Nodulation and Growth of Shepherdia × utahensis ‘Torrey’

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NODULATION AND GROWTH OF SHEPHERDIA ×UTAHENSIS ‘TORREY’

By

Ji-Jhong Chen

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Plant Science

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2020
ABSTRACT

Nodulation and Growth of *Shepherdia × utahensis* ‘Torrey’

by

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Utah State University, 2020

Major Professor: Dr. Youping Sun
Department: Plants, Soils, and Climate

*Shepherdia × utahensis* ‘Torrey’ (hybrid buffaloberry) (Elaeagnaceae) is presumable an actinorhizal plant that can form nodules with actinobacteria, *Frankia* (a genus of nitrogen-fixing bacteria), to fix atmospheric nitrogen. However, high environmental nitrogen content inhibits nodule development and growth. As a newly created hybrid, the nodulation capability of *S. × utahensis* ‘Torrey’ is unclear. Therefore, the objectives of this research were: 1) to compare the nodulation of plants inoculated with soils from Greenville Research Farm at Utah State University, North Logan, UT, or Mohave County, AZ, grown in substrate containing high or low organic matter, and irrigated with nutrient solution at pH 6.5 or 7.5; 2) to investigate the impacts of nitrogen concentrations on the nodulation of *S. × utahensis* ‘Torrey’, and 3) to study the diversity of *Frankia* strains in nodules of *S. × utahensis* ‘Torrey’. The results showed that nodules formed at the 5th week after plants were inoculated with soil from Greenville Research Farm, grown in pure perlite, and irrigated with a nutrient solution at pH 7.5, while nodules formed at the 12th week after plants were inoculated with soil from Mohave
County, grown in a primarily peat moss substrate, and irrigated with a nutrient solution at pH 6.5. Controlled-release fertilizer (CRF, 15N–3.9P–10K) at 2.9 g·L⁻¹ or NH₄NO₃ at 2 mM inhibited nodule formation of S. ×utahensis ‘Torrey’. Plant growth of inoculated S. ×utahensis ‘Torrey’ topdressed with 2.1 g·L⁻¹ CRF was similar to uninoculated plants topdressed with the manufacturer’s prescribed rate at 3.2 g·L⁻¹. Phylogenetic analysis showed that Frankia strains in nodules of S. ×utahensis ‘Torrey’ had high similarity with those in nodules from Elaeagnaceae and Rhamnaceae, suggesting that Frankia strains in nodules of S. ×utahensis ‘Torrey’ in our study potentially have nitrogen-fixing ability. Furthermore, Frankia strains from S. ×utahensis ‘Torrey’ were similar to those reported in the nodules of S. argentea. According to our results, nodules form earlier when S. ×utahensis ‘Torrey’ plants are in the substrates that are similar to the habitats of its parents. In addition, nodulated plants need less nitrogenous fertilizer to sustain acceptable visual quality and have minimal nitrate nitrogen runoff.
PUBLIC ABSTRACT

Nodulation and Growth of *Shepherdia × utahensis* ‘Torrey’

Ji-Jhong Chen

*Shepherdia × utahensis* ‘Torrey’ (Elaeagnaceae) is a hybrid of two native actinorhizal plants in the Intermountain West, *S. argentea* (silver buffaloberry) and *S. rotundifolia* (roundleaf buffaloberry). Due to actinorhizal symbiosis, atmospheric nitrogen (N$_2$) can be converted to ammonium, a bioavailable form. Actinorhizal plants have great value in sustainable nursery production and urban landscape use. However, nitrogen fertilizer negatively affects the nodulation of actinorhizal plants. As a newly developed hybrid, both the symbiont identity and nodule formation of *S. × utahensis* ‘Torrey’ remain largely unknown. Therefore, experiments were conducted to investigate the nodule formation of *S. × utahensis* ‘Torrey’ inoculated with field soils from the Greenville Research Farm at Utah State University, North Logan, UT, or Mohave County, AZ. Further, *S. × utahensis* ‘Torrey’ inoculated with field soils from the Greenville Research Farm were topdressed with controlled-released fertilizer (CRF) at eight application rates or irrigated with nutrient solutions at two nitrogen levels to study the impacts of nitrogen levels on nodule formation of *S. × utahensis* ‘Torrey’. Nodules from *S. × utahensis* ‘Torrey’ were used to identify the symbiont using *nifH* sequence and phylogenetic analysis. The results of our study showed that plants in a low organic substrate (e.g., perlite) and irrigated with a nutrient solution at pH 7.5 developed nodules
7 weeks earlier than in a commercial substrate (e.g., Metro-Mix® 820) irrigated with a nutrient solution at pH 6.5. Nodulated plants need less nitrogenous fertilizer to maintain plant growth and quality compared with uninoculated plants. However, *S. × utahensis* ‘Torrey’ plants had fewer nodules when the nitrogen level of the fertilizer increased, and nodulation was completely inhibited when applying 2.9 g·L⁻¹ CRF or 2 mM ammonium nitrate to plants. Phylogenetic analysis suggested that the symbiont of *S. × utahensis* ‘Torrey’ could also induce nodules on plants in the Elaeagnaceae and Rhamnaceae to fix atmospheric nitrogen. According to the results in this research, to induce nodules earlier, *S. × utahensis* ‘Torrey’ plants should be grown in a substrate with low organic-matter content and irrigated with a nitrogen-free nutrient solution at a relatively high pH when they are inoculated with *Frankia*-contained soil. Fertilizer lower than the manufacturer’s recommended rate may be applied to *S. × utahensis* ‘Torrey’ plants, if inoculated with field soils, to promote nodule formation for nitrogen fixation and reduce nitrogen runoff.
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Ji-Jhong Chen
# CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT .......................................................... iii</td>
</tr>
<tr>
<td>PUBLIC ABSTRACT ................................................ v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENT ................................................ vii</td>
</tr>
<tr>
<td>LIST OF TABLES .................................................... x</td>
</tr>
<tr>
<td>LIST OF FIGURES .................................................. xi</td>
</tr>
</tbody>
</table>

## CHAPTER

### I. INTRODUCTION, PREVIOUS WORK, AND LITERATURE REVIEW ....... 1

- Biology of Actinorhizal Symbiosis .................................................. 2
- Infection and Nodulation of Frankia .................................................. 3
- Nitrogen Fixation of Actinorhizal Symbiosis ....................................... 5
- Factors Affecting Nodulation of Actinorhizal Plants ............................. 7
- Diversity of Frankia Strains ............................................................ 10
- Shepherdia × utahensis ‘Torrey’ ....................................................... 11
- Research Objectives ........................................................................... 13
- Literature Cited ................................................................................. 14

### II. NODULATION OF SHEPHERDIA × UTAHENSIS ‘TORREY’ IN A COMMERCIAL GROWING SUBSTRATE AND A LOW ORGANIC MATTER SUBSTRATE ........................................... 26

- Abstract ............................................................................................. 26
- Introduction ......................................................................................... 27
- Materials and Methods ....................................................................... 29
- Results .................................................................................................. 31
- Discussion ........................................................................................... 32
- Conclusions ......................................................................................... 38
- Literature Cited .................................................................................. 38

### III. NODULATION AND PLANT GROWTH OF SHEPHERDIA × UTAHENSIS TOPDRESSED WITH CONTROLLED RELEASE FERTILIZERS ......................................................... 48

- Abstract ............................................................................................. 48
Introduction .................................................................................................................49
Materials and Methods .................................................................................................52
Results ..........................................................................................................................55
Discussion ......................................................................................................................59
Conclusions ...................................................................................................................65
Literature Cited ...............................................................................................................66

IV. PHYLOGENETIC ANALYSIS OF FRANKIA STRAINS IN THE
    ROOT NODULES OF SHEPHERDIA × UTAHENSIS ‘TORREY’
    USING NIFH GENE AMPLIFICATION .................................................................76

    Abstract .....................................................................................................................76
    Introduction ...............................................................................................................77
    Materials and Methods .........................................................................................81
    Results ......................................................................................................................83
    Discussion ...............................................................................................................84
    Conclusions ............................................................................................................86
    Literature Cited ......................................................................................................87

V. CONCLUSIONS .......................................................................................................92

APPENDIX ..................................................................................................................94

    Appendix I: Four sequences obtained from nodules of Shepherdia
        × utahensis ‘Torrey’ .......................................................................................95
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>List of actinorhizal plants native to the Intermountain West area and their habitat as well as soil properties.</td>
</tr>
<tr>
<td>2-1</td>
<td>Plant height, 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 <em>Shepherdia × utahensis</em> ‘Torrey’.</td>
</tr>
<tr>
<td>2-2</td>
<td>Chi-square analysis of the nodulation of <em>Shepherdia × utahensis</em> ‘Torrey’ irrigated with Hoagland’s solution with (N+) or without (N-) 2 mM ammonium nitrate (NH$_4$NO$_3$) at the second harvest.</td>
</tr>
<tr>
<td>2-3</td>
<td>Chi-square analysis of the nodulation of <em>Shepherdia × utahensis</em> ‘Torrey’ inoculated with (F+) or without (F-) <em>Frankia</em> at the second harvest.</td>
</tr>
<tr>
<td>3-1</td>
<td>Plant growth, number of shoots, leaf area, dry weight (DW) of leaf, stem, and root, and number of nodules of <em>Shepherdia × utahensis</em> ‘Torrey’ inoculated with field soils and irrigated with nitrogen (N)-free nutrient solutions plus or minus 2 mM ammonium nitrate (NH$_4$NO$_3$) for eight weeks.</td>
</tr>
<tr>
<td>4-1</td>
<td>List of ten <em>Frankia</em> strains that have the highest similarity of <em>nifH</em> gene to each of four query sequences (SU1, SU2, SU3, and SU4) obtained from nodules of <em>Shepherdia × utahensis</em> ‘Torrey’.</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure                         Page

1-1  The anatomy of lateral root and actinorhizal nodule lobe...........................................24

1-2  Shepherdia × utahensis ‘Torrey’ plants at Greenville Research Farm at Utah State University .................................................................25

2-1  Root nodules observed in the soil sample collected from the root zone of a wild Shepherdia rotundifolia..........................................................45

2-2  Nitrate-nitrogen (NO₃-N) concentration of leachate solution collected after Shepherdia × utahensis ‘Torrey’ were irrigated in the experiment 1. ..........................................................................................................................46

2-3  Regression analyses of number of nodules, fresh weight of the largest nodule, and diameter of the largest nodule of Shepherdia × utahensis ‘Torrey’ plants. ..............................................................................................................47

3-1  Nitrate-nitrogen (NO₃-N) concentration in leachate recorded after Shepherdia × utahensis ‘Torrey’ plants were irrigated.................................71

3-2  Plant growth, number of shoots, leaf area, leaf dry weight, stem dry weight, and root dry weight of Shepherdia × utahensis ‘Torrey’ .................72

3-3  Shepherdia × utahensis ‘Torrey’ plants inoculated with soil containing infective Frankia and topdressed with 0 to 8.4 g·L⁻¹ controlled-release fertilizer (CRF, 15N–3.9P–10K) and the uninoculated plant that received the manufacturer’s prescribed rate of 3.2 g·L⁻¹........................................73

3-4  The photosynthesis rate (Pₚₐ), stomatal conductance (gₛ), transpiration rate (E), and nitrogen content of shoot of Shepherdia × utahensis ‘Torrey’ ................................................................................................................74

3-5  The number of nodules per plant and nodule dry weight of Shepherdia × utahensis ‘Torrey’ ..........................................................................................75
CHAPTER I

INTRODUCTION, PREVIOUS WORK, AND LITERATURE REVIEW

Utah is the second driest state in the United States (U.S.) (Hakala, 2014). Unfortunately, over 65% of residential water in Utah is taken to irrigate landscapes (Utah Division of Water Resources, 2014). Due to the public awareness of sustainability, there is an urgent need for low-water-use landscapes.

Using native plants in landscapes has long been considered a viable way to conserve water (Love et al., 2009). Owing to unique morphological and physiological characteristics, such as small leaves, hairy leaves, and deep roots, native plants adapt to local environment with low precipitation, high light intensity, and saline soil (Mee et al., 2003). Moreover, due to the increased awareness and interest in growing and using native plants in landscapes (Brzuszek et al., 2010), it is an opportune time to select and develop native plants for landscapes in the Intermountain West.

The American West has diverse actinorhizal plant species, half of which are native to the western U.S. (80 species) (Paschke, 1997). Actinorhizal plants can establish symbioses with _Frankia_, actinobacteria, to fix atmospheric nitrogen. Native actinorhizal plants have great potential for residential landscapes in the Intermountain West. In Utah, several native actinorhizal plants are recommended for low-water landscaping (Mee et al., 2003), including, _Ceanothus velutinus_ (snowbrush ceanothus), _Cercocarpus ledifolius_ (curl-leaf mountain mahogany), _Cercocarpus ledifolius_ var. _intricatus_ (little-leaf mountain mahogany), _Cercocarpus montanus_ (alder-leaf mountain mahogany), _Shepherdia argentea_ (silver buffaloberry), and _Shepherdia rotundifolia_ (roundleaf
buffaloberry) (Table 1-1).

**Biology of Actinorhizal Symbiosis**

*Frankia* is in the Actinomycetes order and exists in soils and root nodules of actinorhizal plants (Schwencke and Caru, 2001). Actinorhizal symbiosis was first observed in 1886 (Quispel, 1990). In 1964, scientists using an electron microscope finally confirmed that Actinomycetes inhabit the nodules of actinorhizal plants (Becking, 1970). Unfortunately, due to the slow growth of *Frankia* (doubling time of 15 to 48 hours or more) and contamination issues, *Frankia* strains are hard to isolate from soil or nodules, which hindered the early studies of actinorhizal symbiosis (Huss-Danell, 1997). In 1978, the first isolated *Frankia* strain was obtained by Callaham et al. (1978). Recently, numerous reports associated with actinorhizal symbiosis have been published as comparative sequence analyses were innovated.

Since the first discovery of actinorhizal plants, over 200 species of dicotyledonous plants in 24 genera have been shown to establish symbiosis with *Frankia* (Schwencke and Caru, 2001). Actinorhizal plants can be found in all continents except Antarctica (Benson et al., 2004). The actinorhizal symbioses are famous for their nitrogen-fixation ability and the associated benefits of stress tolerance. With symbiotic association, atmospheric nitrogen can be fixed into ammonium, a bioavailable form accessible to plants (Huss-Danell, 1997). Hence, ecologically, actinorhizal plants are pioneer plants thriving in nitrogen-deficient soils (Paschke, 1997), which create a favorable growing environment for other plants and promote plant growth of adjacent plants (Wheeler and
Miller, 1990). With the benefits from symbiosis, actinorhizal plants were used for afforestation in mine-tailing areas (Lumini et al., 1994). Another advantage of actinorhizal symbiosis is improving the growth of host plants in a high saline environment (Ng, 1987). Oliveira et al. (2005) reported that *Alnus glutinosa* (black alder) inoculated with *Frankia* had better plant growth than those inoculated or uninoculated with arbuscular mycorrhizal fungi in alkaline and saline soils.

Early research with actinorhizal symbioses focused on the uses of actinorhizal plants for timber production, afforestation, and soil acclimation (Lumini et al., 1994; Schwencke and Caru, 2001; Wheeler and Miller, 1990). Recently, nitrogen leaching has been shown to cause significant environmental contamination during nursery production (Urbano, 1989). As a result, there is an urgent need to improve the nitrogen-use efficiency of nursery plants. Nodulated actinorhizal plants have been recommended for nursery production because of their nitrogen-fixation capacity (Beddes and Kratsch, 2010). Beddes (2008) suggested several actinorhizal plant species, including *Alnus maritima* (seaside alder), *Purshia mexicana* (Mexican cliffrose), and *Shepherdia argentea*, have great potential for urban landscaping.

**Infection and Nodulation of Frankia**

Infection and nodulation of plants by *Frankia* have been well studied and reviewed by Benson and Silvester (1993) and Huss-Danell (1997). The nodulation of actinorhizal symbiosis starts with infection by *Frankia* hyphae. Prior to infection, chemical signals exchange to trigger gene expression allowing symbiotic bacteria to
over-ride the pathogen defense responses of host plants. *Frankia* then infects host plants via either root hair infection or intercellular penetration (Benson and Silvester, 1993). The method of infection depends on the host species. Research has revealed that a *Frankia* strain infects *Myrica* via root hair, but infects *Elaeagnus* via intercellular penetration (Miller and Baker, 1986; Racette and Torrey, 1989).

When infecting via root hair, hyphae penetrate the cell wall, and root hairs deform and branch. After that, hyphae invade into the cortical cells of the host plant. When infecting via intercellular penetration, root hairs do not deform, and hyphae infect cortical cells directly. But, in either way, hyphae stimulate the formation of a nodule primordium from the pericycle cells of the host plant, and nodules grow until emerging through the root periderm (Huss-Danell, 1997). The duration of nodule formation is different among actinorhizal plant species. For example, after inoculation, *Alnus* plants produced nodules at 2 to 3 weeks, but *Ceanothus* plants showed nodules at 8 to 10 weeks (Jeong and Nyrold, 2001; Wall and Huss-Danell, 1997).

Because of nodule primordium growing from pericycle cells, the morphology of nodules of actinorhizal plants is similar to that of lateral roots (Fig. 1-1) (Pawlowski and Bisseling, 1996). Although actinorhizal nodules are morphologically different among plants, they are perennial and typically in coralloid structure, and develop from nodule lobes (Huss-Danell, 1997). Actinorhizal nodules are covered with one or several lenticels at the outer layer to improve air absorption (Huss-Danell, 1997). Meristem cells at the tip of nodules can only divide basipetally, while vascular tissues locating at the center of the nodule lobes surrounding by cortex cells transport nitrogen fixed by *Frankia* (Fig. 1-1)
(Pawlowski and Bisseling, 1996). Cortical cells can be divided into three zones, including an infection zone, a nitrogen fixation zone, and a senescence zone. The infection zone is the zone containing cortical cells that just developed from the meristem and are infected by Frankia. The nitrogen fixation zone is below the infection zone and consists of cortical cells containing vesicles to carry out nitrogen fixation. The senescence zone is the region in which cortical cells lose nitrogen-fixation ability, hyphae, and vesicles will degrade (Pawlowski and Bisseling, 1996).

 Nitrogen Fixation of Actinorhizal Symbiosis

Nitrogen fixation takes place in the vesicles of Frankia in most host plants except those in the nodules of Allocasuarina and Casuarina, which fix nitrogen in the hyphae (Huss-Danell, 1997; Laplaze et al., 2000). In in vitro culture, free-living Frankia form vesicles in a nitrogen-deficient environment, which indicates that Frankia can perform nitrogen fixation without symbiotic host plants (Schwencke and Caru, 2001). In symbiosis biology, effective nodules are those with nitrogen-fixing capacity (Benson and Silvester, 1993). Generally, vesicles produced from Frankia in vitro are less than in symbiosis in vivo (Huss-Danell, 1997). However, ineffective nodules occur occasionally. Bosco et al. (1992) found that Frankia strains can induce effective nodules in Elaeagnus but produce ineffective nodules in Alnus (Bosco et al., 1992).

Nitrogen fixation is a high energy consumption process; therefore, it relies on photosynthetic products to maintain the growth and development of symbiotic Frankia. Photosynthetic products are transported to nodules in sucrose form, and ATP is produced
during respiration and then used for nitrogen fixation (Huss-Danell, 1997). However, oxygen used in respiration is lethal to the nitrogen-fixing enzyme nitrogenase in *Frankia* and must be compartmentalized to reduce the effect (Persson and Huss-Danell, 2009). For *Frankia*, vesicles are the structures that protect nitrogenase from oxygen damage by enveloping the enzyme with single or multiple hopanoid-rich lipid layers, and the thickness of lipid layers of vesicles is associated with the environmental oxygen level (Murray et al., 1985; Parsons et al., 1987; Persson and Huss-Danell, 2009).

The nitrogenase of *Frankia* in the symbiotic nodules of actinorhizal plants catalyzes dinitrogen reduction reaction to convert atmospheric nitrogen to ammonia that is protonated to form ammonium in cells (Persson and Huss-Danell, 2009). Ammonium is the nitrogen compound that can be directly used by plants. The reaction of nitrogen reduction is:

\[
N_2 + 12 - 24ATP + 8e^- + 8H^+ + Mg^{2+} \rightleftharpoons 2NH_3 + 12-24ADP + 12-24Pi + H_2
\]

\[
2NH_3 + 2H^+ + pH = 7 \rightleftharpoons 2NH_4^+
\]

This reaction was tested using the \(^{15}\)N\(_2\) labeling technique in *Myrica* and *Alnus* (Huss-Danell, 1997). After ammonium is produced, it is converted to amino acids via the glutamine synthetase/glutamine oxoglutarate aminotransferase (GA/GOGAT) pathway (Lundberg and Lundquist, 2004).

Actinorhizal plants translocate the nitrogen fixed by *Frankia* with citrulline, glutamine, and/or asparagine, or mixing compounds in the xylem (Persson and Huss-
Danell, 2009). It is more energetically efficient for actinorhizal plants to use these compounds to translocate nitrogen (Schubert, 1986). For example, nitrogen is transported via asparagine for *Ceanothus americanus* (New Jersey tea), but via citrulline for most alder plants (Persson and Huss-Danell, 2009).

With *Frankia* nitrogen fixation, soil sampled from *Ceanothus*-occupied areas had a lower C:N ratio compared with those dominated by pine trees (Johnson et al., 2012). In addition, it has been revealed that nodulated *Ceanothus sanguineus* (redstem ceanothus) fixes about 50 kg nitrogen per hectare per year (Binkley and Husted, 1983), while nodulated *Ceanothus velutinus* (snowbrush ceanothus) produces 80 kg nitrogen per hectare per year (Cromack et al., 1979). Huss-Danell (1997) reviewed the protocols to test the nitrogen fixation of actinorhizal plants. The acetylene reduction assay is one of the most frequently used methods to quantify biological nitrogen fixation activity because it can rapidly, non-destructively, and economically provide nitrogen fixation information (David et al., 1980; Flett et al., 1975). This assay involves reducing acetylene (C$_2$H$_2$) to ethylene (C$_2$H$_4$) by nitrogenase, but its reliability is questionable since acetylene may have indirect effects on the microbial metabolism that results in a decline in nitrogenase activity (Johnson et al., 1997; Tjepkema and Schwintzer, 1992). $^{15}$N related techniques are common methods to assay nitrogen fixation and study the nitrogen assimilation process (Huss-Danell, 1997). Practically, researchers need to consider resources, such as labor and equipment, to choose a suitable method for studying the nitrogen-fixing capacity of actinorhizal plants.
Factors Affecting Nodulation of Actinorhizal Plant

Nitrogen content. Even low nitrogen concentrations negatively affect nodulation, and the inhibition effect is amplified with increasing nitrogen levels (Huss-Danell, 1997; Kohls and Baker 1989). For instance, nodules of Coriaria arborea (tutu) and Hippophae rhamnoides (seaberry) decreased when plants were grown in a substrate spiked with nitrate (Bond and Mackinosh, 1975). In addition, Baker et al. (1997) found nodule growth and activity of Myrica gale (sweetgale) decreased when internal nitrogen levels increased. Nodulation of Alnus glutinosa, Casuarina cunninghamiana (river oak), and Myrica cerifera (bayberry) were inhibited when irrigated with a solution containing 1 mM nitrate (Kohls and Baker, 1989). For Ceanothus griseus (Carmel ceanothus), NH₄NO₃ at 0.714 mM significantly reduced the nodules, while nodulation was completely inhibited at 2.68 mM NH₄NO₃ (Thomas and Berry, 1989). Nitrogen compounds can have multiple effects on actinorhizal plants with varying responses among plant species. For instance, the inhibitory effects on the nodulation of Casuarina cunninghamiana were found more significant when applying ammonium than nitrate (Zhang and Torrey, 1985). Therefore, it is important to determine the nitrogen levels for efficient nodulation in different actinorhizal plants.

Mineral nutrients and pH. Mineral nutrients have been studied for their effects on the nodulation of actinorhizal plants. Iron (Fe) is a critical co-factor in nitrogenase and hemoglobin, two important proteins in the metabolic and biosynthetic processes of both actinorhizal plants and microorganisms (Huss-Danell, 1997; Santi et al., 2013). The slow growth of symbiotic Frankia inhibited nodulation of Alnus japonica (Japanese alder)
when plants were in an iron-deficient environment (Burgess and Peterson, 1987).

Molybdenum (Mo) is also a component of nitrogenase, and molybdenum deficiency has
led to both reduced nodulation and nitrogen-fixation capacity (Bond and Hewitt, 1961).

Hewitt and Bond (1966) observed molybdenum deficiency had negative effects on
nitrogen-fixation of Alnus and Casuarina. Cobalt (Co) is also essential for actinorhizal
plants since the demand for cobalt increased on nodulated Alnus glutinosa, Casuarina
cunninghamiana, and Myrica gale (Hewitt and Bond, 1966). In addition, nodulation of
Alnus incana (grey alder) was stimulated under elevated phosphate concentrations (Huss-
Danell, 1997). Nodulation of actinorhizal plants was related to calcium due to the effect
of calcium on pH (Tisa and Ensign, 1987). However, the effect of pH on nodulation of
actinorhizal plants depends on species and is associated with the availability of mineral
nutrients. For example, the optimal pH for the nodulation of Alnus glutinosa was 5.5,
while a pH less than 4.5 inhibited nodule formation of Alnus incana (Berry and Torrey,
1985). More nodules were found on Alnus rubra (red alder) grown in a substrate at pH
4.5 than at pH 5.6 or 7.2 (Crannell et al., 1994).

Soil moisture. The influence of soil moisture on actinorhizal plants has been
studied (Kratsch and Graves, 2004; Pratt et al., 2006; Sundstrom and Huss-Danell, 1987).

Drought stress lowers osmotic potential, reduces water availability, and results in
symptoms such as wilting and stunted growth. Drought limited the nodulation of
Ceanothus in southern California chaparral (Kummerow et al., 1978). Ceanothus was
also found to have a low net photosynthetic rate at low water potential and limited
nodulation because the symbiont of actinorhizal plants relies on the photosynthetic
products from the host plant (Thomas and Davis, 1989). Drought also led to poor nodulation, as water helps *Frankia* infect the host (Dunn et al., 1985). On the other hand, flooding is also a restriction on the nodulation of actinorhizal plants. Kratsch and Graves (2004) found that *Alnus maritima* in well-drained soils had higher leaf nitrogen content and greater nodulation compared with partial flooding, which showed that flooding has negative effects on nodulation.

**Diversity of Frankia Strains**

Compared with rhizobium-legume symbiosis, actinorhizal symbiosis occurs on highly diverse plant species. Phylogenetic research is important to identify symbiotic compatibility between plants and microorganisms of actinorhizal symbiosis (Benson et al., 2004). Reviewed by Benson and Silvester (1993), the phylogenetic system of *Frankia* classifies symbionts according to their host plants, and early studies investigated the diversity of *Frankia* using morphology and anatomy of nodules. Host specificity is also used to study the diversity and compatibility of *Frankia*. For instance, cross-infectivity studies showed that *Frankia* strains were classified into four host-specific groups (HSGs), in which HSG 1 contains strains in nodules of *Alnus*, *Comptonia*, and *Myrica* plants, HSG 2 contains strains in *Casuarina* and *Myrica* plants, HSG 3 contains strains in Elaeagnaceae and *Myrica* plants, while HSG 4 contains strains in Elaeagnaceae plants only (Baker, 1987; Du and Baker, 1992).

Due to the advances in DNA sequencing, the phylogeny of *Frankia* strains has been recently investigated using comparative sequence analyses with genes such as 16S rRNA,
nif gene, or gyrB (Benson et al., 2004). For instance, Normand et al. (1996) conducted comparative sequence analyses using 16S rRNA, and their results were similar to the results of Baker (1987) and Du and Baker (1992). Nouioui et al. (2011) further investigated infective Frankia strains using gyrB, nifH, and glnII genes and found unculturable strains are all in the same cluster, which is similar to that reported by Normand et al. (1996) and Clawson et al. (2004). After reviewing comparative sequence analyses using 16S rRNA gene and nif genes, Benson et al. (2004) concluded that the infective Frankia comprises three major groups, in which group 1 includes Frankia strains in nodules on plants in Betulaceae, Casuarinaceae, and Myricaceae; group 2 includes Frankia strains that form nodules on plants in Coriariaceae, Datiscaceae, Rosaceae and Ceanothus of the Rhamnaceae family, and Group 3 contains Frankia strains that form effective nodules on plants in Elaeagnaceae, Myricaceae, Rhamnaceae, and Gymnostoma of the Casuarinaceae family, and those without nitrogen-fixation ability in the nodules of Betulaceae, Rosaceae families, some genera in Casuarinaceae family and Ceanothus of the Rhamnaceae.

_Shepherdia xutahensis ‘Torrey’_

One of the obstacles to establish drought-tolerant native plants in urban landscapes in the Intermountain West is poor adaptability and high mortality (Edmondson et al., 2011), which may be attributed to the differences in soils between habitat and landscape. Native plants grow in well-drained soil in the Intermountain West (Mee et al., 2003). Soil with adequate drainage is recommended for growing native plants in urban landscapes in the Intermountain West, such as _Agave parryi_ (Parry’s agave),
Aquilegia caerulea (Colorado blue columbine), Eriogonum niveum (snow buckwheat),
Eriophyllum lanatum (woolly sunflower), and Hesperaloe parviflora (texas red yucca)
(Parkinson et al., 2003). Mee et al. (2003) reviewed native plants that are difficult to
establish in landscapes in the Intermountain West.

Shepherdia rotundifolia is an evergreen and drought-tolerant shrub native to the
Four-Corners region of the southwestern U.S. It has round downward-cupped leaves
covered with densely packed stellate trichomes (Beddes and Kratsch, 2009; Sriladda et
al., 2016). Similar to other drought-tolerant plants in the Intermountain West, S.
rotundifolia has an attractive appearance and strong drought tolerance, but shows poor
performance and low survival rates in low-water landscapes (Sriladda et al., 2016). To
improve the adaptability of Shepherdia in residential landscapes, S. ×utahensis ‘Torrey’
(Fig. 1-2) was created by hybridizing S. argentea and S. rotundifolia, two native
Shepherdia plants in the Intermountain West (Sriladda et al., 2016). Shepherdia argentea
is a deciduous shrub in riparian areas with narrow grey-green leaves (Mee et al., 2003).
Compared with S. rotundifolia, S. argentea tolerates a wide range of soil types but is less
aesthetic (Sriladda et al., 2016).

Shepherdia ×utahensis ‘Torrey’ has intermediate genetic and morphological
characteristics between S. argentea and S. rotundifolia (Sriladda et al., 2016). Further, S.
×utahensis ‘Torrey’ inherited the aesthetic leaf qualities (silver-blue color and revolute
margins) from S. rotundifolia and thus has an appealing appearance in residential
landscapes (Sriladda et al., 2016). Moreover, the stellate trichomes on the leaves help
reflect solar radiation and reduce water loss; therefore, helping it thrive in a dry
environment. *Shepherdia × utahensis* ‘Torrey’ can also tolerate wet, fertile substrate, or disturbed soil (Sriladda et al., 2016).

*Shepherdia × utahensis* ‘Torrey’ is presumably an actinorhizal plant and may have the potential to form a symbiotic association with *Frankia*. Nodulation of actinorhizal plants in genera *Alnus*, *Ceanothus*, and *Purshia* is mostly investigated (Walls and Zamora, 2001). These studies may help elucidate the nodulation of native actinorhizal plants in the Intermountain West. However, little effort has been made to investigate nodulation of *S. argentea* and *S. rotundifolia*, as well as other actinorhizal plants native to the Intermountain West. Additionally, multiple factors affect their nodulation (Huss-Danell, 1997), and the optimal condition for nodule development varies among plant species. The Intermountain West has alkaline soils with low organic matter content and low cation exchange capacity (Heaton and Koenig, 2010), as well as drought conditions accompanied by strong solar radiation (Mee et al., 2003). Therefore, further investigation should be conducted to better understand nodulation of actinorhizal plants native to the Intermountain West.

As a newly created plant, whether *S. × utahensis* ‘Torrey’ forms nodules or not and how to efficiently induce the nodulation are yet to be investigated. Research-based information on the nodulation of *S. × utahensis* ‘Torrey’ is needed to promote this plant for the Green Industry.

**Objectives of Research**

The optimal conditions to induce nodulation of *S. × utahensis* ‘Torrey’ may be
different from its parents. In this study, the first objective was to study plant growth and nodulation of S. × utahensis ‘Torrey’ in conditions that mimic either the nursery environments or the natural habitats of S. rotundifolia. The second objective was to investigate the optimal controlled-release fertilizer levels to produce nodulated S. × utahensis ‘Torrey’ plants in nursery production. The third objective of this study was to look into the phylogenetic relationship between Frankia strains in S. × utahensis ‘Torrey’ nodules and Frankia strains reported in the literature.

Two experiments were conducted to study nodulation of S. × utahensis ‘Torrey’ grown in substrates containing primarily peat moss or perlite and irrigated with nutrient solutions at pH 6.5 or 7.5 to mimic soil texture, structure, and pH in nursery production and the natural habitat of S. rotundifolia, respectively. The third experiment was to study the nodulation of inoculated S. × utahensis ‘Torrey’ plants receiving 0 to 8.4 g·L⁻¹ controlled-release fertilizer 15N-3.9P-10K, while plant growth of nodulated plants was compared with those uninoculated plants receiving the manufacturer’s prescribed rate of 3.2 g·L⁻¹ to study the benefit of actinorhizal symbiosis on plant growth. Lastly, an experiment was conducted to sequence nifH genes of Frankia strains extracted from symbiotic nodules and run phylogenetic analysis to compare the Frankia strains in nodules with those Frankia strains reported in the literature.

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Table 1-1. List of actinorhizal plants native to the Intermountain West area and their habitat as well as soil properties. 

<table>
<thead>
<tr>
<th>Species (Common name)</th>
<th>Habitat</th>
<th>Soil properties</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ceanothus martini</em> (Utah mountain-lilac)</td>
<td>CO, NV, UT</td>
<td>Medium texture soil with pH 6.5, deep and dry and well-drained, low to medium organic matter.</td>
</tr>
<tr>
<td><em>Ceanothus velutinus</em> (snowbrush ceanothus)</td>
<td>CA, NM, WA</td>
<td>Medium texture soil with pH 6.5-7.0, deep, dry and well-drained, low to medium organic matter.</td>
</tr>
<tr>
<td><em>Cercocarpus ledifolius</em> (curl-leaf mountain mahogany)</td>
<td>CA, MT, NM, WA</td>
<td>Coarse to rocky sandy loam soil texture with pH 6.0-7.0, deep to shallow, dry and well-drained, low organic matter.</td>
</tr>
<tr>
<td><em>Cercocarpus ledifolius</em> var. <em>intricatus</em> (little-leaf mountain mahogany)</td>
<td>CA, CO, NM, UT</td>
<td>Medium to coarse sandy loam soil with pH 7.0-7.5, dry and well-drained, low organic matter.</td>
</tr>
<tr>
<td><em>Cercocarpus montanus</em> (alder-leaf mountain mahogany)</td>
<td>CA, MT, NM, WA</td>
<td>Coarse to rocky soil with pH 6.5-7.5, deep, dry, and well-drained.</td>
</tr>
<tr>
<td><em>Shepherdia argentea</em> (silver buffaloberry)</td>
<td>CA, MT, NM, WA</td>
<td>Medium to coarse or fine soil with pH 7.0-8.0, deep, moist, and well-drained.</td>
</tr>
<tr>
<td><em>Shepherdia rotundifolia</em> (roundleaf buffaloberry)</td>
<td>AZ, UT</td>
<td>Rocky soil, very well-drained.</td>
</tr>
</tbody>
</table>

z Adopted from Mee et al., (2003).
Fig. 1-1. The anatomy of lateral root (A) and actinorhizal nodule lobe (B) adopted from Pawlowski (2009). The vascular tissue is indicated as a bold black line in the center of lateral root and actinorhizal nodule lobe. The apical meristem is indicated as ‘M’, and can divide basipetally for lateral root elongation and acropetally for root cap formation. However, in actinorhizal nodule lobes, the apical meristem can only divide basipetally. The periderm is indicated as the gray region, and the actinorhizal nodule lobe can be separated into three zones: infected zone (zone 1), nitrogen fixation zone (zone 2), and senescence zone (zone 3).
Fig. 1-2. *Shepherdia ×utahensis* ‘Torrey’ plants at Greenville Research Farm at Utah State University (North Logan, UT).
CHAPTER II

NODULATION OF SHEPHERDIA ×UTAHENSIS ‘TORREY’ IN A COMMERCIAL GROWING SUBSTRATE AND IN A LOW ORGANIC MATTER SUBSTRATE

Abstract

Shepherdia ×utahensis ‘Torrey’, a hybrid of S. argentea (silver buffaloberry) and S. rotundifolia (roundleaf buffaloberry), has great potential for xeriscape use. It is an actinorhizal plant with the ability to fix atmospheric nitrogen (N₂) in root nodules. However, how to effectively induce nodules in S. ×utahensis ‘Torrey’ using soil containing Frankia, a genus of actinobacteria, was unclear. In this study, S. ×utahensis ‘Torrey’ formed symbiotic nodules when grown in a Metro-Mix® 820 substrate inoculated with soils collected from the root zone of a wild population of S. rotundifolia in Mohave County, AZ or in a low organic-matter substrate amended with soils from an S. ×utahensis ‘Torrey’ plantation in North Logan, UT. In a Metro-Mix® 820 substrate, plants irrigated with quarter-strength nitrogen (N)-free Hoagland’s solution with 2 mM ammonium nitrate (NH₄NO₃) at pH 6.5 appeared healthy but no nodules formed, while plants treated with an identical solution without NH₄NO₃ exhibited growth inhibition and showed nodules at the 12th week after experiment initiation. When quarter-strength N-free Hoagland’s solution was applied, nodules formed in five weeks when S. ×utahensis ‘Torrey’ plants were grown in a low organic-matter substrate, and the pH of the irrigated solution was at 7.5. This research demonstrates that S. ×utahensis ‘Torrey’ has the ability to form nodules and may be used in sustainable landscapes, but nodulation may be inhibited at 2 mM NH₄NO₃. Furthermore, this research provides an efficient protocol for
inoculating *S. × utahensis* ‘Torrey’ with *Frankia* for the Green Industry.

**Introduction**

Actinorhizal plants are able to fix atmospheric nitrogen (N\(_2\)) through symbiosis with *Frankia*, a genus of actinobacteria, and have great potential for urban landscapes (Kratsch and Graves, 2004). Because of their ability to fix N\(_2\), actinorhizal plants thrive in nitrogen (N)-deficient conditions (Johnson et al., 2012). Plant growth and development could also be improved when the symbiotic association is established. For instance, Schwencke and Caru (2001) reported that artificial inoculation to induce symbiotic nodules in nursery conditions could improve the survival rate and growth of actinorhizal plants. *Alnus maritima* (seaside alder) inoculated with 30 ml of soil collected from a wild population had better nutrient status when they were grown at low levels of controlled-release fertilizer (CRF) (Beddes and Kratsch, 2010). However, excessive N inhibits the nodulation of actinorhizal plants (Laws and Graves, 2005). The nodulation of *A. maritima* was prevented by either 2.7 g·L\(^{-1}\) of 15N-3.9P-10K controlled-release fertilizer or 4 mM ammonium nitrate (NH\(_4\)NO\(_3\)). Therefore, to successfully induce symbiotic nodules in nursery production, it is important to study the nodulation of inoculated actinorhizal plants irrigated with nutrient solutions with different N levels.

*Shepherdia argentea* (silver buffaloberry) and *S. rotundifolia* (roundleaf buffaloberry) are native actinorhizal plants in the Intermountain West (Mee et al., 2003). *Shepherdia argentea* can tolerate a wide range of soil conditions (Sriladda et al., 2016), while *S. rotundifolia* has strong drought tolerance and narrower soil 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* ‘Torrey’ is an interspecific hybrid of S. argentea and S. rotundifolia with the tolerance of wet and disturbed soil and drought stress (Sriladda et al., 2016). It has high potential for low-water landscape use. The genetic, physiological, and morphological traits of *S. × utahensis* ‘Torrey’ have been studied by Sriladda et al. (2016). As a progeny of an actinorhizal species, *S. × utahensis* ‘Torrey’ may be able to establish symbiotic associations with *Frankia*. However, few studies have investigated the nodulation of *Shepherdia*. Moreover, previous studies associated with the nodulation of actinorhizal plants focused on *Alnus* (alders), which are actinorhizal plants native to the eastern United States (Beddes and Kratsch; Huss-Danell et al., 1982; Laws and Graves, 2005). Due to different soil physical and chemical properties in the Intermountain West (Heaton and Koenig, 2010; Mee et al., 2003; Sriladda et al. 2014), characteristics of the nodulation of *S. × utahensis* ‘Torrey’ might be different to the nodulation of *Alnus*. Two experiments were conducted in our study.

In experiment 1 (Expt. 1), *S. × utahensis* ‘Torrey’ plants were grown in a commercial growing substrate containing primarily peat and bark and irrigated with N-free nutrient solution spiked with 0 or 2 mM ammonium nitrate at pH 6.5 to mimic nursery conditions to study plant growth as well as nodulation. In experiment 2 (Expt. 2), *S. × utahensis* ‘Torrey’ plants were grown in a soilless substrate with low organic-matter content and irrigated with N-free nutrient solution at pH 7.5 to study the nodulation of actinorhizal plants in a condition that mimics the native habitat of *S. rotundifolia*. 
Materials and Methods

Expt. 1. On 22 Mar. 2019, terminal cuttings (≈10 cm) of *S. × utahensis* ‘Torrey’ were collected from the Greenville Research Farm at Utah State University (USU) (North Logan, UT) (41.765741, -111.813175). Leaves at the bottom of the cuttings were removed, leaving two to three pairs of leaves at the top. Cuttings were dipped in 8,000 mg·L⁻¹ indole-3-butyric acid (IBA) (Hormodin® 3; OHP, Mainland, PA) and stuck in a soilless substrate containing 80% perlite (Hess perlite, Malad City, ID) and 20% peat moss (Canadian sphagnum peat moss; SunGro Horticulture, Agawam, MA). On 15 May 2019, nodule-free rooted cuttings were transplanted into 3.8-L injection-molded, polypropylene containers (PC1D-4; Nursery Supplies, Orange, CA) filled with a MetroMix®820 substrate (SunGro Horticulture, Agawam, MA), which is primarily peat moss. Plants were irrigated with deionized (DI) water until the experiment was initiated.

On 17 June 2019, a factorial experimental design was set up with (F+) or without (F-) *Frankia* inoculation and with (N+) or without nitrogen (N-) application. A total of 84 uniform *S. × utahensis* ‘Torrey’ plants were used with 22 plants in each of F+N+ and F+N- groups and 20 plants in each of F-N+ and F-N- groups. The plants with inoculation were topdressed with 30 mL 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. 2-1), while the plants without inoculation did not receive soil treatment. The plants with N treatment were irrigated with quarter-strength N-free Hoagland’s solution (Hoagland and Arnon, 1950) plus 2 mM NH₄NO₃ at pH 6.5, while those without N treatment were irrigated with the identical solution minus NH₄NO₃.
Ten plants in each treatment were randomly selected and destructively harvested on 29 July 2019 (seven weeks after experiment initiation, first harvest), and the remaining plants were harvested on 4 Sept. 2019 (twelve weeks after experiment initiation, second harvest). Plant height was recorded from the surface of the substrate to the highest terminal bud at the initiation of the experiment and both harvest dates. The number of shoots (> 5 cm) was also recorded at the initiation of the experiment and both harvest dates. A Soil Plant Analysis Development (SPAD)-502 instrument (Minolta Camera, Osaka, Japan) was used to measure relative chlorophyll content of each plant at both harvest date and averaged values of five randomly selected mature leaves per plant from canopy were recorded. 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 three days, and shoot dry weight (DW) was recorded. Roots were harvested, washed by deionized (DI) 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 the leachate solution was recorded on 2 Sept. 2019 using a pH meter (LAQUA Twin; Horiba, Kyoto, Japan).

**Expt. 2.** On 6 Aug. 2019, sixty nodule-free *S. × utahensis* ‘Torrey’ plants propagated using the same method mentioned above were transplanted into 656-ml cone-tainers (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 field soils collected from the rhizosphere of an *S. × utahensis* ‘Torrey’ plant at the Greenville Research Farm were 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 terminated on 18 Nov. 2019. One plant per block was randomly selected and harvested weekly to study the nodulation of *S. × utahensis* ‘Torrey’ plants. At harvest, the number of nodules was counted, the diameter and fresh weight of each nodule were measured.

*Experimental design and statistical analyses.* The Expt. 1 was in a factorial design, while the Expt. 2 was a randomized complete block design with 20 and 4 blocks, respectively. A two-way analysis of variance (ANOVA) procedure was used to test the effects of N treatment and *Frankia* inoculation on the growth and nodulation in Expt. 1, while one-way ANOVA procedure was used to test the effect of time on nodulation in Expt. 2. A Chi-square test was conducted using PROC FREQ procedure to test the nodulation of *S. × utahensis* ‘Torrey’. Mean separation among treatments was adjusted using Tukey-Kramer method for multiplicity at $\alpha = 0.05$ in Expt. 1. In Expt. 2, regression analyses between time and nodule number, diameter, and fresh weight were conducted. All statistical analyses were performed using PROC Mixed procedures in SAS Studio (SAS Institute, Cary, NC).

**Results**

In Expt. 1, *S. × utahensis* ‘Torrey’ plants irrigated with quarter-strength N-free Hoagland’s solution with 2 mM NH$_4$NO$_3$ had greater NO$_3$-N concentration in leachate than those without NH$_4$NO$_3$ at both harvest dates (Fig. 2-2). At the termination of the experiment, the pH leachate solution was $6.2 \pm 0.1$ (mean ± se) and $5.8 \pm 0.1$ (mean ± se)
for plants irrigated with quarter-strength N-free Hoagland’s solution plus or minus 2 mM NH₄NO₃, respectively. No nodules were observed at the 7th week, but nodules were found on plants in F+N- and F-N- groups at the 12th week (Table 2-1). Eight out of 12 plants in F+N- group formed nodules, while four out of 10 plants in F-N- group had nodules (Table 2-2 and 2-3). According to Chi-square analysis, nodulation was affected by 2 mM NH₄NO₃ in quarter-strength N-free nutrient solution (P < 0.0001). On the other hand, although nodulation was greater for those in F+N- group than in F-N- group, inoculation did not affect nodulation (P = 0.32).

At both harvest dates, 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 NH₄NO₃ increased compared to those irrigated with the same solution but without NH₄NO₃ (Table 2-1). Furthermore, the number of shoots of plants in the F+N+ group increased as compared with those in F-N+ group at the first harvest, whereas the shoot DW of plants in F+N+ group increased compared with those in F-N+ group at the second harvest.

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

**Discussion**

Inoculation is a preferred practice in nursery production to induce symbiotic

BESIDES, THE SUBSTRATE USED IN EXPT. 1 MIGHT RETAIN N IONS AT CATION EXCHANGE SITE AND CAUSE INCREASED N TO INHIBITED NODULATION. PEAT MOSS HAVE HIGH CEC RANGING FROM 108 TO 162 cmol$^+$.kg$^{-1}$ (ALTLAND ET AL., 2014; GLENN ET AL., 2000; RIPPY AND NELSON, 2007). AS CATION EXCHANGE CAPACITY (CEC) ALLOWS EXCHANGEABLE CATIONS TO ADHERE TO SOIL/SUBSTRATE PARTICLE SURFACE (TAIZ ET AL., 2015), THE AMMONIUM (NH$_4^+$) IN THE NUTRIENT SOLUTION IS RETAINED IN SUBSTRATE TO AFFECT NODULATION OF ACTINORHIZAL PLANTS. DUE TO THE
potential nitrogen buildup in the substrate, nodule formation of actinorhizal plants is limited, although irrigated with nutrient solution containing a low N level. The buildup of N in substrate affecting nodule formation has been reported by Beddes and Kratsch (2010). Hence, extra precautions should be taken to maintain N levels of irrigated solution and substrate with high CEC when producing nodulated actinorhizal plants in nursery conditions.

Nodulation was observed seven weeks earlier in Expt. 2 compared with Expt. 1, which might be caused by the difference of substrate and pH of nutrient solutions used in the study. The commercial growing substrate contains peat moss, an organic material containing over 20% organic carbon (Canada Soil Survey Committee, 1978). Organic matter in commercially-used growing substrate increases water-holding capacity (Hudson et al., 1994). However, soils in the Intermountain West are different from the commercial growing substrate and contain low organic matter because of the desert climate and low plant coverage (Heaton and Koenig, 2010). The study by Sriladda et al. (2014) reported that soil organic matter in the habitat of wild S. rotundifolia in Utah was between 0.7 to 8.7%. Therefore, soils from the habitats of S. argentea and S. rotundifolia were well-drained (Mee et al., 2003). 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. Sriladda et al. (2016) grew S. × utahensis ‘Torrey’ in a calcined clay, a low organic-matter substrate. Therefore, although peat moss is a commonly used in nursery production, a substrate containing low organic matter may be better for inducing nodulation of S. × utahensis
'Torrey' plants.

Both Expt. 1 and 2 were not controlled experiments of pH. However, the pH of the irrigated solution might also explain the discrepancy in nodule formation between Expt. 1 and 2. The pH of the growing substrate is affected by the pH of the irrigated solution. Moreover, nodulation of actinorhizal plants is associated with the pH of substrate (Huss-Danell, 1997). The optimal pH for nodulation of *Alnus glutinosa* and *Alnus incana* (grey alder) was 5.5, while the 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 at pH 4.5 than at pH 5.6 or 7.2 (Crannell et al., 1994). *Shepherdia argentea* and *S. rotundifolia*, parents of *S. × utahensis* ‘Torrey’, are native to the Intermountain West regions with alkaline soil (Mee et al., 2003). *Shepherdia rotundifolia* thrives in soil pH ranging from 6.5 to 7.9 (Sriladda et al., 2014), while *S. argentea* was found in soil pH between 7.0 to 8.0 (Mee et al., 2003). In our study, nodules formed 7 weeks earlier on *S. × utahensis* ‘Torrey’ in Expt. 2 (pH 7.5) than in Expt. 1 (pH 6.5), which indicates, similar to its parents, an alkaline environment may be better for the nodulation of *S. × utahensis* ‘Torrey’. But *Shepherdia × utahensis* ‘Torrey’ could also establish a symbiotic association with *Frankia* in acid soil.

Our study demonstrated that N inhibited the nodulation of *S. × utahensis* ‘Torrey’, but further research is needed to determine the optimal N level or fertilizer level for effectively producing nodulated *S. × utahensis* ‘Torrey’. Although increased nitrogen levels improve plant growth, they also decrease nodule number (Laws and Graves, 2005). Beddes and Kratsch (2010) reported that the shoot dry weight, plant height, and leaf area
of *A. maritima* increased when levels of controlled-release fertilizers (CRFs) increased, but their nodule number decreased. However, high fertilizer levels may cause excessive nitrogen runoff to contaminate groundwater and higher fertilizer cost (Poole and Conover, 1989). Plant quality of *S. × utahensis* ‘Torrey’ plants without nitrogen treatment was poor, although nodulation showed on these plants. Laws and Graves (2005) also reported that *A. maritima* without nitrogen treatment had the greatest nodule number, but showed irregular shoot shape and yellowing leaves. Consequently, it is important to determine proper nitrogen concentrations or fertilizer application rates for *S. × utahensis* ‘Torrey’ to produce nodulated plants with acceptable visual quality and minimal nitrogen leachate (Beddes and Kratsch, 2010).

Nodule formation also occurred in the rhizosphere of plants in the F-N- group. According to the previous publication, infective *Frankia* strains persist in locations outside the habitat of host plants such as the rhizosphere of non-host plant stands or places that host plants have disappeared from for a long time (Benecke, 1969; Smolander and Sundman, 1987; Wollum et al., 1968). Jeong and Myrold (2001) reported that *Ceanothus velutinus* (snowbrush ceanothus), *Ceanothus sanguineus* (redstem ceanothus), and *Ceanothus integerrimus* (deerbrush) formed nodules when inoculated with soils collected from a site of predominantly *Pseudotsuga menziesii* (Douglas-fir) for more than 100 years. In addition, 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). In our study, no difference showed in the nodulation of *S. × utahensis* ‘Torrey’
between the inoculated and uninoculated plants. Because of little airflow occurring in greenhouse and inoculated plants kept apart for uninoculated plants, cross-contamination was prevented. Therefore, infective *Frankia* strains might exist in the non-sterilized commercial substrate used in our study. But further research is needed to grow an uninoculated *S. × utahensis ‘Torrey’* in the same commercial growing substrate to study whether it contains infective *Frankia* stains.

In addition, a future study investigating the nitrogen-fixing capacity of nodules is needed. Although nodules appeared on the plants in F+N- and F-N- groups, the nitrogen-fixing ability of the nodules was unknown. *Frankia* strains capable of forming nodules in the host plant are referred to as infective stains, while *Frankia* capable of forming symbiotic nitrogen fixation are known as effective strains (Benson and Silvester, 1993). In this study, *Frankia* strains in nodules were infective, but their effectivity was unknown. The nitrogen-fixing ability of root nodules has been studied using acetylene-reduction assays, and the protocol has been reviewed (Laws and Graves, 2005). Further research is needed to test the nitrogen-fixation ability of *Frankia* strains in *S. × utahensis ‘Torrey’* nodules.

**Conclusions**

Our research studied the nodulation of *S. × utahensis ‘Torrey’* grown in two conditions, in a commercial growing substrate irrigated with a nutrient solution at pH 6.5 to mimic nursery condition or in a low organic-matter substrate irrigated with a solution at pH 7.5 to mimic native habitat. Results showed the nodulation of *S. × utahensis*
‘Torrey’ was restricted by 2 mM NH₄NO₃ or accumulation. Nodules formed earlier when S. × utahensis ‘Torrey’ is grown in a low organic-matter substrate and irrigated with quarter-strength N-free Hoagland’s solution at pH 7.5. These results suggested that the nodulation of S. × utahensis ‘Torrey’ was improved when in a condition similar to natural habitat. Further research is needed to study the phylogenetic characteristics and nitrogen-fixing ability of the Frankia strains in the nodules of S. × utahensis ‘Torrey’.

**Literature Cited**


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Thomas, K.A. and A.M. Berry. 1989. Effects of continuous nitrogen application and
nitrogen preconditioning on nodulation and growth of *Ceanothus griseus* var.

stand age to nodulation of *Ceanothus velutinus*. For. Sci. 14:114-118.
Table 2-1. 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 set up in 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.

<table>
<thead>
<tr>
<th>First Harvest</th>
<th>Second harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height (cm)</td>
</tr>
<tr>
<td>F+N+</td>
<td>34.6 a</td>
</tr>
<tr>
<td>F+N-</td>
<td>24.7 b</td>
</tr>
<tr>
<td>F-N+</td>
<td>29.8 a</td>
</tr>
<tr>
<td>F-N-</td>
<td>22.9 b</td>
</tr>
</tbody>
</table>

Footnotes:

*Plants were harvested at 7 (first harvest) and 12 (second harvest) weeks after experiment initiation. Nodules were not observed at the first harvest but at the second harvest. Plants were grown in a commercial soilless substrate containing primarily peat moss.*

*y* Means within a column with same lowercase letters are similar according to the Tukey-Kramer method for multiplicity at \( \alpha = 0.05 \).

*x* Nodules were not observed.
Table 2-2. Chi-square analysis of the nodulation of *Shepherdia × utahensis* ‘Torrey’ irrigated with Hoagland’s solution plus (N+) or minus (N-) 2 mM ammonium nitrate (NH$_4$NO$_3$) at the second harvest.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Count</th>
<th>Nodulation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Unnodulated</td>
<td>Nodulated</td>
</tr>
<tr>
<td>N+</td>
<td>24</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>54.6</td>
<td>0</td>
<td>54.6</td>
</tr>
<tr>
<td>N-</td>
<td>8</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>18.2</td>
<td>27.3</td>
<td>45.5</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>12</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>72.7</td>
<td>27.3</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 2-3. Chi-square analysis of the nodulation of *Shepherdia ×utahensis* ‘Torrey’ with (F+) or without (F-) *Frankia* inoculation as measured at the second harvest.

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>Nodulation</th>
<th>Unnodulated</th>
<th>Nodulated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>F+</td>
<td>Count</td>
<td>16</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>% of total</td>
<td>36</td>
<td>18</td>
<td>54.6</td>
</tr>
<tr>
<td>F-</td>
<td>count</td>
<td>16</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>% of total</td>
<td>36</td>
<td>10</td>
<td>45.5</td>
</tr>
<tr>
<td>Total</td>
<td>Count</td>
<td>32</td>
<td>12</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>% of total</td>
<td>72.7</td>
<td>27.3</td>
<td>100</td>
</tr>
</tbody>
</table>
Fig. 2-1. 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).
Fig. 2-2. Nitrate-nitrogen (NO₃-N) concentration of leachate solution collected after

*Shepherdia × utahensis* ‘Torrey’ was irrigated in experiment 1. Four treatments
were set up in 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. Plants were harvested at 7 weeks
(first harvest) and 12 weeks (second harvest) after experiment initiation. The error
bars represent standard errors of five samples. Same lowercase letters are similar
according to the Tukey-Kramer method for multiplicity at α = 0.05.
Fig. 2-3. Regression analyses of the number of nodules (A), fresh weight of the largest nodule (B), and diameter of the largest nodule (C) of *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. Four plants were randomly chosen and harvested weekly. Nodules were found in the fifth week after experiment initiation.
CHAPTER III

NODULATION AND PLANT GROWTH OF SHEPHERDIA × UTAHENSIS ‘TORREY’ TOPDRESSED WITH CONTROLLED-RELEASE FERTILIZER

Abstract

Shepherdia × utahensis ‘Torrey’ (‘Torrey’ hybrid buffaloberry) is an actinorhizal plant, which can fix atmospheric nitrogen (N\(_2\)) in symbiotic root nodules with Frankia. Actinorhizal plants with N\(_2\)-fixing capacity are valuable in sustainable nursery production and urban landscape use. However, whether nodule formation occurs in S. × utahensis ‘Torrey’ and its interaction with nitrogen fertilization remains largely unknown. Increased mineral nitrogen (N) in fertilizer or in nutrient solution might not only inhibit nodulation of S. × utahensis ‘Torrey’ but also lead to excessive N leaching. In this study, S. × utahensis ‘Torrey’ plants inoculated with soils containing Frankia were irrigated with an N-free nutrient solution with or without added 2 mM ammonium nitrate (NH\(_4\)NO\(_3\)) or with 0.0 to 8.4 g·L\(^{-1}\) controlled-release fertilizer (CRF, 15N–3.9P–10K) to study nodulation and plant morphological and physiological responses. Plant performance of inoculated plants treated with various amounts of CRF was compared with uninoculated plants treated with the manufacturer’s prescribed rate. Plant growth, gas exchange parameters, and shoot N content increased quadratically or linearly along with increasing CRF application rate (all \(P < 0.01\)). All parameters did not increase significantly at CRF doses greater than 2.1 g·L\(^{-1}\). Further, the number of nodules per

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plant decreased quadratically \((P = 0.01)\) with increasing CRF application rates, and nodulation was completely inhibited at 2.9 g·L\(^{-1}\) CRF or by NH\(_4\)NO\(_3\) at 2 mM. According to our results, the nodulation of \(S. \times utahensis\) ‘Torrey’ was sensitive to N in the nutrient solution or in increasing CRF levels. Furthermore, plant growth, number of shoots, leaf area, leaf dry weight, stem dry weight, root dry weight, and N content of shoots of inoculated \(S. \times utahensis\) ‘Torrey’ plants treated with 2.1 g·L\(^{-1}\) CRF were similar to uninoculated plants treated with the manufacturer’s prescribed rate. Our results show that \(S. \times utahensis\) ‘Torrey’ plants inoculated with soil containing \(Frankia\) need less CRF than the prescribed rate to maintain plant quality, promote nodulation for N\(_2\)-fixation, and reduce N leaching.

**Introduction**

Nursery production of native plants has increased tremendously because of rising interest in using native plants in urban gardens and landscapes (Thomas and Schrock, 2004). The growing interest in native plants is due to their ornamental potential, including aesthetic appearance, bio-diversity, and, most important, water conservation (Hooper et al., 2008). In the Intermountain West (IMW), native plants are used in low water-use landscapes with sustainable and low water-use features (Mee et al., 2003). However, it is difficult to establish native plants in disturbed and poorly drained soils (Edmondson et al., 2011; Mee et al., 2003). Native plants in the IMW, such as \(Arctostaphylos patula\) (greenleaf manzanita), \(Artemisia nova\) (black sagebrush), \(Ceratoides lanata\) (winterfat), and \(Cercocarpus montanus\) (alderleaf mountain mahogany), are susceptible to overwatering and wet rooting substrates (Mee et al., 2003).
Parkinson et al. (2003) also reported that adequate drainage was essential for growing native plants in the IMW, for example, *Agave parryi* (Parry’s agave), *Aquilegia caerulea* (Colorado blue columbine), *Eriogonum niveum* (snow buckwheat), and *Eriophyllum lanatum* (woolly sunflower).

A similar scenario was discovered with *Shepherdia* species (buffaloberry). Anecdotal evidence strongly suggests that *Shepherdia rotundifolia* (roundleaf buffaloberry), a drought-adapted native plant native to the IMW, has high mortality in the nursery and landscape settings (Sriladda, 2011). To enhance the adaptability of *Shepherdia* to wet and poorly drained soils while maintaining drought tolerance, *S. × utahensis* ‘Torrey’, an interspecific hybrid of *S. rotundifolia* and *S. argentea* (silver buffaloberry), was created by Sriladda et al. (2016). This hybrid has potential for use in low-water landscapes because of tolerance to drought conditions, as well as occasionally wet soils found in residential landscape environments (Sriladda et al., 2016). Further, being an actinorhizal plant species, *S. × utahensis* ‘Torrey’ may be able to form a symbiotic association with *Frankia* to fix atmospheric nitrogen (*N*₂) in its root nodules (Sriladda, 2011). This biological *N*₂-fixing capacity reduces the need for nitrogenous fertilizers of nodulated actinorhizal plants, solving two primary concerns of the nursery industry, mineral nitrogen (N) runoff and leaching to groundwater (Urbano, 1989). For example, nodulated *Alnus maritima* (seaside alder) had better fertilizer-use efficiency than uninoculated plants when topdressed with controlled-release fertilizer (CRF, 15N–3.9P–10K) (Beddes and Kratsch, 2010). In another study, when irrigated with N-free nutrient solution supplemented with added NH₄NO₃, nodulation improved N-use efficiency of *Alnus incana* (gray alder) (Sellstedt and Huss-Danell, 1986). In the study by
Laws and Graves (2005), nodulated *A. maritima* sustained plant vigor and quality with less NH$_4$NO$_3$ concentration than uninoculated plants. Therefore, actinorhizal plants with symbiotic nodules have commercial potential in nursery production and urban landscapes (Kratsch and Graves, 2004).

Slow-release fertilizer (SRF) and CRF deliver mineral nutrients (mainly N) by gradually releasing them to plants and have been widely used in nursery production (Adams et al., 2013; Beddes and Kratsch, 2010), but excessive N fertilization reduces nodulation (Huss-Danell, 1997). Also, nodulation of actinorhizal plants is inhibited when nutrient solutions contains excessive N. For example, *A. maritima* exhibited decreased nodule activity when the NH$_4$NO$_3$ concentration of nutrient solution increased from 0 to 8.0 mM (Laws and Graves, 2005). In addition, Beddes and Kratsch (2010) reported that *A. maritima* plants had decreased nodule numbers when CRF (15N–3.9P–10K) levels increased from 0 to 1.8 g·L$^{-1}$, and 3.6 g·L$^{-1}$ completely inhibited nodulation. Although N fertilization significantly influences nodulation, the effects of fertilizers on plant growth and nodule development of *S. × utahensis* ‘Torrey’ is largely unknown. Research investigating the effects of CRF and its application rate on nodulation is needed to inform best practices for nurseries.

The objectives of this research were to 1) investigate the impacts of NH$_4$NO$_3$ added to N-free nutrient solution and CRF on nodule formation and plant growth of *S. × utahensis* ‘Torrey’ inoculated with field soils and 2) to determine CRF application rates that maintain acceptable plant quality with minimal nitrate-nitrogen (NO$_3$-N) leaching. In addition, the effect of inoculation on growth and gas-exchange parameters was studied by
comparing inoculated *S. × utahensis* ‘Torrey’ plants treated with 0 to 8.4 g·L\(^{-1}\) CRF with uninoculated plants treated with the manufacturer’s prescribed rate of 3.2 g·L\(^{-1}\).

**Materials and Methods**

*Shepherdia × utahensis* ‘Torrey’ plants were clonally propagated using cuttings collected from the Utah State University (USU) Greenville Research Farm (North Logan, UT) on 16 July 2019. Except for the two to three pairs of leaves at the top, bottom leaves of cuttings were removed. Cuttings then were dipped in 1,000 mg·L\(^{-1}\) indole-3-butyric acid (IBA) (Hormodin® 1; OHP, Mainland, PA) and stuck in a tray filled with perlite (Hess perlite, Malad City, ID). Trays were kept on a mist bench in a greenhouse with temperatures set at 25/20 °C (day/night). On 1 Oct. 2019, nodule-free rooted cuttings were transplanted into 3.8-L injection-molded, polypropylene containers (PC1D-4; Nursery Supplies, Orange, CA) filled with calcined clay (Turface MVP™; Profile Products, Buffalo Grove, IL), an inorganic growing substrate designed for IMW native plants (Beddes and Kratsch, 2009). Plants were irrigated with deionized water prior to the experiment initiation.

The experiment was initiated on 15 Oct. 2019, and plants of uniform size were randomly assigned to 12 groups. In previous studies, soil collected from the rhizosphere of an actinorrhizal plant has been used as a source of *Frankia* inocula (Tortosa and Cusato, 1991). In this study, soils (≈ 8 L) collected from the rhizosphere of *S. × utahensis* ‘Torrey’ (41.765741, -111.813175) at Greenville Research Farm, which can induce symbiotic nodules on *S. × utahensis* ‘Torrey’ containing *Frankia* (unpublished data), was used to inoculate plant receiving various levels of CRF or N-free nutrient solution with or
without NH$_4$NO$_3$. Plants in groups 1 to 10 were inoculated with 50 ml of field soil layered on the surface of substrate. For groups 1 to 8, each plant was topdressed with 0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 g of Osmocote 15N–3.9P–10K [Osmocote® Plus 15-9-12; Israel Chemicals, Tel Aviv-Yafo, Israel (hereafter referred to as CRF)], resulting in CRF levels at 0, 0.1, 0.3, 0.5, 1.1, 2.1, 4.2, and 8.4 g·L$^{-1}$, respectively. Plants in groups 9 and 10 were irrigated with 500 ml modified N-free nutrient solution (nutrient solution) (Bugbee, 2004) with and without added 2 mM NH$_4$NO$_3$, respectively, at pH 7.5 every other day, resulting in a NO$_3$-N concentration at 114.9 ± 6.8 mg·L$^{-1}$ and 13.0 ± 1.1 mg·L$^{-1}$ (mean ± standard error), respectively. Plants in group 11 had no inoculation but received 3.2 g·L$^{-1}$ of CRF following the manufacturer’s recommended application rate for the plant in a 3.8-L container. Plants in group 12 were not inoculated or fertilized to confirm whether non-inoculated, unfertilized plants form nodules. Except for the plants in groups 9 and 10, plants were irrigated with 500 ml tap water every other day. A saucer was placed under a container before irrigation to collect leachate for measurements. A NO$_3$-N meter (LAQUA Twin; Horiba, Kyoto, Japan) and a pH meter (LAQUA Twin; Horiba, Kyoto, Japan) were used to record NO$_3$-N concentration and pH of solution. The NO$_3$ concentration in the leachate of plants treated with CRF was calculated using the difference between NO$_3$ concentration in irrigated water and in leachate to correct the variation from background nitrogen.

Gas-exchange parameters, including leaf net photosynthesis rate ($P_n$), stomatal conductance ($g_s$), and transpiration rate ($E$), were recorded for plants treated with 0, 2.0, 8.0, and 32.0 g of CRF one week before the experiment was terminated. The parameters were recorded using a Portable Photosynthesis System with a PLC3 Universal Leaf
Cuvette (CIRAS-3; PP Systems, Amesbury, MA) on a sunny day between 1000 and 1400 HR. Within the cuvette, the intensity of photosynthetic photon flux density was set at 1000 μmol·m⁻²·s⁻¹ with 38% red, 37% green, and 25% blue light provided from light-emitting diodes, while the carbon dioxide and leaf temperature were controlled at 400 μmol·mol⁻¹ and 25 °C, respectively.

The experiment was terminated on 8 Dec. 2019. Plant height was recorded at the initiation and termination of the experiment to determine growth. Before all plants were destructively harvested, the number of shoots (longer than 5 cm) was recorded for each plant. All S. × utahensis ‘Torrey’ plants were destructively harvested, and leaf area was recorded using a leaf area meter (LI-3000; LI-COR Biosciences, Lincoln, NE). After roots were harvested and washed using deionized water, plants were checked for nodules. Leaves, stems, and roots were dried in an oven at 80 °C for three days, and the dry weight was recorded. Dry leaf and stem samples were grounded and analyzed at the USU Analytical Laboratories for N content with an elemental analyzer (vario MAX cube; Elementar Analysensysteme GmbH, Langenselbold, Germany).

The experiment used a randomized complete block design with ten blocks for all groups. An analysis of variance (ANOVA) procedure was used to test the effects of treatments on plant morphological and physiological responses. Means separation among treatments was adjusted using Tukey-Kramer method for multiplicity at α = 0.05. All statistical analyses were carried out using PROC Mixed procedure in a SAS Studio (SAS Institute, Cary, NC).
**Results**

**Leachate**

The nutrient solution with or without 2 mM NH₄NO₃ led to leachate NO₃-N concentrations of 191.2 mg·L⁻¹ and 19.4 mg·L⁻¹, respectively, during the experiment. With those plants treated with CRF, NO₃-N concentration in leachate increased as the applied CRF levels increased (Fig. 3-1). The NO₃-N concentration of leachate collected from inoculated plants receiving 2.1 to 8.4 g·L⁻¹ of CRF increased quadratically during the experiment (all \( P < 0.002 \), all \( r^2 > 0.58 \)) (data not shown), but inoculated plants receiving 0 to 1.1 g·L⁻¹ CRF and uninoculated plants receiving manufacturer’s prescribed rate did not. The NO₃-N concentration of leachate increased linearly as the amount of CRF rose from 0 to 8.4 g·L⁻¹ (all \( P < 0.0001 \), all \( r^2 > 0.84 \)) (data not shown). The NO₃-N concentration of leachate from plants treated with 0 to 2.1 g·L⁻¹ CRF was 80% to 28% less than the NO₃-N concentration of leachate from uninoculated plants treated with the manufacturer’s prescribed rate (Fig. 3-1). On the other hand, inoculated plants treated with 4.2 g·L⁻¹ and 8.4 g·L⁻¹ CRF had 142% and 279% greater NO₃-N leachate concentration, respectively, than uninoculated plants treated at the manufacturer’s prescribed rate.

**Plant growth, number of shoots, and leaf area**

Plant growth of *S. × utahensis* ‘Torrey’ plants irrigated with a nutrient solution plus 2 mM NH₄NO₃ was three times greater than plants treated with the same solution without NH₄NO₃ (Table 3-1). Regression analysis indicated a quadratic relationship between CRF and plant growth (\( P = 0.002; R^2 = 0.95 \)) (Fig. 3-2), and the plant growth is shown in Fig. 3-3. Plant growth of inoculated *S. × utahensis* ‘Torrey’ plants treated with
1.1 to 8.4 g·L⁻¹ CRF increased one to six times compared with plants treated with 0 g·L⁻¹ CRF. A quadratic trend showed between CRF and the number of shoots of *S. ×utahensis* ‘Torrey’ (*P* = 0.003; *R²* = 0.95) (Fig. 3-2). Except for the plants receiving 0.3 g·L⁻¹ CRF, *S. ×utahensis* ‘Torrey’ plants treated with 0.1 to 8.4 g·L⁻¹ CRF had one to four times more shoots than plants treated with 0 g·L⁻¹ CRF. Leaf area of *S. ×utahensis* ‘Torrey’ irrigated with the nutrient solution plus 2 mM NH₄NO₃ was four times as much as leaf area of plants irrigated with the same solution without NH₄NO₃ (Table 3-1). When plants were fertilized with increasing amounts of CRF, a quadratic relationship was found between leaf area and CRF (*P* = 0.007; *R²* = 0.98) (Fig. 3-2). When *S. ×utahensis* ‘Torrey’ plants were treated with 1.1 to 8.4 g·L⁻¹ CRF, leaf area was one to three times greater than those treated with 0 g·L⁻¹ CRF. No significant difference was found on these parameters between uninoculated *S. ×utahensis* ‘Torrey’ plants treated with the manufacturer’s prescribed rate and the inoculated plants treated with 1.1 g·L⁻¹ and 2.1 g·L⁻¹ CRF.

**Dry weight of leaf, stem, and root**

Leaf dry weight of *S. ×utahensis* ‘Torrey’ plants irrigated with a nutrient solution plus 2 mM NH₄NO₃ was two times greater than plants treated with the same nutrient solution minus NH₄NO₃ (Table 1). A quadratic relationship was found between leaf dry weight and CRF amount in inoculated plants (*P* = 0.002; *R²* = 0.97) (Fig. 3-2). Leaf dry weight of *S. ×utahensis* ‘Torrey’ plants treated with 2.1 g·L⁻¹, 4.2 g·L⁻¹, and 8.4 g·L⁻¹ CRF was three, four, and five times, respectively, greater than those treated with 0 g·L⁻¹ CRF. Nutrient solution with 2 mM NH₄NO₃ increased stem dry weight of *S. ×utahensis* ‘Torrey’ plants by 200% compared with plants treated with the same solution without
When inoculated *S. × utahensis* ‘Torrey’ plants were treated with increasing amounts of CRF, a quadratic relationship was found between CRF and stem dry weight (*P* = 0.03; *R*² = 0.98) (Fig. 3-2). Compared with plants treated with 0 g·L⁻¹ CRF, stem dry weight of *S. × utahensis* ‘Torrey’ treated with 4.2 g·L⁻¹ and 8.4 g·L⁻¹ CRF increased two and three times, respectively. *Shepherdia × utahensis* ‘Torrey’ had four times greater root dry weight when irrigated with the nutrient solution with added 2 mM NH₄NO₃ compared with plants irrigated with the same solution minus NH₄NO₃ (Table 3-1). A quadratic relationship showed between CRF and root dry weight of *S. × utahensis* ‘Torrey’ (*P* = 0.01; *R*² = 0.91) (Fig. 3-2), and root dry weight increased about five times for the plants treated with 4.2 g·L⁻¹ and 8.4 g·L⁻¹ CRF compared with plants treated with 0 g·L⁻¹ CRF. These parameters in uninoculated *S. × utahensis* ‘Torrey’ treated with the manufacturer’s prescribed rate were not different from inoculated *S. × utahensis* ‘Torrey’ plants treated with 1.1 g·L⁻¹ and 2.1 g·L⁻¹ CRF (Fig. 3-2).

**Gas exchange and shoot N content**

A positive linear relationship was exhibited between CRF and the *Pₚₙ* of *S. × utahensis* ‘Torrey’ (*P* < 0.0001; *r*² = 0.74) (Fig. 3-4). The *Pₚₙ* of *S. × utahensis* ‘Torrey’ plants treated with 2.1 g·L⁻¹ and 8.4 g·L⁻¹ CRF increased 12 and 16 times, respectively, compared with plants treated with 0 g CRF. The *gₛ* of *S. × utahensis* ‘Torrey’ increased linearly with increasing CRF (*P* = 0.004; *r*² = 0.66) (Fig. 3-4), and the *gₛ* of *S. × utahensis* ‘Torrey’ plants treated with 8.4 g·L⁻¹ CRF was 72% greater than plants treated with 0 g·L⁻¹ CRF. The *E* of *S. × utahensis* ‘Torrey’ had a positive linear trend with increasing CRF (*P* = 0.005; *r*² = 0.69). *Shepherdia × utahensis* ‘Torrey’ plants treated with 8.4 g·L⁻¹ CRF had a 54% increase in *E* compared with plants treated with 0 g·L⁻¹ CRF.
statistical difference was found in P_n, g_s, and E between S. xutahensis ‘Torrey’ plants receiving 2.1 g·L⁻¹ and 8.4 g·L⁻¹ CRF.

The shoot N content of the inoculated S. xutahensis ‘Torrey’ plants increased linearly with CRF (P < 0.0001; r² = 0.84). When S. xutahensis ‘Torrey’ was fertilized with 2.1 g·L⁻¹ and 8.4 g·L⁻¹ CRF, shoot N content increased by 44% and 71%, respectively, compared with the plants treated with 0 g·L⁻¹ CRF. There was no statistical difference in shoot N content between inoculated S. xutahensis ‘Torrey’ treated with 2.1 g·L⁻¹ or 8.4 g·L⁻¹ CRF and uninoculated plants that received the manufacturer’s prescribed rate (Fig. 3-4).

**Nodulation**

Nodules formed on S. xutahensis ‘Torrey’ plants inoculated with Frankia-infected soils in our experiment (P < 0.0001), while plants without inoculation and treated with 0 g·L⁻¹ CRF did not form any nodules (data not shown). Further, uninoculated plants treated with the CRF manufacturer’s recommended rate did not show nodulation. The NH₄NO₃ in the nutrient solution and CRF affected the nodulation of S. xutahensis ‘Torrey’ (both P < 0.0001) (data not shown). When irrigated with the nutrient solution with added 2 mM NH₄NO₃, one nodule showed on one out of ten inoculated S. xutahensis ‘Torrey’ plants (Table 5-1). In contrast, for plants irrigated with the nutrient solution without added NH₄NO₃, all plants exhibited nodulation, with ten nodules per plant on average. For the inoculated plants treated with CRF, the number of nodules per plant decreased as CRF increased (P = 0.01; R² = 0.74) (Fig. 3-5). When inoculated with soils containing infective Frankia strains, S. xutahensis ‘Torrey’ topdressed with 8.4 g·L⁻¹ CRF did not form any nodules, while one out of ten plants treated with 4.2 g·L⁻¹ CRF
had one nodule. According to regression analysis, 2.9 g·L\(^{-1}\) CRF completely inhibited nodulation. The dry weight of nodules increased when CRF levels increased (\(P = 0.006; R^2 = 0.94\)). The dry weight of nodules on plants treated with 2.1 g·L\(^{-1}\) CRF was 13 times greater than plants treated with 0 g·L\(^{-1}\) CRF (Fig. 3-5).

**Discussion**

**Leachate**

Factors such as environmental temperature and physical properties of CRF coating affect the release rate of CRF, but, generally, Osmocote has a relatively rapid release rate initially followed by a steadily decreasing rate of release over time (Adams et al., 2013). A similar pattern also found on leachate NO\(_3\)-N concentrations increased at early stage and then decreased in the leachate of plants that received 2.1 to 8.4 g·L\(^{-1}\) CRF. Nitrate ion is hard to be retained on the cation exchange site of substrate and primarily removed by leachate. Therefore, the change of NO\(_3\)-N concentration in leachate might relate to the release rate of CRF. The trend of rising NO\(_3\)-N concentrations in the leachate at the early stages of the experiment and then declining concentrations at later stages was also reported by Glenn et al. (2000) and Niemiera and Leda (1993). This pattern is also reported in *A. maritima* plants were grown in a substrate containing peat and vermiculite and treated with CRF, NO\(_3\)-N concentration in the leachate increased from 1 to 14 days for those plants that received 3.6 g·L\(^{-1}\) and 7.3 g·L\(^{-1}\) Osmocote 15N-3.9P-10K, and then decreased (Beddes and Kratsch, 2010). The increasing NO\(_3\)-N concentration in growing substrates during the early period of the experiment might also delay nodule formation. Therefore, caution should be taken in nursery production when growing nodulated
actinorhizal plants in substrates with high CEC, such as peat moss, to prevent N accumulation (Beddes and Kratsch, 2010).

Uninoculated plants treated with the manufacturer’s prescribed rate had less NO$_3$-N in leachate than inoculated plants treated with 4.2 g·L$^{-1}$ and 8.4 g·L$^{-1}$ CRF at 11 days after experiment initiation. Since the NO$_3$-N concentration of leachate was affected by the level of CRF, it might result from the higher CRF dosage causing higher concentrations of NO$_3$-N in the leachate, while the irregular NO$_3$-N concentration at the 4$^{th}$ day of the experiment might associate with higher variation at the beginning of the experiment.

**Morphological and physiological responses**

In our study, although morphological and physiological parameters of *S. x utahensis* ‘Torrey’ increased linearly or quadratically with increasing CRF dosage, the parameters did not change significantly when fertilizer doses exceeded 2.1 g·L$^{-1}$ CRF ($\approx$ 0.3 g N·L$^{-1}$). According to Taiz et al. (2015), morphological and physiological responses do not correlate well with nutrient availability when nutrient concentration exceeds a certain range (Taiz et al., 2015). For morphological responses, the shoot dry weight of *Salvia farinacea* (mealycup sage) increased quadratically with Osmocote 39N-0P-0K increasing from 0.5 to 3.0 g N·L$^{-1}$, but increasing the rate of Osmocote 39N-0P-0K did not contribute to shoot dry weight when it exceeded 2.0 g N·L$^{-1}$ (Knowles et al., 1993). Beddes and Kratsch (2010) also reported that leaf area, shoot dry weight, and root dry weight of *A. maritima* did not increase when Osmocote 15N–3.9P–10K was more than 3.6 g·L$^{-1}$ ($\approx$ 0.5 g N·L$^{-1}$). For physiological parameters, $P_n$ of *Abies fraseri* (fraser fir), *Picea glauca* (white spruce), *Picea pungens* (blue spruce), and *Pinus strobus* (eastern
white pine) increased when Osmocote 15N–3.9P–10K ranged from 0.25 to 0.5 g N·L⁻¹, but it did not change when Osmocote 15N–3.9P–10K was greater than 0.5 g N·L⁻¹ (Klooster et al., 2010). In a study by Zhang et al. (2011), the Pₙ of *Hosta clausa* (hosta) did not increase when controlled-release N fertilizer 46N-0P-0K exceeded 0.3 g·L⁻¹ (≈ 0.1 g N·L⁻¹) at 90 and 120 days after treatment. Therefore, it is important to define the minimum application rate of CRF to maintain plant vigor and photosynthesis while reducing fertilizer costs and the potential for groundwater contamination (Poole and Conover, 1989). Based on the results of our study, an effective CRF application rate for inoculated *S. xutahensis* ‘Torrey’ should be topdressed with 2.1 g·L⁻¹ (≈ 0.3 g N·L⁻¹) to maintain plant vigor and reduce NO₃-N leaching.

**Nitrogen content and nodulation**

Nitrogen concentration in plant tissues in our study increased linearly with increasing fertilizer levels. Klooster et al. (2010) reported that the nitrogen content of *A. fraseri, P. glauca, P. pungens*, and *P. strobus* needles increased when Osmocote 15N–3.9P–10K increased from 0.25 and 0.5 g N·L⁻¹. Also, the N content of *Pilea* ‘Silver Tree’, *Aphelandra squarrosa* (zebra plant), *Chamaedorea elegans* (parlor palm), and *Dieffenbachia maculata* ‘Camille’ (‘Camille’ dumb cane) increased linearly from 0.5 to 1.5 g·L⁻¹ (≈ 0.07 to 0.2 g N·L⁻¹) with increasing 14N-6.2P-11.6K slow-release fertilizer level (SRF) (Poole and Conover, 1989). In the study by Knowles et al. (1993), shoot N content of *Salvia farinacea* showed a quadratic trend with increasing Osmocote 39N-0P-0K. The N content of *A. maritima* increased quadratically with increasing application rate of Osmocote 15N–3.9P–10K, but the shoot N contents of nodulated *A. maritima* plants that received 0.9 to 1.8 g·L⁻¹ CRF (≈ 0.1 to 0.3 g N·L⁻¹) were not different from
uninoculated plants that received manufacturer’s prescribed rate of 2.7 g·L\(^{-1}\) CRF (≈ 0.4 g N·L\(^{-1}\)) because of the N\(_2\)-fixing ability of Frankia (Beddes and Kratsch, 2010). Also, although leaf N of A. maritima plants increased along with NH\(_4\)NO\(_3\) levels in the nutrient solution, Laws and Graves (2005) reported that symbiotic nodules with N\(_2\)-fixing ability formed at concentrations of NH\(_4\)NO\(_3\) that ranged from 0.5 to 2 mM. Due to the N\(_2\)-fixation of Frankia enhancing N content with less fertilizer, inducing nodules in novel actinorhizal plants using field soil is a preferred practice in sustainable nurseries, which are concerned with the economic and ecological impact of chemical fertilizers (Kratsch and Graves, 2004).

In our study, soil used for inoculation was collected from the rhizosphere of a S. ×utahensis ‘Torrey’. Soil inoculation is a preferred practice to induce nodules on actinorhizal plants because soils from wild population habitats have dense populations of infective Frankia and host plants are the primary factor in amplifying Frankia populations in the soil (Benson and Silvester, 1993; Schwencke and Caru, 2001). In previous studies, soils collected from the natural habitat of wild populations has been used to induce nodules of actinorhizal plants (Beddes and Kratsch, 2010; Laws and Graves, 2005; Jeong and Myrold, 2001). Our results also suggest that soil collected from North Logan has infective Frankia and can induce symbiotic nodules of S. ×utahensis ‘Torrey’.

On the other hand, nodulation of actinorhizal plants is inhibited by excessive N, and the N sensitivity of nodulation varies among actinorhizal plants (Huss-Danell, 1997). In our study, nodulation of S. ×utahensis ‘Torrey’ was inhibited by 2.9 g·L\(^{-1}\) CRF (≈ 0.4 g N·L\(^{-1}\)) or a nutrient solution with 2 mM NH\(_4\)NO\(_3\). Thomas and Berry (1989) reported
that the nodule number on *Ceanothus griseus* (Carmel ceanothus) was significantly reduced at 0.714 mM NH$_4$NO$_3$ and completely inhibited at 2.68 mM NH$_4$NO$_3$.

Nodulation of *Alnus glutinosa* (black alder), *Casuarina cunninghamiana* (river oak), and *Myrica cerifera* (southern wax myrtle) was inhibited when the concentration of NO$_3^-$ was greater than 1 mM (Kohls and Baker, 1989), but nodule number of *Elaeagnus angustifolia* (Russian olive) was not affected at 3 mM NO$_3^-$. *Purshia mexicana* (Mexican cliffrose) and *Purshia tridentata* (antelope bitterbrush) did not form nodules when exposed to 6 mM NH$_4$NO$_3$ (Righetti et al., 1986). Nodule number decreased linearly when the NH$_4$NO$_3$ concentration increased from 0.25 to 4 mM and was completely inhibited in *A. maritima* at 8 mM NH$_4$NO$_3$ (Laws and Graves, 2005). Nodule number of *A. maritima* declined when Osmocote 15N-3.9P-10K exceeded 1.8 g·L$^{-1}$ ($\approx 0.3$ g N·L$^{-1}$) and was completely inhibited at 3.6 g·L$^{-1}$ ($\approx 0.5$ g N·L$^{-1}$) (Beddes and Kratsch, 2010). Also, nodule dry weight increased with application rates of Osmocote 15N-3.9P-10K between 0 to 0.9 g·L$^{-1}$ ($\approx 0$ to 0.1 g N·L$^{-1}$) (Beddes and Kratsch, 2010). Compared with *A. maritima*, a relatively lower concentration of NH$_4$NO$_3$ in nutrient solution or CRF level prevents the nodule growth of *S. × utahensis* ‘Torrey’. Therefore, *S. × utahensis* ‘Torrey’ may be relatively sensitive to environmental N content.

Apart from N, mineral nutrients in the CRF might also affect the nodulation of *S. × utahensis* ‘Torrey’. Although NO$_3$-N and ammonium-nitrogen (NH$_4$NO$_3$-N) are the primary nutrients in Osmocote 15N-3.9P-10K, it contains mineral nutrients such as phosphate and iron (Adams et al., 2013). Although a negative correlation was shown between N concentration and nodule number in our study, the macro- and micronutrients in CRF may also limit nodulation. This has been documented by Huss-Danell (1997) in a
study that showed phosphate, cobalt, iron, calcium, and sodium affected the nodulation of actinorhizal plants. Especially when using CRF or SRF in the nursery, it is difficult to control the concentration of the macro- and micronutrients released. The macro- and micronutrients in CRF or SRF may also have a co-effect with N on inhibition of nodulation.

With acceptable plant quality and minimal NO$_3$-N concentration in leachate, Beddes and Kratsch (2010) concluded that 0.9 g·L$^{-1}$ Osmocote 15N-3.9P-10K was the proper application rate to produce nodulated A. maritima. In addition, NH$_4$NO$_3$ at 0.5 to 2.0 mM in nutrient solution was recommended by Laws and Graves (2005) for A. maritima with symbiotic nodules, to enhance leaf N as well as maintain plant vigor. In our research, plant growth was similar between the inoculated plants fertilized with 1.1 g and 2.1 g·L$^{-1}$ CRF and the uninoculated plants that received the manufacturer’s prescribed rate. Furthermore, inoculated plants treated with 2.1 g·L$^{-1}$ CRF had a similar tissue N content to the uninoculated plants that received the manufacturer’s prescribed rate. Therefore, 1.1 g·L$^{-1}$ or 2.1 g·L$^{-1}$ CRF may be the proper application rate of S. ×utahensis ‘Torrey’.

The N$_2$-fixation capacity of nodules of S. ×utahensis ‘Torrey’ was undefined in our study. Further investigation is needed to test the N$_2$-fixation of S. ×utahensis ‘Torrey’ using acetylene-reduction assays (Laws and Graves, 2005). Although the N$_2$-fixation assays were not performed, the results that uninoculated plants receiving the manufacturer’s prescribed rate and nodulated plants treated with 2.1 g·L$^{-1}$ CRF had similar shoot N content suggests that enough N$_2$-fixation occurred in the nodulated plants to improve N concentration and plant growth with less fertilizer.
Conclusions

Although growth and physiological responses of *S. ×utahensis* ‘Torrey’ were improved by increasing CRF, increased CRF led to higher NO$_3$-N concentration in the leachate. No significant difference in morphological and physiological parameters and shoot N contents showed for the inoculated plants when CRF dosage exceeded 2.1 g·L$^{-1}$. Further, when compared with the uninoculated plants treated with the manufacturer’s prescribed rate, nodulated plants treated with 1.1 g·L$^{-1}$ CRF had similar morphological and growth responses, and nodulated plants at 2.1 g·L$^{-1}$ had similar shoot N content. The number of nodules of *S. ×utahensis* ‘Torrey’ was inhibited along with increased CRF application rates and was completely inhibited by 2.9 g·L$^{-1}$ of CRF or 2 mM NH$_4$NO$_3$. Therefore, when nodulated *S. ×utahensis* ‘Torrey’ plants are produced in the nursery, CRF lower than 2.9 g·L$^{-1}$ or NH$_4$NO$_3$ level below 2 mM should be applied. Rates between 1.1 g·L$^{-1}$ and 2.1 g·L$^{-1}$ CRF may be sufficient for nodulated *S. ×utahensis* ‘Torrey’ to sustain acceptable visual quality and promote nodulation for N$_2$-fixation with minimal nitrate leachate.
Literature Cited


Table 3-1. Plant growth, number of shoots, leaf area, dry weight (DW) of leaf, stem, and root, and number of nodules of *Shepherdia × utahensis* ‘Torrey’ inoculated with field soils and irrigated with nitrogen (N)-free nutrient solutions (Bugbee, 2004) plus or minus 2 mM ammonium nitrate (NH$_4$NO$_3$) for eight weeks.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant growth (cm)</th>
<th>Shoots (no.)</th>
<th>Leaf area (cm$^2$)</th>
<th>Leaf DW (g)</th>
<th>Stem DW (g)</th>
<th>Root DW (g)</th>
<th>Nodules (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-free Nutrient Solution with 2 mM NH$_4$NO$_3$</td>
<td>8.4</td>
<td>2.7</td>
<td>154.2</td>
<td>1.6</td>
<td>0.6</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>N-free Nutrient Solution</td>
<td>2.2</td>
<td>0.1</td>
<td>34.6</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
<td>10.8</td>
</tr>
</tbody>
</table>

$P$ value **$\gamma$** **$\gamma$** ***$\gamma$*** $*$ $*$ $*$ $*$

$^2$ Plant growth was calculated as the difference between plant height at the initiation (15 Oct. 2019) and termination (8 Dec. 2019) of the experiment.

$^\gamma$ **$\gamma$** = significant at $P \leq 0.01$ and $P \leq 0.001$, respectively.
Fig. 3-1. Nitrate-nitrogen (NO$_3$-N) in the leachate recorded of *Shepherdia × utahensis* ‘Torrey’ plants topdressed with 0 to 8.4 g·L$^{-1}$ controlled-release fertilizer (CRF, 15N–3.9P–10K) were irrigated with tap water. The background nitrogen in tap water was corrected by calculating the difference between NO$_3$-N concentration in leachate and tap water. The error bars represent the standard errors of five samples.
Fig. 3-2. Plant growth, number of shoots, leaf area, leaf dry weight, stem dry weight, and root dry weight of *Shepherdia × utahensis* ‘Torrey’ inoculated with soil containing infective *Frankia* and topdressed with 0 to 8.4 g·L\(^{-1}\) controlled-release fertilizer (CRF, 15N–3.9P–10K) for eight weeks. The round markers represent means of the plants inoculated with *Frankia*, while the square markers represent the uninoculated plants treated with manufacturer’s prescribed rate of 3.2 g·L\(^{-1}\). The error bars represent the standard errors of ten samples. No significant difference was found between the inoculated plants treated with 1.1 g·L\(^{-1}\) and 2.1 g·L\(^{-1}\) CRF and uninoculated plants treated with the manufacturer’s prescribed rate according to Tukey-Kramer method for multiplicity at \(\alpha = 0.05\).
Fig. 3-3. _Shepherdia × utahensis_ ‘Torrey’ plants inoculated with soil containing infective _Frankia_ topdressed with 0 to 8.4 g·L⁻¹ controlled-release fertilizer (CRF, 15N–3.9P–10K) and the uninoculated plant that received the manufacturer’s prescribed rate of 3.2 g·L⁻¹. Inoculated _S. × utahensis_ ‘Torrey’ plants that received 1.1 g·L⁻¹ and 2.1 g·L⁻¹ CRF had similar plant vigor to uninoculated plants that received the manufacturer’s prescribed. (Photos were taken at the eighth week of the experiment.)
Fig. 3-4. The photosynthesis rate ($P_n$), stomatal conductance ($g_s$), transpiration rate ($E$), and nitrogen content of shoot of *Shepherdia × utahensis* ‘Torrey’ topdressed with 0 g·L$^{-1}$, 0.5 g·L$^{-1}$, 2.1 g·L$^{-1}$, and 8.4 g·L$^{-1}$ of controlled-release fertilizer (CRF, 15N–3.9P–10K) for eight weeks. The error bars represent standard errors of four measurements or samples. The round markers represent means of the plants inoculated with *Frankia*, while the square marker in the shoot nitrogen content represents the uninoculated plants that received the manufacturer’s prescribed rate of 3.2 g·L$^{-1}$ CRF. No significant difference was found in the nitrogen content of shoots between the inoculated plants treated with 2.1 g·L$^{-1}$ CRF and uninoculated plants treated with the manufacturer’s prescribed rate according to Tukey-Kramer method for multiplicity at $\alpha = 0.05$. 
Fig. 3-5. The number of nodules per plant and nodule dry weight of *Shepherdia × utahensis* ‘Torrey’ inoculated with soil containing infective *Frankia* and topdressed with 0 to 8.4 g·L⁻¹ controlled-release fertilizer (CRF, 15N–3.9P–10K) for eight weeks. The error bars represent standard errors of ten samples.
CHAPTER IV

PHYLOGENETIC ANALYSIS OF FRANKIA STRAINS IN THE ROOT NODULES OF SHEPHERDIA ×UTAHENSIS ‘TORREY’ USING NIFH GENE AMPLIFICATION

Abstract

*Shepherdia ×utahensis* ‘Torrey’ (hybrid buffaloberry) is an actinorhizal plant that can form symbiotic nodules with *Frankia*. However, no research has been conducted to investigate the *Frankia* in the nodules of *S. ×utahensis* ‘Torrey’. In addition, the effectiveness (nitrogen-fixing ability) of the *Frankia* strains in nodules is also unknown. In this study, *S. ×utahensis* ‘Torrey’ plants grown in a commercial growing substrate or pure perlite were inoculated with soil samples from wild *Shepherdia* plants to trap *Frankia*. The nodules of *S. ×utahensis* ‘Torrey’ plants were sampled to extract DNA, and nitrogenase (*nifH*) gene sequences were amplified using PolF/PoIR primers. Four 300-bp fragments (SU1, SU2, SU3, and SU4) of query sequences were obtained from nodules. The *nifH* gene sequence amplification in our study may suggest that *Frankia* strains in the root nodules of *S. ×utahensis* ‘Torrey’ have the potential to fix atmospheric nitrogen. When compared with *nifH* gene sequences reported in the literature using Basic Local Alignment Search Tool (BLAST), over 90% similarity to the *nifH* of *Frankia* was obtained. The *Frankia* strains in the nodules shared similar *nifH* sequences with those within the same host-specific group of *Shepherdia*. Further, *Frankia* strains with similar *nifH* genes have been reported in nodules of *Shepherdia argentea* (silver buffaloberry). In addition, *Frankia* strains belong to the cluster 3 infective strains, consisting of the Elaeagnaceae and Rhamnaceae infective *Frankia*, showed high similarity to the query
sequences. In summary, *Frankia* strains in nodules of *S. xutahensis* ‘Torrey’ have consistent phylogenetic characteristics to previously reported Elaeagnaceae-infective *Frankia* and share similar *Frankia* strains to its parents.

**Introduction**

Actinobacteria, *Frankia*, form symbiosis with host plants, actinorhizal plants, to induce nitrogen-fixing nodules that benefit plants in thriving in nitrogen-deficient areas (Huss-Danell, 1997). *Frankia* in symbiotic nodules are highly diverse, but most of the *Frankia* have not been cultured *in vitro* (Schwencke and Caru, 2001). Therefore, to study the diversity of infective *Frankia* strains (which are *Frankia* strains that are able to induce nodules), host plants are grown in substrates incorporated with field soils collected from their habitats to trap *Frankia* (Benson et al., 2004; Huss-Danell, 1997).

For instance, infective *Frankia* strains were trapped via growing *Ceanothus velutinus* (snowbrush ceanothus), *Ceanothus sanguineus* (redstem ceanothus), and *Ceanothus integerrimus* (deer brush) in a soilless substrate and irrigated with soil suspensions that were extracted from soils collected from two stands dominated by *C. velutinus* (host plant) and *Pseudotsuga menziesii* (Douglas-fir) (non-host plant) (Jeong and Myrold, 2001). In another study, the population of *Alnus*-infective *Frankia* was investigated when *Alnus incana* (gray alder) and *Lupinus nootkatensis* (Alaska lupine) were grown in soils collected from natural habitats (Myrold and Huss-Danell, 1994).

Phylogenetic analysis is conducted to investigate the diversity of *Frankia* strains in nodules of host plants (Benson and Clawson, 2000). Based on the results of cross-infectivity studies using liquid inoculation, *Frankia* strains were classified into four host-
specificity groups (HSGs) according to their host plants (Baker, 1987; Du and Baker, 1992). In this system, *Frankia* strains that induce nodules on plants in *Alnus, Comptonia* and *Myrica* are grouped in HSG 1, those induce nodules on plants in *Casuarina* and *Myrica* are classified in HSG 2, those induce nodules on plants in Elaeagnaceae and *Myrica* are included in HSG 3, while those only induced nodules on plants in Elaeagnaceae are in HSG 4 (Baker, 1987; Du and Baker, 1992). Although this system predicts the infectivity of *Frankia*, it is impossible to determine the HSG groups of all *Frankia* strains since several *Frankia* strains are unable to be cultured *in vitro*, and cross-boundary infectivity occurs among HSGs (Huss-Danell, 1997).

Recently, researchers use comparative sequence analyses (e.g., 16S rRNA, *nif*H, *rec*A, and *gln*II) to investigate the phylogeny of *Frankia* strains in nodules (Pawlowski and Bergman, 2007). This approach doesn’t require successful isolation of *Frankia* strains and is thus an important way to study symbiotic microbes in nodules since *Frankia* is hard to isolate (Huss-Danell, 1997; Schwencke and Caru, 2001). For instance, *Frankia* strains in nodules of *Ceanothus velutinus, Ceanothus sanguineus*, and *Ceanothus integerrimus*, which are difficult to culture, were investigated using comparative sequence analyses (Jeong and Myrold, 2001). They observed common *Frankia* strains in nodules of ceanothus species inoculated with different soil samples (Jeong and Myrold, 2001). Vanden Heuvel et al. (2004) compared 23S rRNA gene sequence of *Frankia* strains in the nodules of actinorhizal plants in the California west of the Sierra Nevada crest, and found low diversity of infective *Frankia* strains of multiple host plants such as *Ceanothus* plants and *Datisca glomerata* (durango root) plants. Comparative sequence analysis is also important to identify other microorganisms in the nodule of actinorhizal
plants. According to Huss-Danell (1997), actinorhizal plants formed nodules without *Frankia* because the fungus *Penicillium nodositatum* can also induce nodules that are incapable of fixing atmospheric nitrogen.

Normand et al. (1996) compared 16S rDNA of infective *Frankia* strains from different host plants and classified them into four groups, group 1 contained *Casuarina*-infective and *Alnus*-infective strains with nitrogen-fixing ability, group 2 included *Frankia* from *Dryas, Coriaria*, and *Datisca*, group 3 had *Elaeagnus*-infective strains, and group 4 comprised of non-nitrogen-fixing strains and *Alnus*-infective strains. Currently, phylogenetic research of *Frankia* using 16s rRNA, nitrogen fixation (*nif*) gene, or other genes has been well recognized by most researchers who agree that all infective *Frankia* strains can be classified in either of three groups. (Benson et al., 2004; Normand et al., 1996; Schwencke and Caru, 2001). According to the review study of Benson et al. (2004), group 1 contains *Frankia* strains from nodules of plants in Betulaceae, Myricaceae, and Casuarinaceae, group 2 contains *Frankia* strains from nodules of plants in Coriariaceae, Datiscaceae, Rosaceae and *Ceanothus* of the Rhamnaceae, while group 3 contains effective *Frankia* strains, which can fix atmospheric nitrogen, from nodules of plants in Myricaceae, Rhamnaceae, Elaeagnaceae and *Gymnostoma* of the Casuarinaceae and non-effective strains from Betulaceae, Rosaceae, and genera in Casuarinaceae except *Gymnostoma* and genera in Rhamnacea except *Ceanothus*.

*NifH* gene is commonly used in comparative sequence analysis that encodes the iron (Fe) protein of nitrogenase and is present in all N$_2$ fixers (Poly et al., 2001). With its conservative structure, Young et al. (1992) reported that *nifH* gene is used to study the phylogenetic relationship of nitrogen-fixing microorganisms. Also, the phylogenetic tree
constructed by nifH genes is highly consistent with those from 16S rRNA (Young et al., 1992). Therefore, the phylogenetic relationships of nitrogen-fixing microorganisms, including Frankia, have been largely studied using nifH gene (Boulygina et al., 2002; Poly et al., 2001). To sequence nifH, PolF and PolR primers are commonly used universal primers that amplify a 360-bp fragment of nifH gene (Nouioui et al., 2011). The nifH gene has also been sampled to study the diversity, host specificity, and distribution of Frankia in symbiotic nodules of actinorhizal plants (Benson et al., 2004; Mirza et al., 2009; Nouioui et al., 2011).

With high diversity and nitrogen-fixing capacity via symbiosis with Frankia, actinorhizal plants are popular in sustainable landscapes to reduce nitrate leaching (Beddes and Kratsch, 2010; Kratsch and Graves. 2004). In the Intermountain West, S. × utahensis ‘Torrey’ is a hybrid of two native actinorhizal plants [S. argentea (silver buffaloberry) and S. rotundifolia (roundleaf buffaloberry) ] (Mee et al., 2003; Sriladda et al., 2016). Shepherdia × utahensis ‘Torrey’ has potential for use in urban landscape and forms nodules when nitrogen concentration is low in the environment. However, few studies have been conducted on Frankia from Shepherdia plants in the Intermountain West. Frankia strains in the nodules of S. × utahensis ‘Torrey’ might be similar to those observed in the nodules of its parents, S. argentea and S. rotundifolia. The objective of this study was to investigate the diversity of Frankia strains in nodules of S. × utahensis ‘Torrey’ using comparative sequence analyses of polymerase chain reaction (PCR)-amplified nifH gene fragments.
Materials and Methods

Soil samples. Two soil samples were used as inocula in our study. One soil sample was collected from the rhizosphere of a wild *S. rotundifolia* at Mohave County, AZ (36.881550, -112.895690), while the other soil sample was collected from the root zone of an *S. × utahensis* ‘Torrey’ plant at the Greenville Research Farm at Utah State University (USU, North Logan, UT) (41.765741, -111.813175). Sterilized tools were used during soil collection in each location to avoid cross-contamination. The soil samples were transported in an ice cooler and were sieved with a 2-mm-mesh soil sieve to remove gravel and plant debris. The soil samples were then stored in a refrigerator at 4 °C.

Plant materials. Terminal cuttings were collected from *S. × utahensis* ‘Torrey’ plants at the USU Greenville Research Farm. Leaves of cuttings were removed except two to three pairs at the top. Cuttings were dipped in indole-3-butyric acid (IBA) (Hormodin® 3; OHP, Mainland, PA) at 0.8% and stuck in a soilless substrate containing 80% perlite (Hess perlite, Malad City, ID) and 20% peat moss (Canadian sphagnum peat moss; SunGro Horticulture, Agawam, MA).

On 10 June 2019, 84 nodule-free rooted cuttings were transplanted into 3.8-L injection-molded, polypropylene containers (PC1D-4; Nursery Supplies, Orange, CA) filled with a commercial growing substrate (Metro-Mix®820; SunGro Horticulture, Agawam, MA) and irrigated with deionized (DI) water before inoculation. On 17 June 2019, plants were inoculated with 30 mL soil collected from Mohave County, AZ, and irrigated with 500 mL of quarter-strength nitrogen-free Hoagland’s solution at pH 6.5 every other day. On 4 Sept. 2019, nodules were collected from the inoculated plants and
stored in 95% ethanol at -20 °C until DNA extraction.

On 6 Aug. 2019, 60 nodule-free rooted cuttings were transplanted into 656-ml cone-tainers (D40H; Stuewe and Sons, Tangent, OR) filled with perlite (Malad City, ID). Plants were inoculated with 30 ml of soil samples collected from the USU Greenville Research Farm. Plants were irrigated with a quarter-strength nitrogen-free Hoagland’s solution at pH 7.5. On 4 Nov. 2019, nodules were collected and stored in 95% ethanol at -20 °C until DNA extraction.

**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, Waltham, MA), 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 mins, 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 with a 5-min extension at 72 °C for the last cycle. PCR products were analyzed by gel electrophoresis on 1% agarose gel in TAE buffer after staining with ethidium bromide at 0.5 µg·ml⁻¹.
**Sequence analyses.** The amplified *nif*H gene fragments were purified using Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific™ GeneJET™; Thermo Fisher Scientific, Waltham, MA) following manufacturer’s instructions. The purified amplicons were sequenced in the Center for Integrated BioSystems at Utah State University (Logan, UT), utilizing ABI PRISM™ 3730 DNA Analyzer with ABI BigDye™ terminator (Applied Biosystems, Foster City, CA).

**Phylogenetic analyses.** SeqMan Pro (DNASTAR, Madison, WI) was used to check the mapped reads of sequences manually. Nucleotide sequences were aligned using the ClustalW algorithm (Thompson et al., 1994) and manually trimmed in Molecular Evolutionary Genetics Analysis (MEGA) (Kumar et al., 2018). The generated sequences were analyzed and compared using BLASTN (Baker et al., 1990).

**Results**

Four query sequences, approximately 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 BLASTN, SU1 had 98% similarity with the *nif*H gene of *Frankia* strain NRRLB-16306 (accession no. JF273735.1) (Table 4-1). High similarity (98%) also showed between SU1 and the *nif*H gene of *Frankia* strains G2 (accession no. HM026367.1), Cg70.1 (accession no. HM026362.1), R43(2009) (accession no. FJ477447.1), and CeSl15 (accession no. FJ477443.1). Besides, over 97% similarity of *nif*H gene was found between the *Frankia*
strains in our study and uncultured *Frankia* clone T2P1-7 (accession no. LT840168.1) (data not shown).

The *nifH* gene sequences, SU2 and SU3, had 91% and 99% similarity, respectively, to the *nifH* gene of *Frankia* strain BMG5.12 (accession no. AJ545031). *Frankia* strains FMc5 (accession no. KP342119.1), FMc4 (accession no. KP342118.1), FMc3 (accession no. KP342117.1), FMc2 (accession no. KP342116.1), FMc1 (accession no. KP342115.1), BMG5.15 (accession no. JF273726.1), BMG5.1 (accession no. AJ545034.1), BMG5.2 (accession no. AJ545032.1), and Cc1.17 (accession no. EU862917.1) had 90% and 98% similarity with SU2 and SU3, respectively. The *nifH* gene of uncultured *Frankia* clone T2P1-7 had 90% and 99% similarity with the SU2 and SU3 (data not shown). The SU4 shared 97% similarity with the *nifH* gene of *Frankia* strain EUN1f (accession no. HM026364.1), G2, Cg70.1, and CeSl5.

**Discussion**

The presence of *nifH* gene has been considered as an indicator of potential nitrogen fixation (Young, 1992). In our study, *nifH* amplifications were obtained from *Frankia* strains in the nodules of *S. × utahensis* ‘Torrey’, suggesting that *Frankia* strains are effective. However, it is unclear if *Frankia* strains in the nodules of *S. × utahensis* ‘Torrey’ have nitrogen-fixing capacity as Benson et al. (2004) revealed that effective *Frankia* strains in nodules might have poor nitrogen-fixing ability. Therefore, further study is needed to investigate the nitrogen-fixation ability of the *Frankia* strains using acetylene reduction assay (Laws and Graves, 2005).

Comparative sequence analyses using *nifH* genes in this study revealed that *nifH*
sequences of Frankia strains obtained from S. × utahensis ‘Torrey’ were similar to those reported in the literature. Nouiou et al. (2011) also conducted comparative sequence analyses of 38 Frankia strains using gyrB, nifH, and glnII 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, Ceanothus in Rhamnaceae, Datiscaceae, and Rosaceae in cluster 2, infective Frankia strains in Elaeagnaceae and Rhamnaceae in cluster 3, and non-infective and/or non-nitrogen-fixing Frankia strains in cluster 4. These results are consistent with reports by Normand et al. (1996). Valdés et al. (2005) conducted a comparative sequence analysis using nifH gene and revealed that microorganisms from Casuarina equisetifolia root had 97% to 98% similarity to those infective Frankia in Casuarina [cluster 1 described by Nouiou et al. (2011)], and 84% to 85% similarity to those uncultured Frankia strains [cluster 2 described by Nouiou et al. (2011)]. The Frankia with highly similar nifH gene, such as Frankia strains R43(2009), CeSI5, NRRLB-16306, and FMc5 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, which had similar nifH gene to the sequences obtained from the nodules of S. × utahensis ‘Torrey’, were in cluster 3 and isolated from root nodules of Elaeagnus angustifolia (Russian olive) and Elaeagnus umbellate (autumn olive), respectively (Gtari et al., 2007; Jamann et al., 1993). Frankia strains obtained from the nodules of S. × utahensis ‘Torrey’ are in line with previous reports that Frankia strains in cluster 3 were able to induce nodules on Elaeagnaceae (Nouioui et al., 2011; Normand et al., 1996; Valdés et al., 2005).

Moreover, Frankia strains with nifH gene sequences highly similar to S.
×utahensis ‘Torrey’ in our study have been reported in the nodules of *S. argentea*, one of the parents of *S. ×utahensis ‘Torrey’*. 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 high similarity (98.3%) to strain EUN1f, while the remaining 7% sequences showed high similarity (99.6%) to strain BMG5.12 (Tekaya et al., 2018). Therefore, *Frankia* strains inhabiting the nodules of *S. ×utahensis ‘Torrey’* might also exist in the nodules of *S. argentea*.

The BLAST results also revealed that *Frankia* strains in our study had *nifH* gene sequences highly similar to those in four genera (*Casuarina, Colletia, Elaeagnus, Morella*) (Table 4-1), and these four genera were not in the same HSG. *Frankia* strains NRRLB-16306, G2, and Cg70.1 were isolated from *Casuarina* (Nouioui et al., 2011). As *Casuarina*-infective *Frankia* strains belong to HSG 2 and Elaeagnaceae-infective *Frankia* strains in either HSG 3 or HSG 4, it might suggest cross-boundary infectivity existing in *Frankia* strains in the nodules of *S. ×utahensis ‘Torrey’*. The cross-boundary infectivity of *Frankia* strains has been reviewed by Huss-Danell (1997). Therefore, *Frankia* strains in the nodules of *S. ×utahensis ‘Torrey’* might infect diverse plants to form symbiotic nodules.

**Conclusions**

A preliminary phylogenetic analysis of *Frankia* in root nodules of *S. ×utahensis ‘Torrey’* was conducted in this study. Four *nifH* sequences were obtained from nodules of
S ×utahensis ‘Torrey’. Phylogenetic analysis supported the conclusion that *Frankia* strains in nodules of *S. ×utahensis* ‘Torrey’ had high similarity with those in Elaeagnaceae and Rhamnaceae that are infective, suggesting that *Frankia* strains in nodules of *S. ×utahensis* ‘Torrey’ in our study potentially have nitrogen-fixing ability. Furthermore, *Frankia* strains from *S. ×utahensis* ‘Torrey’ might be similar to those reported in the nodules of *S. argentea*. Comparative sequence analyses with different genes (e.g., *gyr*B, *gln*II, and 16S rDNA) are needed to further clarify the phylogeny of *Frankia* in *S. ×utahensis* ‘Torrey’ nodules.

**Literature Cited**


in host plant root nodules is independent of abundance or relative diversity of

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In: G. Stacey, R.H. Burris, and H.J. Evans (eds.). Biological Nitrogen Fixation.
Chapman and Hall, New York, NY.
Table 4-1. List of ten *Frankia* strains that have the highest similarity of *nifH* gene to each of four query sequences (SU1, SU2, SU3, and SU4) obtained from nodules of *Shepherdia × utahensis* ‘Torrey’.

<table>
<thead>
<tr>
<th>Query</th>
<th>Strains</th>
<th>Similarity (%)</th>
<th><em>nifH</em> accession number</th>
<th>Origin of isolation</th>
</tr>
</thead>
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<td>NRRLB-16306</td>
<td>98</td>
<td>JF273735.1</td>
<td>Casuarina</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>98</td>
<td>HM026367.1</td>
<td><em>Casuarina equisetifolia</em></td>
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<td>Cg70.1</td>
<td>98</td>
<td>HM026362.1</td>
<td><em>Casuarina glauca</em></td>
</tr>
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<td></td>
<td>R43(2009)</td>
<td>98</td>
<td>FJ477447.1</td>
<td><em>Morella</em> (syn: <em>Myrica</em>)</td>
</tr>
<tr>
<td></td>
<td>CeS15</td>
<td>98</td>
<td>FJ477443.1</td>
<td><em>Morella pensylvanica</em> (syn: <em>Myrica pensylvanica</em>)</td>
</tr>
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<td>96</td>
<td>KP342119.1</td>
<td><em>Morella</em></td>
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<td>EUN1f</td>
<td>96</td>
<td>HM026364.1</td>
<td><em>Elaeagnus umbellata</em></td>
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<td>96</td>
<td>KP342117.1</td>
<td><em>Morella</em></td>
</tr>
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<td>FMc2</td>
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<td>KP342116.1</td>
<td><em>Morella</em></td>
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<td><em>Morella</em></td>
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<td></td>
<td>FMc4</td>
<td>90</td>
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<td><em>Morella</em></td>
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<td></td>
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<tr>
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<td>90</td>
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<td><em>Colletia cruciata</em></td>
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</table>
CHAPTER V

CONCLUSIONS

In this project, experiments have been conducted to study optimal nodule formation conditions and the impacts of controlled-release fertilizer and ammonium nitrate on nodulation. Additionally, a phylogenetic experiment was conducted to study the diversity of symbionts of Shepherdia × utahensis ‘Torrey’ inoculated with soil collected from noduled Shepherdia in Mohave County, AZ and Greenville Research Farm at Utah State University (USU, North Logan, UT).

When S. × utahensis ‘Torrey’ plants were grown in a low organic-matter substrate containing pure perlite and irrigated with nitrogen-free nutrient solution at pH 7.5, nodules formed 7 weeks earlier than the plants growing in a commercial growing substrate containing mostly peat and bark and irrigated with nitrogen-free nutrient solution at pH 6.5. In addition, 2 mM ammonium nitrate completely inhibited the nodule formation of S. × utahensis ‘Torrey’ plants in commercial growing substrates or calcined clays.

Controlled-release fertilizer (CRF, 15N–3.9P–10K) application at rates greater than 2.9 g·L⁻¹ completely inhibited the nodulation of S. × utahensis ‘Torrey’ according to regression analyses. When plants were inoculated with soils containing Frankia, less CRF was needed to sustain plant quality of S. × utahensis ‘Torrey’ compared with uninoculated plants receiving the manufacturer’s prescribed rate. The proper CRF rate for producing nodulated S. × utahensis ‘Torrey’ is topdressed with 2.1 g·L⁻¹. Although acetylene reduction assays are yet to be performed to investigate the nitrogen-fixing
capacity of the nodules produced in this research, the fact that nodulated plants sustained plant vigor with less fertilizer might suggest that nitrogen fixation occurred in nodules.

The results of the preliminary phylogenetic analysis conducted in our research showed that four $nifH$ gene sequences obtained from the nodules of $S. \times utahensis$ ‘Torrey’ showed over 90% similarity to the $nifH$ sequence of $Frankia$ from nodules of the plants in Elaeagnaceae and Rhamnaceae. Also, since high similarity showed between $nifH$ genes of $S. \times utahensis$ ‘Torrey’ and $S. argentea$, common $Frankia$ strains might share in both $S. \times utahensis$ ‘Torrey’ and its parents. Further, as $nifH$ gene amplifications obtained from the nodules of $S. \times utahensis$ ‘Torrey’, infective $Frankia$ strains of $S. \times utahensis$ ‘Torrey’ might have the potential of nitrogen-fixing ability.
APPENDIX
Appendix I

Four sequences obtained from nodules of *Shepherdia × utahensis* ‘Torrey’.

SU1  1  RRWTWW-CTGMMATSGAAG--CCAGACCTCGGTC-ATCGAGCTCGCCGCCGAGAAGGGCT
SU2  1  TCGGTTTTGTCTGTTGAAGGGCCAGGGCTTTGTTCTTGCTCGCTGGGATACCGGGGT
SU3  1  ASYWYWKKKYMYWGGAATGTC-AGAACCTCGGTC-ATCCAGCTCGYTGCCGAGAGGGGT
SU4  1  SGGCCTACTGCTTGGAGCCGATAGCC-AGACCTCGGTC-ATCGAGCTGGCCGCCAAGAG-AAGGT

SU1  57  CGGTGAGGACCTGG-AGCTCAAGAGGTCCTCGCGAGGGCCATCGGTCATCGGACCTCC
SU2  61  CTTCCAGAGACC--GCAGCTCGACGAGGTCCTTGAGGCTATTGGCCGCAATCAAGTG
SU3  59  CCGTCGAGGACCT-GGAGCTCGACGAGGTCCTGAGGCCAGGGCAGTGGGCGATCAAGTG
SU4  58  TCGGTGAGGACCTGCGAGCTCGAGGACGTGCTGCGAGGGCGGCGTGGGCAATCGAAGTG

SU1  116  GTCGAGTCTGTCGCTGGCCGAGCGGGGTCTCGCTGGCCTCGCCCGCGTCGTCATACCCCTC
SU2  120  GTCGAGTCTGTCGCTGGCCGAGCGGGGTCTCGCTGGCCTCGCCCGCGTCGTCATACCCCTC
SU3  118  GTCGAGTCTGTCGCTGGCCGAGCGGGGTCTCGCTGGCCTCGCCCGCGTCGTCATACCCCTC
SU4  118  GTCGAGTCTGTCGCTGGCCGAGCGGGGTCTCGCTGGCCTCGCCCGCGTCGTCATACCCCTC

SU1  176  ATCACCTACTGGAAGGAGGCGCCGCCTACGAGAACCCTCGACTGTCACCTACGACGTC
SU2  180  ATCACGTTCCTGAGAGGCGCCGCGTCCGCTATAGC-ACACCTCGGATTCCGTCACCTACGACGTC
SU3  178  ATCACGTTCCTGAGAGGCGCCGCGTCCGCTACGAGAACCCTCGACCTGTCACCTACGACGTC
SU4  178  ATCACGTTCCTGAGAGGCGCCGCGTCCGCTACGAGAACCCTCGACTGTCACCTACGACGTC

SU1  236  CTCGCGTACGGTCTGCGATGCCGATCGGCAGGGCAGGGAGGGAAGCAGGCCACAGG
SU2  239  CTCGCGTACGGTCTGCGATGCCGATCGGCAGGGCAGGGAGGGAAGCAGGCCACAGG
SU3  238  CTCGCGTACGGTCTGCGATGCCGATCGGCAGGGCAGGGAGGGAAGCAGGCCACAGG
SU4  238  CTCGCGTACGGTCTGCGATGCCGATCGGCAGGGCAGGGAGGGAAGCAGGCCACAGG

SU1  296  ATCTACATCGTACCCTCGGCGGAATGATGCGSATYA-
SU2  298  ATCTACATCGTACCCTCGGCGGAATGATGCGSATAAA
SU3  298  ATCTACATCGTACCCTCGGCGGAATGATGCGSATYYA
SU4  298  GATCTACATCGTACCCTCGGCGGAATGATGCGSATATA