increasing its drought tolerance when soils were not too dry in semi-arid regions. It has also been shown that AM fungi can enhance the nutrition of plant communities in semi-arid grasslands under conditions of moisture sufficiency in early summer (Yang et al., 2010). Here, *M. sativa* also increased the P uptake by whole species mixtures but only at the end of the growing season in the wet year (2008) when soil P was high, and at the vegetative growth stage in 2009, irrespective of soil P. This was likely driven by higher *M. sativa* biomass production and by the higher P uptake by this legume relative to *D. purpurea*, when soil moisture was less limiting to plant production.

In contrast, *M. sativa* increased the nutrient concentration (N and P) of total native grass tissues compared to those grown without legumes, but only early in the growing season of 2009. The higher N concentration of native grasses mixes in presence of *M sativa* may also contribute in part to ameliorate the N concentration of total forage, in early summer. In addition, this increase in nutrient concentration did not result in an increase in total nutrient uptake. It appears that early in the season, grass productivity was not limited by N and P availability, but rather by light, water and other soil nutrients. The higher nutrient concentrations in the tissues of native grasses when *M. sativa* was present in July 2009 contributed to improving the nutritive value of native grass herbage, when most grass species (cool-season grasses) were active, early in the summer.

Increased N concentrations in native grass tissues when *M. sativa* was present (without a reduced $\delta^{15}N$ value in grass tissues), indicates that the N nutrition of the native grasses was related to additional soil N (N sparing) that was not used by *M. sativa*, since this legume relies more on fixed N than soil N. A previous study showed that soil N sparing that was not consumed by legumes can play an important role in improving the N concentration in neighbouring plant species since legumes are less competitive for soil N than non-legume species (Temperton et al., 2007). Additionally, it has been suggested that the roots of N-fixing legumes may secrete acid phosphatase enzymes that improve soil P availability, which can enhance the P nutrition of neighbouring plants (Li et al., 2007; Houlton et al., 2008). As with *M. sativa* biomass, P concentration measured in native grass tissues in early summer was positively correlated with AM fungal diversity in the mixture of *M. sativa* and native grasses (Klabi et al., 2015). This may suggest that the contribution of *M. sativa* to the increased P concentrations in native grass mixes may be related to the higher AM fungal diversity promoted by this legume, since AM fungi are known to improve plant P nutrition (Smith and Read, 2008).

High soil P reduced the $\delta^{15}N$ of *D. purpurea* tissues early in the growing season of 2009, likely indicating that this warm-season legume had high P requirements for carrying out N fixation

(Hayat et al., 2008). High soil P availability is considered to be important for promoting nodule growth and nitrogenase activity (Olivera et al., 2004; Schulze and Drevon, 2005; Divito and Sadras, 2014). The increase in nitrogen fixation by this legume at high soil P may suggest that the growth and proliferation of this legume in species mixtures was limited by P availability.

At the end of the growing season, the presence of legumes contributed to increased N concentrations in a few individual native species, but this varied based on year, legume species, and soil P fertility. At low soil P fertility, the warm-season legume *D. purpurea* increased the N concentration in the tissues of the cool-season grass *E. canadensis* in the wet year (2008), while this effect was not observed at high soil P or in 2009. It seems that the accumulation of N in the tissues of *E. canadensis*, in the presence of *D. purpurea* at low soil P, occurred when the abundance of this grass was P-limited. In addition, the influence of soil nutrient availability on the N nutrition of this grass within communities may also depend on competitive interactions between plant species for soil nutrients. However, the low soil P availability could reduce the nitrogen fixation of *D. purpurea*, which might increase the competition between this legume and *E. canadensis* for soil N source, since *D. purpurea* might became more dependent on soil N uptake that was favoured by the grass *E. canadensis*. In addition, the growth of cool season grass may have reinitiate in the fall of the wet year 2008, when soil moisture became available, and thus may have lead to increase its competition for soil N, in the mixtures. It has been shown that the effect of soil N availability on plant N nutrition depends on others factors, such as soil P availability and plant competition (Aerts and Chapin III, 1999; Güsewell, 2004; Blanke et al., 2012). In the drought year of 2009, the warm-season grass *B. gracilis* benefitted from increased N concentration in its tissues when grown with *M. sativa*. This warm-season grass may then contribute to increasing the nutritive value of forage during the hottest periods of summer, when *M. sativa* was present. This result is in agreement with Schellenberg et al. (2012), who showed that the inclusion of warm-season grasses in a mixture containing cool-season grasses can enhance the nutritive

value of forage by increasing its protein content during late summer periods in semi-arid regions. In addition, the improvement of N concentration in *B. gracilis* tissues when *M. sativa* was present at the end of growing season, without affecting the $\delta^{15}N$ value of this grass, indicates that the N nutrition of the native species was related to soil N sparing, since the N nutrition of legumes was based more on fixed N. The warm-season grass (*B. gracilis*) also had a higher P concentration in its tissues, when soil P was high. This suggests that the abundance of this warm-season grass is more limited by nutrient availability than water availability in late summer. However, warm-season grasses are highly drought-resistant (Stout, 1992). These two native grass species (*E. canadensis* and *B. gracilis*) responded differently to the addition of legumes or phosphorus at plant maturity, and this may depend on competitive interactions between native species, legume identity, water availability, and/or climatic variation in native grasslands.

In contrast to native grasses, the $\delta^{15}N$ value of the introduced *B. biebersteinii* was lower when grown with *M. sativa* at the end of growing season in 2008 and in July 2009. This suggests that this grass was able to directly use the fixed N that was transferred from this legume for its own N nutrition. The introduced grasses and native grasses then acquired N from different soil sources or forms that contained different isotopic compositions, when co-grown with the legume *M. sativa.* This could be related to the different morphology and requirements of these grasses, especially the introduced grass, which has a deeper root system compared to the typically shallow-rooted native grass species. In addition, the concentration of P was higher in the tissues of the introduced grass than those of the native grasses when grown alone at the end of the growing season in 2008, and also at the vegetative growth phase of 2009. This may be due to higher nutrient demand by this introduced grass, under conditions of moisture sufficiency, since those effects were not observed at the end of the growing season in the drought year of 2009, compared to the wetter year of 2008. This may rely in part on the fact that this introduced species was

limited more by water availability than soil nutrients during the hotter portion of mid-summer, and may even have entered into dormancy under the extreme drought conditions of 2009.

2.7 Conclusions

Our study shows that the inclusion of the cultivated legume *M. sativa* in forage grasslands can increase herbage and nitrogen uptake, especially under water-limiting conditions of 2009. This suggests that *M. sativa* is highly adapted to the climate and soil of semi-arid pastures, whereas *D. purpurea* was less productive due to the naturally slow establishment, at the time of seeding. In addition, *M. sativa* helped to improve the nutritive value of native grass mixes by increasing the concentration of nutrients in their tissues (N and P) in July 2009 (a drought year) and the N concentration of the warm-season grass *B. gracilis* at the end of the growing season in 2009. Inclusion of *D. purpurea* may have contributed to increased N concentrations in the tissues of the cool-season grass *E. canadensis* in late summer of 2008 (the wet year), but this likely depended on others factors such as soil P fertility, soil moisture and plant competiton. The N nutrition of native grasses grown with *M. sativa* was essentially dependent on high soil N sparing, which is the N not used by the legumes since most of their N requirements are met by fixed N_2. High soil P seems to be beneficial for the nutrition and growth of warm-season species (*D. purpurea* and *B. gracilis*) under different conditions. *M. sativa* appears to be an effective legume for promoting high nutritive value of forage in seeded no-input native pastures early in the summer, and also of the warm-season *B. gracilis* in seeded mixtures under severe drought conditions. However, a longer field trial may be needed to test the effect of *M. sativa* on the productivity and nutritionnel content of native grasses species in well established pastures, in particulary late in the summer. It also appears important to test the true impact of *D. purpurea* on native forage production and nutrition, since the establishment of this legume was extremely slow, and the abundance of this native legume increases with time.

2.8 Acknowledgments

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Chapitre 3: Plant assemblage composition and soil P concentration differentially affect communities of AM and total fungi in a semi-arid grassland

Ce chapitre a fait l'objet de la publication suivante dans une revue scientifique internationale:

Klabi R, TH Bell, C Hamel, MP Schellenberg, A Iwaasa, A Raies, M St-Arnaud. 2015. Plant assemblage composition and soil P concentration differentially affect communities of AM and total fungi in a semi-arid grassland. FEMS Microbiology Ecology 91: Online 2014-12-05 (HYPERLINK "http://dx.doi.org/10.1093/femsec/fiu015" dx.doi.org/10.1093/femsec/fiu015).

Les résultats de ce chapitre ont aussi été présentés dans les conférences suivantes:

*Rim Klabi, Terrence H. Bell, Chantal Hamel, Alan Iwaasa, Michael P. Schellenberg, Aly Raies, and Marc St-Arnaud. Legume planting and phosphorus addition differentially affect non-AM fungi and AM fungi communities in a semi-arid grassland. In 50th Canadian Botanical Association (CBA) conférence, Institut de recherche en biologie végétale, Montréal, QC. 15-18 juin 2014.

*Rim Klabi, Chantal Hamel, Alan Iwaasa and Aly Raies. Seasonal variation and temporal dynamics of arbuscular mycorrhizal (AM) and non-AM endophytes in different plant communities of native forage stands. In Canadian Society of Soil Science & Canadian Society of Agronomy Conference, University of Saskatchewan, Saskatoon, SK. 20-24 June 2010.

3.1 Préface

Dans le chapitre précédent, on a déterminé l'effet de l'inclusion de légumineuses fixatrices d'azote et de la fertilité en phosphore du sol sur la productivité et la valeur nutritive des graminées indigènes. Les résultats ont montré que la présence de *M. sativa* a augmenté les teneurs en nutriments (N et P) des tissus des graminées indigènes au début de l'été, ainsi que la teneur en azote des tissus de la graminée de saison chaude *B. gracilis* à la fin de l'été en condition de sécheresse intense. Par contre, l'augmentation de la fertilité en phosphore du sol n' a pas influencé la productivité des plantes. Dans ce chapitre, il nous apparaissait important d'étudier l'influence de ces pratiques sur la diversité et la structure des populations des champignons mycorhiziens à arbuscule et des champignons totaux, qui peuvent en retour avoir un impact sur la productivité et la nutrition du fourrage. Les données sur la diversité des communautés fongiques présentées dans ce chapitre ont aussi été utilisées au chapitre 2 pour déterminer que le changement de diversité des CMA dans le mélange de *M. sativa* et de graminées indigènes était positivement corrélé avec la productivité et la nutrition azotée de la légumineuse *M. sativa,* ainsi qu'avec la teneur en phosphore trouvée dans les tissus des graminées indigènes au début de l'été.

3.2 Abstract

Adding inorganic P- and N-fixing legumes to semi-arid grasslands can increase forage yield, but soil nutrient concentrations and plant cover may also interact to modify soil fungal populations, impacting short- and long-term forage production. We tested the effect of plant assemblage (seven native grasses, seven native grasses + the cultivated N-fixing legume *Medicago sativa*, seven native grasses + the native N-fixing legume *Dalea purpurea* or the introduced grass *Bromus biebersteinii* + *M. sativa*) and soil P concentration (addition of 0 or 200 P_2O_5 kg ha^{-1} at sowing) on the diversity and community structure of arbuscular mycorrhizal (AM) fungi and total fungi over two consecutive years, using 454-pyrosequencing of 18S rDNA and ITS amplicons. Treatment

effects were stronger in the wet year (2008) than the dry year (2009). The presence of an N-fixing legume with native grasses increased AM fungal diversity in 2008 and 2009, while the interaction between soil P concentration and plant assemblage modified total fungal community structure in 2008. Excluding interannual variations, which are likely driven by moisture and plant productivity, AM fungal communities in semi-arid grasslands appear to be primarily affected by plant assemblage composition, while the composition of other fungi is more closely linked to soil P.

Keywords: plant assemblage; phosphorus; AM fungi; total fungi; communities; semi-arid grassland; 454-pyrosequencing

3.3 Introduction

Arbuscular mycorrhizal (AM) fungi and non-AM fungi colonize plant roots, and represent a large proportion of all soil and rhizosphere fungi in prairie grasslands (Jumpponen and Trappe, 1998; Mandyam and Jumpponen, 2008; Khidir et al., 2010; Porras-Alfaro et al., 2011). Typically, AM fungi confer nutritional benefits to their host plant by facilitating plant uptake of phosphorus (P) and other nutrients (e.g. Zn, N, etc.), while plants reciprocate by supplying carbon (C) and energy to their fungal partners (Smith and Read, 2008; Smith and Smith, 2012). The diverse assemblages of endophytic fungi in plant roots and the surrounding soil are usually referred to as dark septate endophytes (DSE), and form part of the non-AM fungal community (Porras-Alfaro et al., 2008; Porras-Alfaro et al., 2011). Non-AM fungi such as DSE are known to transform organic forms of nutrients to inorganic forms that are plant available (Mandyam and Jumpponen, 2005; Upson et al., 2009; Mandyam et al., 2010), and can increase plant resistance to pathogen attacks, drought and heat (Mandyam and Jumpponen, 2005).

Both AM and non-AM fungi can influence the diversity and productivity of plant communities in semi-arid regions. These groups may interact directly with plants, forming parasitic, mutualistic or commensal relationships (van der Heijden et al., 1998; Schulz and Boyle, 2005; van der Heijden and Scheublin, 2007; Mandyam et al., 2012). The nature of these relationships can vary between plant species (van der Heijden et al., 1998; Kageyama et al., 2008) and is dependent on nutrient availability (Bethlenfalvay et al., 1983; Johnson, 2010). Recently, Rillig et al. (2014) demonstrated that interactions between AM and non-AM fungi may influence plant community structure by shifting the relative abundance of plant species, and as a corollary, plant productivity.

Plant identity may also influence fungal communities, and AM fungi in particular. Scheublin et al. (2004) determined that leguminous and non-leguminous species harbored distinct AM fungal communities in a natural dune grassland. Legumes may preferentially associate with certain AM fungal species that are particularly effective at acquiring P, or other nutrients that are essential to N-fixation, while other AM fungal taxa may simply be attracted to the high N concentrations found in legume nodules (Scheublin and van der Heijden, 2006). Few studies have focused on the effects of plant identity on the structure of non-AM fungal communities in arid regions, although Khidir et al. (2010) demonstrated that different grass species shared the same cohort of root endophytic fungi, and this cohort was distinct from that observed in non-grass species such as *Yucca glauca*.

In the prairie ecozone, the addition of P-fertilizer and/or legumes to pastures composed of warm- and cool-season native grass species can be essential to plant establishment and forage yield (Tilman et al., 2001; Schellenberg and Banerjee, 2002; Temperton et al., 2007; Nyfeler et al., 2009). Legumes may increase soil nitrogen (N) as a by-product of their symbiotic associations with N-fixing bacteria (Mosier, 2002), which can increase the productivity of neighboring plant species (Temperton et al., 2007). Planting legumes or modifying P concentrations within grass

assemblages may also directly influence soil fungal communities, and as a consequence, plant-fungal interactions. Plants growing in extremely fertile environments may not require symbiotic fungal partners to obtain essential nutrients. In such cases, they may reduce C allocation to AM fungi, thus decreasing the abundance of these obligate biotrophs (Kiers et al., 2011; Smith and Smith, 2012). The abundance of AM fungi following N influx may also depend on soil P availability. High soil N:P ratios increase plant C allocation to AM fungi, and Johnson et al. (2003) observed that high N inputs to P-deficient soils increased the growth of AM fungal hyphal networks in semi-arid grasslands, while the addition of N to soil with sufficient P reduced hyphal development. In contrast, non-AM fungi do not acquire C through a plant-fungal interface specialized for nutrient exchange (Peterson et al., 2008) and, as a result, they may be differentially affected by increased nutrient availability. Chagnon and Bradley (2013) recently showed that elevated soil P had no effect on non-AM fungi when AM fungi were absent from roots, but when AM fungi were present, increasing soil P increased non-AM fungi colonization. This suggests that interactions between AM and non-AM fungi can be modulated by soil P. Recent studies have also shown that N addition to grasslands did not alter the structure or richness of fungal endophytic communities in rhizospheric soil or the degree of root colonization by non-AM fungi (Mandyam and Jumpponen, 2008; Porras-Alfaro et al., 2011). Nitrogen addition has also been shown to either increase or decrease endophytic fungal diversity, depending on plant response to N fertilization (Dean et al., 2013).

In this study, we conducted a field experiment in semi-aride prairies to understand how soil P concentration and grassland plant composition, in particular the presence or absence of N-fixing legumes, affect the structure and diversity of fungal populations. We examined four plant assemblage treatments: (1) a mix of seven grass species that are native to the region, (2) the native grass mix grown with the cultivated N-fixing legume *Medicago sativa*, (3) the native grass mix grown with the native N-fixing legume *Dalea purpurea* and (4) the introduced grass *Bromus*

biebersteinii grown with *M. sativa.* The mix of *B. biebersteinii* and *M. sativa* is commonly used in cultivated semi-arid zones of the Canadian Prairies. These plant assemblage treatments were crossed with P fertilization, with each plant assemblage receiving either 0 or 200 kg ha^{-1} of P_2O_5 as triple super-phosphate at seeding, two years prior to this experiment. We harvested soil samples in two consecutive years (2008 and 2009), extracted total DNA, used PCR amplification to target either the AM fungal community (18S rDNA) or total fungal community (ITS) and sequenced these amplicons using 454-FLX Titanium technology. In forage stands, the inclusion of N-fixing legumes and/or the addition of P fertilizer are common practices to reduce N and P limitations on plant yield. However, these treatments may also modify soil fungal diversity and function. We hypothesized that soil P concentration and the presence of legumes in grassland soils would differentially affect assemblages of AM fungi and total fungi. Specifically, we tested (1) the effect of soil P concentration on the root colonization, diversity and community structure of soil AM fungi and of other fungi, (2) the effect of plant assemblage composition on the same parameters, (3) whether a native plant species assemblage promoted increased fungal diversity relative to assemblages that included cultivated or non-native forage plant species, and (4) whether the response of AM fungi and total fungi varied over two consecutive years.

3.4 Materials and methods

3.4.1 Site description

This study was conducted in a field experiment located at the South Farm of the Semiarid Prairie Agricultural Research Centre in Swift Current, Saskatchewan, Canada (50°16′ N 107°44′ W) that was seeded in 2006. The site had been planted with barley (*Hordeum vulgare* L.) in 2005. The soil is a loamy Brown Chernozem containing 9.23 mg kg^{-1} soil of NO_3^--N, 26.63 mg kg^{-1} soil of PO_4^--P and 282.85 mg kg^{-1} soil of K at seeding. The mean temperature at the site during the growing season (1 April-30 September) was 12.7 °C in 2008 and 13.3 °C in 2009. The 2008

growing season was characterized by above-normal precipitation (358.8 mm vs the 1981-2010 average of 269.4 mm), whereas precipitation in 2009 (182.5 mm) was below normal, according to a weather station located at less than 1000 m from the study site (Figure 1).

3.4.2 Experimental design

Treatments were arranged in a 4×2 (plant assemblage x P concentration) factorial design in four complete randomized blocks, giving a total of 32 plots. Each plot was 5.48×7.32 m, and was separated from other plots by a distance of 1.83 m in all directions. Plant assemblages were composed of (1) a mix of seven native grass species, (2) the same native grass mix plus the cultivated legume *M. sativa* L. (alfalfa), (3) the same native grass mix plus the native legume *D. purpurea* Vent. (purple prairie clover), and (4) the introduced forage grass *B. biebersteinii* Roem. & Schult. (meadow brome) plus *M. sativa*. The native grass mixture included five cool-season species, *Elymus canadensis* L. (Canada wildrye), *E. trachycaulus* (Link) Gould ex Shinners ssp. *subsecundus* (Link) (awned wheatgrass), *Pascopyrum smithii* (Rydb.) Barkworth & D.R. Dewey (western wheatgrass), *E. lanceolatus* (Scribn. & J.G. Sm.) Gould ssp. *lanceolatus* (northern wheatgrass), *Nassella viridula* (Trin.) Barkworth (green needlegrass) and two warm-season species, *Bouteloua gracilis* (Kunth.) Lag. ex Griffiths (blue grama) and *Schizachyrium scoparium* (Michx.) Nash (little bluestem). The native grass and legume seeds were developed by Agriculture and Agri-Food Canada for Ducks Unlimited Canada Inc., and were provided by Ducks Unlimited Canada Inc. (Saskatchewan Provincial Office).

Phosphorus fertilizer, in the form of triple super-phosphate (P_2O_5), was applied at either 0 or 200 kg P_2O_5 ha^{-1}, and was incorporated into the top 10 cm of soil by raking at the time of plant establishment in 2006 (i.e. two years prior to initial sampling), to establish the low- and high-soil P treatments. Soil samples were taken from all plots from the top 15 cm of soil, and plant available P was determined by extracting 2.5 g soil with $NaHCO_3$ (Olsen et al., 1954). Measurements in

2009 of 16.5 ± 2.9 and 28.7 ± 8.8 mg kg^{-1} soil of available P in the low- and high-P plots respectively, showed that the P fertilization treatments applied in 2006 created two different ($P < 0.001$) concentrations of soil P that persisted over the experimental period. Additionally, available soil P, as measured in 2009, was not affected by plant assemblage composition (data not shown).

3.4.3 Root and soil sampling

Roots and soil were sampled from each plot on 25 August in 2008 and 2009, using a soil corer (5 cm Ø × 15 cm length). Two core samples were randomly taken from the top 15 cm of soil in each plot and pooled to produce a single composite sample for each plot. Roots were manually separated from the soil, washed, cleared in boiling 10% KOH solution for 10 min, stained in a 5% black ink-vinegar solution (Vierheilig et al., 1998) within a day of sampling, and the percentage of colonization by AM and non-AM fungi was determined under a dissecting microscope at 400×, using the gridline-intersect method (Giovannetti and Mosse, 1980). The soil was stored in small plastic bags at -20 °C prior to molecular analysis.

3.4.4 Soil DNA extraction, PCR amplification and pyrosequencing analysis

Total DNA was extracted from 0.25 g soil samples using a MoBio PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. A nested PCR was used to target AM fungi through amplification of an 18S rDNA fragment. In a first round of PCR, each DNA sample was diluted 1/10 and amplified separately with the universal fungal primer pair NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') (White et al., 1990). In a second round, PCR products from the first round were diluted 1/100 and amplified using the primer set AMV4.5NF (5'-AAGCTCGTAGTTGAATTTCG-3') and AMDGR (5'-CCCAACTATCCCTATTAATCAT-3')

(Sato et al., 2005; Dai et al., 2012) that were coupled with one of 32 unique tags from the extended MID set recommended by Roche Diagnostics (Roche, 2009), as well as the required 454 sequencing adaptors. These primers are known to amplify ~300 bp fragments of AM fungal 18S rDNA from the four known AM fungal orders, which are the *Glomerales*, *Diversisporales*, *Archaeosporales* and *Paraglomerales* (Lumini et al., 2010). PCR reactions were performed in 20 µL volumes containing 16 µL of Platinum® PCR SuperMix High Fidelity (Invitrogen, Life Technologies), 2.6 µL of ddH_2O, 0.2 µL of each primer (20 pmol μl^{-1}) and 1 µL of extracted DNA. Conditions for the first PCR reaction were: 3 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 51°C and 1 min at 72°C, and a final elongation step of 7 min at 72°C. The second PCR round consisted of 10 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, with a final elongation step of 9 min at 72°C.

The primer set ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') was used to amplify a ~400 bp fragment of the fungal ITS region (Buée et al., 2009). These primers also contained the required 454 sequencing adaptors, and unique MID tags. PCR reactions were performed in final volumes of 20 µL, containing 16 µL of PCR SuperMix High Fidelity (Invitrogen, Life Technologies), 2.6 µL of ddH_2O, 0.2 µl/L of each primer (20 pmol μL^{-1}) and 1 µL of extracted DNA. The PCR conditions were 5 min at 94°C, 35 cycles of 30 s at 94°C, 45 s at 55°C and 1 min at 72°C, and a final elongation step of 10 min at 72°C. PCR products were verified using agarose gel electrophoresis (1.0% w/v agarose), purified using the QIAquick PCR Purification Kit (Qiagen), quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Life Technologies), combined into pools of 32 samples, and sequenced at the Laboratory for Advanced Genome Analysis in Vancouver, British Columbia, using one half sequencing plate on the 454 GS-FLX Titanium sequencing platform and using lib-A chemistry (Roche, Branford, CT, USA).

3.4.5 Sequence classification and OTU analysis

AM fungal 18S rDNA and ITS pyrosequencing data were processed in Mothur (v.1.31.2) (Schloss et al., 2011). Sequence reads were initially filtered for length and quality using a 50 bp moving window, with the command 'trim.seqs' and the following parameters: maxambig = 0, maxhomop = 8, bdiffs = 1, pdiffs = 2, qwindowaverage = 30, qwindowsize = 50, minlength = 200. Reads were reduced to unique sequences for further analysis with the 'unique.seqs' command. The AM fungal sequences were aligned to the Mothur implementation of the Silva eukaryotic database using 'align seqs' (ksize = 9, align = needleman, gapopen = -1). Sequences that aligned poorly were discarded to retain only an overlapping region for all AM fungal sequences with 'screen.seqs' (start = 15960, optimize = end, criteria = 90) and 'filter.seqs' (vertical = T, trump = .). To further eliminate sequencing error and chimeric sequences, we used the 'pre.cluster'(diffs = 2),'chimera.uchime' and 'remove.seqs' commands. In addition, sequences that did not belong to the phylum *Glomeromycota* were removed with 'classify.seqs' [using the Silva eukaryotic database, but with the AM fungal 18S rRNA database provided by Krüger et al. (2012) and the 'get.lineage' command (taxon = Eukaryota; Fungi; Glomeromycota)]. This is required since the primers used are not specific for AM fungal amplification, and can amplify 18S rDNA sequences from other phyla. A distance matrix for AM fungi was generated using 'dist.seqs' and operational taxonomic units (OTUs) were formed with average-neighbor clustering at 97% similarity using the 'cluster.split' command. The most abundant representative sequences from each OTU were generated using the 'get.oturep', 'classify.otu', and 'create database' commands, and the AM fungal taxonomic position of each OTU was identified by BLASTing consensus sequences against the MaarjAM database (http://maarjam.botany.ut.ee).

Fungal ITS sequences were processed as above, with the following differences. Following initial trimming, unaligned fungal ITS sequences were reduced to only the first 199 bases using 'chop.seqs', and chimeric sequences were removed using 'chimera.uchime'. ITS sequences were

pre-clustered at 99% similarity and clustered into OTUs at 97% similarity with CD-HIT (Li and Godzik, 2006) using the cd-hit-est command, with a word size of eight nucleotides and default parameters. In addition, the ITS fungal sequences were classified against the UNITE/QIIME 12_11 ITS reference database (alpha release, accessed February 2013) which was reformatted for Mothur. Taxonomic affiliation of representative ITS sequences for OTUs were assigned using the UNITE database (http://unite.ut.ee) or NCBI database (http://www.ncbi.nlm.nih.gov).

The sequence data generated in this study will be deposited in the NCBI Sequence ReadArchive and are available under the SRA project number SRP046836.

3.4.6 Statistical analysis

Statistical analyses were performed using the statistical language R v.3.0.2. (http://www.R-project.org), except where otherwise indicated. Treatment effects were analyzed separately for 2008 and 2009. We tested the effects of plant assemblage composition and soil P concentration as fixed effects (using blocks as random effects) on the community structure of AM and total fungi in 2008 and 2009 with a PERMANOVA on community Bray-Curtis distances, using the 'adonis' function in 'vegan'. The AM and total fungal datasets were additionally normalized using the Hellinger transformation prior to all PERMANOVA analysis. A principal coordinate analysis (PCoA) was used to visualize treatment effects on community composition. PCoA ordinations were calculated using the 'pcoa' function in Mothur and were visualized in R. All singleton sequences were removed from the datasets, and the number of sequences per sample was reduced to 81 reads for AM fungi, and 374 reads for total fungi using the `subsample' command in Mothur, to create equally sized communities for each sample. For AM fungal community analysis, we used only three blocks in 2008, due to the low number of AM fungal sequences in some samples. The effect of treatments on the relative abundance of AM fungal families and the relative abundance of total fungal classes and orders was tested by PERMANOVA analysis. The

significant effects detected by PERMANOVA were revealed by two-way ANOVA with blocks as random effects, to determine which fungal groups were affected. The relative abundance of AM and total fungi at each taxonomic level was calculated as the number of reads corresponding to a specific taxonomic group divided by the total number of reads in that sample. The impact of treatments on Shannon diversity and root colonization estimates were also tested by two-way ANOVA. Mean differences were tested for significance with the Tukey's range test using JMP 8 software. We also compared communities that were reduced to the 20 most abundant AM fungal OTUs or the 50 most abundant total fungal OTUs between treatments using UPGMA clustering. The hierarchical clustering results were visualized with a heatmap of OTU abundance data using 'heatmap.2' from the library 'gplots' in R. Distribution patterns of dominant AM or total fungal taxa across treatments were analyzed with an indicator species analysis using the 'multipatt' function in the 'indicspecies' package in R (Cáceres and Legendre, 2009; De Cáceres et al., 2010). This analysis determines which OTUs are associated with particular treatments based on measurement of an indicator value (IndVal) that is defined as the product of two quantities, called A and B. Quantity A is the mean abundance of the species in the target treatment group divided by the sum of the mean abundance values over all treatment groups, whereas quantity B is the relative frequency of occurrence (presence–absence) of the species inside the target treatment group (Dufrêne and Legendre, 1997). The statistical significance of this association index was tested by a Monte Carlo permutation test (α=0.05, 999).

3.5 Results

Treatment effects were generally stronger in the wet year 2008 (higher precipitation than normal during the growing season), than in the drought year of 2009 (lower precipitation than normal during the same period) (Tables 1-3).

3.5.1 AM and non-AM fungal root colonization

Microscopic observation revealed that non-AM fungi were more abundant than AM fungi in forage plant roots. Non-AM fungi, identified by melanized structures such as inter- and intracellular hyphae with septa and microsclerotia (Figure S1), colonized 45.7 ± 8.7% of plant root lengths in 2008 and 64.4 ± 7.8% in 2009. In contrast, root colonization by AM fungi was 29.0 ± 9.7% in 2008 and 35.7 ± 11.4% in 2009. AM fungal colonization was reduced significantly when soil P was high, in 2008 (Table 1 and 2), while root colonization by non-AM fungi was not affected by treatments (Table 1 and 3).

3.5.2 Effect of plant assemblage composition and soil P concentration on the composition and diversity of AM and total fungal OTUs in soil

After initial quality filtering, we obtained a total of 72 142 18S rDNA sequences and 51 377 ITS sequences. The number of reads ranged from 81 to 8882 reads per sample for AM fungi and from 374 to 2133 reads per sample for total fungi. After subsampling the data to equalize the number of sequences per sample, AM fungal sequences were clustered (97% similarity) into 86 OTUs and total fungal sequences were clustered into 901 OTUs. PERMANOVA analyses of the OTU matrices indicated that plant assemblage significantly affected AM fungal community structure in both years (Table 2), while total fungal community structure was significantly shifted by soil P concentration, but only in the wet year 2008 (Table 3), with a significant interaction between plant assemblage and soil P concentration. Ordinations of PCoA showed marginal separation of the AM fungal communities across the different plant assemblages in 2008 and 2009 (Figure 2A). The first and second PCoA axes explained 16.9 and 15.0% of the variance in 2008, and 15.2 and 14.7% in 2009. In contrast, total fungal communities from different soil P treatments were distinct depending on plant assemblage composition, but in 2008 only (Figure 2B), although

the variance explained by the first two PCoA axes was low (10.7 and 8.9% in 2008 and 11.0 and 7.2% in 2009).

Shannon diversity was calculated to compare shifts in AM and total fungi between treatments (Table 2 and 3). An interaction between plant assemblage and soil P concentration significantly affected AM fungal diversity in 2008. In P-amended plots, the addition of either *M. sativa* or *D. purpurea* to the native grass assemblage increased AM fungal diversity relative to native grasses alone, while *D. purpurea* planting also increased AM fungal diversity in low-P plots (Figure 3A). In 2009, AM fungal diversity was affected by plant assemblage composition, irrespective of soil P concentration, with the planting of either legume species within the native grass mixture leading to increased AM fungal diversity (Figure 3B). AM fungal diversity in *B. biebersteinii* + *M. sativa* plots was low, and was similar to that of the native grasses without legume addition. In contrast, plant assemblage and soil P concentration had no effect on total soil fungal diversity (Table 3), which was similar among treatments in both years.

UPGMA clustering of the most abundant AM fungal OTUs in 2008 showed that AM fungal communities from native grass mixes with legumes were similar, except when the native grasses were growing with *M. sativa* in low-P soil (Figure 4A). In the ITS data, the community structure of total fungi was distinct in plots with *B. biebersteinii* + *M. sativa* and low P, in 2008 (Figure 4B). AM fungal communities from plots planted with *B. biebersteinii* + *M. sativa* in low- and high-P soil formed a cluster with the native grass mix without legumes in low-P soil (Figure 4A). In contrast, there was no discernible pattern in clustering in 2009.

3.5.3 Taxonomic affiliation

Overall, 86 AM fungal OTUs were identified from soil samples in 2008 and 2009. All AM fungal OTUs identified were closely related to virtual taxa from the MaarjAM database (http://maarjam.botany.ut.ee). Of these, 28 belonged to the *Claroideoglomeraceae* (2008: 57.58%

of AM fungal reads; 2009: 53.48%), 37 to the *Glomeraceae* (2008: 26.79%; 2009: 32.45%), including 3 from the genus *Funneliformis* and 34 from the genus *Glomus*, 3 belonged to the *Paraglomeraceae* (2008: 4.63%; 2009: 13.31%) and 5 to the *Diversisporaceae* (2008: 9.61%; 2009: 0.12%). The remaining 13 OTUs had similarity to known sequences that was lower than 95%, and could thus not be identified with reasonable confidence to a specific family within the *Glomeromycota*. In 2008, there was a significant effect of plant assemblage composition (Table 2), shifting the proportion of AM fungal sequences at the family level between treatments (Figure 5A), while this effect was not detected in 2009. The *Claroideoglomeraceae* and *Glomeraceae* were the two AM fungi families that were significantly influenced by plant assemblage composition (Table S1). The *Claroideoglomeraceae* was the most abundant family observed in the presence of *B. biebersteinii* + *M. sativa,* while the relative abundance of *Glomeraceae* was very low under this plant assemblage, as compared to the native grass mix with legumes. In addition, the relative abundance of *Glomeraceae* was higher in the native grass mix with *D. purpurea* compared to the native grass mix without legumes (Table S2).

Analysis of the ITS fungal sequences revealed 901 total fungal OTUs at 97% similarity across soil samples from 2008 and 2009. Among those OTUs, 309 belonged to the phylum *Ascomycota* (41.97% of total fungal reads), 194 to the *Basidiomycota* (23.96%), 21 to the *Chytridiomycota* (1.30%), 15 to the *Zygomycota* (1.04%) and 7 to the *Glomeromycota* (0.55%), while the remaining OTUs (31.18%) could not be identified to the phylum level. PERMANOVA analysis showed that the relative abundance of total fungal sequences at the class and order levels were not affected by treatments in 2008 and 2009 (Table 3). The main fungal classes were the *Dothideomycetes* (2008: 19.53% of total fungal reads; 2009: 14.61%), the *Agaricomycetes* (2008: 12.85%; 2009: 14.05%), the *Tremellomycetes* (2008: 11.20%; 2009: 7.54%) and the *Sordariomycetes* (2008: 7.13%; 10.28%) (Figure 5B).

At the order level, unclassified *Ascomycota* formed the largest group, while *Pleosporales* was the most abundant identified order (2008: 18.64%; 2009: 13.90%) (Figure S2), and was represented exclusively by the genera *Ulocladium, Alternaria, Phoma* and *Epicoccum* (Figure S3A) which include known plant endophytes. The order *Agaricales* (2008: 9.69%; 2009: 9.88%) was also abundant. The most common genera related to the *Agaricales* were *Nolanea, Clitopilus, Cyathus* and *Conocybe* (Figure S3B). In addition, the *Filobasidiales* (2008: 7.92%; 2009: 4.14%; represented primarily by the genus *Cryptococcus*), the *Hypocreales* (2008: 3.40%; 2009: 3.68%; represented primarily by the genera *Fusarium* and *Gibberella*), and the *Sordariales* (2008: 2.30%; 2009: 3.29%; represented primarily by the genus *Schizothecium*) were also common. Other orders such as the *Chaetothyriales, Helotiales, Capnodiales* and *Xylariales* were detected, but at lower relative abundances (Figure S2).

3.5.4 Effect of legumes and soil P concentration on distribution of OTUs between treatments

Taxonomic assignment to the species level for the most abundant AM and total fungal OTUs is shown in Table S3 and S4, respectively. *Claroideoglomus* sp. (VTX00193, VTX00056), *C. claroideum* (VTX00279), *Glomus* sp. (VTX00113) and *Paraglomus* sp. (VTX00281) were the most frequently identified virtual AM fungal taxa (2008: 52.06% of AM fungi reads; 2009: 59.10%), while the most common fungi found in the soils were related to *Ulocladium* sp. (*Pleosporales*) (2008: 7.65% of fungal reads; 2009: 5.56%) and *Exophiala* sp. (*Chaetothyriales*) (2008: 2.42%; 2009: 2.33%) which are known as endophytic taxa (Ragazzi et al., 2003; Sieber, 2007; Lv et al., 2010; Khan et al., 2011), followed by *Nolanea strictior* (*Agaricales*) (2008: 2.97%; 2009: 4.26%). In addition, the two most abundant saprotrophic fungi, *Cryptococcus adeliensis* (*Filobasidiales*) (2008: 5.61%; 2009: 2.59%) and *C. aerius* (*Filobasidiales*) (2008: 2.74%; 2009: 2.89%) were also dominant.

Indicator species analysis showed that certain AM fungi (virtual taxa) were significant indicators of grass assemblages + legumes, but the specific OTUs identified by this analysis depended on legume identity and year of sampling (Table 4). OTU5 (*Glomus* sp. VTX00113; Table S3) was an indicator of the native grass mix + legumes in both years. In the drought year of 2009, OTU8 (*Claroideoglomus* sp. VTX00402) became another significant indicator of the native grass mix + legumes, whereas OTU9 (also similar to *Claroideoglomus* sp. VTX00402) was mainly associated to native grasses alone or *B. biebersteinii* + *M. sativa*. Additionally, OTU13 (*Claroideoglomus* sp. VTX00278) was significantly associated with native grasses + *D. purpurea*.

Analysis of the dominant fungal OTUs demonstrated that OTU13 [*Clitopilus passeckerianus* (FJ770409); Table S4], OTU38 [*Apodus deciduus* (AY681199)] and OTU16 [Uncultured *Ascomycota* (FN295256)] were significant indicators of plots with low-P soil, especially in 2008. In addition, two non-AM fungal OTUs were significantly associated with particular plant assemblages, but only in 2008. In all legume treatments, OTU16 [Uncultured *Ascomycota* (FN295256)] was dominant. In contrast, OTU69 [*Cadophora* sp. (JN995648)] was a significant indicator of *B. biebersteinii* + *M. sativa*.

Table 1 Root colonization percentages of AM fungi as influenced by plant assemblage and soil P concentration, over two consecutive years.

Plant assemblage	2008 Root colonization[a]	2009 Root colonization[a]
Grass	33.5 a	39.1 a
Grass + *M. sativa*	28.8 a	30.9 a
Grass + *D. purpurea*	26.0 a	39.4 a
B. biebersteinii + *M. sativa*	27.6 a	33.3 a
Soil P fetility		
Low P	33.3 b	33.6 a
High P	24.6 a	37.8 a

[a]Means are not significantly different ($p \leq 0.05$, ANOVA; n = 3 (2008); n = 4 (2009)) when followed by the same letter (a, b) according to Tukey's range tests.

Table 2 P-values for AM fungal root colonization, relative abundance at the family level, Shannon index and community structure according to ANOVA or PERMANOVA (n = 3 in 2008 and n = 4 in 2009).

Source	2008				2009			
	Root colon.	Relative abundance - families	Shannon index	Community structure	Root colon.	Relative abundance - families	Shannon index	Community structure
Plant. assem	0.251	0.013	**<0.001**	**0.037**	0.331	0.211	**<0.001**	**0.001**
Soil P	**0.003**	0.742	**0.02**	0.323	0.292	0.378	0.201	0.651
Plant. assem× soil P	0.077	0.185	**0.001**	0.393	0.468	0.869	0.840	0.064

Plant assem.: plant assemblage; root colon.: root colonization. Bolded value are significant.

Table 3 P-values for non-AM fungi root colonization, relative abundance at the class and order level, Shannon index and community structure according to ANOVA or PERMANOVA (n = 4).

Source	2008					2009				
	Root colon.	Relative abundance class level	Relative abundance order level	Shannon index	Community structure	Root colon.	Relative abundance class level	Relative abundance order level	Shannon index	Community structure
Plant assem.	0.065	0.449	0.785	0.849	0.213	0.554	0.222	0.456	0.566 ^{ns}	0.176 ^{ns}
Soil P	0.477	0.062	0.297	0.334	**0.004**	0.462	0.743	0.107	0.610 ^{ns}	0.759 ^{ns}
Plant assem × soil P	0.405	0.504	0.621	0.188	**0.021**	0.476	0.745	0.646	0.201 ^{ns}	0.960 ^{ns}

Plant assem.: plant assemblage; root colon.: root colonization. Bolded value are significant.

Table 4 Indicator species analysis for the 20 most dominant AM fungi and the 50 most dominant non-AM fungal OTUs in response to plant assemblage and soil P concentration. Indicator Values (IndVal) were tested for significance by Monte Carlo permutation tests (α =0.05, 999 permutations).

	Treatment	Indicator species (at 95% similarity)
AM fungi 2008	Grass	none
	Grass + *M. sativa*	*Glomus* sp. (VTX00113)*
	Grass + *D. purpurea*	*Glomus* sp. (VTX00113)*
	B. biebersteinii + *M. sativa*	none
	Low soil P	none
	High soil P	none
AM fungi 2009	Grass	none
	Grass + *M. sativa*	*Claroideoglomus* sp. (VTX00402)**, *Glomus* sp. (VTX00113)**
	Grass + *D. purpurea*	*Claroideoglomus* sp. (VTX00402, VTX00278)**, *Glomus* sp. (VTX00113)**
	B. biebersteinii + *M. sativa*	*Claroideoglomus* sp. (VTX00402)**
	Low soil P	none
	High soil P	none
Non-AM fungi 2008	Grass	none
	Grass + *M. sativa*	Uncultured *Ascomycota* (FN295256)*
	Grass + *D. purpurea*	Uncultured *Ascomycota* (FN295256)*
	B. biebersteinii + *M. sativa*	*Cadophora* sp.(JN995648)**, Uncultured *Ascomycota* (FN295256)*
	Low soil P	*Clitopilus passeckerianus* (FJ770409)*, Uncultured *Ascomycota* (FN295256)**, *Apodus deciduus* (AY681199)**
	High soil P	none
Non-AM fungi 2009	Grass	none
	Grass + *M. sativa*	none
	Grass + *D. purpurea*	none
	B. biebersteinii + *M. sativa*	none
	Low soil P	none
	High soil P	none

*significant at 5%; **significant at 1%.

Table S1 ANOVA results for the proportion of AM families (n = 3)

Source	2008			
	Claroideoglomeraceae	*Glomeraceae*	*Paraglomeraceae*	*Diversisporaceae*
Plant assemblage	**0.002**	**0.001**	0.560	0.273
Soil P concentration	0.337	0.484	0.176	0.550
Plant assemblage X Soil P concentration	**0.002**	0.784	0.195	0.733

Bolded value are significant.

Table S2 Proportion of *Claroideoglomeraceae* and *Glomeraceae* as influenced by plant assemblage

Plant assemblage	2008	
	Claroideoglomeraceae[a]	*Glomeracea* [a]
Grass	58.2 ab	17.5 ab
Grass + *M. sativa*	32.3 a	37.9 bc
Grass + *D. purpurea*	32.9 a	52.7 c
B. biebersteinii + *M. sativa*	83.1 b	0.4 a

[a]Means are not significantly different ($p \leq 0.05$, ANOVA; n = 3) when followed by the same letter (a, b, c) according to Tukey's range test.

Table S3 The twenty most dominant AM fungi OTU detected from soil samples and their taxonomic affiliation according to the closest BLAST matches in Maarjam database. All AMF OTU were related to virtual taxa.

OTU	Family	Most closely related taxa	Molecular Virtual taxon	Similarity (%)
OTU4	*Claroideoglomeraceae*	*Claroideoglomus claroideum*	VTX00279	96
OTU1	*Claroideoglomeraceae*	*Claroideoglomus* sp.	VTX00193	98
OTU2	*Claroideoglomeraceae*	*Claroideoglomus* sp.	VTX00056	98
OTU13	*Claroideoglomeraceae*	*Claroideoglomus* sp.	VTX00278	99
OTU8	*Claroideoglomeraceae*	*Claroideoglomus* sp.	VTX00402	96
OTU10	*Glomeraceae*	*Funelliformus mosseae*	VTX00067	99
OTU5	*Glomeraceae*	*Glomus* sp.	VTX00113	99
OTU6	*Glomeraceae*	*Glomus* sp.	VTX00199	97
OTU7	*Glomeraceae*	*Glomus* sp.	VTX00143	99
OTU11	*Glomeraceae*	*Glomus* sp.	VTX00172	99
OTU14	*Glomeraceae*	*Glomus* sp.	VTX00114	99
OTU16	*Glomeraceae*	*Glomus* sp.	VTX00143	99
OTU17	*Glomeraceae*	*Glomus* sp.	VTX00199	98
OTU18	*Glomeraceae*	*Glomus* sp.	VTX00219	98
OTU23	*Glomeraceae*	*Glomus* sp.	VTX00172	99
OTU12	*Diversisporaceae*	*Diversispora* sp.	VTX00054	97
OTU15	*Diversisporaceae*	*Diversispora* sp.	VTX00354	98
OTU22	*Diversisporaceae*	*Diversispora* sp.	VTX00354	98
OTU3	*Paraglomeraceae*	*Paraglomus* sp.	VTX00281	99
OTU9	*Claroideoglomeraceae*	*Claroideoglomus* sp.	VTX00402	Low similarity

Table S4 The fifty most dominant fungal OTU detected from soils samples and their taxonomic affiliation according to the closest BLAST matches in UNITE or NCBI database.

OTU	Phylum	Order	Most closely related taxa	Accession number	Similarity (%)
OTU1	Ascomycota	*Pleosporale*	*Ulocladium* sp.	HQ829119	99
OTU9	Ascomycota	*Pleosporale*	*Phaeosphaeria* sp.	DQ402526	88
OTU12	Ascomycota	*Pleosporales*	*Alternaria* sp.	GU187964	100
OTU66	Ascomycota	*Pleosporales*	*Phoma medicaginis var. medicaginis*	DQ109960	100
OTU14	Ascomycota	*Pleosporales*	*Phoma sclerotioides*	EU265670	100
OTU17	Ascomycota	*Pleosporales*	*Epicoccum nigrum*	KC867291	99
OTU29	Ascomycota	*Pleosporales*	*Pleosporales* sp.	HQ649945	86
OTU11	Ascomycota	*Hypocreales*	*Gibberella* sp.	EU552132	99
OTU22	Ascomycota	*Hypocreales*	*Fusarium equiseti*	AY147368	100
OTU6	Ascomycota	*Chaetothyriales*	*Exophiala* sp.	EU035420	91
OTU8	Ascomycota	*Chaetothyriales*	Uncultured *Exophiala*	GU055730	100
OTU26	Ascomycota	*Helotiales*	*Helotiales* sp.	JN859275	95
OTU69	Ascomycota	*Helotiales*	*Cadophora* sp.	JN995648	100
OTU133	Ascomycota	*Helotiales*	*Helotiales* sp.	JN859275	95
OTU15	Ascomycota	*Sordariales*	*Schizothecium carpinicola*	AY999118	90
OTU38	Ascomycota	*Sordariales*	*Apodus deciduus*	AY681199	96
OTU89	Ascomycota	*Sordariales*	*Schizothecium carpinicola*	AY999118	96
OTU72	Ascomycota	*Xylariales*	*Anthostomella leucospermi*	EU552100	97
OTU87	Ascomycota	*Orbiliales*	*Dactylellina lobata*	AF106524	78
OTU101	Ascomycota	*Pezizales*	*Pezizales* sp.	AJ969618	96
OTU18	Ascomycota	*mitosporic Ascomycota*	*Tetracladium* sp.	AJ890435	99
OTU3	Ascomycota	-	Uncultured *Ascomycota*	EU489976	98
OTU16	Ascomycota	-	Uncultured *Ascomycota*	FN295256	97
OTU47	Ascomycota	-	*Ascomycota* sp.	AJ972820	94
OTU4	Basidiomycota	*Agaricales*	*Nolanea strictior*	EF421109	100
OTU13	Basidiomycota	*Agaricales*	*Clitopilus passeckerianus*	FJ770409	100
OTU24	Basidiomycota	*Agaricales*	*Conocybe coprophila*	AY194540	93
OTU39	Basidiomycota	*Agaricales*	*Cyathus stercoreus*	FJ478125	100
OTU58	Basidiomycota	*Agaricales*	*Typhula maritima*	AB430447	98
OTU70	Basidiomycota	*Agaricales*	*Coprinellus curtus*	AY461834	100
OTU71	Basidiomycota	*Agaricales*	*Conocybe velutipes*	JF907832	100
OTU83	Basidiomycota	*Cantharellales*	*Ceratobasidium* sp.	DQ102444	94
OTU27	Basidiomycota	*Cantharellales*	Uncultured *Minimedusa*	GU055543	100
OTU53	Basidiomycota	*Cantharellales*	Uncultured *Ceratobasidium*	FM866376	92
OTU62	Basidiomycota	*Corticiales*	*Sistotrema* sp.	KC514807	84
OTU2	Basidiomycota	*Filobasidiales*	*Cryptococcus adeliensis*	JX188117	100
OTU5	Basidiomycota	*Filobasidiales*	*Cryptococcus aerius*	AB032666	100
OTU49	Basidiomycota	*Filobasidiales*	*Cryptococcus albidosimilis*	JQ768933	100
OTU7	Zygomycota	*Mortierellales*	*Mortierella alpina*	AJ271629	96

OTU20	Chytridiomycota	-	Uncultured *Chytridiomycota*	EU489945	100
OTU30	Fungi incertae sedis	-	*Zygomycete* sp.	EU428774	97
OTU10	-	-	Uncultured soil fungus	EU826876	100
OTU19	-	-	Uncultured fungus	AM113724	100
OTU23	-	-	Uncultured fungus	AM113724	95
OTU33	-	-	Uncultured fungus	FJ820546	100
OTU36	-	-	Uncultured soil fungus	EU480270	89
OTU42	-	-	Uncultured fungus	FJ386926	100
OTU65	-	-	Uncultured soil fungus	DQ980575	95
OTU74	-	-	Uncultured fungus	FN397310	94
OTU114	-	-	Uncultured soil fungus	EU806298	92

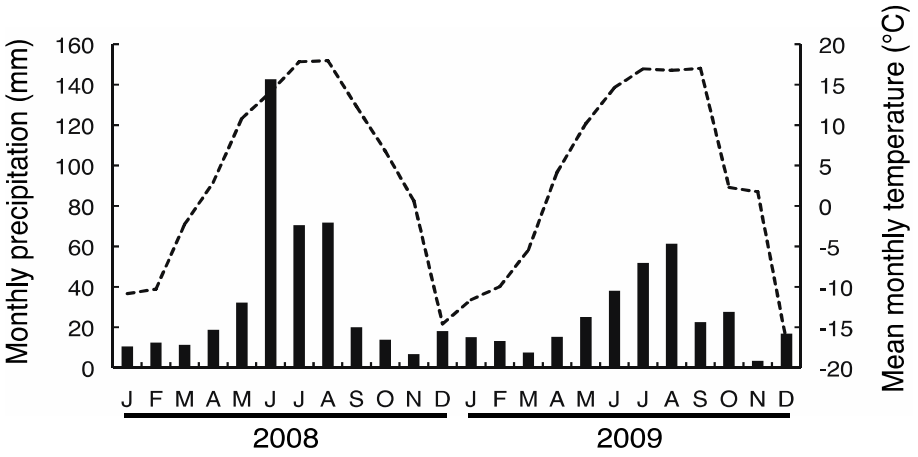


Figure 1 Average monthly temperature (dotted line) and precipitation (bar) during the two consecutive study years.

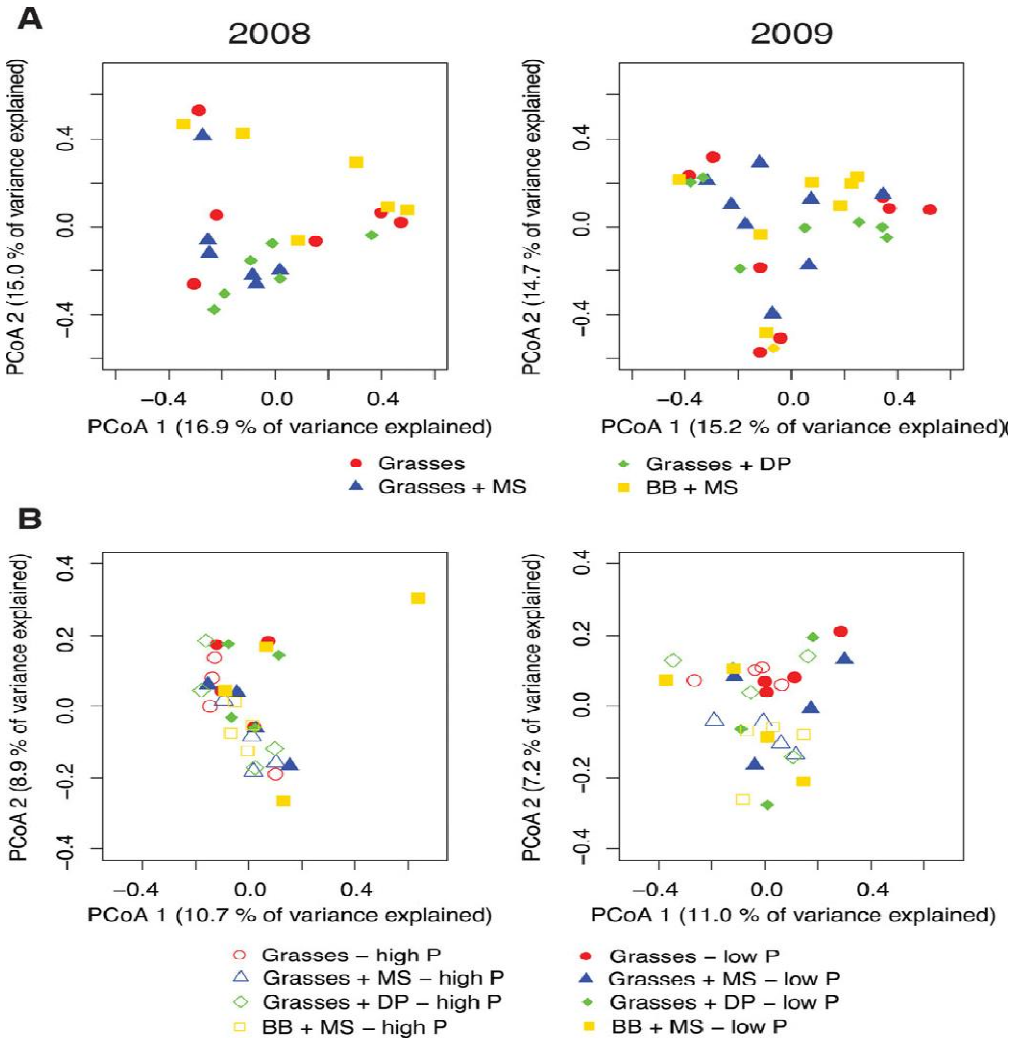


Figure 2 PCoA of (A) AM fungal community composition as influenced by plant assemblage and (B) total fungal community composition as influenced by the interaction between plant assemblage and soil P concentration, in 2008 and 2009. PERMANOVA analysis shown significant differences in AM fungi community in 2008 (n = 3, P = 0.037), in 2009 (n = 4, P = 0.001), and in total fungal community in 2008 (n = 4, P = 0.021). MS = *M. sativa,* DP = *D. purpurea*; BB = *B. biebersteinii.*

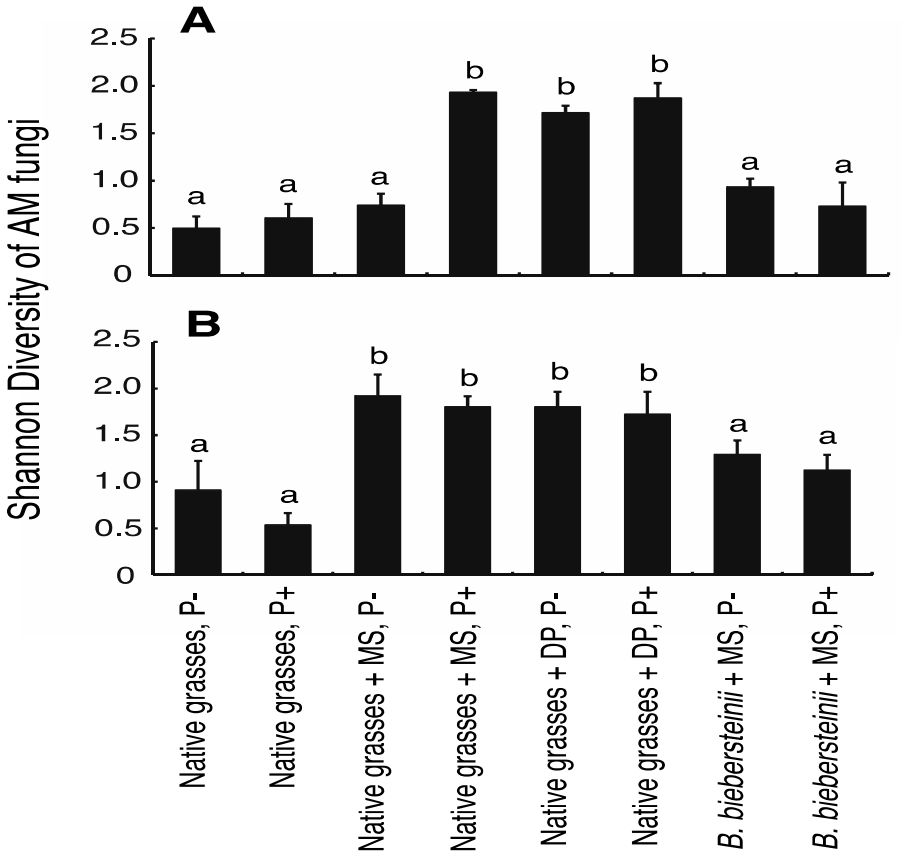


Figure 3 Shannon index of AM fungi as influenced by the interaction between plant assemblage and soil P concentration in 2008 (A) and by plant assemblage alone in 2009 (B), according to ANOVA analysis ($P = 0.001$, $n = 3$ (2008), $P < 0.001$, $n = 4$ (2009)). Different letters over columns indicate significant differences according to Tukey's range test. MS = *M. sativa,* DP = *D. purpurea*; P- = low-soil P concentration; P + = high-soil P concentration.

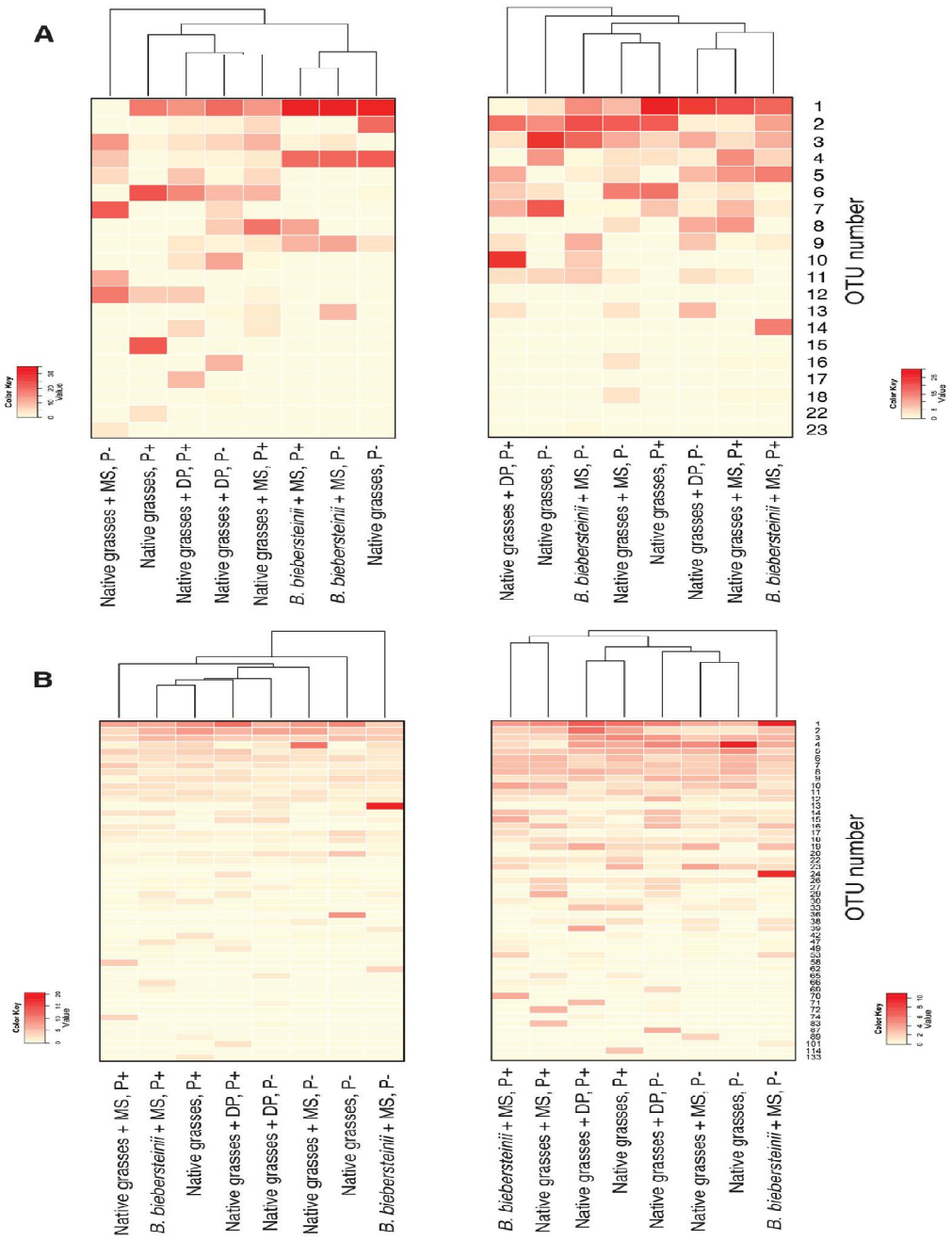


Figure 4 Heatmap showing the relative abundance of the 20 most dominant AM fungi (A) and the 50 most dominant non-AM fungi (B) in low- and high-P soils planted with different forage plant assemblages in 2008 and 2009. The hierarchical clustering tree of treatments reflects the similarity in fungal community composition based on UPGMA clustering. The color of each heatmap rectangle indicates the mean abundance of each OTU in one treatment, as shown in the legend. MS = *M. sativa,* DP = *D. purpurea*; P- = low-soil P concentration; P+ = high-soil P concentration. See Supplemental Table 3 and 4 for the identity of the OTUs.

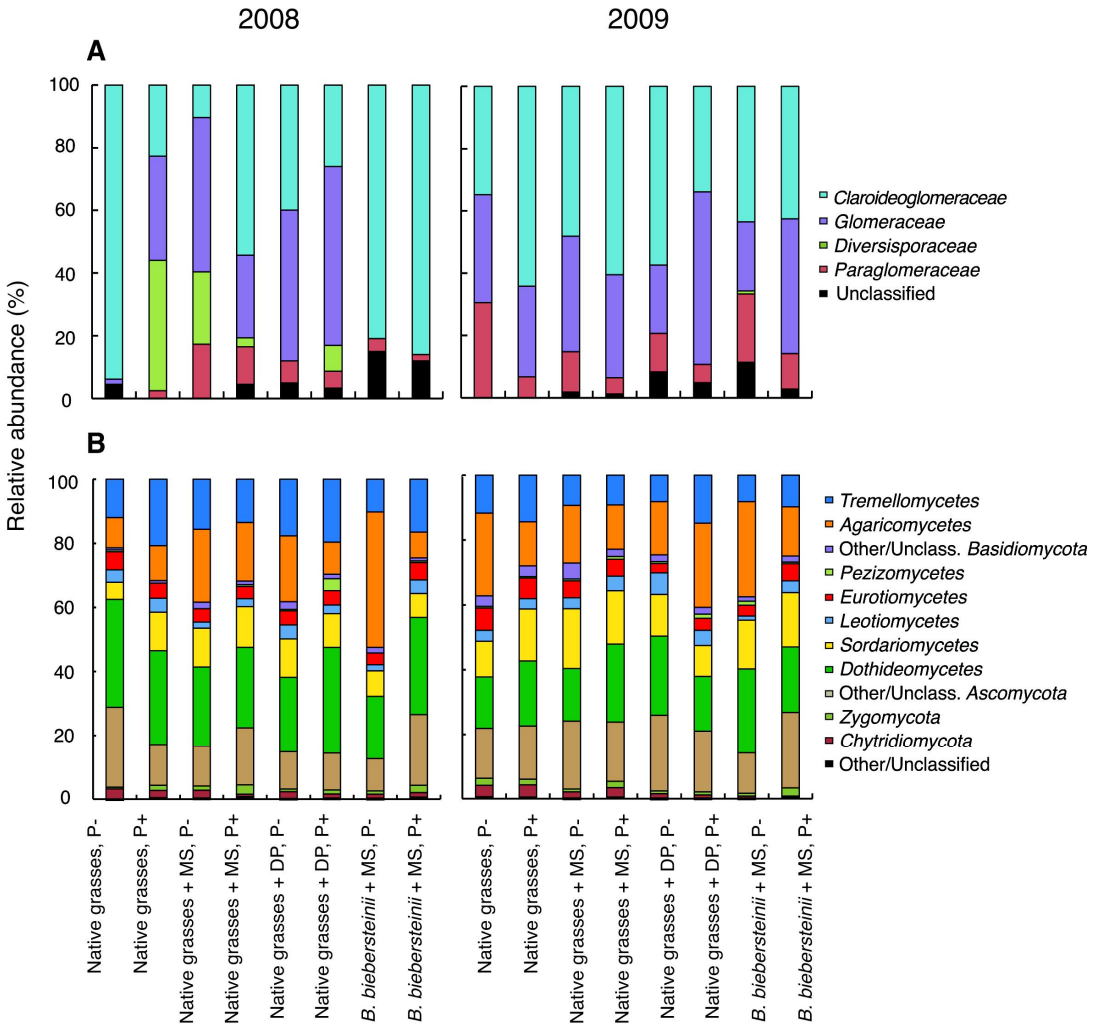


Figure 5 Relative abundance of (A) AM fungi families and (B) the major classes of total fungi detected in low- and high-P soil underlying different plant assemblages in 2008 and 2009. MS = *M. sativa,* DP = *D. purpurea*; P- = low-soil P concentration; P+ = high-soil P concentration.

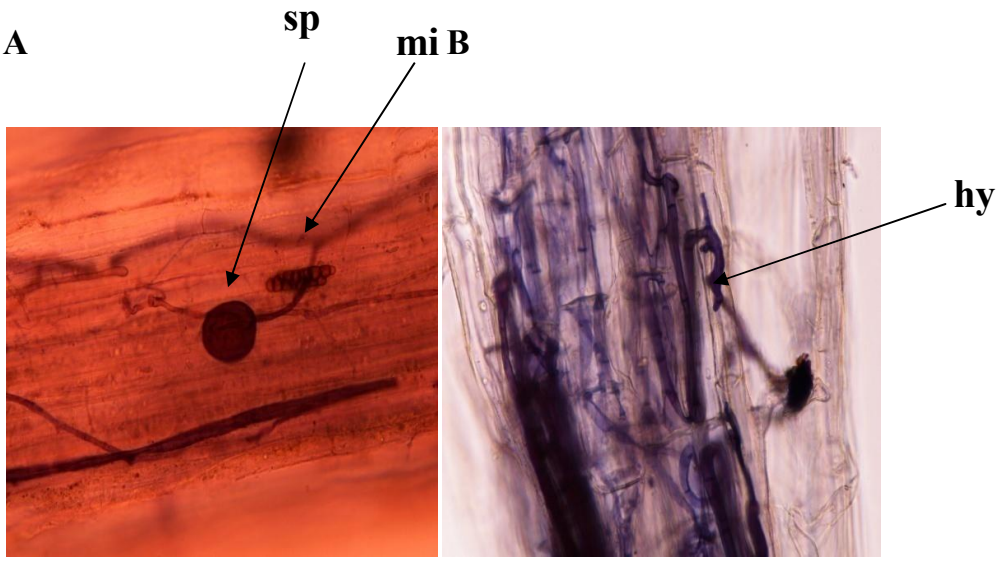


Figure S1 Arbuscular rmycorrhizal fungi (AM fungi) and non-AM fungi structures found in plant roots. (A) Plant root showing an intraradical spore of an AM fungus (sp) and a microsclerotia of a non-AM fungus (mi), and (B) a close-up of the root tissues showing the non-AM fungal hyphae with septa (hy).

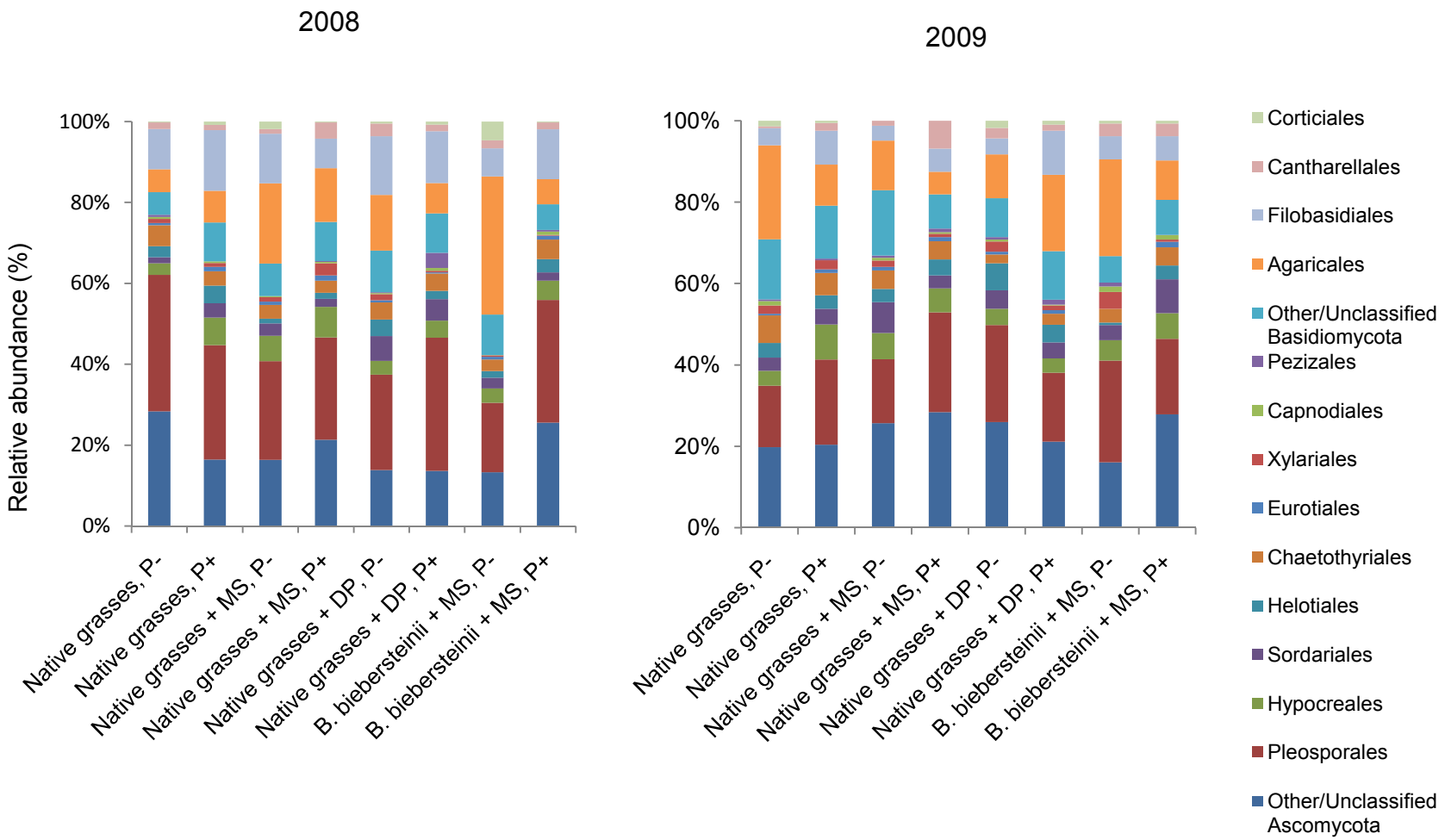
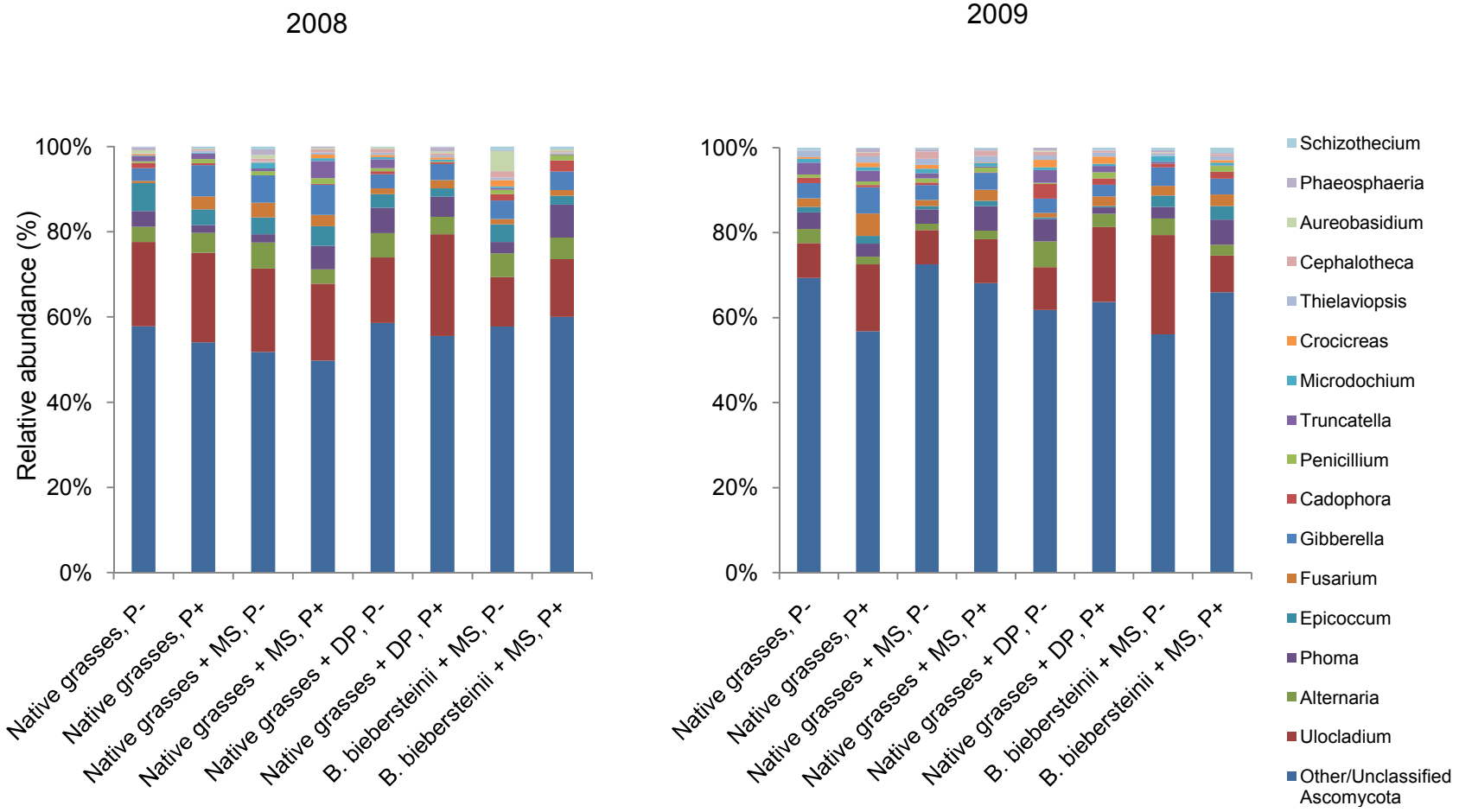


Figure S2 Relative abundance of the major orders of total fungi detected in low- and high-P soil underlying different plant assemblages in 2008 and 2009. MS = *M. sativa,* DP = *D. purpurea*; P- = low-soil P concentration; P+ = high-soil P concentration.

A


2008
2009
Relative abundance (%)
100%
80%
60%
40%
20%
0%
Native grasses, P-
Native grasses, P+
Native grasses + MS, P-
Native grasses + MS, P+
Native grasses + DP , P-
Native grasses + DP , P+
B. biebersteinii + MS, P-
B. biebersteinii + MS, P+
Schizothecium
Phaeosphaeria
Aureobasidium
Cephalotheca
Thielaviopsis
Crocicreas
Microdochium
Truncatella
Penicillium
Cadophora
Gibberella
Fusarium
Epicoccum
Phoma
Alternaria
Ulocladium
Other/Unclassified Ascomycota

B

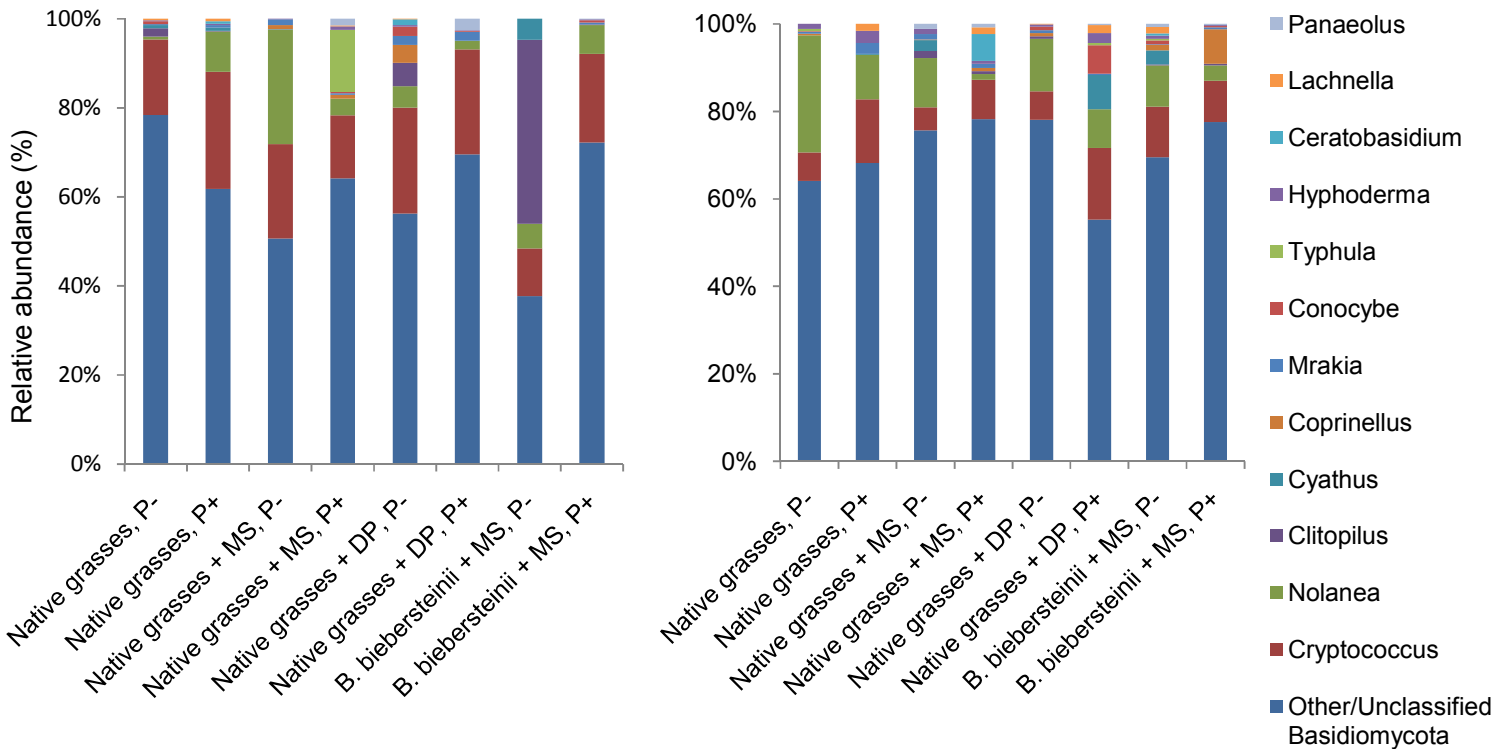


Figure S3 Relative abundance of the major genera of total fungi in Ascomycota (A) and Basidiomycota (B) phylums, detected in low- and high-P soil underlying different plant assemblages in 2008 and 2009. MS = *M. sativa,* DP = *D. purpurea*; P- = low-soil P concentration; P+ = high-soil P concentration.

3.6 Discussion

Our results show that forage plant identity and P addition differentially affected root colonization, community structure, and diversity of AM and total fungi in the underlying soil in a semi-arid grassland. The community structure of AM fungi was significantly affected by plant assemblage composition in both years, while total fungi were most strongly impacted by P addition in 2008 only. These results are consistent with previous studies, which showed that plant assemblage composition can play an important role in structuring AM fungal communities (Bainard et al., 2014). We also demonstrate interactions between plant assemblage and P treatment on both AM fungal diversity and total fungi community in 2008. Interestingly, high soil P increased AM fungal diversity in soils planted with a mix of native grasses and the N-fixing legume *M. sativa*, as compared to native grasses alone, while the native grass mix + the N-fixing legume *D. purpurea* promoted AM fungal diversity regardless of P treatment. In contrast, in 2008, high soil P had a negative impact on AM fungal root colonization, irrespective of plant assemblage composition, while the abundance of non-AM fungal structures in roots was not modified by plant assemblages or P concentration. This suggests that the influence of plant assemblage composition on soil fungal communities may be controlled by soil P, but also likely relies on others environmental factors. The divergent effects of treatments on root colonization and fungal composition in soils also suggest that the fungal communities of roots and nearby soil are dissimilar.

3.6.1 AM fungi (18S rDNA)

The cultivated legume *M. sativa* has been shown to be well adapted to the soil and climate of southwest Saskatchewan (Schellenberg and Banerjee, 2002). High biomass production and/or N2-fixation by *M. sativa* may lead to requiring more P in response to competition by native grasses, when compared with the native and less productive *D.*

purpurea, which was present at a lower relative abundance in grass communities due to its slow establishment (Appendix 2). In 2008, a lower abundance of *M. sativa* was observed when grown with the native grass assemblage as opposed to *B. biebersteinii* (Appendix 2). The highest concentration of P was also observed in the tissues of legumes grown with native grasses assemblages rather than with the non-native *B. biebersteinii* (Klabi et al. unpublished data), which suggests that higher concentrations of P are required by this legume to remain competitive alongside native grasses. Biological N_2-fixation of legumes is an energy-intensive process that is often enhanced by Paddition (Hayat et al., 2008), since P availability is critical for nodule development and function due to the high-energy requirement of nitrogenase activity (Israel, 1987; Drevon and Hartwig, 1997; Olivera et al., 2004; Schulze and Drevon, 2005; Divito and Sadras, 2014). AM fungi have high N requirements (Scheublin et al., 2004; Hodge et al., 2010), which could explain their strong response to this plant functional group, and Porras-Alfaro et al. (2007) demonstrated that N fertilization increased AM fungal diversity in the roots of *B. gracilis* in a semi-arid grassland. In contrast, planting of either legume species within the native grass mix in 2009 led to increased AM fungal diversity, irrespective of soil P concentration.

In contrast to the diversity of AM fungi in soil, the extent of root mycorrhizal colonization was reduced at high soil P concentrations in 2008, when soil moisture was high. Several studies have reported that high concentrations of soil extractable P are linked to reduced AM fungal colonization and development (Johnson et al., 2003; Chagnon and Bradley, 2013). This may mean that AM fungi taxa which colonize roots to a greater extend are also more sensitive to soil P concentrations, or that host plants are inhibiting further root colonization when sufficient P is available, while the growth of AM fungi mycelia in the soil would not be affected. AM fungal distribution is also known to be affected by soil moisture and seasonal variations in climate and plant dynamics (Yang et al., 2010), and the annual

divergence seen in this study suggests that some or all of these factors interacted with soil P concentration to influence AM fungal diversity and root colonization.

In addition to N outputs by legumes, the observed differences in AM diversity may be linked to preferential interactions between AM fungi and particular plant species. Different plant species may be able to select the AM symbioses that most benefit them, while AM fungi may similarly invest in plants that allot more resources to root-associated microbes (Kiers et al., 2011). Host plant identity apparently plays an important role in shifting the structure and diversity of AM fungi (Vandenkoornhuyse et al., 2003; Scheublin et al., 2004), and interactions between plant host and AM fungi seem to be regulated by plant and AM fungal genotypes (Klironomos, 2003; Smith et al., 2011). AM fungal diversity can be a determinant of plant diversity (and vice versa) in grassland communities (van der Heijden et al., 1998), and Daniell et al. (2001) showed that in arable soils, the roots of crops in monoculture had low-AM fungal diversity. In our study, the increased diversity of AM fungi in the native grass mix + legumes treatments may be partly a function of the higher number of plant species that are present in these soils, leading to a wider array of potential AM fungal hosts. The N-fixing legumes may also preferentially associate with AM fungal taxa that improve the acquisition of nutrients that are essential to N_2-fixation, particularly P, while the native grass species (which were heavily colonized) may be more prone to associate with native AM fungi than the introduced grass *B. biebersteinii*. Increased N availability in the soil may also stimulate C allocation by native grasses to their fungal symbionts (Johnson et al., 2003), and different grass species may supply C compounds of varying composition and quality (Millard and Singh, 2010; Hodge and Fitter, 2013), which may select for AM fungal taxa with different growth rates (Brachmann and Parniske, 2006) and carbohydrate demands (Lerat et al., 2003), thus increasing AM fungal diversity.

At the family level, *Claroideoglomeraceae* and *Glomeraceae* were the only AM fungal groups that were significantly affected by plant assemblages in 2008. *Claroideoglomeraceae* was most abundant in soils underlying the *B. biebersteinii* + *M. sativa* assemblage, while the highest abundance of *Glomeraceae* was observed in the native grass mix + *D. purpurea* assemblage. As members of *Claroideoglomeraceae* and *Glomeraceae* were the most abundant taxa in prairies soils, shifts in these groups among different plant assemblage were expected. However, changes in these two groups may have also induced changes in the proportions of other groups that were not of sufficient magnitude to be highlighted by the statistical analysis. According to the indicator species analysis, legumes planted within the native grass mix promoted a few specific AM fungal taxa over two consecutive years. Those taxa, *Glomus* VTX00113 and *Claroideoglomus* VTX00402 and VTX00278, are considered to be indicators of either N-enriched soil or efficient P-acquisition (Scheublin et al., 2004; Thonar et al., 2011). *Glomus* VTX00113 is closely related to the known strains *Glomus fasciculatum* (BEG53) and *Rhizophagus intraradices* (unidentified cultures) (cf. MaarjAM database) (Öpik et al., 2010; Davison et al., 2011). *Rhizophagus intraradices* was associated with high N concentrations in legumes plants and with colonization of root nodules (Scheublin et al., 2004). Additionally, *R. intraradices* may be affected by changes in the carbohydrate status of plants (Ijdo et al., 2010; Saravesi et al., 2013).

3.6.2 Total fungi (ITS)

In contrast, the composition of total soil fungi was modified by soil P concentration. A significant interactive effect of P addition and plant assemblage on fungal composition was detected, although only in 2008, without significant changes to fungal diversity or non-AM fungal root colonization. Our ITS sequences were largely represented by the *Ascomycota*

(44.35%), primarily the *Pleosporales*, which were dominated by *Ulocladium* sp., a likely endophyte (Ragazzi et al., 2003), and the *Basidiomycota* (25%), particularly the *Agaricales* and *Filobasidiales*, the latter of which was represented by the genus *Cryptococcus*, which is a known saprotrophic genus. Non-AM fungi and endophytes have been shown to produce extracellular enzymes for degrading organic matter, indicating that these fungi may have saprophytic functions (Mandyam and Jumpponen, 2005; Porras-Alfaro and Bayman, 2011).

Plant P uptake in 2008 was affected by the interaction between soil P concentration and plant assemblage, without an effect on overall plant productivity. Higher P uptake was measured in plant tissues at high-soil P concentrations when *M. sativa* was present, when compared to native grasses growing without legumes (Klabi et al., unpublished data). This suggests that shifts in soil fungi communities structure at high P concentrations may be partly attributed to the changes in plant P status observed in the *M. sativa* treatments, as this can alter carbon inputs to soil through modification of root exudation or litter deposition (Singh and Pandey, 2003; Millard and Singh, 2010). Our results agree with Beauregard, Hamel and St-Arnaud (2010), who reported a shift in soil fungal communities following P addition. However, the three most abundant OTUs, which belong to the orders *Agaricales* [*Clitopilus passeckerianus* (FJ770409)], *Sordariales* (*Apodus deciduus*) and uncultured *Ascomycota* (FN295256), were dominant in low-P soils. Fertilization can directly reduce enzymatic activity, and may result in the decreased function of certain fungi (Singh and Pandey, 2003; Bais et al., 2006; Neumann and Römheld, 2007). The weak response to plant composition in the ITS data suggests minimal plant specificity outside of the *Glomeromycota.* Two non-AM fungal OTUs were indicative of specific plant assemblages, however, according to our indicator species analysis. One OTU, classified as uncultured *Ascomycota* (FN295256), was indicative of the presence of legumes. Another, closely related to *Cadophora* sp. (JN995648) (*Helotiales*), was indicative of soils planted with the introduced species *B. biebersteinii* and

the cultivated *M. sativa*. This putative endophyte may be able to mineralize nutrients from plant tissues or N-rich litter, as was shown previously (Caldwell et al., 2000).

The lack of an interactive effect of P and plant assemblage on fungi in 2009 likely results from differences in precipitation and plant productivity. Soils were well saturated from spring to sampling time in 2008, whereas the 2009 growing season was very dry, and plant productivity was also higher in 2008 than in 2009 (Klabi et al., unpublished data). In addition, soil moisture was lower when *M. sativa* was present within plant assemblages, in 2008 (Appendix 3). Moisture availability and plant productivity are important factors influencing the composition of soil fungi, and here a shift in fungal species probably occurred when the environment shifts from being P-limited to moisture- or plant productivity-limited. Our observations also concur with numerous previous reports (Hamel et al., 2006; Hawkes et al., 2011; Yang et al., 2012a; 2012b; 2012c; 2013; Ellouze et al., 2013) in showing the overriding effect of moisture availability on soil microbial dynamics and function in semi-arid environments.

3.7 Conclusions

Our hypothesis that soil P addition and legume planting would differentially affect AM and total fungal communities was supported, while our expectation that native plant assemblages would lead to increased soil fungal diversity was not. Plant assemblage composition, particularly the presence or absence of legumes, is a key factor in determining AM fungal diversity in the soils underlying forage plants. This effect can be modified by soil P concentration, but likely depends on competitive interactions between legumes and native grasses, as well as other interactions with the environment. The abundance of root-inhabiting AM fungi is also modified by soil P concentration. In contrast, the community composition of non-AM fungi in the soil, which consist primarily of endophytes and saprophytic fungi, was

driven by the interaction between soil P and plant assemblages in 2008, especially when *M. sativa* was present. The absence of this effect in 2009 suggests that other environmental factors, such as soil moisture and plant productivity, are also important. Overall, this result suggests that AM fungi will be mainly modified by typical forage production practices such as legume planting, whereas non-AM fungi appear to be influenced in part by plant productivity and other environmental parameters such as soil moisture. Future studies should determine whether these changes lead to communities that interact more or less positively with forage plants, and whether further treatments are required to optimize soil microbial communities for plant production.

3.8 Acknowledgments

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Chapitre 4: Interaction between legume and arbuscular mycorrhizal fungi identity alters the competitive ability of warm-season grass species in a grassland community

Ce chapitre a fait l'objet de la publication suivante dans une revue scientifique internationale:

Klabi R, C Hamel, MP Schellenberg, A Iwaasa, A Raies, M St-Arnaud. 2014. Interaction between legume and arbuscular mycorrhizal fungi identity alters the competitive ability of warm-season grass species in a grassland community. Soil Biology & Biochemistry 70: 176–182 (doi:10.1016/j.soilbio.2013.12.019).

Les résultats de ce chapitre étaient présentés lors de la conférence suivante:

*Rim Klabi, Chantal Hamel, Michael P. Schellenberg, Alan Iwaasa, Aly Raies, and Marc St-Arnaud. L'interaction entre les légumineuses et les champignons mycorhiziens à arbuscule change la capacité compétitive des espèces les moins dominantes dans les communautés des graminées. Colloque Mycorhizes 2012, Montréal, 5 Octobre 2012.

4.1 Préface

Dans les chapitres précédents, les résultats de l'expérience au champ ont montré que la structure des communautés de champignons totaux qui sont dominés par les endophytes et les champignons saprophytes était fortement influencée par la teneur en P du sol, en 2008 (année humide), alors que la présence des légumineuses dans le mélange de graminée indigènes a permis d'augmenter la diversité des champignons mycorhiziens à arbuscules. Ce changement était positivement corrélé avec la productivité et la quantité totale d'azote trouvées chez *M. sativa* et aussi avec la teneur en phosphore trouvée dans les tissus des graminées indigènes. Ceci suggère que l'addition de *M. sativa* peut contribuer à augmenter les espèces bénéfiques des champignons mycorhiziens à arbuscules qui sont impliquées dans l'augmentation de la productivité et la nutrition du fourrage dans les prairies indigènes, au début de l'été. Le chapitre 3 a permis de mieux clarifier et comprendre l'influence des CMA et les légumineuses sur la dynamique et la coexistence des graminées indigènes, en testant les effets des CMA (*Glomus* sp. et *Glomus cubense*) et des légumineuses fixatrices d'azote (*M. sativa* ou *D. purpurea*) et de leurs interactions sur la capacité compétitive des graminées de saison fraîche et des graminées de saison chaude.

4.2 Abstract

Arbuscular mycorrhizal fungi (AMF) and N_2-fixing legumes can alter the community structure of grasses. However, the effect of AMF, N_2-fixing legumes, and their interaction on the dynamics of prairie grass communities remains unclear. The aim of this study was to clarify the influence of two AMF (*Glomus cubense* and *Glomus* sp.) and two legumes (*Medicago sativa* and *Dalea purpurea*) on the competitive relationship between three native cool-season (*Elymus canadensis*, *Elymus trachycaulus* ssp. *subsecundus*, and *Elymus lanceolatus* ssp. *lanceolatus*) and two native warm-season species of grasses (*Schizachyrium*

scoparium and *Bouteloua gracilis*). Results show that AMF and legumes altered the community structure of the grasses. *G. cubense* favoured the productivity of warm-season *B. gracilis* when growing with *M. sativa*. This might be related to a negative impact of *G. cubense* on the nitrogen-fixing activity of *M. sativa* and to a lower N-use efficiency of *E. canadensis* and *E. lanceolatus* ssp. *lanceolatus* under competition. This suggested an increased ability of *B. gracilis* to use the available N resource as affected by more competitive species, whereas *Glomus* sp. reduced the competitive ability of this grass when associated with *M. sativa*. The decrease in *B. gracilis* biomass was thus likely caused by enhancement of P uptake by *M. sativa* over this grass. *Glomus* sp. was beneficial to *S. scoparium*, another warm-season species, in the absence of legumes, and this may be attributed to improved P-use efficiency of this grass under competition with cool season-grasses. In contrast, AMF and legumes were not beneficial for the cool season grasses. *G. cubense* depressed the growth of *E. trachycaulus* ssp. *subsecundus*, and *M. sativa* decreased nutrient uptake by cool-season native grasses. This study shows that beneficial effect of the arbuscular mycorrhizal symbiosis on the coexistence of warm-season grasses with more competitive cool-season grasses depends on the identity of the AMF symbiont, the presence of legume species, and nitrogen resource availability that was affected by the most competitive species or P-use efficiency of warm season species.

Keywords: competition; *Glomus cubense; Glomus* sp.; legume; N_2-fixation; nutrient uptake; nutrient-use efficiency; prairie grass dynamics

4.3 Introduction

The AMF supply soil nutrients, particularly nitrogen (N) and phosphorus (P) to their host plant, while the plant reciprocates by providing carbon and energy to the fungus (Smith and Read, 2008). The presence of arbuscular mycorrhizal fungi (AMF) in plant communities can influence plant coexistence (Hart et al., 2003; Stein et al., 2009; Chagnon et al., 2012) by altering their competitive relationship (Scheublin et al., 2007; Wagg et al., 2011). The influence of AMF on plant community structure may result from the different effects of AMF on competing plant species, which can be negative, neutral or positive (Johnson et al., 1997; Hart et al., 2003; Klironomos, 2003; Smith et al., 2011), depending on the degree of physiological compatibility existing between the fungal symbiont and host plant (van der Heijden et al., 1998), nutrient availability (Johnson, 2010), and plant species interactions (Mariotte et al., 2013). The variation in the effects of AMF on plant growth can be explained by the variation in the C cost for the nutrient benefit provided in different AMF-plant associations (Smith et al., 2011).

The different responses of plants to the AMF symbiosis in a plant community can promote plant coexistence and hence increase plant community productivity (Wagg et al., 2011; Mariotte et al., 2013). However, Daisog et al. (2012) have shown that AMF may alter the coexistence of crops with agricultural weed species differing in their mycorrhizal status. Previous studies demonstrated that prairie grasses had different responses to AMF and thus that AMF influence plant community structure in the prairie (Wilson and Hartnett, 1998). Research shows that the warm-season grasses are more responsive to AMF than cool-season grasses (Hetrick et al., 1988; Wilson and Hartnett, 1998; Hoeksema et al., 2010) because they have coarser and less branched root systems, relying on AMF for nutrient uptake (Hetrick et al., 1991; Hartnett and Wilson, 2002).

Pastures composed of warm-season and cool-season native grass species provide good quality forage for early and late season grazing (Tilman et al., 2001; Schellenberg et al., 2012). The inclusion of N_2-fixing legumes with native grass mixtures at seeding may result in the establishment of self-sustaining and permanent pastures in the Prairie ecozone. The inclusion of N_2-fixing legumes in forage stands increases plant productivity (Temperton et al., 2007; Nyfeler et al., 2009) particularly through the transfer of fixed N from legumes to grasses (Temperton et al., 2007). This N transfer can take place through legume root exudation (Jalonen et al., 2009) or decomposition and mineralisation of legume tissues (Ledgard and Steele, 1992; Sierra and Desfontaines, 2009). Uptake and translocation of N by AMF hyphal networks may facilitate this transfer of N (Bethlenfalvay et al., 1991; Miller and Allen, 1992; He et al., 2003; Smith and Read, 2008).

Several studies have shown that AMF influence the competitive relationship between legumes and neighbouring grasses by favouring the legume component in mixes where the legume is more responsive to AMF than the grass (Scheublin et al., 2007; Wagg et al., 2011), but in other cases, AMF increased the competitive ability of grass rather than of the legume component, favouring grass uptake of the N_2-fixed and released by the legume (Hamel et al., 1991). This suggests that the effect of AMF on the competition between grass and legumes depends on the relative efficiency of the symbiosis formed by the grass and legume component of a plant community (Hamel et al., 1991; Hamel et al., 1992; Scheublin et al., 2007). However, the effect of the N_2-fixing legumes and the AMF on plant growth and nutrition of forage mixtures containing warm- and cool-season native grasses is poorly understood.

The aim of this study was to clarify the influence of AMF and legumes on the competitive relationship between native grasses. Specifically, we tested (1) if the interactive effect of N_2-fixing legumes and AMF alters the competitive ability of prairies grasses, and (2)

if the identity of the AMF and neighbouring N_2-fixing legumes determines the competitive ability of warm- and cool season prairies grasses in a plant community.

4.4 Materials and methods

4.4.1 Experimental design and biological material

The experiment had a randomized 3×3 factorial design with five repetitions. The first factor, inoculation, had three levels: plant communities were non-inoculated, inoculated with *Glomus cubense* Rodr. & Dalpé (INCAM-4 = DAOM 2411981) or inoculated with *Glomus* sp. (INCAM-8), a non-described species (Dalpé, personal communication). Both AMF isolates were graciously provided by Dr. Yakelin Rodriguez, Instituto Nacional de Ciencias Agrícolas, San José de las Lajas Cuba. The second factor also had three levels, the inclusion of *Medicago sativa* L. (alfalfa), *Dalea purpurea* Vent. (purple prairie clover) or the absence of legume in the plant communities. The treatment combinations were applied on pot-grown stands composed of three cool-season grasses, *Elymus canadensis L.* (Canada wildrye), *Elymus trachycaulus* (Link) Gould ex Shinners ssp. *subsecundus* (Link) (awned wheatgrass), *Elymus lanceolatus* (Scribn. & J.G. Sm.) Gould ssp. *lanceolatus* (northern wheatgrass) and two warm-season grasses, *Bouteloua gracilis* (Kunth.) Lag. ex Griffiths (blue grama) and *Schizachyrium scoparium* (Michx.) Nash (little bluestem). The grass and legume seeds were provided by Ducks Unlimited Canada Inc, Saskatchewan Provincial office.

4.4.2 Germination of seeds

Seeds of the native grasses *E. canadensis* and *S. scoparium* needed a cold treatment to relieve dormancy. They were then placed between two wet filter papers in Petri dishes at 5ºC for two weeks in the dark and after that exposed to light at room temperature for one week until germination. The seeds of *E. trachycaulus* ssp. *subsecundus*, *E. lanceolatus* ssp.

lanceolatus, *B. gracilis,* and of the legumes *M. sativa* and *D. purpurea* were simply placed on wet filter papers in Petri dishes in the dark for two days to allow germination. Germinated seeds were transferred into small pots filled with vermiculite and were watered daily with distilled water for two weeks, after which the seedlings were transferred into the experimental pots as described below.

4.4.3 Growth conditions

The 10-L pots were filled with 10 kg dry weight of pasteurized soil (90 °C, 1h). The Brown Chernozemic soil was a loamy sand with pH of 6.5, EC of 0.48 ms cm^{-1} containing 19.7 µg of NH_4-N, 14.1 µg of NO_3-N, 21.9 µg of PO_4-P and 357 µg of K per g soil after pasteurization. The soil was taken from the Bulin site located near Swift Current, Saskatchewan. Designated pots received 6 g of AMF inoculum consisting of chopped leek roots infected by one of the mycorrhizal isolates, or of autoclaved roots. The AMF inocula were placed 5 cm below the soil surface. *M. sativa* was inoculated with 0.2 g of *Sinorhizobium meliloti* (Nitragin Gold) and *D. purpurea* with 0.2 g of *Rhizobium* sp. isolated from *Onobrychis* sp. (Nitragin type F). The rhizobium strains were used as dry powdered culture. Mixtures composed of the five grass species (five plants of single species), or of the five grass species and a legume species (six plants of single species) were grown in each pot in a growth chamber with 16 h photoperiod per day, a temperature cycle of 23 °C day/19 °C night, and 60% relative humidity. For the mixture of native grass species and legumes, the legume specie was planted in the centre of each pot, while each native grass specie was randomly planted around the legume specie. After 4 weeks, each pot was fertilized twice a week with 500 mL of a modified Long Ashton nutrient solution containing: 750 mg KNO_3, 200 mg NaH_2PO_4 H_2O, 244 mg $MgSO_4$, 950 mg $Ca(NO_3)_2 4H_2O$, 1.7 mg $MnSO_4$, 0.25 mg

$CuSO_4$ $5H_2O$, 0.30 mg $ZnSO_4$ $7H_2O$, 3.0 mg H_3BO_3, 5.0 mg NaCl, 0.09 mg $(NH_4)_6Mo_7O_{24}$ $4H_2O$ and 32.9 mg NaFe-EDTA per L. Pots were watered as required with distilled water.

4.4.4 Harvest, sampling and analysis

After five months of growth, plants were cut at ground level, dried separately at 40 °C for 3 days, weighed to determine drymass and the dry plants were ground. A subsample of ground plant material was digested (Varley, 1966) and the concentration of N (Noel and Hambleton, 1976) and P in plant tissues was measured (Milbury et al., 1970) on a segmented flow auto analyser (Technicon, AAII System, Tarrytown, NY). Uptake of P and N by each species was calculated by multiplying plant biomass by the concentration of each individual nutrient. Nitrogen- and phosphorus-use efficiencies were calculated as total aboveground biomass divided by total nutrient uptake. A second subsample of ground plant material was reduced to powder in a bead miller before placing 0.006-g samples in tin capsules, in preparation for the measurement of the abundance of ^{14}N and ^{15}N in plant tissues on a Carlo Erba 17 NA1500 elemental CN analyzer coupled to an Optima isotope ratio mass spectrometer. Roots were harvested from each pot, washed, cleared in 10% KOH solution, stained in a 5% black ink-vinegar solution (Vierheilig et al., 1998) and the percent colonization by AMF was determined under a dissecting microscope at 40× magnification, using the gridline-intersect method (Giovannetti and Mosse, 1980). We have extended the growing period up to five months because the native warm season species have a slower rate growth and lower establishment than cool season grasses and they need more time to be productive. In addition, this may be also needed to ensure that all native species, in particular the warm season species, are well colonized by AMF.

4.4.5 Estimation of N-transfer between legumes and grasses

The analysis of nitrogen isotopic ratio $\delta^{15}N$ in plant tissue allowed estimation of N acquisition from soil and air (Gubsch et al., 2011). The $\delta^{15}N$ value (i.e. the isotopic nitrogen composition of the sample relative to that of atmospheric nitrogen expressed in ‰) was calculated according to Equation (1) (Robinson, 2001):

$$\delta^{15}N = \left((R_{sample} - R_{atm})/R_{atm}\right) \times 1000 \quad (1)$$

Where R_{sample} is the sample $^{15}N/^{14}N$ isotope ratio and R_{atm} is the $^{15}N/^{14}N$ ratio of atmospheric N_2 (R_{atm} = 0.00366). The $\delta^{15}N$ of atmospheric N_2 by definition is 0 ‰. The $\delta^{15}N$ of a N_2-fixing plant is usually close to the atmospheric $\delta^{15}N$ since legumes are capable of fixing nitrogen through a symbiosis with N_2-fixing bacteria (Hogberg, 1997). However, the $\delta^{15}N$ of grasses growing without legumes reflects the $\delta^{15}N$ of the soil N, which is usually higher than that of atmospheric N_2 (Shearer and Kohl, 1986). Consequently, $\delta^{15}N$ values closer to 0 ‰ (the atmospheric $\delta^{15}N$ value) in non-N_2-fixing plants growing with a N_2-fixing plants compared to that of non-fixing plants growing in absence of N_2-fixing plants, indicate that fixed N was transferred from the legumes to non-N_2-fixing plants (Temperton et al., 2007).

4.4.6 Estimation of N_2-fixation by the natural abundance method

The proportion of nitrogen derived from the atmosphere (Ndfa) in the N_2-fixing plants (*M. sativa* and *D. purpurea*) was calculated by comparing the isotopic composition of the N_2-fixing legumes tissues with that of a non-fixing reference plant deriving its N solely from plant available soil nitrogen. It was calculated according to Equation (2) (Shearer and Kohl, 1986):

$$\%Ndfa = ((\delta^{15}N_{ref-}\delta^{15}N_{fix})/(\delta^{15}N_{ref-}\delta^{15}N_{atm})) \times 100 \quad (2)$$

where $\delta^{15}N_{ref}$ is the $\delta^{15}N$ of the non-N_2-fixing reference plants. In the present study, it was the mean of the $\delta^{15}N$ in all grasses grown without legumes. $\delta^{15}N_{fix}$ is the $\delta^{15}N$ of N_2-fixing plants.

$\delta^{15}N_{atm}$ is the $\delta^{15}N$ of the N_2-fixing plant when relying on atmospheric N_2 as the sole N source. It reflects the isotope discrimination which occurs during N_2-fixation in the specific legume (Hogberg, 1997; Evans, 2001). The $\delta^{15}N_{atm}$ of legumes was measured in a separate growth chamber experiment with four replicates, where each species of legumes inoculated with the appropriate rhizobia were grown under 16 h of light per day, a temperature cycle of 23 °C day/19 °C night, and 60% relative humidity, in a 1 L pot filled with 500 g of washed coarse sand and irrigated twice a week with 200 mL of a N-free modified Long Ashton nutrient solution containing: 750 mg KCl, 200 mg NaH_2PO_4 H_2O, 244 mg $MgSO_4$, 950 mg $CaCl_2 2H_2O$, 1.7 mg $MnSO_4$, 0.25 mg $CuSO_4$ $5H_2O$, 0.30 mg $ZnSO_4$ $7H_2O$, 3.0 mg H_3BO_3, 5.0 mg NaCl, 0.09 mg $(NH_4)_6Mo_7O_{24}$ $4H_2O$ and 32.9 mg NaFe-EDTA per L. Pots were watered with distilled water as needed. Plants were harvested after three months, dried, ground and analysed for $\delta^{15}N$ as described previously.

4.4.7 Statistical analyses

The effect of legumes and inoculation on the structure of grass plant communities was analyzed by multivariate analysis of variance (MANOVA). The competitive ability of each species was expressed by the percentage of biomass contributed by each grass species relative to total biomass of grasses per pot. The difference between treatment means was tested using the Wilks' lambda test. The significant effects detected by MANOVA were revealed by factorial analyses of variance (ANOVA) that was performed separately on each grass species, in order to identify affected species. The effect of legumes and AM fungi on nutrient uptake, the concentration of nutrients in grass tissues, the $\delta^{15}N$ of the different grasses, the % Ndfa of legume plants were separately tested by 2-way ANOVA. The significance of differences between treatment means was tested with the Tukey's range test. Analyses were conducted using the JMP 8 software.

4.5 Results

4.5.1 Plant competitive ability, nutrient uptake, nutrient concentration and arbuscular mycorrhizal colonization

The level of root colonization by AMF was similar in the different legume treatments. *Glomus* sp. colonized plant roots to a higher percentage (45%) than *G. cubense* (19%). Non-inoculated plants were not colonized. The interaction between legumes and inoculation affected significantly the competitive relationships between different native grasses (Fig. 1). However, the legume and inoculation treatments influenced the competitive ability of the two warm-season grass species, *S. scoparium* and *B. gracilis*, while not substantially affecting the cool-season grass species (Table 1). *G. cubense* improved the percentage of above-ground biomass contributed by *B. gracilis* (Table 1) and the nutrient uptake (Table S1 and S2) when it was grown in presence of *M. sativa* compared to the non-AMF treatment, whereas the presence of this legume reduced the biomass of *B. gracilis* (Table 1) and the nutrient uptake by this grass (Table S1 and S2) in plant communities inoculated with *Glomus* sp. as compared to the non-legume treatment. The competitiveness of the other warm-season grass, *S. scoparium*, was also influenced by this interaction. Inoculation with *Glomus* sp. increased the proportion of *S. scoparium* biomass (Table 1) compared to the non-mycorrhizal treatment and nitrogen uptake (Table S1) by this grass in the mix, but only in the absence of legumes,

In contrast, the cool-season *E. trachycaulus* ssp. *subsecundus* was affected by inoculation irrespective of the legume treatment. *G. cubense* significantly reduced the competitive ability of *E. trachycaulus* ssp. *subsecundus* (Table 1) and phosphorus uptake (Table S2) by this grass, while inoculation had no effect on the competitive ability of *E. canadensis* or *E. lanceolatus* ssp. *lanceolatus* (Table 1). The concentration of nitrogen in different species of grasses was affected by legume treatments. *M. sativa* enhanced the concentration of N in grass tissues (Table 2). The concentration of phosphorus in cool-season

grasses and in *S. scoparium* tissues was also influenced by the presence of legumes. The tissues of these grasses had higher concentration of P in the presence of *M. sativa* than in another legume treatment. There was no effect of inoculation or *D. purpurea* presence on the concentration of N and P in grasses.

The concentration and content of phosphorus in *M. sativa* tissue was also influenced by inoculation treatment, and were higher when this legume was inoculated with *Glomus* sp. (Table S3).

4.5.2 Nutrient-use efficiency

The legumes and inoculation treatments influenced the nitrogen-use efficiency of cool-season grasses. *M. sativa* decreased the nitrogen-use efficiency of *E. canadensis* in non-inoculated plant communities or those inoculated with *G. cubense* or *Glomus* sp., while this legume reduced the nitrogen-use efficiency of *E. lanceolatus* ssp. *lanceolatus*, either uninoculated or inoculated with *G. cubense*, compared to the non-legume treatment. In contrast to *M. sativa*, *D. purpurea* increased the N-use efficiency of cool season-grasses inoculated with *Glomus* sp. compared to non-legume treatments (Table S4).

The P-use efficiency of *S. scoparium* was also affected by the presence of legumes and AMF. It was higher when this grass was inoculated with *Glomus* sp. in the absence of legumes compared to legume treatments. *Dalea purpurea* increased the P-use efficiency of this grass, but only in the absence of AMF. In contrast, *M. sativa* reduced the P-use efficiency of cool season grasses, irrespective of the AMF treatment (Table S5).

4.5.3 Nitrogen fixation and $\delta^{15}N$ isotopic composition

Inoculation had no effect on the isotopic composition of N in different species of grasses. However, the presence of legumes significantly modified the $\delta^{15}N$ of *E. trachycaulus*

ssp. *subsecundus*. Both legumes decreased the value of $\delta^{15}N$ measured in this grass compared to the legume-free treatment, indicating that there was N-transfer from the legumes to *E. trachycaulus* ssp. *subsecundus* (Table 3). A significant interaction between legume and inoculation was detected in the amount of N_2-fixed by legumes (Fig. 2). It indicated that *G. cubense* reduced N_2-fixation in *M. sativa*, but not in *D. purpurea*.

Table 1 Contribution of different grass species to total grass biomass as influenced by the presence of a legume and by inoculation.

Grasses	Proportion of the grass biomass (%)[a]					P value		
	Legume	Control	*Glomus cubense*	*Glomus* sp.	Mean	Leg[b]	AMF	Leg*AMF
E. canadensis	No legume	70.3	69.6	58.5	66.1 a	0.547	0.170	0.177
	M. sativa	51.4	66.1	71.1	62.9 a			
	D. purpurea	59.6	75.6	70.6	68.6 a			
	Mean	60.5 x	70.4 x	66.7 x				
E. trachycaulus ssp. *subsecundus*	No legume	21.2	19.5	27.7	22.8 a	0.656	**0.019**	0.169
	M. sativa	38.2	20.2	19.7	36.0 a			
	D. purpurea	31.4	12.0	23.7	22.3 a			
	Mean	30.2 y	17.2 x	23.7 xy				
E. lanceolatus ssp. *lanceolatus*	No legume	5.2	7.3	7.7	6.8 a	0.585	0.606	0.767
	M. sativa	8.4	8.5	7.6	8.2 a			
	D. purpurea	5.8	8.8	2.8	5.8 a			
	Mean	6.4 x	8.2 x	6.1 x				
S. scoparium	No legume	0.8 ax	1.1 axy	3.1 by	1.7	0.041	0.132	**0.012**
	M. sativa	0.8 ax	0.6 ax	0.8 ax	0.7			
	D. purpurea	0.9 ax	1.7 ax	0.8 ax	1.1			
	Mean	0.8	1.1	1.6				
B. gracilis	No legume	2.5 ax	2.4 ax	2.9 bx	2.6	0.434	0.024	**<0.001**
	M. sativa	1.2 ax	4.6 ay	0.9 ax	2.20			
	D. purpurea	2.3 ax	2.0 ax	2.0 bx	2.1			
	Mean	2.0	3.0	1.9				

[a]Means are not significantly different ($p \leq 0.05$, ANOVA; $n = 5$) when followed by the same letter (a, b) within a column or (x, y) within a row, according to the Tukey's range test. Bolded values are significant.

[b]Leg = legumes.

Table 2 Concentration of nitrogen and phosphorus of each grass species as influenced by the presence of legumes.

	Concentration of nitrogen[a] (mg g^{-1} DM) Grass species					Concentration of phosphorus[a] (mg g^{-1} DM) Grass species				
	EC[b]	ET	EL	SS	BG	EC	ET	EL	SS	BG
No legumes	9.3 a	15.8 a	14.1 a	10.2 a	14.8 a	1.0 a	1.9 a	1.4 a	1.2 a	2.0 a
MS	14.3 b	19.3 b	19.1 b	14.4 b	19.8 b	1.7 b	2.3 b	2.1 b	1.6 b	2.0 a
DP	8.3 a	13.9 a	12.2 a	9.1 a	14.7 a	0.9 a	1.8 a	1.3 a	1.1 a	1.7 a
P value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.109

[a]Within each grass species, means are not significantly different ($p \leq 0.05$, ANOVA; n = 5) when followed by the same letter (a, b) according to Tukey's range test. Bolded values are significant.

[b]EC = *E. canadensis*; ET = *E. trachycaulus* ssp. *subsecundus*; EL = *E. lanceolatus* ssp. *lanceolatus*; BG = *B. gracilis*; SS=*S. scoparium*; MS = *M. sativa* and DP = *D. purpurea*.

Table 3 Nitrogen isotopic ratio ($\delta^{15}N$) of each grass species as influenced by the presence of legumes.

	$\delta^{15}N$[a]				
	Grass species				
	E. canadensis	*E. trachycaulus* ssp. *subsecundus*	*E. lanceolatus* ssp. *lanceolatus*	*S. scoparium*	*B. gracilis*
No legumes	12.2 a	14.0 b	12.3 a	17.7 ab	12.0 a
MS	12.5 a	11.3 a	10.6 a	13.6 b	11.5 b
DP	11.6 a	12.2 a	11.2 a	10.9 a	10.7 a
P value	0.522	**0.003**	0.059	**0.012**	0.134

[a]Within each grass species, means (n = 5) are not significantly different ($p \leq 0.05$, ANOVA; n = 5) when followed by the same letter (a, b), according to Tukey's range test. Bolded values are significant.

Table S1 Nitrogen uptake by each grass species as influenced by the presence of a legume and by inoculation.

Grasses		Nitrogen uptake (g)[a]				P value		
	Legume	Control	*Glomus cubense*	*Glomus* sp.	Mean	Leg[b]	AMF	Leg*AMF
E. canadensis	No legume	60.8	67.0	56.2	61.3 b	**<0.001**	0.131	0.722
	M. sativa	15.5	21.9	18.1	18.5 a			
	D. purpurea	55.6	79.4	47.9	62.0 b			
	Mean	44.0 x	56.1 x	40.7 x				
E. trachycaulus ssp.	No legume	33.6 abx	27.7 bx	42.5 bx	34.7	<0.001	0.002	**0.029**
subsecundus	*M. sativa*	16.5 ay	7.8 ax	5.6 ax	10.0			
	D. purpurea	46.4 by	18.9 bx	26.7 bxy	30.7			
	Mean	32.2	18.2	25.0				
	No legume	7.7	9.1	11.3	9.3 b	**<0.001**	0.737	0.739
E. lanceolatus ssp.	*M. sativa*	3.7	2.3	2.6	2.8 a			
lanceolatus	*D. purpurea*	6.3	12.1	3.3	7.2 ab			
	Mean	5.9 x	7.8 x	5.7 x				
S. scoparium	No legume	1.0 bx	1.1 ax	2.5 bx	1.5	<0.001	0.266	**0.017**
	M. sativa	0.3 ax	0.2 ax	0.2 ax	0.2			
	D. purpurea	0.9 abx	1.4 ax	0.8 ax	1.0			
	Mean	0.7	0.9	1.1				
B. gracilis	No legume	4.4 bx	3.3 ax	3.7 bx	3.8	<0.001	0.005	**0.001**
	M. sativa	0.5 ax	1.7 ay	0.3 ax	0.8			
	D. purpurea	3.6 bx	3.4 ax	2.8 bx	3.2			
	Mean	2.8	2.8	2.3				

[a]Means are not significantly different ($p \leq 0.05$, ANOVA; n = 5) when followed by the same letter (a, b) within a column or (x, y) within a row, according to the Tukey's range test. Bolded values are significant.

[b]Leg =legumes.

Table S2 Phosphorus uptake by each grass species as influenced by the presence of a legume and by inoculation.

Grasses		Phosphorus uptake (g)[a]				P value		
	Legume	Control	*Glomus cubense*	*Glomus* sp.	Mean	Leg[b]	AMF	Leg*AMF
E. canadensis	No legume	6.7	6.3	6.3	6.4 b	**<0.001**	0.378	0.527
	M. sativa	1.9	2.6	2.3	2.3 a			
	D. purpurea	5.2	7.8	6.8	6.6 b			
	Mean	4.6 x	5.6 x	5.2 x				
E. trachycaulus ssp. *subsecundus*	No legume	4.8	3.1	5.1	4.3 b	**<0.001**	**<0.001**	0.075
	M. sativa	2.2	0.9	0.7	1.2 a			
	D. purpurea	5.3	2.5	4.4	4.0 b			
	Mean	4.1 y	2.1 x	3.4 xy				
E. lanceolatus ssp. *lanceolatus*	No legume	0.8	0.8	1.2	0.9 b	**<0.001**	0.749	0.692
	M. sativa	0.5	0.3	0.3	0.3 a			
	D. purpurea	0.6	1.2	0.4	0.7 b			
	Mean	0.6 x	0.8 x	0.6 x				
S. scoparium	No legume	0.1	0.1	0.3	0.2 b	**<0.001**	0.539	0.219
	M. sativa	0.03	0.02	0.01	0.02 a			
	D. purpurea	0.1	0.2	0.1	0.1 b			
	Mean	0.1 x	0.1 x	0.1 x				
B. gracilis	No legume	0.5 bx	0.5 bx	0.53 cx	0.48	<0.001	0.003	**<0.001**
	M. sativa	0.1 ax	0.2 ay	0.02 ax	0.07			
	D. purpurea	0.4 bx	0.4 bx	0.34 bx	0.37			
	Mean	0.30	0.3	0.29				

[a]Means are not significantly different ($p \leq 0.05$, ANOVA; $n = 5$) when followed by the same letter (a, b, c) within a column or (x, y) within a row, according to the Tukey's range test. Bolded values are significant.

[b]Leg =legumes.

Table S3 P concentration (mg g^{-1}) and P uptake (g) by legumes species as influenced by inoculation.

	M. sativa		*D. purpurea*	
Inoculation[a]	P concentration	P uptake	P concentration	P uptake
Control	1.3 a	7.8 a	1.8 a	0.4 a
Glomus cubense	1.2 a	5.9 a	2.1 a	0.5 a
Glomus sp.	1.8 b	11.2 b	2.0 a	0.4 a
P value	**0.002**	**0.018**	0.133	0.744

[a]Means (n = 5) are not significantly different ($p \leq 0.05$, ANOVA; n = 5) when followed by the same letter (a, b), according to Tukey's range test. Bolded values are significant.

Table S4 Nitrogen-use efficiency of each grass species as influenced by the presence of a legume and by inoculation.

Grasses		N-use efficiency[ab]				P value		
	Legume	Control	*Glomus cubense*	*Glomus* sp.	Mean	Leg[c]	AMF	Leg*AMF
E. canadensis	No legume	1.3 bx	1.0 bx	1.1 bx	1.1	<0.001	0.032	**0.002**
	M. sativa	0.8 ay	0.7 ax	0.7 axy	0.7			
	D. purpurea	1.1 bx	1.2 bx	1.7 cy	1.3			
	Mean	1.1	0.9	1.1				
E. trachycaulus ssp. *subsecundus*	No legume	0.7 ax	0.6 abx	0.6 ax	0.6	<0.001	0.104	**0.032**
	M. sativa	0.6 ax	0.5 ax	0.5 ax	0.5			
	D. purpurea	0.7 ax	0.7 bx	0.9 by	0.8			
	Mean	0.6	0.6	0.7				
E. lanceolatus ssp. *lanceolatus*	No legume	0.8 bx	0.8 bx	0.6 ax	0.7	<0.001	0.645	**0.021**
	M. sativa	0.6 ax	0.5 ax	0.5 ax	0.5			
	D. purpurea	0.8 bx	0.8 bx	0.9 bx	0.8			
	Mean	0.7	0.7	0.7				
S. scoparium	No legume	1.0	1.0	1.1	1.0 b	**<0.001**	0.126	0.478
	M. sativa	0.6	0.8	0.7	0.7 a			
	D. purpurea	1.1	1.1	1.2	1.1 b			
	Mean	0.1 x	0.1 x	1.0 x				
B. gracilis	No legume	0.7	0.7	0.7	0.7 b	**<0.001**	0.050	0.850
	M. sativa	0.5	0.5	0.5	0.5 a			
	D. purpurea	0.7	0.7	0.8	0.7 b			
	Mean	0.6 x	0.6 x	0.7 x				

[a]N-use efficiency of each species was calculated as total aboveground biomass divided by total nitrogen uptake.

[b]Means are not significantly different ($p \leq 0.05$, ANOVA; $n = 5$) when followed by the same letter (a, b, c) within a column or (x, y) within a row, according to the Tukey's range test. Bolded values are significant.

[c]Leg =legumes**.**

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Table S5 Phosphorus-use efficiency of each grass species as influenced by the presence of a legume and by inoculation.

Grasses		P-use efficiency[ab]				P value		
	Legume	Control	*Glomus cubense*	*Glomus* sp.	Mean	Leg[c]	AMF	Leg*AMF
E. canadensis	No legume	12.3	10.2	9.4	10.7 b	**<0.001**	0.188	0.709
	M. sativa	6.7	5.6	5.3	5.8 a			
	D. purpurea	12.1	12.2	11.8	12.0 b			
	Mean	10.3 x	9.3 x	8.8 x				
E. trachycaulus ssp. *subsecundus*	No legume	5.0	5.6	5.3	5.3 b	**<0.001**	0.842	0.247
	M. sativa	4.2	4.6	4.3	4.4 a			
	D. purpurea	6.0	5.1	6.1	5.7 b			
	Mean	5.1 x	5.1 x	5.2 x				
E. lanceolatus ssp. *lanceolatus*	No legume	7.7	8.4	6.2	7.4 b	**<0.001**	0.440	0.081
	M. sativa	4.6	4.8	4.8	4.7 a			
	D. purpurea	7.1	7.5	8.1	7.6 b			
	Mean	6.5 x	6.9 x	6.4 x				
S. scoparium	No legume	6.9 ax	8.2 ax	9.7 bx	8.3	<0.001	0.419	**<0.001**
	M. sativa	6.2 ax	6.3 ax	6.3 ax	6.2			
	D. purpurea	12.2 by	8.9 axy	7.2 ax	9.4			
	Mean	8.4 x	7.8 x	7.7 x				
B. gracilis	No legume	6.3	4.7	5.8	5.6 a	0.196	0.579	0.618
	M. sativa	4.6	5.1	5.1	5.0 a			
	D. purpurea	5.9	5.7	6.3	6.0 a			
	Mean	5.6 x	5.2 x	5.7 x				

[a]P-use efficiency of each species was calculated as total aboveground biomass divided by total phosphorus uptake.

[b]Means are not significantly different ($p \leq 0.05$, ANOVA; n=5) when followed by the same letter (a, b, c) within a column or (x, y, z) within a row, according to the Tukey's range test. Bolded values are significant.

[c]Leg =legumes.

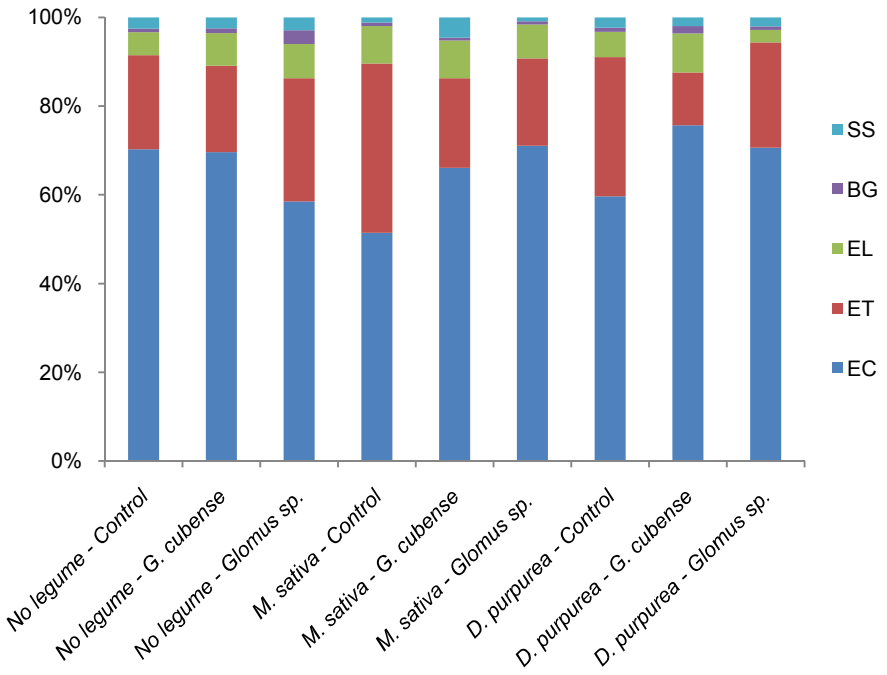

Figure 1 Percentage of biomass contributed by different native grasses growing with and without legumes, in presence or absence of AMF in each pot. The interaction between species, legumes and AMF was significant according to Manova ($p = 0.013$, $n = 5$). EC = *E. canadensis*; ET = *E. trachycaulus* ssp. *subsecundus*; EL = *E. lanceolatus* ssp. *lanceolatus*; SS=*S. scoparium*; BG = *B. gracilis.*

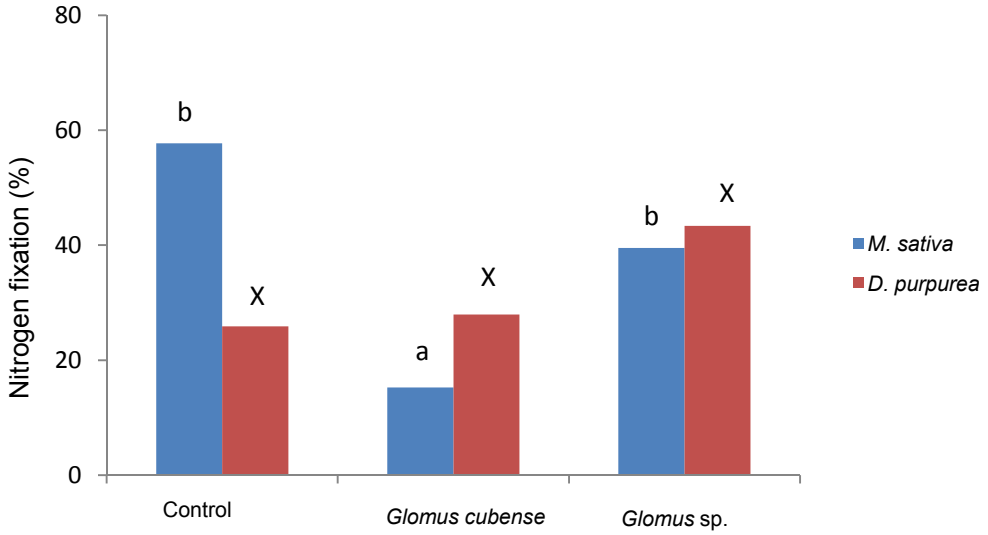

Figure 2 Percentage of nitrogen derived from the atmosphere in *M. sativa* and *D. purpurea* species as influenced by AMF inoculation. Means (n = 5) are not significantly different ($p \leq 0.05$, ANOVA; n = 5) when followed by the same letter (a, b) for *M. sativa* or (X, Y) for *D. purpurea*, according to Tukey's range test.

4.6 Discussion

Our results clearly show that the presence and identity of legumes and AMF are key factors determining the coexistence of warm-season and cool-season grasses in plant communities.

G. cubense was favourable to the nutrition and productivity of *B. gracilis* and this effect might be related to a negative impact of this AMF on the N_2-fixing activity of *M. sativa* and on N-use efficiency of more competitive species such as *E. canadensis* and *E. lanceolatus* ssp. *lanceolatus*. However, the greater N uptake by *B. gracilis* was unrelated to fixed N_2 transfer since the $\delta^{15}N$ of *B. gracilis* remained higher in presence of legumes or to sparing N because we did not observe the increase in nitrogen fixation that could ameliorate soil N sparing. The lower nitrogen fixation caused by this fungus could increase the competition between grasses and *M. sativa* for N sources due to lower N sparing because this legume might become more dependent on soil N. It is likely that the reduction of N-use efficiency of cool season-grass through *G. cubense* could also contribute to increasing the N availability for *B. gracilis* when in competition with *M. sativa*. Our results are in contrast with the findings of Gubschet al. (2011) and Temperton et al. (2007) showing that the grasses in competition benefited from fixed N_2 transferred from legumes or the sparing soil N may played an important role for plant N nutrition in the presence of N_2-fixing plants. However, the negative effect of *G. cubense* on N_2 fixing activity of *M. sativa* may also result from the fact that this fungus was not sufficient to provide the large amount of phosphorus that was required for nitrogen fixation (Mortimer et al., 2008). It has been suggested that the N_2 fixing legume might prefer to associate with specific AMF more efficient in supplying phosphorus (Scheublin et al., 2004).

Hoeksema et al. (2010) showed that AMF were more beneficial to grasses than N_2-fixing plants. Limited amounts of C to allocate to both the AMF and rhizobia can result in antagonistic interactions between the two symbionts of an N_2-fixing legume (Bethlenfalvay et al., 1985; Hoeksema et al., 2010). The reduction of N fixation might be due to the high C cost provided to support AMF colonisation since AMF is the primary sink of host C in dual symbiosis, which resulted in delayed nodule growth and thus can lead to a reduction in C available for nodule development after AMF establishment (Mortimer et al., 2008). In another case, however, a legume (*Lotus*) was strongly dependent on AMF, causing a growth depression in the associated grass (*Festuca*) (Scheublin et al., 2007).

As was the case in our study, the identity of AMF was shown to alter plant community structure (van der Heijden et al., 2006; Scheublin et al., 2007). In contrast to *G. cubense*, *Glomus* sp. negatively impacted productivity and nutrient uptake by *B. gracilis* and favoured *M. sativa* by increasing the percentage of P in plant tissue as well as total P uptake. These results suggest that the *Glomus* sp. benefited *M. sativa* at the expense of *B. gracilis*. It seems here that this fungus preferentially supplies soil P to *M. sativa* reducing P availability to *B. gracilis*. The N_2-fixing activity of legumes may often depend on AMF for nitrogen fixation activity by improving P nutrition (Azcón et al., 1991; Mortimer et al., 2008). *Bouteloua gracilis* may also lack compatibility with *Glomus* sp. Different AMF taxa vary in their effects on plant productivity (van der Heijden et al., 1998). It has been shown that plants associated with different AMF could have different growth responses ranging from mutualism to parasitism (Klironomos, 2003) depending on nutrient availability (Johnson, 2010). The two warm season grasses

of the plant community, *B. gracilis* and *S. scoparium*, responded to legume presence and inoculation, but their competitive ability was favoured by different conditions. *Glomus* sp., rather than *G. cubense,* increased the competitive ability by *S. scoparium* over cool-season grasses, and then only in the absence of legume. The growth promotion of *S. scoparium* was mainly attributed to improved P-use efficiency through *Glomus* sp., when the legume was absent. The different growth pattern of the warm-season grasses in plant assemblages was determined by the identity of AMF, the presence or absence of legumes and nutrient-use efficiency of dominant competitor or of warm-season species.

In contrast to warm-season grasses, cool-season grasses did not benefit from AMF. The competitive ability of the cool season grass *E. trachycaulus* ssp. *subsecundus* was reduced in the presence of *G. cubense* irrespective of legumes. The reduction of growth of the dominant competitor *E. trachycaulus* ssp. *subsecundus* mediate plant coexistence. Similarly, Mariotte et al. (2012) showed that the growth of dominant species was more negatively affected by AMF than subordinate species and thus reduced the competitiveness of the dominant species toward the other species in the plant community. Daisog et al. (2012) reported that AMF increased the competitive ability of a non-mycorrhizal plant species (*Chenopodium album*) when grown in competition with a mycorrhizal host species (*Solanum nigrum*) and noted that this concur with a higher biomass and N uptake by *C album*. Similarly, growth reduction and P uptake of the AMF-host *Zea mays* was more severe when in competition with the non-host *C. album* than with the host-species *S. nigrum* in presence of AMF, thus increasing the ability of non-host species to coexist with AMF host-species in a community.

On the other hand, the legumes were also unfavourable to cool-season grasses. *M. sativa* decreased nutrient uptake by cool season grasses, even though this legume played a significant role in increasing the percentage of nutrients in these grass tissues. The increase in the percentage of nutrients in grass tissues was not converted into biomass or total nutrient uptake. This may be due to competition between grasses and *M. sativa* for other factors such as light and water (Tilman et al., 1997), especially since *M. sativa* is characterized by high biomass production and extensive canopies that could reduce the light for neighbouring grasses.

4.7 Conclusions

Our data have shown that AMF and N_2-fixing-legumes regulate grass community structure by favouring the productivity of warm-season species over cool-season species. The growth promotion of *B. gracilis* through *G. cubense* was mainly attributed to a more efficient use of the soil N under competition with *M. sativa* and may thus be due to higher nitrogen availability as affected by cool-season species (*E. canadensis* and *E. lanceolatus* ssp. *lanceolatus*) and to a lower N_2-fixing activity that could increase the competition between this legume and *B. gracilis* for soil N uptake. In contrast, *S. scoparium* benefited from *Glomus* sp. only in the absence of legumes and may thus be related to higher P-use efficiency through this fungus. The growth promotion of warm-season grasses depended on the ability of these warm-season species to receive an additional benefit provided by AMF that varied with fungal identity, presence or absence of certain legume species, nutrient resource availability and nutrient use-efficiency of more competitive species or of certain warm-season species under different conditions.

4.8 Acknowledgements

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Conclusion générale et perspectives

L'objectif à long terme de ces travaux est d'obtenir un système de pâturage formé de graminées indigènes productif, nutritif et durable, par l'utilisation de légumineuses fixatrices d'azote et l'augmentation de la fertilité en phosphore du sol, afin d'assurer une bonne alimentation des animaux au long de la saison de pâturage dans les régions semi-arides. L'augmentation de la fertilité du sol et la couverture végétale peuvent interagir avec les populations de champignons du sol et les modifier, ce qui peut en retour influencer à court ou à long terme la productivité et la valeur nutritive du fourrage.

Les résultats obtenus au champ nous ont permis de clarifier l'effet des légumineuses (*M. sativa*, légumineuse cultivée fixatrice d'azote ou *Dalea purpurea*, légumineuse indigène fixatrice d'azote) et l'ajout de P inorganique au sol sur la productivité et la valeur nutritive des graminées indigènes, comparé avec celles de la graminée introduite *B. biebersteinii* en association avec le *M. sativa* et d'identifier l'influence de ces pratiques sur la diversité et la structure des communautés de CMA et des champignons totaux colonisant le sol des prairies. Ces résultats montrent que l'inclusion de *M. sativa* améliore la valeur nutritive des graminées indigènes par l'augmentation des teneurs en azote et en phosphore de leurs tissus au début de l'été, lorsque la croissance de la plupart des graminées indigènes, en particulier les graminées de saison fraîche est active. En plus, cette légumineuse est capable d'augmenter la teneur en azote des tissus de la graminée de saison chaude *B. gracilis* en conditions de sécheresse intense, typique de la fin de l'été. Cela indique que la gestion des prairies semées avec les graminées indigènes basée sur l'intégration de la légumineuse fourragère cultivée *M. sativa* peut être un moyen efficace d'améliorer la valeur nutritive du fourrage

et la production du bétail tout au long de la saison, par l'augmentation des teneurs en azote du fourrage. L'inclusion de *D. purpurea* a aussi permis d'augmenter la teneur en azote des tissus de la graminée de saison fraîche *E. canadensis* à la fin de la période de croissance de l'année humide, mais seulement dans les sols non fertilisés. Il semble que la faible disponibilité du phosphore ait réduit la quantité d'azote fixé par la légumineuse *D. purpurea*, ce qui pourrait augmenter la compétition de la graminée *E. canadensis* pour l'acquisition de l'azote du sol au détriment celle de la légumineuse, puisque cette dernière était probablement devenu dépendante à l'azote du sol. En plus, la croissance de cette graminée de saison fraîche pourrait être rétablie en automne de l'année humide 2008 et lorsque la disponibilité de l'eau devient favorable pour leur croissance, ce qui pourrait favoriser leur compétition avec *D. purpurea* pour l'azote du sol.

L'augmentation de la teneur en azote des tissus des graminées indigènes en présence de *M. sativa*, sans réduction de la valeur de $\delta^{15}N$ signifie que la nutrition azotée de ces graminées était fortement liée à la plus grande disponibilité d'azote du sol (N sparing) en présence de la légumineuse fixatrice d'azote qui dépendait fortement de l'azote atmosphérique pour sa croissance (Temperton et al., 2007). Par contre, *M. sativa* réduisait le $\delta^{15}N$ mesuré chez la graminée introduite dans des conditions d'humidité suffisante, ce qui montre que cette graminée était capable d'utiliser l'azote fixé par *M. sativa,* contrairement aux graminées indigènes. D'autre part, l'augmentation des teneurs en nutriments des tissus des graminées indigènes en présence de *M. sativa,* trouvée au début de la période de croissance de l'année sèche, n'ont pas entraîné une augmentation de la biomasse ou des quantités totales de nutriments chez ces graminées. Ces observations suggèrent que la productivité des graminées n'était pas limitée par l'azote ou le

phosphore, mais peut être par la lumière ou d'autres nutriments du sol, au début de l'été. De plus, la biomasse des graminées indigènes en mélange avec ou sans légumineuse était identique à celle de la graminée introduite. Lors de cette étude, il a été démontré que l'addition de P à l'ensemencement dans un système de pâturage n'ait pas d'effet sur la productivité de l'ensemble des graminées indigènes, au début et à la fin de l'été. De plus, la productivité des espèces de saison chaude qui sont naturellement caractérisées par une croissance lente et une productivité faible semble de ne pas être amélioré par l'augmentation de la fertilité en phosphore du sol.

Cette étude souligne aussi l'importance de l'addition des légumineuses dans le système de pâturage, en particulier de l'addition de *M. sativa,* sur la modification de la structure et la diversité des communautés fongiques du sol et sur le rôle de ces dernières dans la productivité fourragère. L'inclusion des légumineuses a permis d'augmenter la diversité des champignons mycorhiziens à arbuscules, particulièrement dans les mélanges contenant les graminées indigènes, mais cet effet pourrait varier selon la teneur en phosphore du sol et probablement selon la présence d'autres facteurs tel que les interactions compétitives entre les graminées indigènes et les légumineuses et l'humidité du sol. L'augmentation de la diversité des CMA induite par l'inclusion de *M. sativa* dans les prairies indigènes était corrélée positivement avec la productivité et la quantité totale d'azote trouvée chez *M. sativa* et aussi avec la teneur en phosphore des tissus des graminées indigènes, au début de l'été. Cela suggère que *M. sativa* pourrait contribuer à augmenter la croissance des taxons de CMA fonctionnels et bénéfiques pour la productivité et la valeur nutritive du fourrage dans les praires indigènes, en conditions d'humidité suffisante typique du début de l'été. Par contre la structure des communautés

de champignons totaux, dont la plupart étaient dominées par des champignons endophytes et saprophytes, était influencée par l'interaction entre la teneur en phosphore du sol et le mélange de plantes, en conditions d'humidité élevée favorable à la productivité des plantes.

Les résultats obtenus dans les expériences en chambre de culture nous ont permis de mieux comprendre l'effet des légumineuses et des champignons mycorhiziens à arbuscules sur la dynamique des communautés de graminées indigènes. Ils montent que les légumineuses et les CMA entrainent une modification de la structure des communautés de graminées indigènes des prairies en favorisant la productivité des espèces de saison chaude au détriment des espèces de saison fraîche. L'amélioration de la productivité de la graminée *B. gracilis* par le *Glomus cubense* a été attribuée à l'utilisation accrue des ressources en azote disponibles en présence de *M. sativa* et ceci était due à la diminution de l'efficacité d'utilisation de l'azote de certaines espèces de saison fraîche, nommément *E. canadensis* et *E. lanceolatus* ssp. *lanceolatus* et à la réduction de l'activité fixatrice de l'azote par le *M. sativa*. Par contre, le *Glomus* sp. avait un effet bénéfique sur le *S. scoparium*, une espèce de saison chaude, mais seulement en absence de légumineuses, ce qui pourrait être lié à une amélioration de l'efficacité d'utilisation du phosphore par cette graminée en compétition avec des graminées de saison fraîche.

La différence de taux de la croissance des espèces de saison chaude et des espèces de saison fraîche pourrait aussi expliquer la dynamique et la compétition de différentes espèces des graminées au sein d'une communauté, selon la théorie de la sélection r/K (Grime, 1977). En effet, les espèces de saison fraîche à stratégie K ont une croissance et une absorption rapide des nutriments du sol, en particulier l'eau, ce qui peut favoriser leur

productivité durant la phase précoce de leur établissement, et quand les conditions d'humidité du sol deviennent défavorables pour leur croissance, ces espèces ont la tendance à entrer dans un état de dormance. Par contre, les espèces de saison chaude ayant une stratégie r ont une croissance lente et elles sont fortement tolérantes au manque d'eau, ce qui peut augmenter leur productivité et leur coexistence avec les graminées de saison fraîche, durant la phase tardive de leur établissement. D'ailleurs, l'effet positif de certaine espèces de CMA sur l'amélioration de la coexistence des graminées de saison chaude pourrait être lié à l'augmentation de leur tolérance au manque d'eau dans certaines conditions, après cinq mois de croissance des plantes.

Avec les connaissance acquises dans ce projet, il serait donc nécessaire d'impliquer la légumineuse *M. sativa* dans les futurs programmes de gestion de systèmes de pâturage à base des graminées indigènes, au vu de sa grande importance écologique. Il faudrait aussi analyser à long terme les effets bénéfiques de cette légumineuse dans l'amélioration de la productivité et la valeur nutritive des pâturages bien établis, en particulier à la fin de la période de croissance quand la valeur nutritive de la plupart des graminées indigènes diminue. Puisque *D. purpurea* a permis d'augmenter la diversité des CMA dans le mélange de graminées indigènes, il semble aussi important de tester l'effet à long terme de cette légumineuse sur la productivité du fourrage et évaluer les fonctions de la diversité de CMA trouvés dans le mélange de graminées indigènes avec *D. purpurea*, puisque cette légumineuse nécessite plus de temps pour être productive. Il est également intéressant de noter que les taxons de CMA bénéfiques stimulés par *M. sativa* dans la prairie indigène semi-aride pourraient être utilisés comme inoculum, suite à des pratiques de gestion appropriées pour une production durable du fourrage.

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Appendix 1 Relative abundance of native grasses species, in each species mixtures

	Relative abundance of each native grass (%)[a]					
September 2008	EL+PS[¥]	ET	**NV**	EC	SS	**BG**
Native grasses	14.0	15.1	31.6	9.0	8.8	21.6
Native grasses +*M. sativa*	13.4	14.3	31.2	16.8	5.3	19.3
Native grasses + *D. purpurea*	17.3	14.2	29.2	8.8 a	9.3	20.9
September 2009	EL+PS	ET	**NV**	EC	SS	**BG**
Native grasses	44.6	25.8	3.3	11.6	5.9	8.9
Native grasses +*M. sativa*	46.1	30.8	4.3	9.4	2.2	7.0
Native grasses + *D. purpurea*	40.7	27.6	3.9	11.5	7.7	8.5

[a]The relative abundance of native grasses species from each plot was determined visually.

[¥]EL+PS=*E. lanceolatus* ssp. *lanceolatus* + *P. smithii*; ET=*E. trachycaulus* ssp. *subsecundus*; NV=*N. viridula*, EC=*E. canadensis*; SS=*S. scoparium*; BG=*B. gracilis*.

Appendix 2

Relative abundance of legumes expressed as % of total sown species determined in September 2008 and 2009, as influenced by species mixtures (Unpublished data)

Species mixtures	Legumes	Relative abundance of legumes (%)[ab]	
		2008	2009
Native grasses + *M. sativa*	*M. sativa*	30.6 b	34.3 b
Native grasses + *D. purpurea*	*D. purpurea*	1.9 a	0.7 a
B. biebersteinii + *M. sativa*	*M. sativa*	44.0 c	35.4 b
P value		**<0.001**	**<0.001**

[a]The relative abundance of legumes from each plot was determined visually.

[b]Within each column, means are not significantly different ($p \leq 0.05$, ANOVA; n = 12) when followed by the same letter (a, b, c) according to Tukey's range. Bolded value are significant.

Appendix 3

The soil moisture measured at soil depth (from 0 to 15 cm), as influenced by species mixtures, (Unpublished data)

Plant mixtures	Soil moisture (%)[ab]	
	2008	2009
Native grasses	13.9 b	6.2 b
Native grasses + *M. sativa*	12.8 a	6.0 b
Native grasses + *D. purpurea*	14.2 b	6.1 b
B. biebersteinii + *M. sativa*	12.5 a	5.8 a
P value	**<0.001**	**0.018**

[a]The soil moisture content was calculated as the weight lost after drying 25 g of soil at 105°C until a constant weight was achevied, and expressed on a percent basis.

[b]Within each column, means are not significantly different ($p \leq 0.05$, ANOVA; n = 12) when followed by the same letter according to Tukey's range tests. Bolded value are significant.