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Seasonal variation in mycorrhizal fungi colonizing roots of *Allium tricoccum* (wild leek) in a mature mixed hardwood forest

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Abstract The community of arbuscular mycorrhizal (AM) fungi colonizing roots of the forest herb *Allium tricoccum* Ait. (wild leek) was examined to assess whether colonization varied seasonally and spatially within the forest. Whole plants were collected to coincide with observed phenological stages, and the perennial tissue (i.e., the bulb) was used to analyze total C, N, and P over the growing season. AM fungal community composition, structure, and abundance were assessed in roots by terminal restriction fragment length polymorphism analysis and quantitative PCR. It was found that *A. tricoccum* rDNA co-amplified using the general AM primers NS31/AM1, and a new primer for qPCR was designed that discriminated against plant DNA to quantify AM colonization. Community structure of AM fungi did not vary seasonally, but did change spatially within the forest, and AM fungal communities were correlated with the presence of overstory tree species. Fungal colonization of roots, however, did change seasonally with a maximum observed in late winter and early spring following leaf emergence. Maximum AM fungal colonization was associated with declines in bulb N and P, suggesting that leaf emergence and growth were responsible for both declines in stored nutrients and increases in AM fungal colonization. Plant N and P contents increased between late summer and early spring while C contents remained unchanged. The observed increase in nutrient content during a time when *A. tricoccum* lacks leaves indicates that the roots or AM fungi are metabolically active and acquire nutrients during this time, despite an absence of photosynthesis and thus a direct supply of C from *A. tricoccum*.

Keywords *Allium tricoccum* · Hardwood forest · Mycorrhizal fungi · Nutrient content · TRFLP

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Introduction

Many northern hardwood forests contain extensive and diverse herbaceous understory plant communities, which contribute significantly to overall plant community diversity and comprise much of the species richness within forest systems. In addition, it has been estimated that herbaceous plants may contribute as much as 15 % of total litter fall within some forests and may have a large influence on seasonal carbon (C) and nutrient cycling (Gilliam 2007). Despite their importance to forests, the persistence of herbaceous plant populations are threatened by a number of factors including deer herbivory (Côté et al. 2004; Knight et al. 2009), the presence of invasive plant and animal species (Lawrence et al.2002, Stinson et al. 2006), and climate change (Rapacz et al. 2014). In addition, many forest herbs have limited seed dispersal, grow and reproduce slowly through vegetative organs, and recover slowly from disturbance (Keddy and Drummond 1996), which can impact their persistence following environmental change. Consequently, a greater understanding of the biology of these plants, and how they interact with other organisms that contribute to their persistence in forests, is necessary for long-term management of these plant species and communities.

Of particular interest is the biotic interaction between forest herbs and the mycorrhizal fungi that form mutualistic

relationships with them. Mycorrhizal fungi colonize plant roots and form an extensive network of fungal filaments (hyphae) that explore soil and act as an extension of the plant root system, acquiring nutrients that are exchanged with the plant for carbon (Smith and Read 1997). Arbuscular mycorrhiza (AM) are widespread mutualistic relationships (Smith and Read 1997), maintained by many forest herbaceous plants including *Arisaema triphyllum* (jack-in-the-pulpit), *Erythronium americanum* (trout lily), *Trillium erectum* (wake-robin), *Maianthemum racemosum* (false Solomon's seal), *Podophyllum peltatum* (mayapple) and *Allium tricoccum* (wild leek) (Brundrett and Kendrick 1990ab, DeMars 1996; Lapointe and Molard 1997; Watson et al. 2002). Mycorrhizal colonization has been shown to benefit forest herbs through increases in biomass, growth, and phosphorus (P) acquisition compared to a non-mycorrhizal condition (Helgason et al. 2002). For example, Lapointe and Molard (1997) found that the growth rate of the spring ephemeral, *E. americanum*, was two times higher when colonized by AM fungi. These mycorrhizal benefits, however, may change seasonally, and temporal variation in the ability of plants to acquire P or resist drought has been suggested (Lapointe and Molard 1997). Although it is difficult to generalize why plant resource acquisition could vary seasonally, seasonal variation in mycorrhizal colonization patterns (Brundrett and Kendrick 1990ab) and changes in the identity of mycorrhizal fungal species colonizing the root system could partly explain observed seasonal changes in plant resource acquisition, given the known difference in functional capabilities of AM fungi (Helgason et al. 2002; Maherali and Klironomos 2007).

Understanding patterns of root colonization over time and the identity of the AM fungal colonists is important to understand the effects of environmental change on forest herbs. For example, physical disruption of the soil hyphae can affect root colonization (Merryweather and Fitter 1998), with implications for plant growth and nutrient uptake. Recent work has suggested that earthworm activity can directly disrupt soil hyphae (Lawrence et al. 2002), while the invasive plant *Alliaria petiolata* (garlic mustard) could also affect plant root colonization and AM fungal community structure (Stinson et al. 2006; Burke 2008; Hale et al. 2011), possibly through production of secondary compounds that are toxic to soil fungi (Stinson et al. 2006; Hale and Kalisz 2012), with implications for plant growth performance (Hale et al. 2011). However, seasonal influences on the roots of forest herbs and associated fungal mutualists may vary over the course of the year as AM fungal communities and colonization change. Therefore, a greater understanding of patterns of root colonization over time is necessary in order to predict and mitigate the impacts of environmental disturbance on forest herbs. Further, in many forest herbs, seasonal changes coincide with plant phenological changes with possible effects on the mycorrhiza (Brundrett and Kendrick 1990ab, Lapointe and Molard 1997).

The perennial forest herb *A. tricoccum* (wild leek) is a spring ephemeral that persists for only brief periods of time in the forest understory, before leaf emergence of canopy trees when light availability at the forest floor is highest. *A. tricoccum* has only a short photosynthetic period, leaves persist for 6–8 weeks, before senescing after tree leaf emergence, and reproduction occurs in summer after leaves have senesced. The bulbs persist throughout the year and act as the main storage organ for the plant's nutrients; new roots form on the rhizome at the base of the bulb in autumn and have an annual life cycle (Nault and Gagnon 1988; Lapointe 2001). Consequently, resource capture for *A. tricoccum* is seasonally explicit, and there may be large differences between light and soil resource acquisition (Nault and Gagnon 1988, 1993). Whether varying nutrient allocation patterns over the growth cycle of *A. tricoccum* plants are accompanied by changes to the colonization or community structure of their AM fungal symbionts is largely unknown. For other herbaceous ephemerals, variation has been shown in AM fungal colonization as the plants move through different phenological phases (Brundrett and Kendrick 1990ab, Lapointe and Molard 1997). Since roots of *A. tricoccum* are known to be colonized by AM fungi, with as much as 60 % of the root length possessing mycorrhizal structures (DeMars 1996), variation in fungal root colonization over time may also occur and could be relevant to seasonal variation in soil resource acquisition. However, whether mycorrhizal colonization varies over time for *A. tricoccum*, and whether fungal community composition and structure also varies, is yet largely unexplored.

This study reports on changes in mycorrhizal colonization and AM fungal community structure in the roots of *A. tricoccum*, an important forest wildflower in much of eastern North America. It was predicted that 1) root colonization and community structure would vary seasonally, 2) carbon (C), nitrogen (N), and phosphorus (P) concentrations in the perennial storage organ (bulb) will change seasonally and be associated with alterations in AM fungal colonization and communities, and 3) location of the sampling plot would also affect AM fungal colonization and communities since differences among microsites could also affect fungal taxon distribution.

Sampling was performed over the course of one growing season, and the AM fungal communities analyzed by terminal restriction fragment length polymorphism (TRFLP) profiling and quantitative PCR (qPCR) to examine changes in community structure and root colonization, respectively. A new primer and PCR procedure was developed to overcome amplification of non-target rDNA using common AM primers.

Materials and methods

Site description and plant and soil sampling

The study site is located within a mature 360-ha mixed-meso-phytic forest known as Stebbins Gulch located within the Holden Arboretum in northeastern Ohio, USA (41° 36′ N and 81° 16′ W). Within Stebbins Gulch, the sampling location was an 80-ha, old-growth beech-maple stand and is the same site as in previous work exploring mycorrhizal fungi and soil microbial diversity (Burke et al. 2009, 2012). The stand is dominated by an overstory of *Fagus grandifolia* (American beech), *Acer saccharum* (sugar maple), and *Liriodendron tulipifera* (tulip popular), a sub-canopy dominated by *F. grandifolia*, *A. saccharum*, and *Lindera benzoin* (spice-bush), and a herbaceous understory comprised mostly of spring ephemerals and dominated by *A. tricoccum* (wild leek) and *Dicentra canadensis* (squirrel corn). The site is characterized by acidic, moderately drained silt loam soil with a mean pH of 4.0 ± 0.1 (measured in H_2O), and it receives precipitation averaging 116 cm per year, including an average of 287 cm of snowfall per season (28.7 cm rainfall equivalents). For more detailed information about the field site, see Burke et al. (2009, 2012).

A 100-m transect was established in early spring 2011, with eight sampling plots measuring 0.5×1.0 m (0.5 m^2) in areas dominated by *A. tricoccum* at least 5 m apart. The presence or absence of canopy trees within 3 m of each patch of *A. tricoccum* was noted and patches were sampled eight times between May 2011 and April 2012, coinciding with observable phenological stages, including: vegetative stage, leaf senescence, flowering, fruiting, seed set, dormancy, and leaf emergence and expansion. On each date, three to four whole plants from each plot were gently dug from the soil, placed in plastic bags, and kept in a cooler on ice until transport to the laboratory. In the laboratory, each plant was separated into leaf/stem, bulb, and root. *A. tricoccum* leaves and reproductive structures are ephemeral, lasting only a few weeks, whereas root growth occurs throughout the year with new roots produced in autumn (Nault and Gagnon 1988; Lapointe 2001), as the previous year's roots are senescing (Hewins, personal observation). Consequently, although all portions of the plants were dried and weighed, the seasonal nutrient analyses focused on the bulb. This represents the perennial and persistent portion of the plant and constitutes the storage structure; bulbs have a life expectancy of greater than 8 years (Nault and Gagnon 1993). Roots were gently washed of soil and separated into two portions: one portion was placed in a small plastic bag, stored at −70 °C, and reserved for analysis of AM fungal colonization and communities, and another portion was dried, weighed, and reserved for future nutrient analyses.

Plant nutrient analysis

Plant bulbs were dried at 60 °C for 1 week and weighed. Bulbs collected from the same plot and sampling time were dried, weighed, ground, and analyzed together so results represent the composite nutrient content of all bulbs collected within the plot. After coarse grinding, the bulb tissue was further pulverized in a Precellys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) for C, N, and P analysis. Total C and N was estimated through dry combustion on an ECS 4010 CHNSO elemental analyzer (Costech Analytical, Valencia, CA), while total P was determined through acid digestion using sulfuric acid and 30 % hydrogen peroxide (Moore 1992) followed by colorimetric analysis using a modified ascorbic acid method (Kuo 1996). Nutrient data is presented as mass of C, N, or P per bulb where bulb mass is the average mass of the bulbs within that plot (i.e., total mass of all bulbs collected divided by number of bulbs). Bulb N/P ratios are also provided as an indicator of plant physiological nutrient limitation; ratios of greater than 16 suggest plant P limitation, while ratios less than 14 indicate plant N limitation (Koerselman and Meuleman 1996).

Molecular analysis of mycorrhizal fungi

DNA was extracted from *A. tricoccum* roots using a bead beating protocol (Burke 2008). Briefly, 250 mg of roots were placed in a 1.5-mL bead beating tube containing 500 mg of sterile glass beads and 750 μL of 2 % cetyltrimethyl ammonium bromide (CTAB) and beaten for 90 s in a Precellys homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) at 6500 rpm. Extracted genomic DNA was purified by phenol/chloroform extraction, precipitated with 20 % polyethylene glycol 8000 in 2.5 M NaCl and re-suspended in 100 μL TE (Tris EDTA) buffer prior to storage at −20 °C (Burke 2008).

To examine AM fungal community structure, the 18S rDNA was targeted using the universal eukaryotic primer NS31 (Simon et al. 1992) and the AM fungal specific primer AM1 (Helgason et al. 1998). Although this primer set may exclude some members of the *Glomeromycota* (e.g., *Archaeosporaceae*, *Ambisporaceae*, and *Paraglomaceae*), taxa in the *Glomerales* and *Diversisporales* are well represented by the primer set (Lee et al. 2008), which remains a useful tool for examination of AM fungal communities in many plant species. PCR was conducted in 50 μL reaction volumes using 1 μL of purified DNA (approximately 100 ng), 0.2 μM of each primer, 2.0 mM MgCl, 0.2 mM dNTP, 0.15 mg mL^{-1} bovine serum albumin, and 2.0 units GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA) on a PTC 100 Thermal Cycler (MJ Research, Boston, MA, USA). An initial denaturation step of 5 min at 94 °C was followed by amplification for 32 cycles at the following

conditions: 30 s at 94 °C, 60 s at 58 °C, and 90 s at 72 °C. A final 5-min extension at 72 °C completed the protocol. Primers were labeled with the fluorochromes 6-carboxyfluorescein (6FAM) (AM1) and 4, 7, 2′ ,4′ ,5′ ,7′-hexachloro-6-carboxyfluorescein (HEX) (NS31). Although previous work has found this primer set specific for AM fungi, as confirmed through cloning and sequencing of PCR amplicon (Burke 2008; Kluber et al. 2012), a nonspecific band was observed in the present study at approximately 210 bp along with the specific band representing the 18S rDNA region at 550 bp. It was not possible to prevent amplification of the nonspecific band, despite changes in PCR conditions, including annealing temperature alteration or changes in Taq polymerase. Consequently, prior to TRFLP analysis, all the PCR products from samples were run in 1 % agarose gels, and both the specific and nonspecific bands were excised for gel purification using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified PCR amplicons were used for cloning using the Qiagen® PCR Cloning Plus Kit (QIAGEN Inc., Valencia, CA, USA) as per the manufacturer's protocols. Randomly selected colonies were grown overnight in LB (Luria-Bertani) media, and plasmids were purified from cultures using a Wizard® Plus SV Miniprep DNA purification system (Promega). One hundred forty-six clones from three different sampling time points, representing the putatively AM specific PCR product, were analyzed and sequenced, as well as an additional 42 sequences representing the nonspecific 210-bp band. Taxonomic assignment was made by comparing the sequences to EMBL/GenBank/DDBJ database entries using the BLAST tool through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) for the 146 AM clones and through the European Bioinformatics Institute (http://www.ebi.ac.uk/) for the 42 nonspecific sequences. Some of the 146 AM clone sequences had no significant database matches, which resulted in 122 clones that were used to determine taxonomic identity of AM fungi in the leek roots. It was found that 67 % (82 of 122 sequences) of the putatively AM-specific clones were of AM fungal origin (Table 1) with the remaining sequences either good (% ID>98 %) nonspecific matches to other fungi (17 % of sequences) (Table 1) or poor quality (%ID<86 % or % query coverage<10 %) nonspecific matches (15 % of sequences) (data not shown). This suggests that the primer set NS31/AM1 was not specific for AM fungi associated with *A. tricoccum*. 95 % of the sequences (40 of 42 sequences) recovered from the nonspecific 210-bp-long PCR band had greater than 96 % nucleotide identity to 18S rDNA of *Allium victorialis* (accession number HM640714). A closer examination of the 18S rDNA of *A. victorialis* revealed a 14-bp sequence stretch approximately 168 bp downstream from the 3′ end of the NS31 primer that showed some sequence complementarity to the 3′ end of the AM1 primer, but which contained several nucleotide mismatches (Supplemental

Data 1). This sequence stretch probably served as an alternative primer site for the AM1 primer. Including the primers, this region of *A. victorialis* sequence is 209 bp in size (Supplemental Data 1), corresponding to the size of the nonspecific PCR amplicons, which may explain the nonspecific amplification of the *A. tricoccum* 18S rDNA.

Analysis of the root-colonizing AM fungi was thus carried out on gel-extracted PCR products of the putatively specific AM fungal band, but analysis only included those TRFs that could be positively attributed to AM fungi based on predictions of the AM TRFs for recovered clones and those from recent similar studies (Burke 2008; Kluber et al. 2012). In this way, the nonspecific *A. tricoccum* rDNA amplification products would not affect analysis while allowing examination of a sufficiently long PCR product for length polymorphisms. The restriction enzyme *Hinf*I was used to generate TRFLPs from the HEX-labeled NS31 primer, as previously described (Burke 2008; Burke et al. 2011); this primer and restriction enzyme was chosen because in previous work, it was found to discriminate well among AM taxa from the study forest (Burke 2008; Burke et al. 2011). TRFLPs were completed through the Cornell Bioresource Center using an Applied BioSystems 3730×l DNA Analyzer, and profiles were analyzed using the GS600 LIZ size standard and Peak Scanner™ Software (version 1.0, Applied Biosystems 2006).

To quantify root colonization by AM fungi, a new PCR primer was designed approximately 210 bp upstream of the AM1 primer binding site but downstream of the nonspecific 18S rDNA sequence to avoid amplifying *A. tricoccum* rDNA while still using AM1 for mycorrhizal specificity (Fig. 1). The AM-specific primer AM1 (Helgason et al. 1998) was used as the reverse primer, while the forward primer AMG1F (5′–ATAGGGATAGTTGGGGGCAT–3′), designed using the Primer3Plus program (Untergasser et al. 2012), was used to avoid amplification of nonspecific *A. tricoccum* rDNA. Specificity of amplification by the primers AMG1F and AM1 was verified by cloning and sequencing the pooled qPCR products of ten random samples (two from each date and three replicates for each sample=30 total amplifications) with the Wizard® SV Gel and PCR Clean-Up System (Promega). The gel extracted product was ligated into the pDrive Cloning Vector (QIAGEN) using a 5:1 insert/vector ratio and transformed into competent cells of the QIAGEN® PCR Cloning Kit (QIAGEN). Plasmids were purified with the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer's protocol and used for direct sequencing with plasmid primer T7 using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Sequencing was conducted at Cornell University's Biotechnology Resource Center on an Applied BioSystems 3730×l capillary DNA sequencer. The sequences were checked for quality and trimmed to remove primers and regions of poor quality in BioEdit, version 7.1.11

Table 1 Database matches to clones generated with the NS31 and AM1 primer set

Clone accession numbers	No. of clones	Database match description[a]	Match accession number	Percent identity[b]	Query cover (%)[b]
AM Fungal Matches					
LN609583-LN609595	13	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AB178729	97.1	99.2
LN609596	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AF131052	99	100
LN609597-LN609609	13	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AF131053	99.3	99.5
LN609610	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AF437670	99	99
LN609611-LN609612	2	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AF481572	99	99
LN609613	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AF481660	99	100
LN609614-LN609615	2	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AJ418867	99	98.5
LN609616	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AJ418899	99	100
LN609617	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AJ496046	99	100
LN609618-LN609622	5	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AJ496079	99.2	99.6
LN609623-LN609625	3	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AY512345	98.7	99.7
LN609626	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AY512367	99	99
LN609627	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	AY512373	99	100
LN609628	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	FR773844	97	98
LN609629-LN609630	2	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	JQ811204	99	100
LN609631	1	*Glomus irregulare* 18S ribosomal RNA gene and ITS, partial sequence	FJ009612	99	100
LN609632-LN609642	11	*Glomus* mycorrhizal symbiont, 18S ribosomal RNA gene and ITS1, partial sequence	AJ699068	99.2	100
LN609643-LN609658	16	*Acaulospora lacunosa* 18S ribosomal RNA gene, partial sequence	HE610427	98.9	99.2
LN609659-LN609661	3	*Acaulospora mellea* 18S ribosomal RNA gene, partial sequence	JN687473	99	99
LN609662-LN609664	3	Glomeromycota sp. 18S ribosomal RNA gene, partial sequence	JF414185	96	99.7
Non-AM Fungal Matches					
LN609665	1	*Russula compacta* 18S ribosomal RNA gene, partial sequence	AF026582	99	99
LN609666	1	*Russula exalbicans* 18S ribosomal RNA gene, partial sequence	AY293156	99	98
LN609667-LN609676	10	*Coniosporium* sp. 18S ribosomal RNA gene	AJ972863	99	99.1
LN609677-LN609679	3	*Exophiala cancerae* 18S ribosomal RNA gene, partial sequence	KF155198	99	99.3
LN609680-LN609681	2	*Neonectria ramulariae* 18S ribosomal RNA gene, partial sequence	HQ840408	99.5	99
LN609682-LN609683	2	*Bionectria* sp. 18S ribosomal RNA gene, partial sequence	KJ145325	99	99
LN609684	1	*Knufia* sp. 18S ribosomal RNA gene, partial sequence	KF601368	99	98
LN609685	1	Ascomycota sp. 18S ribosomal RNA gene, partial sequence	GU250335	99	100

[a] Best match (excluding uncultured organisms) to the EMBL/NCBI/DDBJ database with a BLASTn search

[b] The % identity and % query coverage are reported here as averages of all clones if the database match was best for more than one clone

(Hall 1999). Species affiliations of the 47 newly generated sequences were determined with BLAST searches through the National Center for Biotechnology Information against databases as described above. All sequences showed high similarity to AM fungi (Table 2) indicating that the primer set successfully amplified AM fungi without amplifying *A. tricoccum* or nontarget microbial groups. With the new primer set, AM fungal taxon distribution was similar to that obtained with NS31-AM1 when only recovered AM clones were examined (see Results section). Consequently, this primer set was used to estimate AM root colonization through qPCR. However, because the primer generates a fairly short amplicon when used with AM1, the amplicon is too short for use in TRFLP analysis since most sequences recovered from the forest system generate TRFs greater than 260 bp when targeting this region of the 18S rDNA (see Burke 2008; Burke et al. 2011).

Mycorrhizal root colonization was quantified in terms of the copy numbers of 18S rDNA, using qPCR. Although qPCR approaches have the potential to bias estimates of AM root colonization toward some fungal taxa due to differences in amplification efficiency, or other known bias typical of PCR approaches, other methods for assessing root colonization are also affected by inherent bias. For example, root staining may not capture viable or active fungal structures within the roots while PLFA methods for estimating AM fungal biomass rely on a marker (16:1w5c) that is also found in some bacterial taxa (see Frostegård et al. 2011 for review). qPCR

was used because it is less likely to overestimate active root colonization, and it has been shown to be a reliable technique for studying AM fungal colonization in roots in other studies (Isayenkov et al. 2004; Alkan et al. 2006). Three replicate qPCR reactions were run for each sample on a MiniOpticon™ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). The 25-μL qPCR reactions contained 1 μL of template DNA, 1× iTaqUniversal™ SYBR® Green Supermix (Bio-Rad), 0.4 μM of each primer, and 0.5 mg/mL of bovine serum albumin. Thermal cycling used 95 °C for 5 min followed by 35 cycles of 95 °C for 15 s, and 62 °C for 1 min, with plate reads after every 62 °C step. The specificity of the qPCR reactions was determined by melting curves (65–95 °C) and by running the amplicons on 2 % agarose gels in addition to the verification check via cloning and sequencing as noted above. Samples were also used for cloning and sequencing of amplified product to further insure specificity as noted above. The gene copy number in the root samples was determined by comparing the quantification cycle (C_q) in the sample reactions to a standard curve using the CFX Manager™ software, version 2.0 (Bio-Rad). Four qPCR runs were conducted, each with their own standard curve. Standard curves were generated using a transformed plasmid containing an AM fungal 18S rDNA sequence that was quantified photospectrometrically with the Quant-it™PicoGreen®dsDNA Reagent (Life Technologies™, Carlsbad, CA). Each run included a five-point standard curve that ranged in value from 10^6 to 10^2 copies. The C_q was determined manually for each run, such that the reaction efficiency and r^2 of the standard curve were optimized. The r^2 for the standard curves ranged from 0.991 to 0.996, and the efficiencies of the runs ranged from 100.0 to 103.7 %. All sample reactions fell within this standard curve, and all no template controls (NTCs) were below the detection threshold. One technical sample replicate was removed as an outlier from the analysis due to high C_q standard deviation (thus two replicate qPCR reactions instead of three were used for this sample), which provided data with standard deviations ranking between 0.031 and 0.565 cycles.

Data and statistical analysis

Differences in plant nutrient content and mycorrhizal colonization were analyzed by ANOVA using SigmaStat 3.5 (Systat Software Inc., CA, USA). All nutrient data passed equal variance tests after transformation. Phenological stage and plot were the independent variables for these analyses, and nutrient content or mycorrhizal colonization were the dependent variables. TRFLP profiles were used to examine the effects of season and space on mycorrhizal community structure. Observed TRFs were used as operational taxonomic units (OTU) and are considered proxy measures of taxa (Feinstein et al. 2009; Carrino-Kyker et al. 2012). Only TRFs confirmed

to represent AM fungi through cloning and sequencing as noted above were included in these analyses. Relative peak area was used as an abundance measure for non-metric multidimensional scaling (NMS) analysis of community structure using PC-ORD 4 (MjM Software, OR), and all data were arcsine-square root transformed prior to analysis. The Sørenson distance with a random starting configuration was used for these analyses. Multi-response permutation procedures (MRPP) were also used to assess effects of season and space on AM fungal community structure (PC-ORD 4, MJM Software, 1999). MRPP is a nonparametric analysis which tests for difference between two or more groups of entities.

Results

Analysis of plant nutrient content

Significant changes were recorded in bulb nutrient content of *A. tricoccum* over the course of the season. Total C content increased significantly (2-way ANOVA; $F=34.0$, $P<0.001$) following leaf senescence in spring and peaked as plants began to flower before declining to its lowest point prior to leaf emergence the following spring (Table 3, Fig. 2). Total N content also increased significantly (2-way ANOVA; $F=22.7$, $P<0.001$) in bulbs following leaf senescence but declined gradually until reaching its lowest level during autumn. N content increased over winter before declining again with leaf emergence in spring (Table 3, Fig. 2). Total P concentration followed a similar pattern, but declined steeply during plant flowering and fruit set, before increasing gradually through fall and winter. P content also declined substantially in bulbs after leaf emergence the following spring (2-way ANOVA; $F=15.9$, $P<0.001$) (Table 3, Fig. 2). Total bulb mass also changed significantly over the season, reaching maximum size during flowering and smallest size during dormancy and spring leaf emergence (2-way ANOVA; $F=33.7$, $P<0.001$) (Table 3, Fig. 3). Significant changes were also found in bulb N/P ratio (2-way ANOVA; $F=8.0$, $P<0.001$), with the highest N/P ratio during seed set and low levels below 14 during vegetative growth and early spring leaf expansion (Table 3, Fig. 3). There was no significant effect of sampling plot on bulb nutrient content (Table 3).

Analysis of AM fungal community structure

Eighteen different AM fungal TRFs were detected among samples, all of which were confirmed to match previously sequenced AM fungal clones (Table 4). NMS ordination produced a three-dimensional solution with a final stress of 12.3 and a cumulative coefficient of determination of 0.920 (Fig. 4). The analysis showed no separation among the samples by season, but communities appeared to separate by
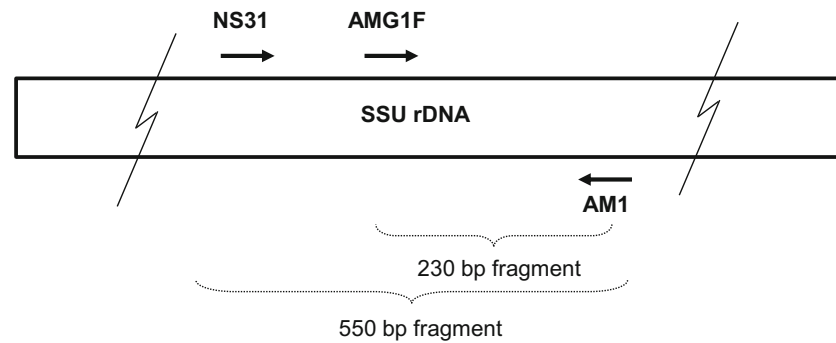
Fig. 1 Relative locations of the primers AM1 (Simon et al. 1992), NS31 (Helgason et al. 1998), and AMG1F (designed in the current study) in the 18S rDNA of AM fungi. The AMG1F primer was designed 1) to avoid nonspecific binding to *Allium tricoccum* DNA; thus it has no complementarity to the *Allium tricoccum* 18S sequence (Supplemental Data 1) and 2) for use in quantitative PCR along with the AM1 primer; thus these primers amplify a small DNA fragment (230 bp). The gene region is not drawn to scale

sampling location (Fig. 4). MRPP analysis confirmed that there was no significant effect of season on the AM communities ($A=0.02$, $P=0.15$), but communities were significantly affected by sampling location ($A=0.11$, $P<0.001$). Tree occurrence had a significant effect on AM fungal communities as revealed by ordination, with *L. tulipifera* positively correlated ($r=0.470$) with axis 3, and *A. saccharum* negatively correlated ($r=-0.520$) with axis 3. These correlations were driven by the presence of *L. tulipifera* near plot 4, and the presence of *A. saccharum* near plots 1, 6, 7, and 8 (plots 2, 3 and 5 were near *F. grandifolia*). No significant correlations were found between the C, N and P content of bulbs and AM fungal community structure.

Eighty two clones were successfully recovered that matched AM fungi using primers NS31 and AM1, with 60 (73 %) sequences showing affinity to the genus *Glomus*, 19 (23 %) affinities to the genus *Acaulospora*, and 3 (4 %) sequences with matches to unresolved Glomeryomycota (Table 1; accession numbers LN609583–LN609664) (As discussed above, an additional 21 clones produced with primers NS31 and AM1 matched with non-AM fungi and these sequences can be accessed with accession numbers LN609665–LN609685). Forty-seven sequences were recovered using the newly designed primer AMG1F for qPCR with AM1, all of which showed affinities to AM fungi (accession numbers LN609686–LN609714). A total of 29 (62 %) sequences matched the genus *Funneliformis*, while 13 (28 %) sequences matched the genus *Glomus* and 5 (10 %) sequences matched *Acaulospora* (Table 2). Therefore, the targets of the AMG1F and AM1 primer pair were similar to those of the primer pair NS31 and AM1, since all clones matched with the genera *Glomus* (or formerly *Glomus* in the case of *Funneliformis*) and *Acaulospora*.

Analysis of AM root colonization

The level of root colonization by AM fungi remained the same over most of the season, but it increased following leaf emergence in spring (Fig. 5, Table 3). Consequently, a significant seasonal change was found in overall AM root colonization (Table 3; $F=4.1$, $P=0.01$); however, no significant effect of plot location on root colonization was observed.

Discussion

Examination of the mycorrhizal fungal communities of *A. tricoccum* has revealed contrasting patterns of root colonization and community structure. As expected, significant seasonal changes in root colonization were found, but mycorrhizal fungal communities appeared unaffected by plant phenology. Previous studies have reported that AM fungal communities can vary across forest plant species with respect to fungal morphology (e.g., Arum versus Paris colonization patterns, Brundrett and Kendrick 1990b) and taxonomic composition (Burke 2008). In a previous study, *Trillium grandiflorum* and *M. racemosum* were found to host different communities of AM fungi, with some species significantly overrepresented in roots of *M. racemosum* (Burke 2008). *T. grandiflorum* and *M. racemosum* have perennial root systems and differ from *A. tricoccum*, which has an annual root system; however, although root growth does occur throughout the year in *A. tricoccum*, there are no abrupt changes in root growth over time in contrast to other spring ephemerals, such as *E. americanum* (Lapointe 2001). In *E. americanum*, root growth begins in the autumn, occurring through winter, with roots senescing in late spring and the rhizome being without roots through summer (Lapointe 2001). This pattern of pulsed root growth and senescence could lead to changes in root colonization and potentially community structure as roots age and C resources available to the AM fungi change. The consistent presence of roots with *A. tricoccum* could prevent clear changes in fungal community structure since the plant would contain roots at all points of the growing season. Since root growth occurs throughout the year, there may be little seasonal difference in C resource availability within roots.

Table 2 Database matches to clones generated with the AMG1F and AM1 primer set

Clone accession number	No. of clones	Database match description[a]	Match accession number	Percent identity[b]	Query cover (%)[b]
LN609686-LN609714	29	*Funneliformis* sp. 18S ribosomal RNA gene, partial sequence	JN859563	99.8	100
LN609715-LN609725	11	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	EU332713	99.8	100
LN609726	1	*Glomus* sp. 18S ribosomal RNA gene, partial sequence	FN429383	100	100
LN609727	1	*Glomus mosseae* 18S ribosomal RNA gene, partial sequence	AJ306438	100	100
LN609728-LN609729	2	*Acaulospora spinosa* 18S ribosomal RNA gene, partial sequence	JX461239	99.5	99
LN609730-LN609731	2	*Acaulospora colombiana* 18S ribosomal RNA gene, partial sequence	KF412636	100	100
LN609732	1	*Acaulospora* sp. 18S ribosomal RNA gene, partial sequence	AB178764	99	100

Clones were recovered from qPCR amplicons and indicate specificity of the primer set for AM fungi found in the study forest. No nonspecific or non-AM fungi were recovered with this primer set in contrast to the NS31and AM1 primer set as shown in Table 1

[a] Best match (excluding uncultured organisms) to the EMBL/NCBI/DDBJ database with a BLASTn search

[b] The % identity and % query coverage are reported here as averages of all clones if the database match was best for more than one clone

This consistent root growth could lead to the consistent presence of some AM fungal taxa with little seasonal change. To our knowledge, there has been little work examining changes in AM fungal community structure of forest herbs over time. Thus, how AM taxa respond to changes in root growth and turnover remains uncertain. The present results are in contrast to those of Helgason et al. (1999), where clear seasonal differences were noted in bluebell (*Hyacinthoides non-scripta*), with *Acaulospora* sp. more abundant in summer and *Scutellospora* sp. more abundant in winter. However, *Glomus* sp. showed no seasonal pattern in bluebell (Helgason et al. 1999). Although *Acaulospora* is present within the root samples of *A. tricoccum*, it occurs in low abundance, and the roots of *A. tricoccum* and the studied forest are dominated by *Glomus* spp. If *Glomus* spp. are insensitive to seasonality, this could explain the lack of an observed seasonal pattern in the AM community structure in *A. tricoccum* roots.

A significant seasonal change in AM fungal colonization of *A. tricoccum* roots was observed based on qPCR, although root colonization seemed fairly consistent across the time of measurement and only increased during leaf elongation in early spring. Other forest herbs have clear seasonal patterns of root colonization, with AM fungal colonization in many species (e.g., *T. grandiflorum* and *M. racemosum*) peaking during vegetative growth before declining through plant senescence (Brundrett and Kendrick 1990a). In *E. americanum*, root colonization increases through winter when only below ground structures are active, before peaking in early spring when leaves are active (Brundrett and Kendrick 1990a; Lapointe 2001). In *A. tricoccum*, AM fungal colonization seems to persist throughout the year, though at a low level in

the absence of leaves and photosynthetic activity. However, once leaves elongate in early spring, root colonization appears to increase rapidly. This is similar to the pattern of *M. racemosum* where AM fungal colonization peaks during vegetative growth (Brundrett and Kendrick 1990a). *A. tricoccum* does not go dormant in summer, despite a lack of photosynthetic tissue. Flowering and seed set occurs during the summer in the absence of leaves, so the plant is metabolically active but drawing on C reserves stored within the bulb (Nault and Gagnon 1988; Lapointe 2001). Nutrient and carbon recycling within spring ephemerals can be very efficient, and it has been suggested that C availability, not nutrients, is the main limitation for the growth of many forest herbs and spring ephemerals (Lapointe 2001). That would suggest that low levels of C are available to support AM fungal colonization in the absence of leaf tissue, and only during leaf elongation and initiation of photosynthesis does sufficient C become available to allow appreciable increases in root colonization.

In fact, there was little relationship between root colonization and bulb C content of *A. tricoccum*, suggesting that mycorrhizal colonization did not deplete C reserves through the summer. Bulb C content declined consistently over the summer, probably due to the cost of reproduction and seed set as well as basal metabolic costs, and began to increase with leaf expansion in spring when mycorrhizal colonization also increased. We suspect that only low levels of AM fungal colonization are present in roots in the absence of leaves and photosynthesis but that the presence of AM fungi within the roots allows for rapid increases in their growth and colonization following leaf elongation when additional C becomes

Table 3 Results of 2-way ANOVA using the general linear model for effects of phenology and plot location on *Allium tricoccum* bulb nutrient content and AM fungal root colonization

| Variable	Source of variation	DF	SS	MS	F	P
Carbon	Phenology	7	2454261.550	350608.793	33.997	*<0.001*
	Plot	7	42715.338	6102.191	0.592	0.759
	Residual	49	505330.450	10312.866		
	Total	63	3002307.339	47655.672		
Nitrogen	Phenology	7	3553.595	507.656	22.719	*<0.001*
	Plot	7	52.071	7.439	0.333	0.935
	Residual	49	1094.918	22.345		
	Total	63	4700.584	74.612		
Phosphorus	Phenology	7	11.683	1.669	15.899	*<0.001*
	Plot	7	0.312	0.0445	0.424	0.883
	Residual	49	5.144	0.105		
	Total	63	17.138	0.272		
NP ratio	Phenology	7	10933.279	1561.897	8.042	*<0.001*
	Plot	7	1468.165	209.738	1.080	0.390
	Residual	49	9516.143	194.207		
	Total	63	21917.587	347.898		
Bulb mass	Phenology	7	14449315.090	2064187.870	34.720	*<0.001*
	Plot	7	261080.458	37297.208	0.627	0.731
	Residual	49	2913131.507	59451.663		
	Total	63	17623527.056	279738.525		
AM root Colonization	Phenology	4	1.613E+015	4.033E+014	4.086	*0.010*
	Plot	7	7.874E+014	1.125E+014	1.139	0.368
	Residual	28	2.764E+015	9.872E+013		
	Total	39	5.165E+015	1.324E+014		

Significant differences are highlighted in italics

available to support the fungi. Since many spring ephemerals acquire most of their nutrients during the spring months (Lapoint 2001), the increase in root colonization at that time could facilitate nutrient uptake. In addition, nutrient uptake in spring can be impeded by cold soil conditions (Lapointe 2001), and there is evidence that soil temperature can affect direct nutrient uptake in the plant roots with cooler temperatures generally resulting in lower rates of nutrient uptake (reviewed in Pregitzer and King 2005). Whether AM fungi could help plants overcome temperature induced limitations to nutrient uptake is unclear, and the effects of soil temperature on roots and mycorrhiza are understudied in general (Pregitzer and King 2005). However, Barrett et al. (2011) found that *Glomus hoi* could colonize and acquire N from organic matter patches at temperatures as low as 10 °C when colonizing *Plantago lanceolata*, suggesting that AM fungi may assist the plant in nutrient acquisition at low soil temperatures. Nevertheless, maintenance of AM fungal colonization in *A. tricoccum* roots over winter during times of low soil temperatures is interesting, especially because low soil temperature during winter has been shown to decrease mycorrhizal

growth (reviewed in Pregitzer and King 2005). For example, LaPointe and Molard (1997) found that the presence of AM fungi in *E. americanum* roots decreased growth in winter and that maintaining the mycorrhizal association increased plant growth in the spring.

The present observations suggest that maintaining mycorrhizal fungal colonization in *A. tricoccum* over the winter benefits the plant by enhancing nutrient acquisition. In *A. tricoccum*, N uptake has been documented to occur over summer, when leaves are absent (Rothstein and Zak 2001), and Nault and Gagnon (1988) observed nutrient uptake through early autumn. The present data suggest, however, that bulb total N and P content is fairly dynamic over the growing season, and declines through summer before increasing through autumn and winter, before a dramatic decline in nutrient content corresponding to leaf elongation. It appears that N and P content is tied closely to phenological stages, with declines during flowering, fruiting and seed set and also following leaf elongation in spring. Although N and P content could increase after seed set through recovery of nutrients from flower scape senescence, previous work has suggested

Fig. 2 Changes in the C, N, and P content of *Allium tricoccum* bulb tissue over the course of one growing season. Relationships to plant phenological stage and sampling data are indicated on the x-axis
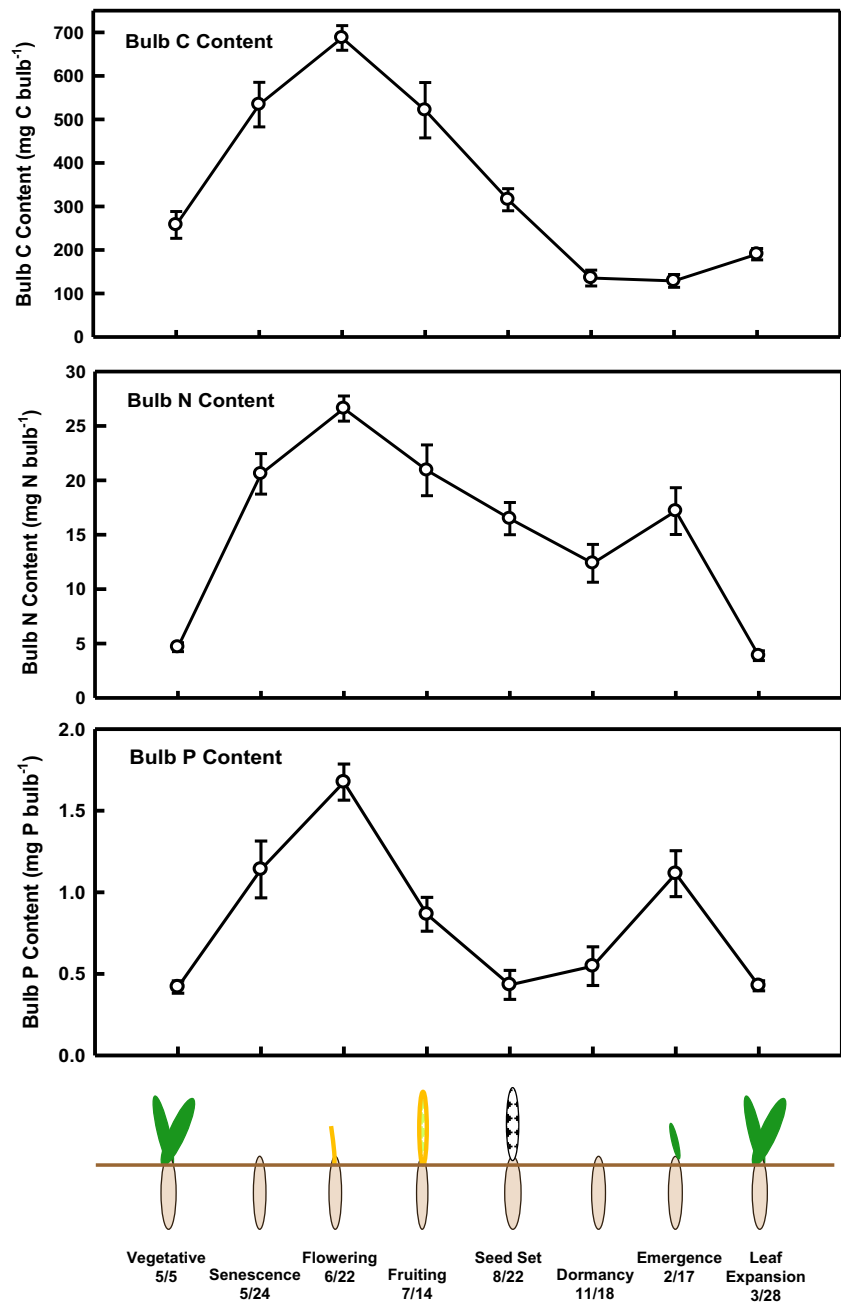
that flower and reproductive structures contain only low over-all levels of plant nutrients (Nault and Gagnon 1988). This suggests that either *A. tricoccum* roots acquired N and P directly from soil, or that mycorrhizal fungi were active within the root systems at this time and facilitated nutrient uptake. Alternatively, nutrient mobilization from senescing roots in autumn when new roots are also growing could be partly responsible for increases in N and P in the bulb. Metabolically active AM fungi could be present in roots between summer and winter, supported by the plant in the absence of photosynthetic tissue, and AM fungi were detected in *A. tricoccum* roots during this time frame. However, how the

plant could support these fungi without an overall reduction in bulb C content during this time is unclear. Although C content declined during fruiting and seed set, it remained fairly constant during the period of nutrient uptake, suggesting that if mycorrhizal fungi assisted in nutrient uptake at this time, there was little effect on bulb C content. Previous work has suggested that C transfer can occur between spring ephemerals and forest trees via the fungal hyphal network that connects different plant species (Lerat et al. 2002). Although this concept is controversial, it is possible that C flow from trees to AM fungi could support a metabolically active population of fungi within the roots of *A. tricoccum*. These fungi could then

Fig. 3 Changes in bulb mass and bulb N/P ratio in *Allium tricoccum* over the course of one growing season. Relationships to plant phenological stage and sampling data are indicated on the x-axis
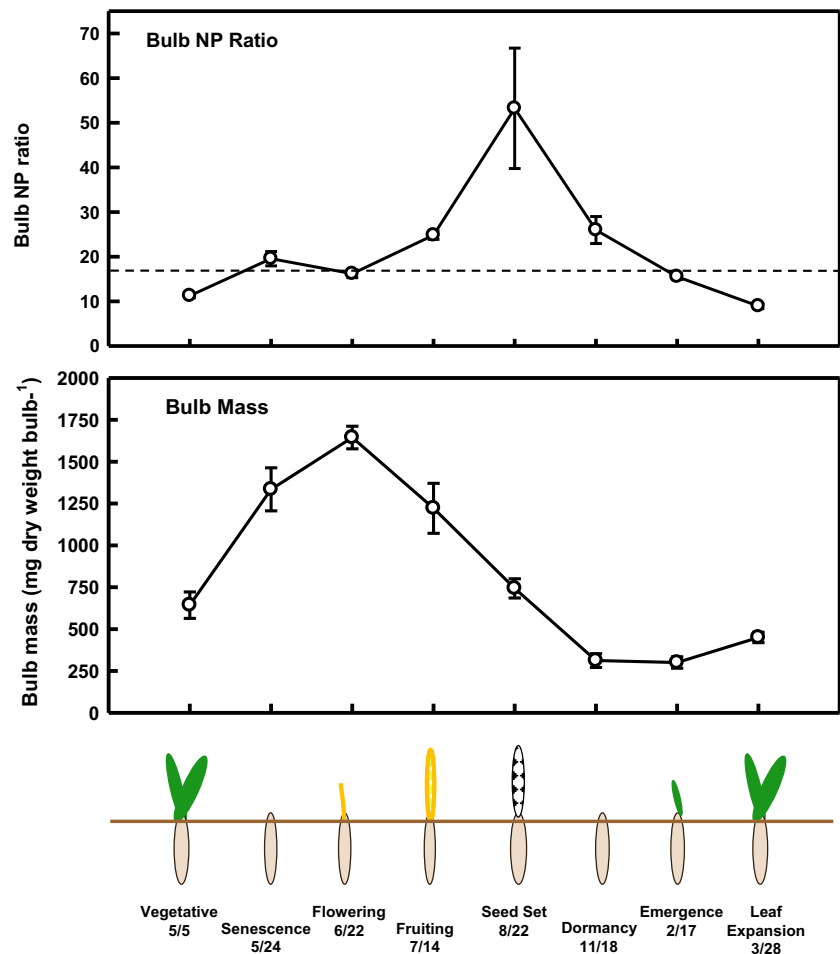
transfer both N and P to the roots, resulting in overall increases in nutrient content.

Of additional interest were changes in N/P ratios over the course of the growing season. Koerselman and Meuleman (1996) found that N/P ratios were a reliable indicator of plant physiological nutrient limitation with ratios of greater than 16 suggesting plant P limitations and ratios less than 14 indicating plant N limitation. The present data suggest that nutrient limitation may change seasonally in *A. tricoccum*, with plants experiencing N limitation during times when leaves are present and P limitation during times of fruiting and seed set. Increases in bulb N and P between seed set and leaf emergence appears to have eliminated nutrient limitation in autumn and winter (N/P ratios between 14 and 16 suggest no limitation or co-limitation), but N limitation may reappear during leaf expansion. This is in contrast to Nault and Gagnon (1988) who observed NP ratios in *A. tricoccum* bulbs of approximately 10 during fruiting and seed set suggesting general N limitation; however, mean dry weight of the bulbs was also greater in that study, averaging 2.6 g per bulb and pH of the study site was 6.4. Since our study site has a pH closer to 4.0, P limitation in

soil may be greater (Walker and Syers 1976), leading to increased plant P limitation during certain times of the life cycle.

Although changes in AM fungal communities were not observed with *A. tricoccum* phenology, changes occurred in community composition in space, with communities within roots correlated with the presence of AM tree species such as *L. tulipifera* and *A. saccharum*. This suggests that canopy trees can affect the AM fungal taxa colonizing the roots of *A. tricoccum*. These results support those of Helgason et al. (1999) who found that overstory trees affected root colonization in bluebell, with *Acaulospora* spp. associated with oaks and *Glomus* spp. with sycamore. Whether trees help to sustain the mycorrhizal fungi in the roots of *A. tricoccum* when C input from photosynthesis is absent will require additional study.

In conclusion, contrasting patterns of AM fungal colonization were observed in *A. tricoccum*, with root colonization responding to changes in plant phenology while fungal community structure did not. There were significant differences in AM fungal community structure between forest patches, with communities correlating with the presence of overstory AM

Table 4 The relative abundance of identified terminal restriction fragments (TRF) of AM fungi from colonized roots of *Allium tricoccum* in an old growth hardwood forest

Plot	Terminal restriction fragments representing AM fungi based on recovered sequence data and prediction of TRFs from same[a]																	
	93	101	102	126	128	138	144	146	187	188	277	278	279	293	491	494	518	519
1	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	38.97 (17.62)	1.05 (1.05)	0.00 (0.00)	28.79 (16.20)	12.68 (8.55)	2.78 (2.78)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	1.40 (1.40)	0.00 (0.00)	1.70 (1.70)	12.64 (11.16)
2	13.45 (8.84)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	49.79 (13.47)	0.59 (0.59)	0.72 (0.72)	12.48 (9.07)	16.59 (5.64)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	1.42 (1.42)	0.00 (0.00)	0.00 (0.00)	4.95 (2.82)
3	0.00 (0.00)	0.00 (0.00)	1.69 (1.69)	0.00 (0.00)	0.00 (0.00)	0.43 (0.43)	0.00 (0.00)	0.00 (0.00)	70.75 (12.40)	2.39 (2.39)	1.22 (1.22)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	9.65 (9.65)	0.41 (0.41)	0.00 (0.00)	13.46 (7.28)
4	12.06 (6.28)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	6.83 (4.42)	0.00 (0.00)	0.00 (0.00)	30.29 (14.77)	23.92 (9.18)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	1.47 (1.47)	4.69 (4.69)	0.00 (0.00)	20.74 (10.45)
5	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	6.78 (6.78)	25.35 (19.37)	0.00 (0.00)	0.00 (0.00)	22.45 (14.48)	0.00 (0.00)	0.55 (0.55)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	13.22 (13.22)	0.00 (0.00)	0.00 (0.00)	31.66 (20.48)
6	19.86 (12.80)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.95 (0.95)	44.42 (11.86)	0.00 (0.00)	0.28 (0.28)	20.07 (9.76)	0.00 (0.00)	1.05 (0.75)	0.49 (0.49)	2.44 (2.44)	0.00 (0.00)	7.79 (3.89)	1.25 (1.25)	0.49 (0.49)	0.91 (0.91)
7	0.00 (0.00)	0.21 (0.21)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	66.95 (13.77)	0.00 (0.00)	0.00 (0.00)	2.70 (2.70)	1.52 (1.52)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	26.96 (13.54)	0.00 (0.00)	0.00 (0.00)	1.67 (1.40)
8	0.00 (0.00)	0.00 (0.00)	0.64 (0.64)	0.18 (0.18)	1.66 (1.42)	59.55 (15.19)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	1.79 (1.79)	26.21 (11.44)	0.00 (0.00)	6.01 (6.01)	3.96 (2.49)

The mean relative abundance of each TRF detected on roots from each plot is shown averaged across all sampling time points. The standard error of the mean is provided in parenthesis. The putative identity of the TRFs as determined through sequence analysis from this study and from previous studies (Burke 2008; Burke et al. 2011) is provided at the end of the table. TRFs are predicted from 18S rDNA sequences

[a] Putative identity of TRFs: 93, *Acaulospora* sp.; 100, *Acaulospora* sp.; 102, *Acaulospora* sp.; 126, *Glomus* sp.; 128, *Glomus* sp.; 138, *Glomus* sp.; 144, *Glomus* sp.; 146, uncultured Glomeromycota; 187, *Glomus* sp.; 188, *Glomus* sp.; 277, *Glomus* sp.; 278, *Glomus* sp.; 279, *Glomus* sp.; 293, *Glomus* sp.; 491, *Scutellospora* sp.; 494, *Paraglomus* sp.; 518–519, some *Acaulospora* sp. and *Glomus* sp. both produce TRFs of this size
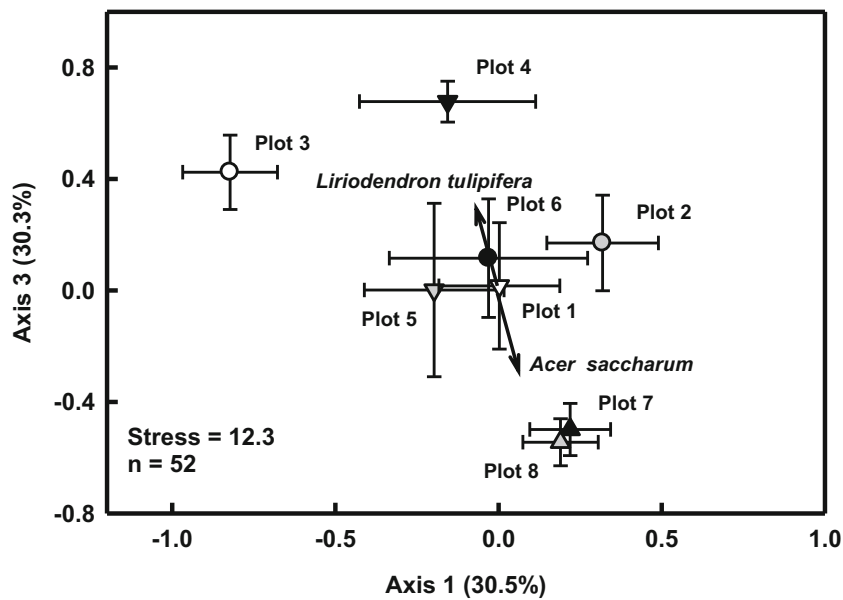
Fig. 4 Non-metric multidimensional scaling ordination of arbuscular mycorrhizal fungal community composition in *Allium tricoccum* roots based on mean values for each sampled plot. *Symbols* represent means and standard errors of axis scores. Significant environmental variables are shown with *vector lengths* indicating the strength and direction of the strongest correlations. The final stress was 12.3 for a three-dimensional solution. Plot identification for each symbol is shown on the figure (*symbol shape* and *color* is meant for visual separation only and does not refer to any relationship among the *plots*)

tree species. This suggests that tree species influence AM fungal communities in *A. tricoccum*. Bulb nutrient content increased from late summer after seed set through winter when *A. tricoccum* lacked leaves and C input, indicating that roots and/or AM fungi are metabolically active despite an absence of photosynthesis and C gain. Nutrient content declined following plant leaf elongation, which was also co-incident with increases in mycorrhizal colonization. These contrasting patterns suggest that the nutrient content of *A. tricoccum* is dynamic over the season, and linked to changes in plant phenology and AM fungal colonization of the root system.

Fig. 5 Mycorrhizal colonization of *A. tricoccum* roots as determined through qPCR of AM fungal 18S rDNA and expressed as copy number per gram of root dry weight. Relationships to plant phenological stage and sampling date are indicated on the x-axis
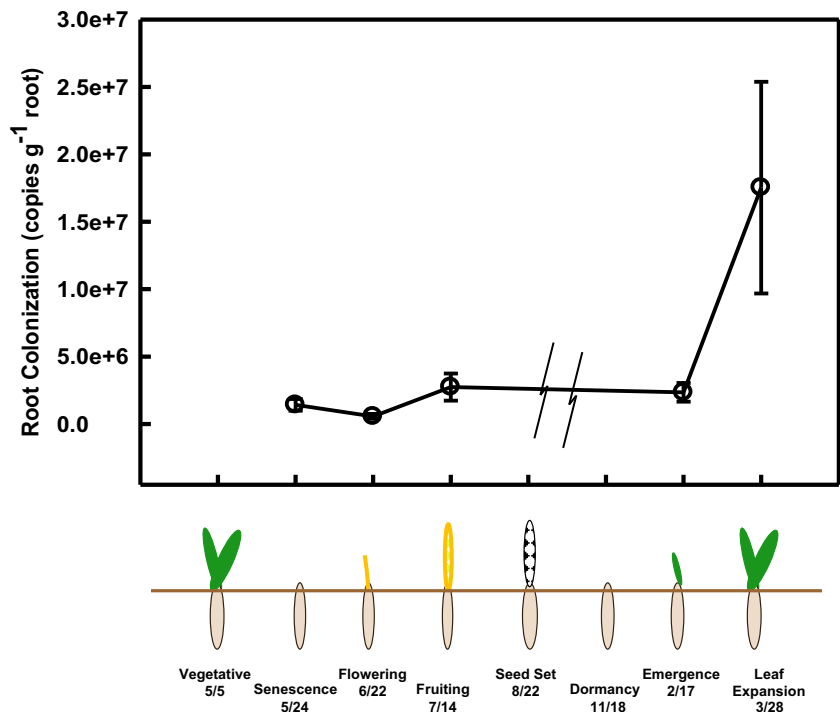
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