Nodulation of *Shepherdia* × *utahensis* ‘Torrey’ and the Diversity of Symbiotic *Frankia* Strains

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Abstract. *Shepherdia* × *utahensis* ‘Torrey’ (hybrid buffaloberry) is an actinorhizal plant that can form symbiotic nodules with the actinobacterial genus *Frankia*. However, little research has been conducted to investigate the presence of *Frankia* in their nodules and the effects on plant growth. In this study, plants were grown in a Metro-Mix® 820 substrate and inoculated with soils collected from Mohave County, AZ, or in a low organic-matter substrate inoculated with soils from North Logan, UT. The presence of *Frankia* was quantified using PolF/PolR primers to amplify their nitrogenase (*nifH*) gene sequences. In the Metro-Mix 820 substrate, plants irrigated with nitrogen (N)-free Hoagland’s solution at pH 6.5 formed nodules at week 12 after experiment initiation, whereas those receiving the same solution with 2 mM ammonium nitrate (NH₄NO₃) appeared healthy, but no nodules formed. In the low organic-matter substrate, nodules formed in 5 weeks when plants were irrigated with N-free Hoagland’s solution at pH 7.5. Four 300-bp fragments of query sequences (SU1, SU2, SU3, and SU4) were obtained from nodules. When compared with *nifH* gene sequences reported in the literature using the Basic Local Alignment Search Tool (BLAST), more than 90% similarity to the *nifH* of *Frankia* spp. was obtained. The *Frankia* strains in the nodules shared *nifH* sequences similar to those of the same host-specific group of *Shepherdia*. Furthermore, *Frankia* strains with similar *nifH* genes have been reported in nodules of *Shepherdia argentea* (silver buffaloberry). Additionally, *Frankia* strains belonging to cluster 3 infective strains consisting of Elaeagnaceae and Rhamnaceae infective *Frankia* showed high similarity to the query sequences. This research demonstrates that nodulation of *S. ×utahensis* is inhibited at 2 mM NH₄NO₃. Apart from N, nodule formation may be associated with the substrate type and pH of the nutrient solution. Based on *nifH* gene sequence amplification, *Frankia* strains in the root nodules may have the potential to fix atmospheric nitrogen (N₂). These *Frankia* strains have signature gene sequence characteristics of Elaeagnaceae-infective *Frankia*, suggesting that *S. ×utahensis* shares *Frankia* strains similar to its parents.

Actinorhizal plants are able to fix atmospheric nitrogen (N₂) through symbiosis with *Frankia*, a genus of actinobacteria, and have great potential for sustainable landscaping (Kratsch and Graves, 2004). Plant growth and development may be improved when the symbiotic association is established, as reported by previous studies (Laws and Graves, 2005; Schwencke and Caru, 2001). However, excessive N may inhibit nodulation of actinorhizal plants. For instance, nodule formation of *Alnus maritima* (seaside alder) was prevented by either 2.7 g·L⁻¹ of 15N–3.9P–10K controlled-release fertilizer (CRF) or 4 mM NH₄NO₃ (Beddes and Kratsch, 2010; Laws and Graves, 2005). Therefore, to successfully induce symbiotic nodules in nursery production, it is important to examine the nodulation of inoculated

actinorhizal plants irrigated with nutrient solutions with different N levels.

Frankia in symbiotic nodules are highly diverse (Schwencke and Caru, 2001). Phylogenetic analyses have been conducted to investigate the diversity of *Frankia* strains in nodules of inoculated actinorhizal plants (Jeong and Myrold, 2001; Myrold and Huss-Danell, 1994). Recently, researchers used comparative sequence analyses (e.g., *glnII*, *nifH*, *recA*, and 16S rRNA) to investigate the phylogeny of *Frankia* strains in nodules (Pawlowski and Bergman, 2007). The phylogenetic research of *Frankia* using nitrogen fixation (*nif*) gene, 16S rRNA, or other genes is recognized by most researchers, who agree that all infective *Frankia* strains can be classified in one of three groups (Benson et al.,

2004; Normand et al., 1996; Schwencke and Caru, 2001). According to the review study by Benson et al. (2004), group 1 contains *Frankia* strains from nodules of plants in Betulaceae, Casuarinaceae, and Myricaceae; group 2 contains *Frankia* strains from nodules of plants in Coriariaceae, Datisceae, Rosaceae, and *Ceanothus* of Rhamnaceae; and group 3 contains effective *Frankia* strains that can fix N₂ from nodules of plants in Elaeagnaceae, Myricaceae, Rhamnaceae, and *Gymnostoma* of Casuarinaceae and noneffective strains from Betulaceae, Rosaceae, and genera in Casuarinaceae, except *Gymnostoma*, and genera in Rhamnaceae, except *Ceanothus*. Comparative sequence analyses are also important to identify other microorganisms in the nodules of actinorhizal plants. According to Huss-Danell (1997), actinorhizal plants formed nodules without *Frankia* because a fungus, *Penicillium nodositatum*, can induce nodules that are incapable of fixing N₂.

Shepherdia argentea and *S. rotundifolia* (roundleaf buffaloberry) are native actinorhizal plants in the U.S. Intermountain West (Mee et al., 2003). *Shepherdia argentea* can tolerate a wide range of soil conditions (Sriladda et al., 2016), whereas *S. rotundifolia* has strong drought tolerance. Although *S. rotundifolia* is more aesthetically appealing compared with *S. argentea* (Mee et al., 2003), *S. rotundifolia* has high mortality in nursery conditions (Sriladda et al., 2016). *Shepherdia* × *utahensis* is an interspecific hybrid of *S. argentea* and *S. rotundifolia* that tolerates wet and disturbed soil and drought stress (Sriladda et al., 2016). It has high potential for low-water landscaping. The genetic, morphological, and physiological traits of this plant have been studied by Sriladda et al. (2016). As an actinorhizal species, it is able to establish symbiotic associations with *Frankia*. However, previous studies associated with actinorhizal plant nodulation have focused on *A. maritima* in the eastern United States (Beddes and Kratsch, 2010; Laws and Graves, 2005), and few studies have investigated the nodulation of *Shepherdia*. Because of different soil chemical and physical properties in the U.S. Intermountain West (Heaton and Koenig, 2010; Sriladda et al., 2014), characteristics of *Shepherdia* nodulation might be different from *Alnus*. *Frankia* strains in the nodules of *S. ×utahensis* might be similar to those in nodules of its parents, *S. argentea* and *S. rotundifolia*.

The objectives of this study were to 1) evaluate plant growth and nodulation of *S. ×utahensis* in conditions that mimic nursery environments and natural habitats of *S. rotundifolia*; and 2) investigate the diversity of *Frankia* strains in nodules using comparative sequence analyses of polymerase chain reaction (PCR)-amplified *nifH* gene fragments.

Materials and Methods

Nodulation of *S. ×utahensis*

Expt. 1. On 22 Mar. 2019, terminal cuttings (≈10 cm) of *S. ×utahensis* were collected from the Utah Agricultural Experiment Station's (UAES) Greenville Research Farm (North Logan, UT) (41.765741, –111.813175).

Fig. 1. Root nodules observed in the soil sample collected from the root zone of a wild *Shepherdia rotundifolia* at Mohave County, AZ (36.881550, -112.895690) (A), and nodules formed on the roots of *Shepherdia xutahensis* 'Torrey' during Expts. 1 (B) and 2 (C).

Leaves at the bottom of the cuttings were removed, leaving two to three pairs of leaves at the top. Cuttings were dipped in 8000 mg·L⁻¹ indole-3-butyric acid (Hormodin 3; OHP, Mainland, PA) and stuck in a soilless substrate containing 80% perlite (Hess Perlite, Malad City, ID) and 20% peatmoss (Canadian sphagnum peatmoss; Sun Gro Horticulture, Agawam, MA). Cuttings were kept on a mist bench with temperatures set at 22 °C in the UAES Research Greenhouse (Logan, UT). On 15 May 2019, nodule-free rooted cuttings were transplanted to 3.8-L injection-molded, polypropylene containers (PC1D-4; Nursery Supplies, Orange, CA) filled with Metro-Mix 820 substrate (Sun Gro Horticulture), which is primarily peatmoss. Plants were irrigated with deionized water before the experiment. All plants were grown in the UAES Research Greenhouse with temperatures set at 25/22 °C (day/night). A heated silicon chip pyranometer (SP-230; Apogee Instruments, Logan, UT) mounted to a weather station at the UAES Greenville Research Farm, ≈1000 m away from the greenhouse, was used to record light intensities. The daily light integral inside the greenhouse, calculated using a light

transmission rate of 68%, was 32.6 ± 9.8 mol·m⁻²·d⁻¹ (mean ± sd) from 17 June to 4 Sept. 2019.

On 17 June 2019, a factorial treatment combination was created with (F+) or without (F-) *Frankia* inoculation and with (N+) or without (N-) N application. A total of 84 uniform plants were used, with 22 plants in each of the F+N+ and F+N- groups and 20 plants in each of the F-N+ and F-N- groups. The plants with inoculation were topdressed with 30 mL of soil collected from the root zone of a wild *S. rotundifolia* plant in Mohave County, AZ (36.881550, -112.895690), with symbiotic nodules observed in the rhizosphere (Fig. 1); the plants without inoculation did not receive the soil topdressing treatment. The plants with N treatment were irrigated with quarter-strength N-free Hoagland's solution (Hoagland and Arnon, 1950) with 2 mM NH₄NO₃ at pH 6.5; those without N treatment were irrigated with the same solution without NH₄NO₃.

Ten plants in each treatment were randomly selected and destructively harvested on 29 July 2019 (7 weeks after experiment initiation; first harvest), and the remaining plants were harvested on 4 Sept. 2019 (12 weeks after experiment initiation; second harvest). Plant heights from the surface of the substrate to the highest terminal bud and number of shoots (>5 cm) were recorded at the initiation of the experiment and on both harvest dates. A soil plant analysis development (SPAD) 502 meter (Minolta Camera, Osaka, Japan) was used to measure the relative chlorophyll content (SPAD reading) of each plant at both harvest dates, and the average values of five randomly selected mature leaves per plant from the canopy were recorded. The leaf area of each plant was recorded using a leaf area meter (LI-3000; LI-COR Biosciences, Lincoln, NE) at both harvest dates. Shoots were dried in an oven at 80 °C for 3 d, and shoot dry weight (DW) was recorded. Roots were harvested, washed with deionized water, and checked for nodulation. The NO₃-N concentration of leachate was recorded using a NO₃-N meter (LAQUA Twin; Horiba, Kyoto, Japan) on 27 July and 2 Sept. 2019. The pH of the leachate solution was recorded only on 2 Sept. 2019, using a pH meter (LAQUA Twin; Horiba).

Expt. 2. On 6 Aug. 2019, 60 nodule-free plants propagated using the aforementioned method were transplanted to 656-mL cone-

ainers (D40H; Stuewe and Sons, Tangent, OR) filled with perlite (Hess Perlite, Malad City, ID) and sorted into four blocks. After plants were transplanted, 30 mL of field soil collected from the rhizosphere of a nodulated *S. xutahensis* plant at the UAES Greenville Research Farm was used to inoculate plants. Plants were irrigated with 250 mL quarter-strength N-free Hoagland's solution at pH 7.5 every other day. The experiment was initiated on 6 Aug. 2019, and it ended on 18 Nov. 2019. One plant per block was randomly selected and harvested weekly to study nodulation. At harvest, the number of nodules was counted and the diameter (mm) and fresh weight (mg) of each nodule were measured. All plants were also grown in the UAES Research Greenhouse, and the daily light integral from 6 Aug. to 18 Nov. 2019 was 23.9 ± 10.6 mol·m⁻²·d⁻¹ (mean ± sd; calculated as described). Supplemental light was provided using 1000-W high-pressure sodium lamps (Hydrofarm, Petaluma, CA) from 0600 to 2200 hr. Lamps were turned on at an average intensity of 130.4 ± 18.0 µmol·m⁻²·s⁻¹ (mean ± sd) at plant canopy level when greenhouse light intensity was less than 544 µmol·m⁻²·s⁻¹.

Phylogenetic analyses of *Frankia* strains

DNA extraction. DNA was extracted from root nodules using a DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany). The quantity and quality of DNA were analyzed using a spectrophotometer (Thermo NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA).

Amplification of *nifH* gene. Polymerase chain reaction (PCR) amplification of *nifH* gene was conducted following the method of Gtari et al. (2007). In brief, primers PolF (5' TGC GAY CCS AAR GCB GAC TC 3') and PolR (5' ATS GCC ATC ATY TCR CCG GA 3') (Poly et al., 2001) were used to amplify *nifH* gene of *Frankia* strains in nodules in a reaction volume of 20 µL containing 10 µL Master Mix (Thermo Fisher Scientific), 1 µL of each primer, 1 µL DNA template, and 7 µL distilled water. The PCR was performed in a thermocycler (Eppendorf Mastercycler; Eppendorf, Hamburg, Germany) under the following conditions: primary denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, and a 5-min extension at 72 °C for the last cycle. PCR products were analyzed by electrophoresis on 1% agarose gel in a TAE buffer after staining with ethidium bromide at 0.5 µg·mL⁻¹.

Sequence analyses. The amplified *nifH* gene fragments were purified using a Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific GeneJET; Thermo Fisher Scientific) according to the manufacturer's instructions. The purified amplicons were sequenced at the Utah State University's Center for Integrated BioSystems (Logan, UT) using an ABI PRISM 3730 DNA Analyzer with an ABI BigDye terminator (Applied Biosystems, Foster City, CA).

Phylogenetic analyses. SeqMan Pro (DNASTAR, Madison, WI) was used to check the mapped reads of sequences

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manually. Nucleotide sequences were aligned using the ClustalW algorithm (Thompson et al., 1994) and manually trimmed off primers using molecular evolutionary genetics analysis (MEGA) (Kumar et al., 2018). The generated sequences were analyzed and compared using BLAST (Altschul et al., 1990).

Experimental design and statistical analyses. Expt. 1 had a completely randomized design with a factorial treatment combination of two factors with 20 blocks, whereas Expt. 2 had a randomized complete block design with 4 blocks. A two-way analysis of variance (ANOVA) procedure was used to test the effects of N treatment and *Frankia* inoculation on growth and nodulation during Expt. 1, whereas a one-way ANOVA procedure was used to test the effect of time on nodulation during Expt. 2. A χ^2 test was conducted using the PROC FREQ procedure in SAS Studio (Version 3.8; SAS Institute, Cary, NC) to test the effects of N or *Frankia* inoculation on the nodulation of *S. ×utahensis*. Mean separation among treatments was adjusted using the Tukey-Kramer method for multiplicity at $\alpha = 0.05$ during Expt. 1. During Expt. 2, regression analyses of time, nodule number, diameter, and fresh weight were conducted. Except for the χ^2 test, all statistical analyses were performed using PROC Mixed procedures in SAS Studio.

Results

Nodulation of *S. ×utahensis*

During Expt. 1, *S. ×utahensis* plants irrigated with quarter-strength N-free Hoagland's solution with 2 mM NH_4NO_3 had a greater $\text{NO}_3\text{-N}$ concentration in the leachate solution at both harvest dates (Fig. 2) than those without NH_4NO_3 . At the time of termination of the experiment, the pH values of the leachate solution were 6.2 ± 0.1 (mean $\pm$ SE) and 5.8 ± 0.1 (mean $\pm$ SE) for plants irrigated with quarter-strength N-free Hoagland's solution with or without 2 mM NH_4NO_3 , respectively. No nodules were observed at week 7, but nodules were found on plants in the F+N– and F–N– groups at

week 12 (Table 1, Fig. 1). Eight of 12 plants in the F+N– group formed nodules, whereas 4 of 10 plants in the F–N– group had nodules (Tables 2 and 3). According to the χ^2 test, nodulation was affected by 2 mM NH_4NO_3 in quarter-strength N-free nutrient solution ($\chi^2 = 19.8$; df = 1; $P < 0.0001$). However, although nodulation was greater for those in the F+N– group than those in the F–N– group, inoculation did not affect nodulation ($\chi^2 = 0.98$; df = 1; $P = 0.32$).

At both harvest dates, the plant height, SPAD reading, number of shoots, leaf area, and shoot DW of plants irrigated with quarter-strength N-free Hoagland's solution with 2 mM NH_4NO_3 increased compared with those treated with the same solution but without NH_4NO_3 (Table 1). Furthermore, the number of shoots of plants in the F+N+ group increased compared with that in the F–N+ group at the first harvest, whereas the height and shoot DW of plants in the F+N+ group increased compared with that in the F–N+ group at the second harvest.

During Expt. 2, nodules were first observed at week 5 after the experiment was initiated (Figs. 1 and 3). Positive correlations were observed between weeks and number of nodules ($P < 0.0001$), nodule diameter ($P < 0.0001$), and fresh weight ($P < 0.0001$) of the largest nodule (Fig. 3).

Phylogenetic analyses of *Frankia* strains

Four query sequences (≈ 300 -bp fragments) were obtained from PCR reactions, including a sequence from the nodule induced with soils from Mohave County, AZ (SU1), and three sequences from three nodules induced with soils from North Logan, UT (SU2, SU3, and SU4).

When compared with sequences reported in the literature using BLAST, SU1 had 98% similarity with the *nifH* gene of *Frankia* strain NRRLB-16306 (accession number JF273735.1) (Table 4). High similarity (98%) was also observed between SU1 and the *nifH* gene of *Frankia* strains G2 (accession number HM026367.1), Cg70.1 (accession number

HM026362.1), R43(2009) (accession number FJ477447.1), and CeSI5 (accession number FJ477443.1). Additionally, more than 97% similarity with the *nifH* gene was found between the *Frankia* strains in our study and uncultured *Frankia* clone T2P1-7 (accession number LT840168.1) (data not shown).

The *nifH* gene sequences SU2 and SU3 had 92% and 99% similarity, respectively, with the *nifH* gene of *Frankia* strain BMG5.12 (accession number AJ545031.1), FMc5 (accession number KP342119.1), FMc4 (accession number KP342118.1), FMc3 (accession number KP342117.1), FMc2 (accession number KP342116.1), and FMc1 (accession number KP342115.1), whereas *nifH* gene of *Frankia* strain BMG5.15 (accession number JF273726.1), BMG5.1 (accession number AJ545034.1), and BMG5.2 (accession number AJ545032.1) had 91% and 98% similarity with SU2 and SU3, respectively. The *nifH* gene of the uncultured *Frankia* clone T2P1-7 (accession number LT840168.1) had 90% and 99% similarity with the SU2 and SU3, respectively (data not shown). The SU4 shared 97% similarity with the *nifH* gene of *Frankia* strain EUN1f (accession number HM026364.1), G2 (accession number HM026367.1), Cg70.1 (accession number HM026362.1), CeSI5 (accession number FJ477443.1), and NRRLB-16306 (accession number JF273735.1).

Discussion

Nodulation of *S. ×utahensis*

Inoculation is a preferred practice in nursery production to induce symbiotic nodules on actinorhizal plants (Schwencke and Caru, 2001). Soils collected from the rhizosphere of wild actinorhizal plants have been used for inducing nodules in previous studies (Beddes and Kratsch, 2010; Jeong and Myrold, 2001; Laws and Graves, 2005). However, increasing N levels decreased the nodule number of inoculated plants (Laws and Graves, 2005). Nodulation of *Purshia mexicana* (mexican cliffrose) and *Purshia tridentata* (antelope bitterbrush) was inhibited at 6 mM NH_4NO_3 during a greenhouse study (Righetti et al., 1986). For *Ceanothus* species, no nodules formed on *Ceanothus griseus* (Carmel ceanothus) at 2.68 mM NH_4NO_3 (Thomas and Berry, 1989). Nodulation of *Alnus glutinosa* (black alder) was inhibited with 2 mM potassium nitrate (Huss-Danell et al., 1982), whereas that of *A. maritima* was not completely inhibited until 4 mM NH_4NO_3 was used (Laws and Graves, 2005). It appears that *S. ×utahensis* may be more sensitive to environmental N levels than *A. maritima*, *C. griseus*, *P. mexicana*, and *P. tridentata* in terms of nodulation, but less sensitive than *A. glutinosa*; however, a direct comparison is needed to study N sensitivity of actinorhizal plants. Because this plant is sensitive to environmental N content, the N concentration in irrigation solutions needs to be monitored to maximize nodule formation when producing nodulated plants.

Research is needed to determine the optimal N level or fertilizer level to effectively produce nodulated plants. Increased N levels

Fig. 2. The nitrate-nitrogen ($\text{NO}_3\text{-N}$) concentration of leachate solution collected after *Shepherdia ×utahensis* 'Torrey' was irrigated during Expt. 1. Four treatments were created with a factorial design with (F+) or without (F–) *Frankia* inoculation and irrigation with quarter-strength nitrogen-free Hoagland's solution with (N+) or without (N–) 2 mM ammonium nitrate at pH 6.5. Plants were harvested at 7 weeks (first harvest) and 12 weeks (second harvest) after experiment initiation. The error bars represent the SE of five samples. The same lowercase letters above column bars within harvest dates denote no significance among treatments according to Tukey-Kramer method for multiplicity at $\alpha = 0.05$.

Table 1. Plant height, relative chlorophyll content [soil plant analysis development (SPAD) reading], number of shoots, leaf area, shoot dry weight (DW), nodulation rate, number of nodules per plant, and nodule DW of *Shepherdia × utahensis* ‘Torrey’. Four treatments were created with a factorial design with (F+) or without (F–) *Frankia* inoculation and irrigated with quarter-strength nitrogen-free Hoagland’s solution with (N+) or without (N–) 2 mM ammonium nitrate at pH 6.5.^z

	First harvest					Second harvest							
	Ht (cm)	SPAD reading	Shoots (no.)	Leaf area (cm ²)	Shoot DW (g)	Ht (cm)	SPAD reading	Shoots (no.)	Leaf area (cm ²)	Shoot DW (g)	Nodulation (%)	Nodules (no.)	Nodule DW (mg)
F+N+	34.6 a ^y	46.3 a	9.3 a	289.1 a	3.8 a	54.2 a	53.6 a	29.7 a	1070.7 a	22.1 a	0 b ^x	— ^w	—
F+N–	24.7 b	27.9 b	4.2 c	102.5 b	1.8 b	31.5 c	28.5 b	6.2 b	257.9 b	4.7 c	67 a	5.8 a	120 a
F–N+	29.8 a	46.9 a	7.0 b	227.8 a	3.8 a	48.6 b	53.2 a	25.4 a	938.7 a	17.8 b	0 b	—	—
F–N–	22.9 b	34.4 b	5.7 c	89.7 b	1.5 b	33.1 c	30.5 b	7.5 b	309.0 b	5.0 c	40 a	0.7 b	160 a

^zPlants were harvested at 7 (first harvest) and 12 (second harvest) weeks after experiment initiation. Nodules were not observed at the time of the first harvest, but they were observed at the time of the second harvest. Plants were grown in a commercial soilless substrate containing primarily peatmoss.

^yMeans within a column with the same lowercase letters are similar among treatments according to the Tukey-Kramer method for multiplicity at $\alpha = 0.05$.

^xMeans with the same lowercase letters within the nodulation column are similar among treatments according to the χ^2 test at $\alpha = 0.05$.

^wNodules were not observed.

Table 2. Chi-square test of the nodulation of *Shepherdia × utahensis* ‘Torrey’ irrigated with Hoagland’s solution with (N+) or without (N–) 2 mM ammonium nitrate (NH₄NO₃) at the time of the second harvest. The overall chi-square statistic (χ^2) is the sum of all of cell χ^2 with their degree of freedom (df) and *P* value.

Treatment		Nodulation		Total
		Not nodulated	Nodulated	
N+	Count	24	0	24
	% of total	54.5	0	54.5
N–	Count	8	12	20
	% of total	18.2	27.3	45.5
Total	Count	32	12	44
	% of total	72.7	27.3	100

$$\chi^2 = 19.8, df = 1, P < 0.0001$$

Table 3. Chi-square test of the nodulation of *Shepherdia × utahensis* ‘Torrey’ with (F+) or without (F–) *Frankia* inoculation as measured at the time of the second harvest. The overall chi-square statistic (χ^2) is the sum of all of cell χ^2 with their degree of freedom (df) and *P* value.

Inoculation		Nodulation		Total
		Not nodulated	Nodulated	
F+	Count	16	8	24
	% of total	36.4	18.2	54.6
F–	Count	16	4	20
	% of total	36.4	9.0	45.4
Total	Count	32	12	44
	% of total	72.8	27.2	100

$$\chi^2 = 0.98, df = 1, P = 0.32$$

decrease the nodule number, but they improve plant growth (Laws and Graves, 2005). During our study, plant quality without N treatment was poor, although nodulation was present. Laws and Graves (2005) also reported that *A. maritima* without N treatment had the greatest nodule number but showed irregular shoot shape and yellowing leaves. Consequently, proper fertilizer application is important for producing nodulated plants with acceptable visual quality and minimal N leaching. A study conducted in Oct. 2019 suggested that 2.1 g·L^{–1} of 15N–3.9N–10K CRF was an appropriate application rate to produce nodulated *S. × utahensis* (Chen et al., 2020).

Interestingly, nodulation was observed 7 weeks earlier during Expt. 2 than during Expt. 1, which may be the result of the differences in substrates and the pH of nutrient solutions. The commercial growing substrate

contains peatmoss, which is a material containing more than 80% organic matter (Brady, 1990). Organic matter in commercially used growing substrates increases the water-holding capacity (Hudson, 1994) and decreases pH (McCauley et al., 2017). However, soils in the Intermountain West are different from the commercial growing substrate and contain low organic matter because of the desert climate and lack of plant coverage (Heaton and Koenig, 2010). Sriladda et al. (2014) reported that soil organic matter in the habitat of wild *S. rotundifolia* in the Intermountain West was between 0.7% and 8.7%. Growing native plants in conditions mimicking native habitats improves their growth. For example, according to Beddes and Kratsch (2009), seed germination of *S. rotundifolia* is optimized in a low organic-matter substrate. Therefore, although peatmoss is commonly used in nursery production, a

Fig. 3. Regression analyses of the number of nodules (A), diameter of the largest nodule (B), and fresh weight of the largest nodule (C) of inoculated *Shepherdia × utahensis* ‘Torrey’ plants grown in pure perlite, a low organic-matter substrate, irrigated with quarter-strength nitrogen-free Hoagland’s solution at pH 7.5 during Expt. 2. Four plants were randomly chosen and harvested weekly. Nodules were found at week 5 after experiment initiation.

substrate containing low organic matter may be better for inducing nodulation of *S. × utahensis* plants.

The pH of the irrigation solution might also explain the discrepancy in nodule formation during Expt. 1 and Expt. 2. Nodulation of actinorhizal plants is associated with

Table 4. List of 10 *Frankia* species that have the highest *nifH* gene similarity with each of four query sequences (SU1, SU2, SU3, and SU4) obtained from nodules of *Shepherdia ×utahensis* ‘Torrey’.

Query	Strains	Similarity (%)	<i>nifH</i> accession number	Origin of isolation
SU1	NRRLB-16306	98	JF273735.1	<i>Casuarina</i>
	G2	98	HM026367.1	<i>Casuarina equisetifolia</i>
	Cg70.1	98	HM026362.1	<i>Casuarina glauca</i>
	R43(2009)	98	FJ477447.1	<i>Morella</i> (syn: <i>Myrica</i>)
	CeSI5	98	FJ477443.1	<i>Morella pensylvanica</i> (syn: <i>Myrica pensylvanica</i>)
	FMc5	96	KP342119.1	<i>Morella</i>
	EUN1f	96	HM026364.1	<i>Elaeagnus umbellata</i>
	FMc3	96	KP342117.1	<i>Morella</i>
	FMc2	96	KP342116.1	<i>Morella</i>
	FMc1	96	KP342115.1	<i>Morella</i>
	BMG5.12	92	AJ545031.1	<i>Elaeagnus angustifolia</i>
	FMc5	92	KP342119.1	<i>Morella</i>
	FMc4	92	KP342118.1	<i>Morella</i>
	FMc3	92	KP342117.1	<i>Morella</i>
	FMc2	92	KP342116.1	<i>Morella</i>
SU2	FMc1	92	KP342115.1	<i>Morella</i>
	BMG5.15	91	JF273726.1	<i>Elaeagnus angustifolia</i>
	BMG5.1	91	AJ545034.1	<i>Elaeagnus angustifolia</i>
	BMG5.2	91	AJ545032.1	<i>Elaeagnus angustifolia</i>
	Cc1.17	91	EU862917.1	<i>Colletia cruciata</i>
	BMG5.12	99	AJ545031.1	<i>Elaeagnus angustifolia</i>
	FMc5	99	KP342119.1	<i>Morella</i>
	FMc4	99	KP342118.1	<i>Morella</i>
	FMc3	99	KP342117.1	<i>Morella</i>
	FMc2	99	KP342116.1	<i>Morella</i>
	FMc1	99	KP342115.1	<i>Morella</i>
	BMG5.15	98	JF273726.1	<i>Elaeagnus angustifolia</i>
	BMG5.1	98	AJ545034.1	<i>Elaeagnus angustifolia</i>
	BMG5.2	98	AJ545032.1	<i>Elaeagnus angustifolia</i>
	Cc1.17	97	EU862917.1	<i>Colletia cruciata</i>
SU3	EUN1f	97	HM026364.1	<i>Elaeagnus umbellata</i>
	G2	97	HM026367.1	<i>Casuarina equisetifolia</i>
	Cg70.1	97	HM026362.1	<i>Casuarina glauca</i>
	CeSI5	97	FJ477443.1	<i>Morella pensylvanica</i>
	NRRLB-16306	97	JF273735.1	<i>Casuarina</i>
	R43(2009)	96	FJ477447.1	<i>Morella</i>
	FMc5	95	KP342119.1	<i>Morella</i>
	FMc3	95	KP342117.1	<i>Morella</i>
	FMc2	95	KP342116.1	<i>Morella</i>
	FMc1	95	KP342115.1	<i>Morella</i>
SU4	FMc5	95	KP342119.1	<i>Morella</i>
	FMc3	95	KP342117.1	<i>Morella</i>
	FMc2	95	KP342116.1	<i>Morella</i>
	FMc1	95	KP342115.1	<i>Morella</i>

substrate pH (Huss-Danell, 1997). The pH of the growing substrate was affected by the irrigation solution pH (Bailey et al., 2000). The optimal pH for nodulation of *Alnus glutinosa* and *Alnus incana* (grey alder) was 5.5, whereas pH less than 4.5 inhibited nodule formation (Berry and Torrey, 1985). However, for *Alnus rubra* (red alder), more nodules were found when plants were grown in a substrate with pH 4.5 than with pH 5.6 or 7.2 (Crannell et al., 1994). *Shepherdia argentea* and *S. rotundifolia*, parents of the *S. ×utahensis*, are native to the Intermountain West regions with alkaline soil. *Shepherdia rotundifolia* thrives in soil pH ranging from 6.5 to 7.9 (Sriladda et al., 2014), whereas *S. argentea* has been found in soil with pH between 7.0 and 8.0 (Mee et al., 2003). Consequently, an alkaline environment may be better for the nodulation of *S. ×utahensis* because nodules formed 7 weeks earlier during Expt. 2 (irrigation solution with pH 7.5) than during Expt. 1 (irrigation solution with pH 6.5); however, a symbiotic association may be established in a slightly acid soil.

Nodule formation also occurred in the rhizosphere of plants in the F–N– group.

Previous publications found infective *Frankia* strains persist in locations outside the habitat of host plants, such as the rhizosphere of nonhost plant stands or places where host plants have disappeared over time (Benecke, 1969; Smolander and Sundman, 1987; Wollum et al., 1968). Jeong and Myrold (2001) reported that *Ceanothus integririmus* (deer brush), *Ceanothus sanguineus* (redstem ceanothus), and *Ceanothus velutinus* (snowbrush ceanothus) formed nodules when inoculated with soils collected from a site dominated by *Pseudotsuga menziesii* (Douglas fir) for more than 100 years. Additionally, Wollum et al. (1968) reported that soils collected from a 300-year-old conifer stand contained *Ceanothus*-infective *Frankia* strains. *Casuarina*, which is native to Australia, formed nodules when first grown in Florida (Benson and Silvester, 1993). During our study, no difference in nodulation was observed between the inoculated and uninoculated plants. This suggests that infective *Frankia* strains might exist in the non-sterilized commercial substrate used in our study because limited airflow occurred in the greenhouse and inoculated plants were kept

apart from uninoculated plants to prevent cross-contamination.

Phylogenetic analysis

The presence of the *nifH* gene has been considered an indicator of potential N₂ fixation (Young, 1992). In our study, *nifH* amplifications were obtained from *Frankia* strains in the nodules of *S. ×utahensis*, suggesting these *Frankia* are effective strains. However, their N₂-fixing capacity is unclear because Benson et al. (2004) revealed that effective *Frankia* strains in nodules might have poor N₂-fixing ability. Therefore, further studies are needed to investigate the possible N₂ fixation abilities of the *Frankia* strains using an acetylene reduction assay or ¹⁵N-labeling techniques (Huss-Danell, 1997; Laws and Graves, 2005).

Comparative sequence analyses using *nifH* genes revealed that *nifH* sequences of *Frankia* strains obtained during our study were consistent with the results of previous research. Nouioui et al. (2011) conducted comparative sequence analyses of 38 *Frankia* strains using *glnII*, *gvrB*, and *nifH* genes and classified all *Frankia* strains into four clusters: infective *Frankia* strains in Betulaceae, Casuarinaceae, and Myricaceae in cluster 1; uncultured *Frankia* strains in Coriariaceae, Datisaceae, Rosaceae, and *Ceanothus* in Rhamnaceae in cluster 2; infective *Frankia* strains in Elaeagnaceae and Rhamnaceae in cluster 3; and noninfective and/or non-N₂-fixing *Frankia* strains in cluster 4. These results are consistent with those of Normand et al. (1996). *Frankia* strains CeSI5, FMc5, NRRLB-16306, and R43(2009), all of which shared a highly similar *nifH* gene with the four sequences (SU1, SU2, SU3, and SU4) obtained during our study, were all classified in cluster 3 (Nouioui et al., 2011; Welsh et al., 2009; Wilcox and Cowan, 2016). In addition, *Frankia* strains BMG5.12 and EUN1f were in cluster 3 and isolated from root nodules of *Elaeagnus angustifolia* (Russian olive) and *Elaeagnus umbellata* (autumn olive), respectively (Gtari et al., 2007; Jamann et al., 1993). *Frankia* strain BMG5.12 has a *nifH* gene similar to sequences SU2 and SU3, whereas *Frankia* strain EUN1f was similar to sequences SU1 and SU4. These results suggested that *Frankia* strains in the nodules of *S. ×utahensis* were similar to those in nodules of plants in Elaeagnaceae (Normand et al., 1996; Nouioui et al., 2011; Valdés et al., 2005).

Frankia strains with *nifH* gene sequences highly similar to those in our study have been observed in the nodules of *S. argentea*, one of the parents of *S. ×utahensis*. Tekaya et al. (2018) obtained uncultured *Frankia* clone T2P1–7 from the nodules of *S. argentea*. *Frankia* strain Cc1.17 had *nifH* gene sequences highly similar to those from the symbiotic nodules of *S. argentea* (Mirza et al., 2009). In another study, 93% of sequences obtained from the nodules of *S. argentea* shared 98.3% similarity with strain EUN1f, whereas the remaining 7% of sequences showed 99.6% similarity to strain BMG5.12 (Tekaya et al., 2018). The *nifH* gene sequences of these strains were similar to those obtained

during our study. Therefore, *Frankia* strains inhabiting the nodules of *S. ×utahensis* are similar to those in the nodules of *S. argentea*.

The BLAST results also revealed that *Frankia* strains in our study had *nifH* gene sequences highly similar to symbionts found in four genera (*Casuarina*, *Colletia*, *Elaeagnus*, and *Morella*) (Table 4), and all four of these genera were not in the same host-specific group (HSG) (Benson and Silvester, 1993). Because *Casuarina*-infective *Frankia* strains belong to HSG 2, and because *Elaeagnaceae*-infective *Frankia* strains belong to either HSG 3 or HSG 4 (Huss-Danell, 1997), cross-boundary infectivity might exist in *Frankia* strains in the nodules of *S. ×utahensis*. The cross-boundary infectivity of *Frankia* strains has been reviewed by Huss-Danell (1997). *Frankia* strains in the nodules of *S. ×utahensis* might infect diverse plants to form symbiotic nodules.

Conclusions

This study showed that nodulation of *S. ×utahensis* was restricted by 2 mM NH_4NO_3 . Nodules formed earlier when plants were grown in a low organic-matter substrate and irrigated with quarter-strength N-free Hoagland's solution at pH 7.5. These results suggest that nodulation was improved when plants were grown in conditions similar to their native, low-fertility, arid habitat. A preliminary genetic analysis of *Frankia* in root nodules was conducted during this study. Four *nifH* sequences were obtained from nodules of *S. ×utahensis*. Results of phylogenetic analyses support that *Frankia* strains in nodules have high similarity to those in *Elaeagnaceae* nodules and suggest that they potentially have N_2 -fixing ability. *Frankia* strains obtained during our study are similar to those observed in *S. argentea* nodules. Further research is needed to study the phylogenetic characteristics and N_2 -fixing ability of *Frankia* strains in the nodules of *S. ×utahensis*. Further comparative sequence analyses involving several genes (e.g., *glnII*, *gyrB*, and 16S rDNA) are needed to clarify the phylogeny of the *Frankia* spp. in *S. ×utahensis* nodules.

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