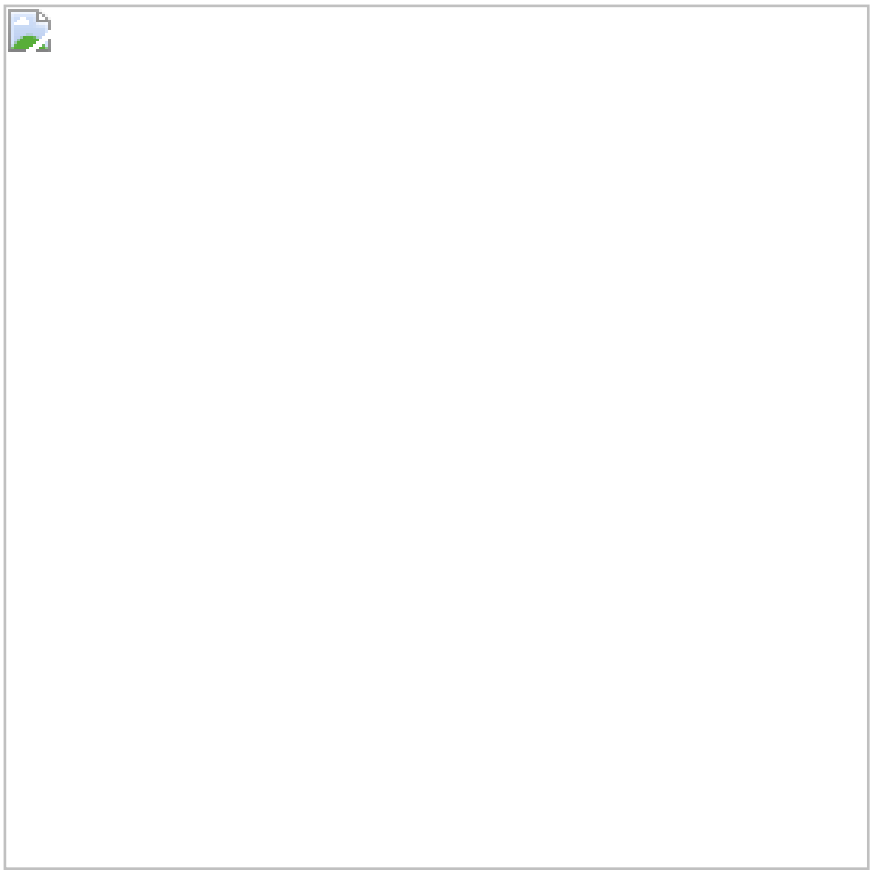

Soil and microbe interactions in two populations of Appalachian black cohosh (Actaea racemosa L.)1

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Soil and microbe interactions in two populations of Appalachian black cohosh (*Actaea racemosa* L.)[1]

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Abstract. Soil and whole plant samples were collected from two natural populations of Appalachian black cohosh (*Actaea racemose* L.) to assess soil-plant-microbe interactions and determine seasonal mineral acquisition by the species. *A. racemosa* is one among medicinal forest plants subject to excessive harvesting, and there is increasing concern over the sustainability of natural populations. Following standard procedures, mineral content and chemistry of soils sampled from the two sites were determined, and monthly (May to August) *A. racemosa* root, stem, and leaf mineral content were analyzed. Fresh tissue samples were processed for isolation and genotyping of associated endophytic organisms, and classical root staining was used to assess presence and extent of the arbuscular mycorrhizal (AM) symbiosis. Results showed that forest soils in the natural habitat of *A. racemosa* are slightly acidic (pH 4.5–6.0; 40–70% base saturation) with high organic matter (6–25%) and microelement content. Significant ($P \leq 0.05$) variation was observed in seasonal concentration of key elements in leaf, stem, and root samples, with high levels of iron (Fe), aluminum (Al), manganese (Mn), and zinc (Zn) sequestered in root tissues. Root colonization by AM fungi was found to be high (85–100%) at both locations throughout the sampling period. A total of 22 fungal and 24 bacterial endophytes were isolated from *A. racemosa* root, shoot, and flower organs. Molecular characterization revealed bacterial isolates to be primarily *Bacillus*, *Pseudomonas*, and *Serratia* spp., whereas fungal isolates included *Alternaria*, *Cadophora, Diaporthe, Penicillium,* and *Volutella* spp. We conclude that potential exists for managed cultivation of black cohosh in arable land. Further, our findings confirm endophytic (mycorrhizal and nonmycorrhizal) associations in *A. racemosa*. We believe associated organisms could play a role in the adaptation of *A. racemosa* to the Appalachian ecosystem and recommend further examination of these relationships.

Key words: *Actaea racemosa*, Appalachian forest ecosystem, arbuscular mycorrhizal symbiosis, endophytic associations, plant nutrition, soil properties

More than 25% of plant species worldwide are listed either as threatened or endangered. The reasons for endangerment are varied, but habitat loss due to development, agriculture (including grazing), extractive industries such as mining and logging, and displacement by invasive species are key drivers (Schemske *et al*. 1994). Other factors include collection for trade, water control, fire, and recreation (off-road vehicles and trampling). Among strategies adopted in conservation of endangered plant species are *in situ* preservation through legislative and physical protections (Maxted *et al*. 2002), seed deposition in gene banks (Guerrant *et al*. 2004), and *ex situ* conservation via managed cultivation (Havens *et al*. 2009).

In the USA, black cohosh (*Actaea racemosa* L., Syn.: *Cimicifuga racemosa* (L.) Nutt.) is one among species classified as "at risk" ("endangered" in Illinois) due to uncontrolled harvesting from natural populations for trade (Predny *et al*. 2006). A member of the Ranunculaceae family, *A. racemosa* is an herbaceous species native to the Appalachian forest ecosystem of eastern North America (Davis *et al*. 2019). Historical records show that Native Americans valued the *A. racemosa* rhizome as treatment for a variety of conditions including gynecological ailments (Predny *et al*. 2006). The practice of harvesting *A. racemosa* for medicine was continued by European settlers, and significant extraction from public lands to meet demand for treatment of menopausal and premenstrual symptoms in women has been reported in the recent past (Chamberlain *et al*. 2002, American Herbal Products Association 2007). According to Davis *et al*. (2019), some of the bioactive compounds found in the black cohosh rhizome are triterpene glycosides, isoflavones, and formononetin.

Several studies on the sustainability of the species considering current harvesting trends have

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been conducted. Small *et al*. (2011) reported that intensive harvesting (removal of up to 66% of rhizomes) results in significant reduction of leaf area, stemming, and mean and maximum plant height, with no evidence of recovery in the first year postharvest. Long-term monitoring of the plots coupled with regression analysis showed that recovery of leaf area and plant height to preharvest levels takes 4–7 yr, provided there was no more plant removal (Small and Chamberlain 2018). To aid development of a more effective inventory and management strategy, Chamberlain *et al*. (2013) developed a model for estimating *A. racemosa* rhizome biomass based on aboveground metrics.

In addition to sustainable management of natural populations, forest farming has been proposed as a tool for offsetting the ecological impact of unsustainable harvesting faced by *A. racemosa* and other nontimber forest species (Baker and Saha 2018). General guidelines for managed cultivation of *A. racemosa* were described by Davis *et al*. (2019). Uniform stands can be established by spring or fall planting of rhizome sections in rich, moist soil, preferably in raised beds. Plantings can also be started from seed, but the seed must first be exposed to a warm/cold/warm cycle before they will germinate (Albrecht and McCarthy 2007). However, various attempts to cultivate *A. racemosa* have uncovered various challenges, including susceptibility to fungal diseases (Thomas *et al*. 2006), significant variation in plant performance depending on site and growing conditions (McCoy *et al*. 2007), and a more than 50% loss of transplanted rhizomes in simulated forest farming (Small *et al*. 2014). Further study is also required on seed treatment and storage in response to dormancy and viability challenges observed by Albrecht and McCarthy (2007).

In a study to assess the effect of canopy opening and soil fertility on growth and synthesis of bioactive components in *A. racemosa* and other medicinal plants, Naud *et al*. (2010) found that increased irradiation, and higher soil pH and fertility correlated with improved plant growth. It is notable that all studies on managed cultivation of *A. racemosa* and other nontimber forest species emphasize site selection as one of the key determinants of success, with soil physical and chemical properties and degree of shading ranking among the most important site factors. Small *et al*. (2014) also identified soil amendment to raise pH as a practice that might improve survival and growth of new plantings. Although there is little information on endophyte and other microbe interactions in *A. racemosa*, many reports have highlighted the important role that symbiotic relationships play in plant adaptation and survival. For example, Zubek *et al*. (2009) reported that inoculation with arbuscular mycorrhizal fungi (AMF) can improve *ex situ* survival and success of some threatened species, including *Senecio umbrosus*. Similarly, Rodriguez *et al*. (2008) have demonstrated what they term habitat-adapted symbiosis whereby similar endophytes can confer different benefits dependent upon biotic or abiotic stresses affecting the host plant.

The observations noted above informed our hypothesis that species such as *A. racemosa* adapted to niche environments (*e.g*., the forest understory) benefit from unique soil-plant-microbe interactions that might need to be replicated for successful managed cultivation. The findings we report in this paper will contribute to better understanding of complex interactions within forest ecosystems. For example, endophytic and other associated microbes, for which there are no reports for *A. racemosa,* could play a significant role in the survival of transplanted plants as observed by Wennström and Ericson (1994). We believe that such information would hasten discovery of husbandry practices optimized for managed cultivation of species threatened by excessive extraction from natural stands. Here, we present results from a study to survey soils and endophytes associated with two populations of *A. racemosa* found in the Appalachian forest ecosystem.

Materials and Methods. SITES. Plant and soil samples were obtained from two Virginia sites located in closed-canopy, mixed-oak stands dominated by *Quercus alba* L. within the George Washington and Jefferson National Forests. The first (Mt. Rogers) in Wythe County (36°45′36.56″N; 81°12′58″W) is at an elevation of about 1,180 m above sea level on a moderately steep north-facing slope. The second (Reddish Knob) is in Augusta County (38°26′33.52″N; 79′15′52″W) at an elevation of about 1,190 m above sea level on a moderately steep southeast-facing slope. Soil cores to a depth of 15 cm were collected from four randomly selected locations within *A. racemosa* patches and transferred into Ziploc® bags. At the

same time, whole *A. racemosa* plants were excavated, with the ball of soil around the root system moistened before potting. Samples were collected monthly from May to August 2011 (Mt. Rogers) and 2012 (Reddish Knob) and immediately transferred to Virginia State University (VSU) for analysis.

Sample Processing and Analysis. Soil samples were air-dried before passing through a 2-mm sieve in preparation for chemical and mineral analysis. In the lab, soils were processed for measurement of elements (phosphorus [P], potassium [K], magnesium [Mg], calcium [Ca], sodium [Na], sulfur [S], Zn, Mn, Fe, copper [Cu], boron [B]) by extraction in Mehlich III solution following methods described by Mehlich (1984). Briefly, 2 g of soil per sample was mixed with 20 ml of Mehlich III solution and extracted by shaking for 5 min on a reciprocating mechanical shaker. After shaking, the suspension was filtered and analyzed for mineral content on an Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES). Soil organic matter (OM) and soluble salts were determined following methods described in the Recommended Chemical Solution Test Procedures Manual for the North Central Region (Brown 2011), and soil pH (1:1) was determined following methods published by the American Society of Agronomy (Thomas 1996).

In the lab, plants were washed and separated into root, shoot, and leaf portions before subsamples were collected for isolation of associated endophytes. At the same time, 20–30 fresh root tips (2.0 ± 0.2 cm) were excised from each plant and preserved in 50% ethyl alcohol pending estimation of mycorrhizal infection. The rest of the root, shoot, and leaf portions were transferred into labeled bags and dried in a convection oven at 70 °C for 48–96 hr (some *A. racemosa* samples had large rhizomes that took longer to dry). Dried samples were ground with a Wiley mill to pass a 2-mm sieve and portions processed by microwave digestion. After filtration and centrifuging, the metal content in plant samples was determined using an ICP-AES as explained in method #6010C (EPA 2007).

Estimation of Mycorrhizal Infection. Root tips were cleared with 10% potassium hydroxide (KOH) (w/v) followed by staining in a 1:1:1 solution of glycerol, lactic acid, and 0.03% Chlorazol Black E as described by Brundrett *et al*. (1996). After destaining in 50% glycerol, root pieces were mounted on slides and mycorrhizal infection quantified under the microscope following the grid-intersect method described by Giovannetti and Mosse (1980).

Isolation of Endophytic Organisms. Healthy leaf, stem, root, and flower sections were washed in running water and then dissected into small segments (< 10 mm in length, or < 10 mm^2 in area). For surface sterilization, all samples were rinsed with 75% ethyl alcohol for 30 sec prior to treatment for 20 min in 10% bleach. Samples were then rinsed five times in sterile water. To ascertain success of the disinfection process, aliquots of the final rinse solution were plated onto potato dextrose agar (PDA) media supplemented with 50 mg/L ampicillin and 15 mg/L tetracycline to prevent bacterial growth. For endophytic isolation, surface-sterilized samples were placed onto PDA media with the same antibiotics as mentioned above and incubated at 23 °C for 3–7 days. After incubation, all isolates recovered from each sample fragment were selected and purified by subculturing onto fresh PDA media. Colony morphology was recorded at this stage.

Extraction of Endophytic Genomic DNA. A total of 22 fungal isolates obtained from *A. racemosa* tissues were subjected to molecular characterization. Genomic DNA was extracted with DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following procedures described by the manufacturer in the User's Handbook. Extracted DNA was dissolved in 50 µl Tris-EDTA (TE) buffer and quantified by Nanodrop 2000 (Thermo Scientific, Waltham, MA). Bacterial genomic DNA was extracted from all 24 isolates using standard procedures described by Park *et al*. (2005).

PCR Amplification and PCR-Based Fingerprinting. In order to genotype all isolates, a DNA fragment of the gene encoding either 18S rDNA for fungal isolates or 16S rDNA for bacterial isolates was amplified using universal primer pairs (universal bacterial primers, forward 8F 5′-AGAGTTTGATCCTGGCTCAG-3′, and reverse primer 1492 5′- GGTTACCTTGTTACGACTT-3′, universal fungal primer, forward S1 5′-ACTGC-GAATGGCTCATTAAATCA-3′, and reverse primer S3 5′-AGTCAAATTAAGCCGCAG-3′) (Khan and Doty 2009). Fifty ng of genomic DNA was used for each amplification reaction. The amplification was performed in a 50 µl reaction

under the following conditions: initial denaturation at 95 °C for 4 min, then followed by 35 cycles of denaturing at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. Five min at 72 °C final extension was added after 35 cycles of amplification. The PCR products were monitored on 1% agarose gel.

The amplified PCR products were then digested with 4-base cutter restriction enzyme MseI for 3 hr at 37 °C, and DNA digestion patterns were resolved on 4% agarose gel. Taking advantage of the fact that 4-base cutter restriction enzymes digest DNA more frequently and yield multiple DNA bands, we could simultaneously monitor more site mutations and group together those with same digestion patterns.

CLONING AND SEQUENCING OF DNA FRAGMENTS. PCR products with distinct digestion patterns were selected for further sequence analysis. The products were cloned into TOPO pCR2.1 vector following manufacturer directions. Plasmid DNAs were isolated from selected clones, and the presence and sizes of insertions were confirmed by EcoRI digestion. A representative of each cloned PCR product was sequenced using M13 forward and reverse primers on ABI 3100 sequencer (Applied Biosystems, Inc., Foster City, CA). Similarity for all representative sequences was analyzed by BLAST comparison against the nonredundant database maintained by the National Center for Biotechnology Information (NCBI 2020).

DATA ANALYSIS. Two-way analysis of variance for fixed effects (with interaction) was performed on soil and plant mineral data using the Proc-ANOVA function in SAS (2020). Sampling time and location were taken as independent variables and means were separated using Tukey's honestly significant difference (HSD) test where significant differences were observed. Pearson correlation (Proc-CORR; SAS 2020) was used to analyze the relationship between soil and plant mineral content.

Results. SOIL CHEMICAL AND MINERAL PROPERTIES. There were no significant ($P \leq 0.05$) differences between the two sites in soil pH and acidity. Cation exchange capacity (CEC) was similarly uniform, except in July when it was significantly lower at both sites when compared with August readings for Reddish Knob, whereas the buffer index was significantly higher at all other sites than at Reddish Knob in August (Table 1). Two-way analysis of variance for fixed effects found that site had a significant effect on buffer index and Ca^{2+} saturation, while the buffer index and K^{+} and Na^{+} saturation varied with time during the growing season. There was no significant interaction between time and place except in the buffer index (Table 1).

Soil organic matter content was similar at both sites during the growing season except in August when significantly ($P \leq 0.05$) higher content was recorded in soil samples from Reddish Knob. There were no differences in soil K content except in June and July when content at both sites was significantly lower than at Reddish Knob in August, and Mg levels were also similar except for a significant difference between May and July in Reddish Knob. Sodium was generally higher in soil samples from Reddish Knob, with data for August being significantly higher than levels recorded for Mt. Rogers throughout the sampling period. For Mn, Fe, and copper (Cu), variations were observed within Reddish Knob where soil Mn content was significantly higher in May than in August, with the opposite being true for Fe. Copper levels were higher in May than in July. No significant differences were observed between and within sites in estimated nitrogen release, and in soil P, Ca, S, Zn, and B content throughout the sampling period (Table 2).

Analysis of variance for fixed effects showed significant differences by site and time in organic matter content, with a significant interaction between the two factors. Similarly, site was a significant factor in Na, S, and Fe content, and the same was found for K, Mg, Ca, Na, Mn, and Cu with time. In addition to organic matter, the interaction between the two factors was significant with respect to Mn and Fe content (Table 2).

PLANT MINERAL CONTENT. Among the three plant parts, the greatest variation in mineral content was recorded in leaf tissue. Clear trends were observed in nitrogen (N), P, K, S, and Na levels that declined over time, with significantly ($P \leq 0.05$) higher levels generally found in May-June than in July–August samples. The opposite was true for Ca, Al, Mn, and B, where levels were highest towards the end of the growing season. No clear trend was present for Mg content in leaf tissue, where the highest content was recorded in the June samples from Mt. Rogers and the lowest in the July

Table 1. Soil chemical properties in soils associated with two black cohosh (*Actaea racemosa* L.) populations found in the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from Mt. Rogers (Site 1) in 2011 and Reddish Knob (Site 2) in 2012.

Parameter	pH	Acidity (meq/100 g)	CEC. (meq/100 g)	Buffer Index	Base saturation (%)				
					K^+	Mg^{2+}	Ca^{2+}	Na^+	H^+
Month and site*									
May									
1	5.63a	3.20a	13.67ab	6.50a	2.00a	12.80a	55.13a	0.43b	29.87a
2	5.57a	3.03a	13.97ab	6.47a	2.40a	12.40a	55.00a	0.67ab	29.73a
June									
1	5.20a	3.00a	8.47ab	6.60a	2.67a	13.63a	45.47a	0.63ab	37.77a
2	5.07a	3.27a	8.80ab	6.60a	2.67a	12.03a	44.20a	0.90ab	40.10a
July									
1	5.00a	2.87a	7.20b	6.63a	3.20a	11.60a	43.50a	0.73ab	41.00a
2	4.90a	3.03a	7.07b	6.67a	3.47a	10.00a	42.03a	1.13a	43.37a
August									
1	5.30a	3.43a	10.40ab	6.57a	2.80a	12.33a	51.77a	0.57ab	32.40a
2	4.53a	3.43a	16.50a	6.10b	2.27a	6.13a	33.30a	0.57ab	57.77a
P values†									
Site	0.2600	0.9402	0.2056	0.0128	0.9002	0.0437	0.2981	0.0145	0.2045
Month	0.1704	0.9741	0.0027	0.0003	0.0471	0.1190	0.2703	0.0133	0.2869
Site × Month	0.6642	0.9453	0.2753	0.0018	0.6106	0.3050	0.5208	0.4067	0.3699

Abbreviations: CEC = cation exchange capacity; ions: K^+ = potassium; Mg^{2+} = magnesium; Ca^{2+} = calcium; Na^+ = sodium; H^+ = hydrogen.

* Within columns, means followed by a different letter are significantly different ($P \leq 0.05$; Tukey's HSD).

† *P* values are significant at $\alpha \leq 0.05$.

samples from Reddish Knob. Similarly, leaf Mn content was significantly higher in July samples from Reddish Knob than in samples collected from both sites in May, and from Mt. Rogers in June, but there were no differences between July and August samples from Reddish Knob on the one hand, and between the rest of the samples on the other. No differences were observed in leaf Cu, Fe, and Zn throughout the growing season. Site was a significant factor in leaf N, K, Mg, S, Mn, Al, and Zn content, whereas time of sampling was significant for all elements except Mg, Cu, and Zn. A significant interaction between the two factors was observed only in leaf N, K, and B content (Table 3).

In stem tissue, N content was similar except in May samples from Reddish Knob, where content was significantly ($P \leq 0.05$) higher than all other samples. In K, a trend similar to that observed in leaf tissue was observed whereby significantly higher levels were present at the beginning of the growing season. Marked differences between sites were found in Ca, Mg, and S stem content with significantly higher Ca levels found in samples from Reddish Knob, while the opposite was observed for Mg and S where levels were higher in samples from the Mt. Rogers site. Samples from Reddish Knob contained higher levels of Mn in August than for both sites in May and June, whereas overall, Cu was significantly higher in stem tissue sampled from Mt. Rogers in May. Stem B was generally the same except for samples from Mt. Rogers in May, and Reddish Knob in July and August that were significantly higher. Site was a significant factor in stem N, Ca, Mg, S, Mn, and Zn content; time of sampling was significant for N, P, K, Mg, Na, Fe, Cu, and B. Interactions between the two factors were significant for stem Mg, S, Cu, and B content (Table 4).

The least variation was observed in root mineral content, with no significant differences found within and between sites in root P, K, Na, Al, Cu, and Zn concentrations. Even where differences were observed, no clear trends could be discerned. Root N content was significantly ($P \leq 0.05$) higher in May samples from Reddish Knob and lower in July samples from Mt. Rogers, while the only variation in Ca content was between May samples from Mt. Rogers and August samples from Reddish Knob. Magnesium concentrations were highest in May samples from Mt. Rogers; S content at both sites was similar in May and significantly higher than June samples from Mt. Rogers and August samples from Reddish Knob. Conversely, June samples from Reddish Knob

Table 2. Organic matter and mineral content in soils associated with two black cohosh (*Actaea racemosa* L.) populations found in the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from Mt. Rogers (Site 1) in 2011 and Reddish Knob (Site 2) in 2012.

	Month and site*								*P* values†		
	May		June		July		August				
Content	1	2	1	2	1	2	1	2	Site	Month	Site × Month
OM (%)	6.77b	12.93b	5.77b	9.40b	5.87b	10.70b	6.90b	25.13a	<0.0001	0.0003	0.0012
ENR (kg/ha)	149.67a	150.00a	145.00a	150.00a	147.57a	150.00a	150.00a	150.00a	0.0816	0.3354	0.3354
Minerals (mg/kg)											
P	49.67a	60.17a	27.10a	43.90a	29.13a	40.43a	38.90a	39.43a	0.2092	0.2320	0.8913
K	96.57ab	108.57ab	82.23b	80.43b	88.33b	91.23b	109.43ab	141.67a	0.1327	0.0028	0.3475
Mg	224.80ab	276.13a	142.80ab	132.13ab	100.20ab	83.23b	148.10ab	122.77ab	0.9873	0.0027	0.6981
Ca	1656.23a	1745.27a	803.77a	828.30a	596.67a	612.10a	1077.23a	1110.23a	0.8629	0.0219	0.9995
Na	11.57d	16.80ab	11.97d	16.77abc	12.10cd	16.97ab	13.87bcd	20.77a	<0.0001	0.0139	0.6664s
S	17.43a	19.77a	15.43a	16.67a	18.13a	20.10a	16.20a	23.67a	0.0327	0.2647	0.3979
Zn	9.60a	11.27a	5.97a	6.97a	4.73a	6.20a	8.33a	7.53a	0.4982	0.0507	0.8821
Mn	178.00ab	203.90a	118.10ab	132.43ab	170.00ab	188.80ab	187.23ab	106.00b	0.6618	0.0068	0.0237
Fe	140.33b	164.00b	151.30b	181.53ab	145.00b	166.90ab	128.67b	281.43a	0.0035	0.1403	0.0351
Cu	2.13ab	2.40a	1.47ab	1.33ab	1.43ab	1.13b	1.50ab	1.50ab	0.7952	0.0018	0.6398
B	0.80a	0.77a	0.33a	0.43a	0.30a	0.47a	0.50a	0.47a	0.6625	0.0712	0.8963

Abbreviations: OM = organic matter; ENR = estimated nitrogen release; P = phosphorus; K = potassium; Mg = magnesium; Ca = calcium; Na = sodium; S = sulfur; Zn = zinc; Mn = manganese; Fe = iron; Cu = copper; B = boron.

* Within rows, means followed by different letters are significantly different ($P \leq 0.05$; Tukey's HSD).

† *P* values are significant at $\alpha \leq 0.05$.

Table 3. Seasonal variation in leaf mineral content in two black cohosh (*Actaea racemosa* L.) populations found within the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from Mt. Rogers (Site 1) in 2011 and Reddish Knob (Site 2) in 2012.

	Month and Site*								*P* values†		
	May		June		July		August				
Content	1	2	1	2	1	2	1	2	Site	Month	Site × Month
%											
N	2.67bc	3.61a	2.37bcd	2.96b	1.97d	2.13cd	1.33e	2.23cd	<0.0001	<0.0001	0.0266
P	0.30ab	0.31a	0.19c	0.20bc	0.14c	0.14c	0.12c	0.15c	0.4588	<0.0001	0.8598
K	3.38a	2.93bc	3.08ab	3.01abc	3.28ab	2.40d	2.65cd	2.28d	<0.0001	<0.0001	0.0008
Ca	0.96bc	0.76c	1.34abc	1.46abc	1.54ab	1.56ab	1.89a	1.87a	0.8285	<0.0001	0.7324
Mg	0.26abc	0.16bc	0.37a	0.18bc	0.26abc	0.09c	0.28ab	0.15bc	<0.0001	0.0952	0.6151
S	0.15ab	0.18a	0.13b	0.15ab	0.11bc	0.12bc	0.08c	0.11bc	0.0031	<0.0001	0.3526
Na	0.02b	0.02b	0.02b	0.03ab	0.03ab	0.03ab	0.03ab	0.04a	0.7892	0.0006	0.7533
ppm											
Fe	68.3a	85.7a	323.7a	166.7a	242.7a	247.3a	195.3a	151.2a	0.2725	0.0249	0.4119
Al	41.6c	36.6c	302.3ab	90.9bc	320.0a	205.3abc	143.3abc	171.4abc	0.0339	0.0015	0.0741
Mn	46.3c	103.4c	102.3c	180.0abc	145.0bc	307.7a	137.7bc	260.5ab	<0.0001	0.0002	0.2602
Cu	14.7a	12.4a	11.3a	12.7a	11.7a	12.0a	9.3a	11.6a	0.6334	0.1453	0.3195
Zn	25.3a	34.5a	26.3a	29.8a	20.7a	26.7a	25.3a	28.8a	0.0148	0.2101	0.7196
B	18.7bc	13.2c	20.7abc	16.7c	25.3abc	32.7a	27.7ab	29.2a	0.8926	<0.0001	0.0058

Abbreviations: N = nitrogen; P = phosphorus; K = potassium; Ca = calcium; Mg = magnesium; S = sulfur; Na = sodium; ppm = parts per million; Fe = iron; Al = aluminum; Mn = manganese; Cu = copper; Zn = zinc; B = boron.

* Within rows, means followed by different letters are significantly different ($P \leq 0.05$; Tukey's HSD).

† *P* values are significant at $\alpha \leq 0.05$.

recorded significantly higher Fe and Mn, whereas B content was highest in May. Site had a significant effect on root N, K, Ca, Mg, Na, and B, and the same was true for time of sampling with respect to N, Ca, Mg, S, Fe, Al, Cu, and B root content. The interaction between site and time of sampling had no significant effect on root mineral content except in S nutrition (Table 5).

Table 4. Seasonal variation in stem mineral content in two black cohosh (*Actaea racemosa* L.) populations found within the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from two sites: Mt. Rogers (Site 1) in 2011 and Reddish Knob (Site 2) in 2012.

	Month and Site*								*P* values†		
	May		June		July		August				
Content	1	2	1	2	1	2	1	2	Site	Month	Site × Month
%											
N	1.08b	1.62a	0.83b	1.01b	0.83b	1.06b	0.78b	1.11b	<0.0001	0.0001	0.1679
P	0.26a	0.26a	0.23a	0.15a	0.19a	0.12a	0.16a	0.13a	0.0556	0.0058	0.4232
K	3.22a	3.30a	2.57abc	2.30abc	2.84ab	2.01bc	1.73c	1.65c	0.0996	<0.0001	0.2275
Ca	0.51a	0.08b	0.47a	0.05b	0.56a	0.06b	0.63a	0.06b	<0.0001	0.1213	0.1308
Mg	0.12c	0.52b	0.12c	0.45b	0.11c	0.72a	0.14c	0.77a	<0.0001	<0.0001	<0.0001
S	0.06c	0.10ab	0.06c	0.09ab	0.06c	0.08c	0.05c	0.16a	<0.0001	0.1034	0.0208
Na	0.02a	0.02a	0.01b	0.01b	0.02a	0.02a	0.01b	0.02a	0.1274	0.0013	0.1908
ppm											
Fe	43.7a	57.0a	100.7a	53.3a	98.33a	97.9a	105.7a	128.5a	0.8559	0.0458	0.4243
Al	38.7a	30.7a	49.0a	27.8a	77.0a	56.5a	46.0a	142.0a	0.6285	0.2824	.02654
Mn	25.7b	72.6ab	29.4b	46.8ab	46.0ab	150.7ab	55.7ab	205.1a	0.0058	0.0632	0.2826
Cu	32.7a	12.3b	10.3b	10.0b	11.0b	9.90b	7.67b	9.00b	0.0536	0.0032	0.0205
Zn	17.0a	28.6a	19.3a	22.8a	23.7a	30.1a	25.0a	32.9a	0.0073	0.1152	0.6823
B	17.0a	13.3ab	13.3ab	9.93b	13.0ab	15.9a	13.0ab	14.1a	0.2147	0.0042	0.0021

Abbreviations: N = nitrogen; P = phosphorus; K = potassium; Ca = calcium; Mg = magnesium; S = sulfur; Na = sodium; ppm = parts per million; Fe = iron; Al = aluminum; Mn = manganese; Cu = copper; Zn = zinc; B = boron.

* Within rows, means followed by a different letter are significantly different ($P \leq 0.05$; Tukey's HSD).

† *P* values are significant at $\alpha \leq 0.05$.

Table 5. Seasonal variation in root mineral content in two black cohosh (*Actaea racemosa* L.) populations found within the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from Mt. Rogers (Site 1) in 2011 and Reddish Knob (Site 2) in 2012.

	Month and Site*								*P* values†		
	May		June		July		August				
Content	1	2	1	2	1	2	1	2	Site	Month	Site × Month
%											
N	1.72bc	2.58a	1.17c	2.03ab	1.13c	1.79abc	1.37bc	1.66bc	<0.0001	0.0033	0.3316
P	0.18a	0.19a	0.17a	0.12a	0.13a	0.13a	0.17a	0.15a	0.4034	0.3444	0.7912
K	1.61a	1.34a	1.15a	1.02a	1.44a	0.97a	1.37a	0.92a	0.0171	0.1709	0.7432
Ca	0.95a	0.77ab	0.72ab	0.74ab	0.69ab	0.56b	0.72ab	0.57b	0.0101	0.0018	0.2802
Mg	0.31a	0.20ab	0.32ab	0.21ab	0.21ab	0.13bab	0.25ab	0.17ab	0.0031	0.0722	0.9468
S	0.09a	0.09a	0.06b	0.08ab	0.07ab	0.07ab	0.08ab	0.06b	0.6552	0.0008	0.0335
Na	0.02a	0.02a	0.02a	0.01a	0.02a	0.02a	0.02a	0.02a	0.0073	0.4208	0.3972
ppm											
Fe	297.0b	181.0b	439.0ab	954.3a	209.6b	199.6b	87.3b	134.1b	0.2334	0.0011	0.0954
Al	625.3a	442.0a	697.6a	1093.3a	535.6a	386.7a	326.6a	261.4a	0.9960	0.0020	0.1296
Mn	415.6ab	570.3ab	379.6ab	1417.3a	553.6ab	508.6ab	322.6b	437.3ab	0.0526	0.1253	0.0855
Cu	16.67a	12.27a	11.67a	17.63a	11.00a	11.47a	10.33a	9.43a	0.8313	0.0477	0.0798
Zn	55.33a	57.93a	49.67a	67.37a	45.33a	39.33a	39.00a	41.20a	0.6155	0.2799	0.7713
B	22.00a	16.23ab	15.00b	12.37b	13.33b	12.40b	15.33b	11.32b	0.0011	0.0002	0.2540

Abbreviations: N = nitrogen; P = phosphorus; K = potassium; Ca = calcium; Mg = magnesium; S = sulfur; Na = sodium; ppm = parts per million; Fe = iron; Al = aluminum; Mn = manganese; Cu = copper; Zn = zinc; B = boron.

* Within rows, means followed by a different letter are significantly different ($P \leq 0.05$; Tukey's HSD).

† *P* values are significant at $\alpha \leq 0.05$.

Generally, macronutrient content was highest in the leaf, intermediate in the root, and lowest in stem samples, while micronutrient levels were highest in root samples.

SOIL AND PLANT MINERAL CONTENT CORRELATIONS. Pearson correlation analysis of soil and plant mineral content data from both sites found relationships between soil and leaf K, Zn, and Cu, stem S, and root Ca and B. Scatter plots show that among micronutrients (Fig. 1), the relationship between soil and leaf K was negative with higher soil levels associated with decreases in tissue content. There was a positive correlation between soil and tissue concentrations for Ca (root) and S (stem), and among micronutrients (Fig. 2), for Cu and Zn (leaf) and B (root). As shown in Table 6, Pearson correlation values for soil and tissue K, Ca, S, Zn, Cu, and B were, respectively, –0.5327 ($P \leq 0.0074$), 0.5066 ($P \leq 0.0115$), 0.5961 ($P \leq 0.0021$), 0.4258 ($P \leq 0.0380$), 0.4983 ($P \leq 0.0132$) and 0.4965 ($P \leq 0.0136$), showing that they were all medium to strong correlations.

MYCORRHIZAL INFECTION. Classical root staining revealed *A. racemosa* roots to be heavily colonized by AMF fungi throughout the season. Infection rates ranged between 85–100% with highest colonization observed in August, when root samples from both the Mt. Rogers and Reddish Knob sites averaged 100% root infection. No significant differences in mycorrhizal infection were observed with time, and between the two locations (Fig. 3).

ENDOPHYTE ISOLATION AND IDENTIFICATION. Isolation of endophytes associated with natural populations of *A. racemosa* yielded 22 fungal and 24 bacterial organisms. Among fungal isolates, nine, six, three, and four organisms were isolated, respectively, from root, stem, leaf, and flower samples, while eight each of bacterial isolates were found to be associated, respectively, with root, stem, and leaf samples. Molecular characterization of isolated endophytes revealed that bacterial isolates were primarily *Bacillus* spp. (root, stem, leaf), *Pseudomonas* (root), and *Serratia* (stem, leaf). Among fungal isolates, *Alternaria* spp. were found to be associated with leaf, flower, and stem; *Cadophora* spp. with root; *Diaporthe* spp. with stem and root; and *Penicillium* and *Volutella* spp. with root samples. When matched with the closest species, bacterial isolates rated between 99–100%, and fungal isolates between 98–99.8% in sequence similarity (Table 7).

Discussion. Examination of chemical and mineral properties of soils associated with two

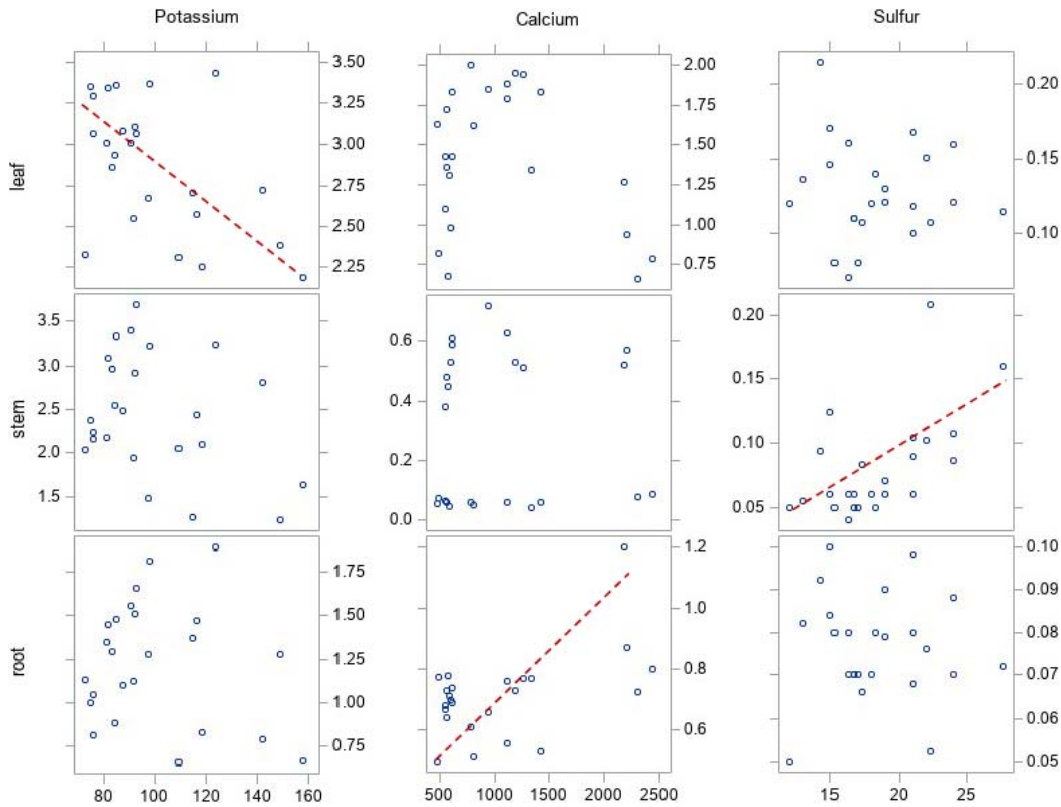

FIG. 1. Scatter plot matrices for three macronutrients where a correlation was observed between soil mineral content and plant nutrition in two black cohosh (*Actaea racemosa*) populations found within the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from Mt. Rogers in 2011 and Reddish Knob in 2012.

natural populations of *A. racemosa* shows a high level of homogeneity. Among chemical properties, pH (4.5–5.6) was lower than in most agricultural soils, with acidity accounting for a third to half of CEC, depending on site and season (Table 1). However, CEC (7–14 meq/100g) was moderately high with Ca^{2+} and Mg^{2+} contributing 50–70% of base saturation. By these measures, the chemistry of *A. racemosa* soils generally mirrors that of unamended arable soils in the greater mid-Atlantic (Daniels and Haering 2015). Similar comparisons with a soil analysis report for a farm in Madison County, Virginia, found *A. racemosa* soils to contain much higher concentrations of Fe and Mn, and to a lesser degree P and Zn, while the agricultural soil recorded higher Ca and Mg, which was reflected in a concomitantly higher pH of 7.1. However, there were no marked differences between the two soil sources for the other elements measured. The positive correlations observed between soil mineral content and plant nutrition suggest growth-limiting deficiency in some mineral elements, but this cannot be verified, and furthermore, most niche species are known to adjust growth to available resources.

Attempts have been made to grow *A. racemosa* in both forest (McCoy *et al*. 2007, Naud *et al*. 2010, Small *et al*. 2014) and field (Thomas *et al*. 2006, McCoy *et al*. 2007) environments with mixed results. In forest cultivation (Naud *et al*. 2010), the species responded negatively to increasing levels of acidity-related elements (pH; Al, Fe, Zn, and hydrogen [H]) than to soil fertility, and positively to increasing irradiance. Furthermore, although bioactivity per unit of biomass increased in conditions that negatively affected growth, better soil and light conditions were associated with a higher cumulative yield in bioactive material. McCoy *et al*. (2007) reported similar results from a 3-yr study comparing cropland, and disturbed and undisturbed forest, with *A. racemosa* grown under 78% shade in an agricultural field,

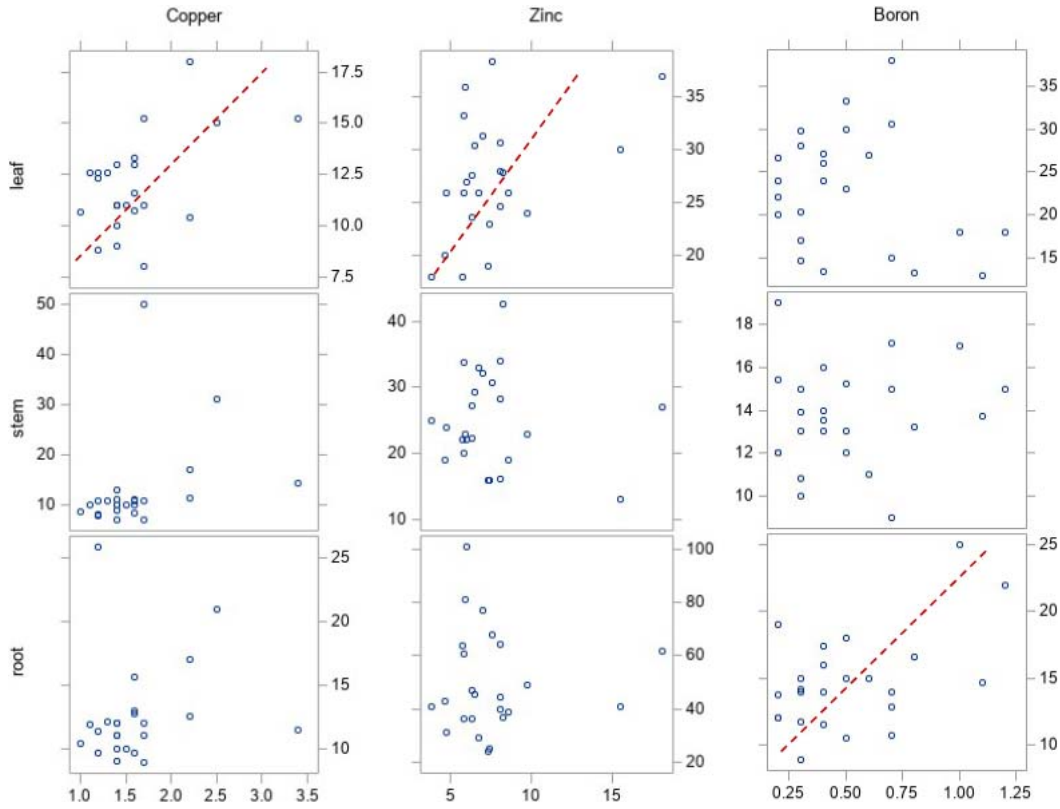

FIG. 2. Scatter plot matrices for three micronutrients where a correlation was observed between soil mineral content and plant nutrition in two black cohosh (*Actaea racemosa*) populations found within the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from Mt. Rogers in 2011 and Reddish Knob in 2012.

Table 6. Correlation between soil mineral content and plant nutrition in two black cohosh (*Actaea racemosa*) populations found within the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from two sites: Mt. Rogers (Site 1) in 2011 and Reddish Knob (Site 2) in 2012.

	Pearson correlation			*P* value*		
Mineral element	Leaf (n = 24)	Stem (n = 24)	Root (n = 24)	Leaf (n = 24)	Stem (n = 24)	Root (n = 24)
Nitrogen	0.0824	0.2481	0.3524	0.7018	0.2425	0.0913
Phosphorus	0.2831	0.1097	−0.0329	0.1801	0.6099	0.8789
Potassium	−0.5327	−0.3207	−0.1541	0.0074	0.1265	0.4721
Calcium	−0.2797	0.0083	0.5066	0.1855	0.9693	0.0115
Magnesium	0.1843	−0.1066	0.3256	0.3885	0.6201	0.1203
Sulfur	−0.0667	0.5961	−0.0422	0.7573	0.0021	0.8448
Sodium	0.2098	0.3207	−0.1223	0.3252	0.1266	0.5691
Iron	−0.2146	0.0084	0.1532	0.3140	0.9688	0.4748
Zinc	0.4258	−0.1055	0.0183	0.0380	0.6237	0.9323
Manganese	−0.1158	−0.1899	−0.1800	0.5900	0.3740	0.3999
Copper	0.4983	0.3304	0.1736	0.0132	0.1148	0.4173
Boron	−0.2345	0.1299	0.4965	0.2700	0.5452	0.0136

* *P* values are significant at $\alpha \leq 0.05$.

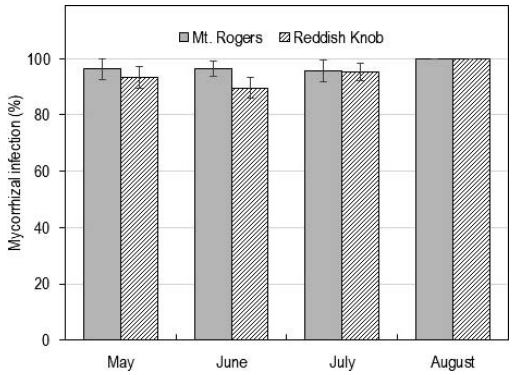

FIG. 3. Within season mycorrhizal infection in black cohosh (*Actaea racemosa* L.). Root samples were obtained from populations at Mt. Rogers (2011) and Reddish Knob (2012) within the George Washington and Jefferson National Forests in Appalachian Virginia.

recording the best performance, second only to a natural population sampled as a control. On the other hand, Thomas *et al*. (2006) and Small *et al*. (2014) reported less-than-satisfactory performance by rhizomes transplanted, respectively, to poorly drained nursery soil under 60% shade or a deciduous forest within the Appalachian ecosystem. In both cases, the rhizomes were attacked by fungal pathogens, and poor drainage and low pH (Small *et al*. 2014) were identified as possible causes of infection and transplant failure.

Variously, findings from the four reports suggest that soil conditions associated with the natural habitats sampled in our study are not ideal for optimal growth of *A. racemosa*. For example, soil analysis data for the three sites tested by McCoy *et al*. (2007) showed that, with the exception of base saturation, soil conditions at the forest and disturbed forest edge sites (where growth was lower than in agricultural soil) were similar to those associated with natural populations of the species. Similarly, poor recruitment among transplants into deciduous forest soils within the Appalachian ecosystem (Small *et al*. 2014), and poor recovery among natural populations after harvest (Small *et al*. 2011) pointed to a highly fragile existence by *A. racemosa* in what is considered its natural habitat.

When considered from the perspective of nutrition, it appears that nutrient requirements in *A. racemosa* are similar to those of a number of agronomic crops. We assume that leaf tissue content for each element measured in our study (averaged for the two locations in June) falls within the sufficiency range for *A. racemosa*. Based on this assumption, we find that macro- and microelement demand by *A. racemosa* closely resembles that of cotton (*Gossypium hirsutum* L.), tobacco (*Nicotiana tabacum* L.), and tomato (*Solanum lycopersicum* L.) according to sufficiency ranges published for cotton by Mitchell and Baker (2000) and for tobacco and tomato by Campbell (2000a, b). This might explain the good performance noted by McCoy *et al*. (2007) in *A. racemosa* grown in arable land, implying that the

Table 7. Identification and species assignment to endophytic microbes isolated from two black cohosh (*Actaea racemosa* L.) populations found within the George Washington and Jefferson National Forests in Appalachian Virginia. Samples were obtained from Mt. Rogers in 2011 and Reddish Knob in 2012.

Endophyte type	Tissue	Genus	Closest species	Sequence identity match (%)
	Leaf	Bacillus	*Bacillus* sp.	99.8
Bacterial		Bacillus	*Bacillus megaterium*	100.0
		Bacillus	*Bacillus cereus*	100.0
		Serratia	*Serratia* sp.	100.0
	Stem	Bacillus	*Bacillus mycoides*	100.0
		Bacillus	*Bacillus thuringiensis*	99.8
		Serratia	*Serratia* sp.	100.0
	Root	Bacillus	*Bacillus mycoides*	99.8
		Pseudomonas	*Pseudomonas fluorescens*	100.0
	Flower	Alternaria	*Alternaria* sp.	98.2
Fungal	Leaf	Alternaria	*Alternaria alternata*	98.4
	Stem	Alternaria	*Alternaria alternata*	99.3
		Diaporthe	*Diaporthe maritima*	99.4
	Root	Cadophora	*Cadophora lacrimiformis*	99.6
		Cadophora	*Cadophora luteo-olivacea*	99.8
		Penicillium	*Penicillium limosum*	99.8
		Diaporthe	*Diaporthe amygdali*	98.8
		Volutella	*Volutella ciliata*	99.6

higher-than-average soil P, Fe, Mn, and Zn associated Appalachian forest soils are not essential for *A. racemosa* survival and growth. This observation is further supported by evidence of nontranslocation to aboveground tissues of Fe, Mn, Zn, and Al proportional to levels sequestered in root biomass.

The observations above lead us to conclude that *A. racemosa* occupies a niche within the Appalachian forest ecosystem to which it is adapted to tolerate biotic and abiotic stressors and to avoid competition from other understory plants. Heavy colonization by AMF along with a prevalence of endophytic associations observed in our study suggest these relationships might aid in nutrient acquisition and stress tolerance in *A. racemosa*. Furthermore, among the fungal (*Cadophora* spp., *Penicillium* spp., *Volutella* spp.) and bacterial (*Bacillus* spp., *Pseudomonas* spp., *Serratia* spp.) endophytic suspects isolated in our study, significant research exists to confirm symbiotic or mutualistic function by members of the family or genus in association with both cultivated and wild plants. For example, excellent *Fusarium* wilt control by a *Cadophora* isolate in melon seedlings inoculated with *Fusarium oxysporum* f. sp. *melonis* has been reported (Khastini *et al*. 2014). According to Knapp *et al*. (2018), the *Cadophora* might be a less well-understood group of root endophytic fungi essential to carbon cycling and ecosystem functioning in stressful environments. Yang *et al*. (2014) and Zhang *et al*. (2017), respectively, report improved nutrient uptake by rice (*Oryza sativa* L.) and peanut (*Arachis hypogaea* L.) colonized by mutualistic *Phomopsis liquidambari*, and amelioration of osmotic stress in soybean (*Glycine max* (L.) Merr.) has been observed in the presence of the endophyte *Penicillium funiculosum* (Khan *et al*. 2011). Finally, among the fungal endophytes isolated from *A. racemosa*, *Volutella ciliata* has been reported to inhabit the rhizosphere of *Cypripedium japonicum* Thunb. (Gang *et al*. 2017) and *Panax notoginseng* (Burkill) F.H. Chen ex C.H. Chow (Miao *et al*. 2016), and to ameliorate the phytotoxic effects of vanillic acid on *Pisum sativum* L. (Vaughan *et al*. 1993).

Similar evidence of beneficial action exists for bacterial endophytes isolated from *A. racemosa*. *Bacillus* spp. have been found to form symbiotic relationships and to contribute to disease resistance in *A. hypogea* (Figueredo *et al*. 2014) and *Brassica rapa* L. (Walker *et al*. 2002). Also, endophytic *Pseudomonas* isolates have been shown to improve plant growth by solubilizing inorganic phosphate (Otieno *et al*. 2015), and to enhance bioavailability and extraction of cadmium (Cd), Zn, and Cu by *Solanum nigrum* L. (Chen *et al*. 2013). Finally, *Serratia plymuthica* is reported to induce resistance against *Pythium ultimum* infection in cucumber (*Cucumis sativus* L.) by sensitizing more rapid and efficient formation of physical and chemical barriers at sites of potential fungal entry (Benhamou *et al*. 2000). These examples of symbiotic function by related species suggest that *A. racemosa* might derive adaptive benefits from associated endophytes essential to its survival in the Appalachian forest ecosystem.

Our work, coupled with previous research, suggests a two-fold approach to the management of *A. racemosa*. Experimental cultivation in field soils (McCoy *et al*. 2007), and the observation that nutrient demand in *A. racemosa* matches that of a number of commonly cultivated species, points to potential for development of agronomic practices for on-farm production. Other innovations, including vegetative propagation protocols published by Fischer *et al*. (2006) could facilitate mass propagation of propagules and the emergence of *A. racemosa* as a commercial cash crop similar to American ginseng (*Panax quinquefolius* L.) (Hsu 2002). For simulated forest farming and ecosystem restoration, it is apparent that more work is needed to understand soil-plant-microbe interactions in *A. racemosa*.

We conclude that soil factors might not be as important as symbiotic relations in *A. racemosa*. Although nutrient uptake by *A. racemosa* did not vary from that of common crops, our findings confirm the ubiquity of mycorrhizal and nonmycorrhizal endophytes living in association with the species. This area of research has barely been addressed for *A. racemosa* and offers ample room for discovery.

A comprehensive discussion by Khare *et al*. (2018) of the multifaceted interactions between plants and microbial endophytes shows how plants exist in myriad symbiotic relations that aid in nutrient acquisition and in defense against pests and diseases. There is increasing evidence that plant-associated organisms provide benefits including ecosystem services (Burges *et al*. 2016), and more time must be devoted to their study as integral to plant communities.

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