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Colonization of Three Alfalfa (*Medicago sativa* L.) Nodulation Genotypes by Indigenous Vesicular-Arbuscular Mycorrhizal Fungi from Soil

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Abstract

Colonization of myc$^-$ alfalfa (*Medicago sativa* L.) nodulation genotypes was accomplished under greenhouse conditions with indigenous VA mycorrhizal fungi from field soil. The greatest percent colonization was found in nod$^+$fix$^+$ roots; however, nod$^+$fix$^-$ roots did become colonized and did produce arbuscules. Under growth room conditions, indigenous VA mycorrhizal fungi from field soil produced very few arbuscules in the nod$^+$fix$^-$ and nod$^-$fix$^-$ roots. The differences in colonization were attributed to the different light intensity or quality, and to the higher temperature of the greenhouse grown plants.

Keywords: vesicular arbuscular mycorrhizae, alfalfa nodulation genotypes, indigenous VA mycorrhizal fungi, temperature, light intensity

1. Introduction

Recently, Duc et al. (1989) and Gianinazzi-Pearson et al. (1991) reported the existence of non-mycorrhizal (termed myc$^-$) *Pisum sativum* L. and *Vicia faba* L. mutants. Furthermore, Bradbury et al. (1991) and Bradbury (1992) have found genotypes of alfalfa (*Medicago sativa* L.) which do not form VA mycorrhizal associations under controlled growth room conditions with five VA mycorrhizal fungal species in pot culture.

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Increases in normal VA mycorrhizal colonization have been demonstrated under higher light intensities (Hayman, 1974; Ferguson and Menge, 1982; Jensen, 1984; Smith and Gianinazzi-Pearson, 1990) and under higher temperatures (Furlan and Fortin, 1973; Hayman, 1974; Smith and Bowen, 1979; Graham et al., 1982; Jensen, 1984; Sieverding, 1988), although evidence suggests that these effects may be host and fungal species specific (Furlan and Fortin, 1977; Sieverding, 1988; Borges and Chaney, 1989; Raju et al., 1990). Increased levels of VA mycorrhizal colonization under high light intensity and temperature has been attributed to an increase in production of root exudates required for fungal growth (Ferguson and Menge, 1982; Graham et al., 1982), or more simply as an increase in fungal activity due to warmer temperatures (Graham et al., 1982).

Indigenous VA mycorrhizal fungi have an increased colonization ability when maintained in the soil from which they were isolated (Stahl and Christensen, 1991). Introduction of VA mycorrhizal fungi to substrates in which they are not naturally found may reduce the receptiveness of plants to VA mycorrhizal colonization (Perrin et al., 1988).

The objective of this study was to investigate the ability of indigenous VA mycorrhizal fungi in soil to colonize three alfalfa nodulation genotypes (nod^+fix^+, nod^+fix^-, and nod^-fix^-) grown under two different environmental conditions.

2. Materials and Methods

Colonization process

Soil was collected from two local field sites: (1) an alfalfa plot at the Ontario Ministry of Agriculture and Food, Elora Research Station (Site A) and, (2) an alfalfa-timothy-clover (*Medicago sativa* L., *Phleum pratense* L., *Trifolium repens* L.) field in North Eramosa Township (Site B). Both sites are located in South-central Ontario; the soil is classified as a clayed Brunisolic Gray Brown Luvisol-London. The soil was sifted to remove stones and plant debris. The moisture content of the soil was determined by weighing four separate small samples of soil, drying the samples to a constant weight, reweighing the soil samples, and determining the weight difference due to moisture loss. With a known moisture content, a calculated volume of water was added to a known volume of soil in the pots in order to adjust the soil to a bulk density of 1 g soil cm^{-3} and a field capacity of 25%. Two 25 cm pots of soil from both sites were used in the greenhouse experiment and two pots of soil from Site A were used in the growth room experiment.

Seeds from each genotype, nod^-fix^-, nod^+fix^- and nod^+fix^+ (described by Bradbury et al., 1991), were surface sterilized in 10% Javex (active ingredient 6% sodium hypochlorite) for 10 min, rinsed several times in sterile distilled water, and allowed to imbibe overnight. Imbibed seeds were placed on moist filter paper in Petri plates and left for 3 days, allowing for the emergence of the radicle and cotyledons. The experiment was arranged such that each pot contained nod^+fix^+, nod^+fix^-, nod^-fix^- alfalfa plants. This eliminated effects due to differences in VA mycorrhizal inoculum level between pots. Fifteen seeds of each of the nod^+fix^+, nod^+fix^-, and nod^-fix^- genotypes were planted in each of the pots. A layer of silica sand was added to the surface of the soil to reduce evaporation, drying and clumping of the surface soil, but still allowing easy watering. Pots in the greenhouse were watered by mass three times per week, during May and June 1991. The temperature during this period was maintained on a 24° C/18° C day/night regime; the light level was incident daylight.

In the growth room experiment pots were maintained at a constant temperature of 21° C, with a 16/8 hr light-dark cycle, during October and November 1991. A mixture of cool white growlux tubes and incandescent bulbs provided 125 $\mu molm^{-2}s^{-1}$ of light at pot level, an irradiance above the compensation point for *Medicago sativa*. Pots in the growth room were watered by mass three times per week.

Colonization assessment

To assess the roots for degree of VA mycorrhizal colonization after 6 weeks, approximately ten plants of each genotype were randomly selected from each pot. Plants were removed from the pots, washed thoroughly, the roots excised, and fixed overnight in formalin-acetic acid-alcohol (FAA). After rinsing with several changes of tap water to remove FAA, the roots were transferred into a 5% (w/v) KOH solution and cleared in an autoclave for 15 min at 121° C. Several rinses with deionized water removed excess KOH. The roots were stained with 0.1% chlorazol black E for 1 hr at approximately 90° C (as in Brundrett et al., 1984). The method developed by McGonigle et al. (1990) and used previously by Bradbury et al. (1991) and Bradbury (1992) was used to determine the level of colonization. The percent colonization for each fungal structure was then determined. Data were analysed using a fixed-effect split-plot model, with pots as the main effect (pots within soil as the error term) and with genotypes as sub-plots. An arcsine square root transformation was applied prior to variance analyses. In the controlled growth room study, the genotype x pot effect was not significant ($P<0.05$); the genotype x pot effect and the residual

error terms were thus pooled. Means were separated by Duncan's Multiple Range Test. The significance level for all tests of hypotheses was established at $\alpha = 0.05$.

3. Results

Greenhouse experiment

Variance analyses of the colonization assessments are summarized in Table 1. There were no significant differences among the three alfalfa genotypes (nod^+fix^+, nod^+fix^- and nod^-fix^-) for all fungal structures studied, suggesting that all genotypes were responding the same to available inocula. The mean percent colonization for each fungal structure in each alfalfa genotype can be seen in Table 2. High incidences of arbuscule formation were observed; genotypes ranged from 28 to 52% of the root colonized (Table 2).

Table 1. Variance analyses of arcsine transformed percent root length incidence of appressoria, arbuscules, vesicles and internal hyphae in the three alfalfa genotypes grown in association with indigenous VA mycorrhizal fungi present in soil collected from two fields (field site) and grown under greenhouse conditions

Source	df	Mean square			
		Appressoria	Arbuscules	Vesicles	Internal hyphae
Soil	1	0.0080 NS	0.1004 NS	0.3530 NS	0.3135 NS
Pot (Soil)	1	0.0018 NS	0.1826 NS	0.0082 NS	0.0165 NS
Genotype	2	0.0120 NS	0.6179 NS	0.1566 NS	0.1140 NS
Soil × genotype	2	0.0153 NS	0.0095 NS	0.0188 NS	0.0331 NS
Geno × pot (soil)	4	0.0266**	0.4008**	0.0405**	0.0380**
Error	99	0.0031	0.0086	0.0034	0.0069

** significant at 0.01 level
NS = not significant

Table 2. Alfalfa genotype dependent variation in percent of root length incidence of appressoria, arbuscules, vesicles and internal hyphae by indigenous VA mycorrhizal fungi from two alfalfa field soils grown under greenhouse conditions

Genotype	Incidence (% root colonized)*			
	Appressoria	Arbuscules	Vesicles	Internal hyphae
nod^-fix^-	8.66	34.77	4.73	12.15
nod^+fix^-	6.89	27.84	3.16	6.68
nod^+fix^+	7.06	52.02	9.03	6.41

* Means within a column are not significantly different using ANOVA ($P<0.05$).

Table 3. Indigenous VA mycorrhizal fungal source dependent variation in percent root length incidence of appressoria, arbuscules, vesicles and internal hyphae in the three alfalfa genotypes grown under greenhouse conditions

VAM fungi source	Incidence (% root colonized)*			
	Appressoria	Arbuscules	Vesicles	Internal hyphae
Site A**	7.1	35.1	3.1	5.5
Site B***	8.0	41.0	8.3	11.4

* Means within a column are not significantly different using Duncan's Multiple Range Test ($P<0.05$).
** Alfalfa field, Elora Research Station.
***Alfalfa-Timothy-Clover field, North Eramosa Township.

No differences in the infectivity were observed between the field soils (Table 3). Inoculum in soil from Site B (alfalfa-Timothy-clover mix field) produced numerically but not significantly, more structures (appressoria, arbuscules, vesicles, and internal hyphae) than did inoculum in soil from the strictly alfalfa field (Site A).

4. Growth room experiment

Results of the analysis of variance are summarized in Table 4. Significant differences were detected among alfalfa genotypes for arbuscule, vesicle and internal hyphae formation.

Growth room grown alfalfa genotype dependent variation can be seen in

Table 4. Variance analyses of arcsine transformed root length incidence of appressoria, arbuscules, vesicles and internal hyphae in the three alfalfa genotypes grown in association with indigenous VA mycorrhizal fungi present in soil collected from a local alfalfa field (Elora Research Station) and grown under controlled growth room conditions

Source	df	Mean square			
		Appressoria	Arbuscules	Vesicles	Internal hyphae
Pot	1	0.0228 NS	0.0098 NS	0.0054 NS	0.0000 NS
Sample (pot)	18	0.0062 NS	0.0113 NS	0.0050 NS	0.0039 NS
Genotype	2	0.0176 NS	0.7325*	0.2400**	0.1626*
Geno × pot	2	0.0148 NS	0.0027 NS	0.0018 NS	0.0067 NS
Error	15	0.0042	0.0049	0.0039	0.0079

* Significant at .05 level
**Significant at .01 level

Table 5. Alfalfa genotype dependent variation in percent root length incidence of appressoria, arbuscules, vesicles and internal hyphae by indigenous VA mycorrhizal fungi from alfalfa field soil (Site A) grown under controlled growth room conditions and comparison of percent root length incidence of indigenous VA mycorrhizal fungi between greenhouse and controlled growth room grown alfalfa nodulation genotypes

		Incidence (% root colonized)*		
Structure	Environment	nod^-fix^-	nod^+fix^-	nod^+fix^+
Appressoria	greenhouse**	9.54 a	5.75 a	6.19 a
	growth room	2.99 bx	3.77 ax	1.24 bx
Arbuscules	greenhouse	33.46 a	23.73 a	49.00 a
	growth room	0.02 bx	0.00 bx	22.24 by
Vesicles	greenhouse	3.51 a	1.08 a	5.67 a
	growth room	0.00 bx	0.00 bx	0.74 by
Internal hyphae	greenhouse	7.02 a	4.75 a	5.96 a
	growth room	0.49 bx	1.13 bx	9.46 by

* Means followed by the same letter (a, b with a column, within a structure, x, y within a row, within an environment) are not significantly different using Student's t Test ($P<0.05$).
**Greenhouse grown plant means were calculated using data obtained from Site A field soil only.

Table 5. There was no difference in percent root length containing arbuscules, vesicles and internal hyphae in the nod^-fix^- and nod^+fix^- genotypes; however, nod^+fix^+ roots produced significantly greater percent root length colonization than the two remaining alfalfa genotypes.

Comparison of greenhouse and growth room experiments

Comparison of results obtained from the greenhouse and growth room grown alfalfa genotypes are also presented in Table 5. Only soil from site A was available for the growth room study, therefore, in order to compare greenhouse and growth room colonization levels, soil B data were eliminated from this analysis. Significant differences were detected between the greenhouse and growth room-experiments for all fungal structures studied (appressoria, arbuscules, vesicles and internal hyphae) for the nod^-fix^-, nod^+fix^- and nod^+fix^+ alfalfa genotypes, with the exception of appressoria on the nod^+fix^- genotype. For all comparisons, except internal hyphae in nod^+fix^+ roots, roots grown under greenhouse conditions produced significantly greater levels of colonization.

5. Discussion

Bradbury et al. (1991) and Bradbury (1992) have shown that the nod^+fix^- and nod^-fix^- alfalfa nodulation genotypes do not develop VA mycorrhizal associations with four *Glomus* species and *Gigaspora margarita* in pot cultures containing turface grown under growth room conditions. Colonization of the nod^-fix^- and nod^+fix^- alfalfa genotypes was achieved under greenhouse and growth room conditions using indigenous VA mycorrhizal fungi in field soil. However, growth room grown alfalfa plants were unable to develop VA mycorrhizal colonization to the same level as those in the greenhouse. Since all roots were subjected to the same preparatory procedures of colonization and assessment, and significant differences in the colonization of roots were observed between growth room and greenhouse grown genotypes in soil, there must be an effect of environmental conditions (i.e. light intensity and temperature) on the VA mycorrhizal colonization process. Because the soil used in all pots originated from the same source, the indigenous VA mycorrhizal fungi present in the soil should be the same. Thus, the differences in colonization cannot be attributed to differences in VA mycorrhizal fungal species.

Since previous studies have indicated that VA mycorrhizal colonization can be increased with higher light intensities and warmer temperatures (Furlan and Fortin, 1973; Hayman, 1974; Furlan and Fortin, 1977; Smith and Bowen, 1979; Ferguson and Menge, 1982; Graham et al., 1982; Jensen, 1984; Sieverding, 1988; Borges and Chaney, 1989; Raju et al., 1990; Smith and Gianinazzi-Pearson, 1990), it is conceivable that the nod^-fix^- and nod^+fix^- alfalfa genotypes are unable to produce certain gene products at the level required by the VA mycorrhizal fungi for colonization, at lower light intensities and temperatures, and of different light quality conditions present in the growth room. Under the higher light intensity and temperature of the greenhouse, substances required for colonization may have been synthesized to a greater extent. In addition to light and temperature, soil physical properties may also affect VA mycorrhizal fungal growth. Introduction of VA mycorrhizal fungi to substrates in which they are not naturally found may reduce their capability for colonization (Perrin et al., 1988). The importance of environmental conditions on the genetic expression of VAM colonization of alfalfa genotypes has been demonstrated but requires further study.

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