

Dinitrogen Fixation in Illinois Bundleflower

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ABSTRACT

Symbiotic N₂ fixation capacity may affect productivity of the perennial legume Illinois bundleflower [*Desmanthus illinoensis* (Michx.) MacMill. ex B.L. Rob. & Fernald]. Our objective was to estimate N₂ fixation of three Illinois bundleflower accessions managed for forage. Herbage yield ranged from 1.02 Mg ha⁻¹ to 3.69 Mg ha⁻¹ in Year 1, and 2.99 Mg ha⁻¹ to 8.27 Mg ha⁻¹ in Year 2. Accessions differed in herbage yield, aboveground N yield, and N₂ fixed at certain locations in Year 1, but not in Year 2. Percentage of N derived from the atmosphere (%Ndfa) varied with location but not with accession in either year. The ¹⁵N natural abundance method gave lower estimates of %Ndfa than the ¹⁵N enrichment method. In yr 1, N₂ fixation ranged from 0 to 30 kg ha⁻¹ N (¹⁵N natural abundance method), 11 to 43 kg ha⁻¹ (¹⁵N enrichment method), and 0 to 50 kg ha⁻¹ N (total N difference method), and in Year 2 these estimates at two locations were 60 to 67 kg ha⁻¹ N, 79 to 127 kg ha⁻¹ N, and 67 to 142 kg ha⁻¹ N, respectively. Differences in N₂ fixation among locations could have been due to rhizobial strains. At the high-yielding location with the greatest N₂ fixation, over one-half of the nodules were occupied by indigenous rhizobial strains, whereas at other locations, strains from commercial inoculant accounted for most nodules. Symbiotic N₂ fixation by Illinois bundleflower could be enhanced by new rhizobial inoculums.

ILLINOIS BUNDLEFLOWER, an herbaceous perennial legume (subfamily Mimosoideae) native to the central USA (Luckow, 1993), may fulfill the need for a persistent, high-quality forage legume to complement warm-season grasses. DeHaan et al. (2003) found that in Minnesota, northern accessions of Illinois bundleflower produce much of their biomass during July and August when the productivity of cool-season grasses and legumes in the region is low.

Illinois bundleflower is compatible in mixtures with a diversity of warm-season grasses. In east-central Texas, established kleingrass (*Panicum coloratum* L.) stands interseeded with 'Sabine' Illinois bundleflower had greater forage yields than kleingrass monocultures in the second, third, and fourth years after seeding (Dovel et al., 1990). Mixtures of Illinois bundleflower accession PI 434011 with switchgrass (*Panicum virgatum* L.), sideoat grama (*Bouteloua curtipendula* Michx.), or indiagrass [*Sorghastrum nutans* (L.) Nash] in northeastern Kansas had higher forage yield and crude protein concentration than grass monocultures in the fourth and fifth years after establishment (Posler et al., 1993). Springer et al. (2001) showed that Sabine Illinois bundleflower was highly compatible in mixture with indiagrass.

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Although Illinois bundleflower is known to contribute N in forage systems (Dovel et al., 1990; Posler et al., 1993; Springer et al., 2001), there is a paucity of information on its N₂ fixation capability. Nodulation has been observed in 90% of the Mimosoideae (de Faria et al., 1989) including Illinois bundleflower (Allen and Allen, 1981). Kulakow et al. (1990) found that mean acetylene reduction rates of 70-d-old seedlings of Illinois bundleflower were comparable with those of soybean [*Glycine max* (L.) Merr.] and alfalfa (*Medicago sativa* L.), but also noted that rates varied significantly among accessions. However, the instantaneous rates of N₂ fixation measured by the acetylene reduction technique cannot be extrapolated to obtain an accurate estimate of total N₂ fixed across a growing season.

Integrated estimates of N₂ fixation can be made by comparing N accumulation with non-N₂-fixing plants (the total N difference technique) or by isotopic N methodologies. The latter depends on a ¹⁵N-labeled soil N pool, which is manifested in higher ¹⁵N concentrations in non-N₂-fixing reference plants dependent on soil N than in N₂-fixing plants, which incorporate both soil and unlabeled atmospheric N. In the ¹⁵N enrichment or isotope dilution technique, the soil N pool is labeled with an ¹⁵N-enriched source, whereas the ¹⁵N natural abundance method relies on the small but measurable elevation of ¹⁵N already present in most soils relative to the atmosphere (Chalk, 1985; Shearer and Kohl, 1986). In this study, we compared both isotope methods and the total N difference method as estimates of N₂ fixation in monocultures of Illinois bundleflower. Our objective was to determine the in-field N₂-fixation of three accessions of Illinois bundleflower managed as a forage crop in pure stands during the first 2 yr after seeding.

MATERIALS AND METHODS

Site and Climate Characteristics

The experiment was conducted at the Sand Plain Research Farm, Becker, MN (45°24' N, 93°53' W), the Rosemount Research and Outreach Center, Rosemount, MN (44°53' N, 93°13' W), and the Southwest Research and Outreach Center, Lamberton, MN (44°15' N, 95°19' W). The soil at Becker was a Hubbard loamy sand (sandy, mixed, frigid Entic Hapludoll). At Rosemount, it was a Waukegan silt loam (fine-silty over sandy, mixed, superactive, mesic Typic Hapludoll). At Lamberton, the soil was a Normania clay loam (fine-loamy, mixed, superactive, mesic Aquic Hapludoll). Soil chemical characteristics for the three sites are reported in Table 1. Previous crops at the three sites were rye (*Secale cereale* L.) at Becker, corn (*Zea mays* L.), and soybean at Rosemount, and oat (*Avena sativa* L.) at Lamberton. Manure had not been applied to the sites within the previous 5 yr. On the basis of soil test results, 390 kg ha⁻¹ K and 4.5 kg ha⁻¹ B were applied at Becker in

Abbreviations: DAP, days after planting; PCR, polymerase chain reaction; PNL, Perennial Native Legume; %Ndfa, percentage of N derived from the atmosphere.

Table 1. Soil characteristics at three experimental sites in Minnesota.[†]

Site	Year	pH	SOM [‡]		Total N	NO ₃ -N		P	K
			g kg ⁻¹			mg kg ⁻¹			
Becker	2000	6.9 [‡]	24	0.49	6.2	54	121		
	2001	6.7	25	0.46	6.1	50	94		
Rosemount	2000	6.9	47	1.88	16.3	31	98		
	2001	6.7	48	1.95	16.8	30	100		
Lamberton	2000	7.4	41	1.64	32.6	19	156		
	2001	7.0	46	1.55	32.6	16	138		

[†] Values reported are means across six replicates at each site. Samples were taken to a depth of 15 cm at planting time in 2000 and when shoots were first visible in 2001.

[‡] SOM, soil organic matter.

March 2000 and 100 kg ha⁻¹ P was applied at Lamberton in May 2000. The background $\delta^{15}\text{N}$ natural abundance of total N in the top 15 cm of the soil at Becker, Rosemount, and Lamberton, as determined by a Europa Scientific Hydra 20/20 mass spectrometer (PDZ Europa, Cheshire, UK)¹, was 7.47 ± 0.12 , 7.85 ± 0.03 , and $7.95 \pm 0.16\%$ (means \pm standard error for six replicates), respectively.

Monthly precipitation totals and average air temperatures from May to September for the three sites are shown in Table 2. Precipitation at Lamberton was near the 30-yr average both years. In the 2000 growing season, precipitation was 175 mm below average at Becker. Precipitation was near average in the 2000 season at Rosemount, but 128 mm above average in July. In the 2001 growing season, precipitation was 127 and 96 mm below the 30-yr mean at Becker and Rosemount, respectively. The plots at Becker received 225 mm of irrigation in both seasons. Air temperatures at all three sites were generally near average in the 2000 season and above average in the 2001 season.

Plant Characteristics

Three accessions of Illinois bundleflower from the University of Minnesota Perennial Native Legume (PNL) collection were used. They were chosen for winter survival, maturation, height, and seed yield using the results collected by DeHaan et al. (2003) for plants grown at Becker and Saint Paul, MN. Plants of PNL534 from Lake Hattie, Stevens County, Minnesota ($45^{\circ}32'$ N, $96^{\circ}05'$ W) had early to midseason maturity, short height, low to moderate aboveground biomass, and low to moderate seed yield. Accession PNL539 from Spirit Lake,

Dickinson County, Iowa ($43^{\circ}29'$ N, $95^{\circ}05'$ W) had midseason maturity, tall height, moderate aboveground biomass, and moderate to high seed yield. PNL541 from Cottonwood Lake, Spink County, South Dakota ($44^{\circ}47'$ N, $98^{\circ}42'$ W) had early-season maturity, moderate height, high aboveground biomass, and high seed yield. All accessions had good winter survival at Becker and Saint Paul. On the basis of 54 phenotypic traits measured, DeHaan et al. (2003) clustered PNL539 and PNL541 together, whereas PNL534 grouped in a different cluster.

Wild senna [*Senna hebecarpa* (Fernald) H.S. Irwin & Barneby], a warm-season perennial legume native to the north-eastern and east-central USA (Irwin and Barneby, 1982), was grown as the non-N₂-fixing reference plant for use in the comparisons involving ¹⁵N. Like many Caesalpinioideae legumes, it is not nodulated (Allen and Allen, 1981), but in Minnesota, it generally exhibits similar emergence times and biomass accumulation patterns as Illinois bundleflower (E. Ristau, 1999, personal communication). Detailed descriptions of the root morphology of *D. illinoensis* and *S. hebecarpa* are lacking.

Field Experiment

The experiment was designed as a split plot at each of three locations with whole plots arranged in randomized complete blocks. There were six replicates per location. The whole-plot treatments were natural abundance and enriched levels of ¹⁵N. The split-plot treatments were the three Illinois bundleflower accessions and wild senna. Each plot measured 6 by 3 m. A 1.5-m-wide alley was maintained around each whole plot to reduce contamination of ¹⁵N natural abundance plots with enriched ¹⁵N.

Seed of the three populations of Illinois bundleflower were from DeHaan et al. (2003). The seed was mechanically scarified with sandpaper and inoculated with 5 g kg⁻¹ of a commercial, peat-based inoculant for *Desmanthus* using 22 mL kg⁻¹ seed of a 10% (w/v) sucrose solution as an adhesive. The

¹ Names are necessary to report factually on available data. However, the USDA and the University of Minnesota neither guarantee nor warrant the standard of the product, and use of the name by the USDA and the University of Minnesota implies no approval of the product to the exclusion of others that may be suitable.

Table 2. Monthly precipitation and average air temperature means and deviation (dev) from 30-yr (1971–2000) mean (dev) at Lamberton, Rosemount, and Becker, MN, May to September 2000 and 2001 (Minnesota Climatology Working Group, 2002).

	Becker				Rosemount				Lamberton			
	2000		2001		2000		2001		2000		2001	
	Mean	Dev	Mean	Dev	Mean	Dev	Mean	Dev	Mean	Dev	Mean	Dev
	Precipitation, mm											
May	61	-32	86	-7	104	4	116	16	165	86	58	-21
June	109	5	102	-2	109	0	113	4	60	-28	57	-31
July	106	-1	50	-57	230	128	26	-76	117	23	167	73
August	40	-67	68	-39	79	-22	42	-59	91	20	39	-32
September	10	-80	68	-22	15	-61	95	19	14	-76	87	-3
	Air temperature, °C											
May	15.4	1.4	14.4	0.4	16.6	1.6	14.7	-0.3	15.5	0.5	15.3	0.3
June	17.6	-2.4	19.7	-0.3	18.3	-1.7	19.4	-0.6	19.2	-0.8	20.3	0.3
July	22.1	0.1	23.0	1.0	21.4	-0.6	22.8	0.8	21.0	-0.1	23.1	1.1
August	22.2	2.2	22.6	2.6	21.2	0.2	22.4	1.4	21.3	0.3	21.5	0.5
September	15.2	0.2	14.9	-0.1	15.7	-0.3	15.4	-0.6	15.4	-0.6	15.3	-0.7

inoculant contained two strains: Nitragin 43A1 and 43C2 (Liphatec, Milwaukee, WI). In 2000, seed was drilled in rows 15 cm apart on 25 May at Becker, 3 June at Lambertton, and 7 June at Rosemount. Seeding rates were 200 live seeds m⁻² for Illinois bundleflower and 510 live seeds m⁻² for wild senna. The higher rate for senna was based on previous data showing lower persistence and a less-open growth form than bundleflower (E. Ristau, 1999, personal communication). Average stand densities of Illinois bundleflower in 2000 were 157, 93, and 72 plants m⁻² at Becker, Rosemount, and Lambertton, respectively. The corresponding densities of wild senna were 256, 171, and 155 plants m⁻².

In the seeding year, 52 mL a.i. ha⁻¹ of imazapic [Plateau, \pm 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-methyl-3-pyridinecarboxylic acid, American Cyanamid Co., Parsippany, NJ] was applied before seedling emergence to control weeds in the Illinois bundleflower plots. In the second year, 68 mL a.i. ha⁻¹ imazapic and 0.65 L a.i. ha⁻¹ pendimethalin [Prowl, *N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine, American Cyanamid Co.] with 1.2 L ha⁻¹ of an N-surfactant blend (Class Prefer-28, Cenex-Land O'Lakes Agronomy Co., Winona, MN) were applied before seedling emergence in the Illinois bundleflower plots. Pendimethalin inhibited growth of any seedlings from shattered first-year seed, ensuring purely 2-yr-old stands. Because wild senna does not tolerate imazapic (E. Ristau, 1999, personal communication), 0.5 L a.i. ha⁻¹ of trifluralin (Treflan, α,α,α -trifluoro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine, Dow AgroSciences, Indianapolis, IN) was incorporated into the soil before planting the wild senna plots. In both years, hand weeding was also performed.

At 20 to 23 days after planting (DAP), a 0.2% (w/v) aqueous solution of 98 atom% ¹⁵N (NH₄)₂SO₄ was applied to a 1.8-m² area in the enriched plots by injection every 7.5 cm between every row for a total application rate of 0.2 g ¹⁵N m⁻². Dinitrogen fixation should not have been inhibited by this low rate of N addition (2 kg N ha⁻¹). The injection cannulas had pores at depths of 7.5 cm and 15 cm. In the second year, the solution was applied to a separate 1.8-m² area in the enriched plots within 28 d after new shoots were visible. In both years, central areas in each plot were chosen for treatment to minimize edge effects.

Plants were harvested at maximum aboveground biomass (approximately 10% seedpod fill). In 2000, this occurred on 9 September at Lambertton, 10 September at Becker, and 14 September at Rosemount. In 2001, harvest dates were 14 August at Rosemount, 20 August at Becker, and 25 to 26 August at Lambertton. Aboveground biomass from a 0.8-m² area within the ¹⁵N-treated area in the enriched plots was clipped at ground level by hand. A 0.8-m² area also was harvested from the ¹⁵N natural abundance plots in a similar manner. Standing first-year stems were mowed and removed from the plots by hand-raking in April and May 2001, before emergence of second-year plants.

Samples were oven-dried for 48 h at 60°C and ground to pass a 0.5-mm screen. Total N concentration was determined using a LECO CN-2000 analyzer (LECO Corporation, St. Joseph, MI). The University of California-Davis Stable Isotope Facility determined isotopic composition of shoots. Shoots with enriched levels of ¹⁵N were analyzed on a Europa Scientific Integra mass spectrometer (PDZ Europa, Cheshire, UK), whereas shoots with natural abundance levels of ¹⁵N were analyzed on a Europa Scientific Hydra 20/20 mass spectrometer. Illinois bundleflower leaf tissue was analyzed for S and Mo concentration using a LECO S-144DR S analyzer (LECO Corporation, St. Joseph, MI) and an Applied Research Laboratories 3560 inductively coupled plasma spectrometer (Thermo ARL, Ecublens, Switzerland), respectively.

Calculations

The yield-independent measure %Nd_{fa} represents the percentage of plant N derived from the atmosphere through N₂ fixation. With natural abundance levels of ¹⁵N,

$$\%Nd_{fa} = 100 \left(\frac{\delta^{15}N_o - \delta^{15}N_t}{\delta^{15}N_o - \delta^{15}N_a} \right), \quad [1]$$

where $\delta^{15}N$ is the per mil (‰) departure from the ¹⁵N concentration of the atmosphere (which is at a constant 0.3663 atom% ¹⁵N, thus $\delta^{15}N_{atmosphere} = 0$ by definition), *o* is the non-N₂-fixing reference plant, *t* is the N₂-fixing plant grown under field conditions in which both soil and atmospheric N are available, and *a* is the N₂-fixing plant grown under conditions in which only atmospheric N is available (Shearer and Kohl, 1986). Discrimination against the heavier ¹⁵N isotope during chemical reactions such as N₂ fixation and N metabolism can lead to ¹⁵N enrichment in certain tissues (nodules), and ¹⁵N depletion in others (shoots) (Boddey et al., 2000). The resulting isotope fractionation is accounted for by the value $\delta^{15}N_a$.

If $\delta^{15}N_o$ is sufficiently large relative to $\delta^{15}N_a$ or the converse, $\delta^{15}N_a$ can be disregarded (Shearer and Kohl, 1986). Thus, at enriched levels of ¹⁵N, Eq. [1] reduces to:

$$\%Nd_{fa} = 100 \left(\frac{\delta^{15}N_o - \delta^{15}N_t}{\delta^{15}N_o} \right) = 100 \left(1 - \frac{\delta^{15}N_t}{\delta^{15}N_o} \right). \quad [2]$$

At enriched levels of ¹⁵N, isotope concentrations are more conventionally expressed as atom% ¹⁵N excess, the departure in atom% ¹⁵N from the atmospheric concentration of 0.3663 atom% ¹⁵N, rather than as per mil departures:

$$\%Nd_{fa} = 100 \left[1 - \frac{\text{atom}\%^{15}\text{N excess (fs)}}{\text{atom}\%^{15}\text{N excess (nfs)}} \right], \quad [3]$$

where *FS* refers to the N₂-fixing system and *NFS* to the non-N₂-fixing system (Rennie, 1984).

These measures of %Nd_{fa} are multiplied by N yield (which is calculated as dry matter yield per unit area \times N concentration in the tissue) of the N₂-fixing plant to give the amount of N₂ fixed per unit area, a yield-dependent measure. Both methods assume that the N₂-fixing and reference plants absorb N from the same pool of soil N across time and that the N₂-fixing and nonfixing crops absorb ¹⁵N-labeled N and the plant-available soil N in the same ratio. An additional estimate of fixed N₂ can be calculated as the difference in N yield between the N₂-fixing plant and the reference plant. This total N difference method assumes that the N₂-fixing and non-N₂-fixing crops absorb equal amounts of soil N (Rennie, 1984).

Nodule Occupancy and ¹⁵N Fractionation

Because isotopic fractionation in plant shoots can vary according to rhizobial strain and host cultivar (Ledgard, 1989), a separate $\delta^{15}N$ was determined for selected rhizobium from each location \times accession combination. For this determination, 10 nodules sampled from each location \times accession combination in August 2001 were surface sterilized in 3% (w/v) NaOCl, rinsed repeatedly, and crushed in yeast-mannitol broth (Somasegaran and Hoben, 1994). Nodule preparations were streaked on yeast mannitol agar plates. Plates were incubated for 3 d at 25°C, then rhizobium were reisolated and resubcultured to ensure purity (Somasegaran and Hoben, 1994). Rep-PCR (polymerase chain reaction) genomic fingerprinting of cultures was performed using the BOXA1 primer 5'-CTACGGCAAGGCGACGCTGACG-3' (Veraslovic et al., 1994; Schneider and de Bruijn, 1996) and the results subjected to cluster analysis using BioNumerics software (BioSystematica, Devon, UK) to produce an unweighted pair group

Table 3. Isotopic fractionation ($\delta^{15}\text{N}_a$) in whole shoots of 72-d-old Illinois bundleflower plants grown in the absence of soil N. Plants were thus dependent solely on fixed N_2 , except for N originally present in the seed.

Accession	Rhizobial strains		Accession mean
	Inoculant	Inoculant + indigenous†	
	‰		
PNL534	$-0.13 \pm 0.15\text{a}\ddagger$	$-0.64 \pm 0.11\text{ab}$	$-0.38 \pm 0.09\text{i}$
PNL539	$-0.74 \pm 0.11\text{b}$	$-1.59 \pm 0.10\text{c}$	$-1.16 \pm 0.08\text{j}$
PNL541	$-0.65 \pm 0.13\text{ab}$	$-0.93 \pm 0.13\text{b}$	$-0.79 \pm 0.09\text{k}$
Strain mean	$-0.51 \pm 0.08\text{p}$	$-1.05 \pm 0.07\text{q}$	

† Indigenous rhizobial strains obtained from Lambertson, MN.

‡ Values reported are least squares means \pm standard error. Means followed by the same letter are not significantly different by the Tukey-Kramer approximate honestly significant difference ($P < 0.05$), with a, b, c for interaction means, i, j, k for overall accession means, and p, q for overall rhizobial strain means. The number of replicates per accession \times rhizobial strain combination varied from 5 to 11, depending on plant survival.

method with arithmetic mean dendrogram with the Pearson correlation as the similarity coefficient.

Isolates were transferred to yeast mannitol broth cultures. Seeds of the three accessions of Illinois bundleflower were chemically scarified and sterilized by soaking in concentrated H_2SO_4 for 5 min and then were washed repeatedly with sterile water. Plants were grown in 15-cm pots previously soaked in 3% NaOCl and filled with silica sand that had been washed with concentrated HCl. Six seeds were planted and then thinned to two per pot. Plants were inoculated at planting with 10 mL of broth culture of either the inoculant strains alone or a mixture of the inoculant strains plus indigenous strains from Lambertson. Plants were watered daily with N-free nutrient solution through capped glass tubes (Somasegaran and Hoben, 1994) and grown at 24°C during the day and 18°C at night, with a daylength of 16 h. Shoots were harvested 72 DAP, dried for 48 h at 60°C , and analyzed for natural abundance of ^{15}N as above. The values of $\delta^{15}\text{N}_a$ from the plants inoculated with the inoculant strain only were used for Becker and Rosemount, and the values of $\delta^{15}\text{N}_a$ from the plants inoculated with the mixture of inoculant and indigenous strains were used for Lambertson (Table 3).

Statistical Analysis

Data were analyzed using the PROC MIXED procedure of SAS 8.2 (SAS Institute, 1996) with all factors fixed except for the blocking variable, which was random and nested within location. To compare each ^{15}N method with the difference method, a separate repeated measures ANOVA was performed for each ^{15}N method, using the ^{15}N method and the difference method as repeated measures. Normality of data was confirmed via normal probability plots of residuals (Oelbert, 2000). Constant variance of data was assessed with plots of raw residuals vs. predicted values across all locations (Oelbert, 2000). Since herbage yield and measures derived from this response (aboveground N yield and N_2 fixed) had nonconstant variance across locations, each location was analyzed separately for these responses. The herbage yield-independent measure %Ndfa had constant variance across all locations and the data from the three locations were combined into a single ANOVA for this response. Pairwise comparison of adjusted (least squares) means was performed using the Tukey-Kramer approximate honestly significant difference at significance level $\alpha = 0.05$ (Oelbert, 2000).

RESULTS AND DISCUSSION

Nodule Occupancy and ^{15}N Fractionation

The rep-PCR results revealed that at both Becker and Rosemount, 26 of the 30 rhizobial isolates grouped with the inoculant strains at $>70\%$ similarity (Fig. 1). In contrast, only 13 of the 28 isolates from Lambertson grouped with the inoculant strains at this level of similarity; the remainder presumably representing indigenous rhizobial strains. Both rhizobial strain ($P < 0.001$) and host accession ($P < 0.001$) affected isotopic fractionation (Table 3). Accession differences were likely due to differences in N partitioning between shoots and roots. Like Ledgard (1989), we observed lower shoot $\delta^{15}\text{N}_a$ for plants inoculated with a mixture of indigenous strains than with the inoculant strains. Thus, we would have

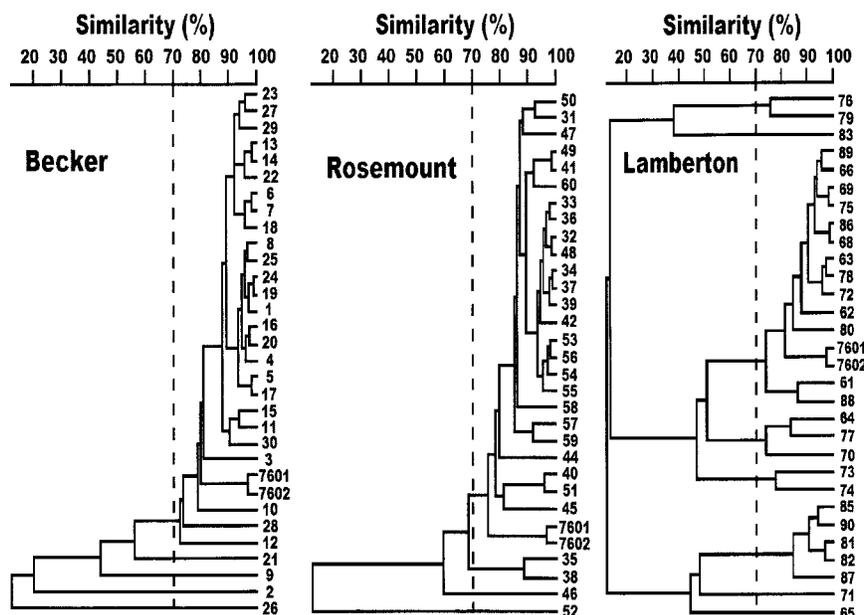


Fig. 1. Dendrogram showing relationships among rhizobial isolates (1–89) from nodules on Illinois bundleflower at three Minnesota locations and Nitragin strains 43A1 and 43C2 based on rep-PCR (polymerase chain reaction) analysis. Vertical dashed line represents 70% similarity among strains.

Table 4. Herbage yield and aboveground N yield for first-year (2000) Illinois bundleflower.[†]

Accession	Herbage yield			Aboveground N yield		
	Becker	Rosemount	Lamberton	Becker	Rosemount	Lamberton
	Mg ha ⁻¹			kg ha ⁻¹		
PNL534	0.65a‡	2.30i	3.45p	11a	45i	76p
PNL539	0.99a	3.24j	3.68p	15a	53i	77p
PNL541	1.42b	3.04ij	3.93p	24b	60i	87p
Location mean	1.02	2.86	3.69	17	53	80
Wild senna	1.99	3.29	1.84	16	46	30

[†] Values reported are least squares means of six replicate plots.

[‡] Means followed by the same letter are not significantly different by the Tukey-Kramer approximate honestly significant difference ($P < 0.05$), with a, b for Becker means, i, j for Rosemount means, and p for Lamberton means.

overestimated N₂ fixation at Lamberton if we had based estimates for %Ndfa on the fractionation value from plants infected only with the inoculant strains rather than with a mixture of the inoculant strains plus indigenous strains. It should be recognized that the inoculant-adjusted values for $\delta^{15}\text{N}_a$ may not reflect the relative occupancy of nodules by different rhizobial strains in the field, which was not estimated in this study. There was no rhizobial strain \times accession interaction ($P > 0.05$).

First Year

The three accessions of Illinois bundleflower differed in herbage yield at Becker ($P < 0.001$) and Rosemount ($P < 0.05$), but not at Lamberton ($P > 0.05$) (Table 4). Herbage yield across all three accessions was lowest at Becker and highest at Lamberton. At Becker, PNL541 had higher herbage yield than the other accessions, whereas at Rosemount, PNL539 had higher herbage yield than PNL534. At Becker and Rosemount, herbage yield of all accessions was lower than that of wild senna, but at Lamberton, the three accessions yielded more than wild senna.

The accessions differed in aboveground N yield at Becker ($P < 0.001$), where PNL541 was the sole accession yielding more N than the non-N₂-fixing wild senna (Table 4). Therefore, no N₂ fixation was detected by the total N difference method for PNL534 and PNL539. At Rosemount and Lamberton, accessions had similar N yield ($P > 0.05$), that averaged across accessions was 53 kg and 80 kg ha⁻¹, respectively. Thus, there were also no differences among accessions at these locations in the estimation of N₂ fixed by the total N difference method, which averaged 7 kg ha⁻¹ at Rosemount and 50 kg ha⁻¹ at Lamberton (Fig. 2).

There was a location \times ¹⁵N method interaction for %Ndfa ($P < 0.05$) (Fig. 3). Averaged across accessions, the %Ndfa for the two ¹⁵N methods differed at Becker and Rosemount but not at Lamberton. The contrast between methods was particularly striking at Becker, where the ¹⁵N enrichment method estimated %Ndfa to be 52 units higher than for the ¹⁵N natural abundance method. The %Ndfa was similar for the three accessions at all locations ($P > 0.05$). With the ¹⁵N natural abundance method, none of the accessions at Rosemount fixed N₂ during the first year of growth, nor did two of the three accessions at Becker.

Dinitrogen fixation was greatest at Lamberton, where 26 and 48 kg N ha⁻¹ were fixed as estimated using natural abundance and enriched levels of ¹⁵N, respec-

tively. Because of low N yield, N₂ fixation at Becker and Rosemount was only 11 and 16 kg ha⁻¹, respectively, according to the ¹⁵N enrichment method, and nearly zero at both locations according to the ¹⁵N natural abundance method. The ¹⁵N methods gave similar results at Lamberton ($P > 0.05$), but not at Becker ($P < 0.05$) and Rosemount ($P < 0.01$). Accessions differed in N₂ fixation at Becker ($P < 0.05$) and Lamberton ($P < 0.05$), but not at Rosemount ($P > 0.05$). At Becker and Lamberton, PNL541 consistently fixed the most N₂ using either ¹⁵N method. Dinitrogen fixation was similar for PNL534 and PNL539.

The difference in %Ndfa between the two ¹⁵N methods at Becker and Rosemount may be due to the small differences in $\delta^{15}\text{N}$ between Illinois bundleflower and wild senna (0.75‰ at Becker and 0.10‰ at Rosemount, vs. 2.33‰ at Lamberton, averaged across accessions). Numerous physical and biological processes other than

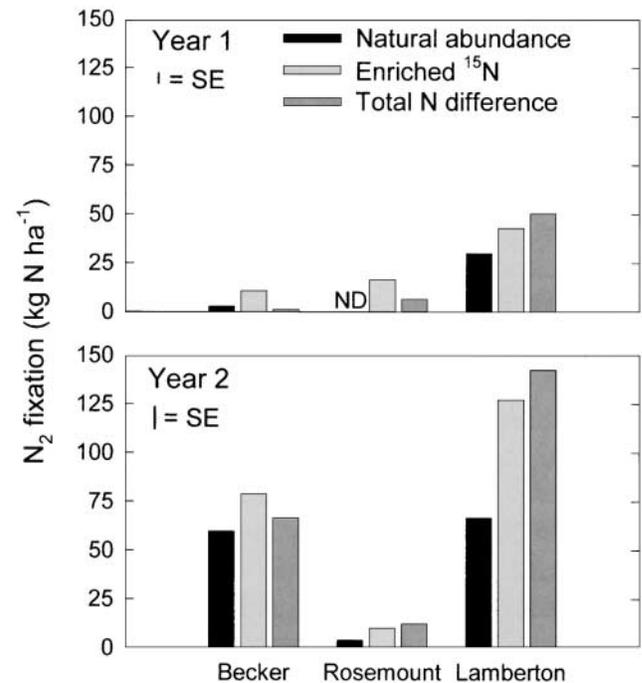


Fig. 2. Cumulative aboveground N in Illinois bundleflower derived from symbiotic N₂ fixation at three Minnesota locations in 2 yr following seeding, based on three methods for estimating N₂ fixation. Bars represent least squares means derived from six replicates of three accessions at each location. Error bars represent the maximum standard error of the least squares means for the three separate models comparing pairs of methods.

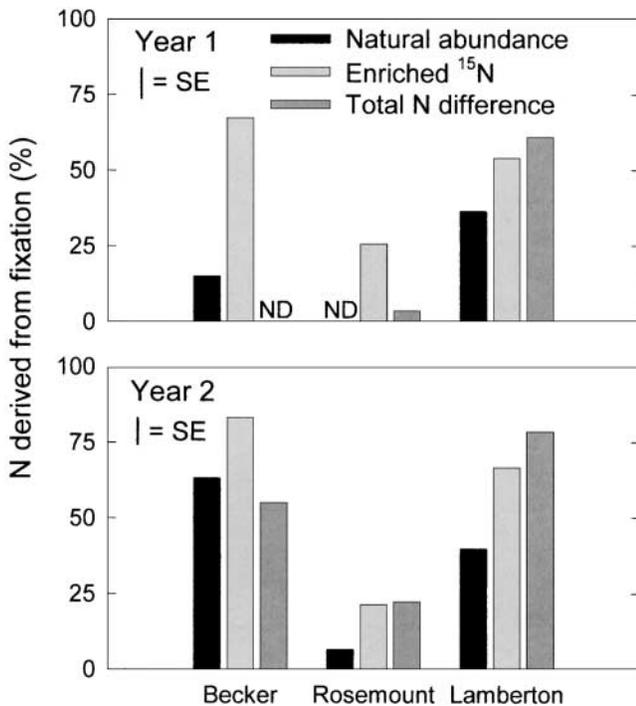


Fig. 3. Reliance of Illinois bundleflower herbage on N derived from symbiotic N₂ fixation at three Minnesota locations in 2 yr following seeding, based on three methods for estimating N₂ fixation. Bars represent least squares means derived from six replicates of three accessions at each location. Error bars represent the maximum standard error of the least squares means for the three separate models comparing pairs of methods.

symbiotic N₂ fixation can fractionate ¹⁵N, and can obscure the ¹⁵N signature of fixed atmospheric N₂ (Handley and Scrimgeour, 1997). The contribution of these fractionation processes becomes important at low levels of ¹⁵N enrichment, and leads to invalidation of the assumption that fixed N₂ and soil-derived N necessarily have unique ¹⁵N signatures (Handley and Scrimgeour, 1997). Therefore, the estimates of %Ndfa by the ¹⁵N natural abundance method probably should be considered less reliable than those given by the ¹⁵N enrichment method in this study.

Estimates of N₂ fixed were similar for the ¹⁵N natural abundance and total N difference methods at Becker and Rosemount ($P > 0.05$), but estimates for the methods differed at Lamberton ($P < 0.001$), where estimates for the total N difference method were higher (Fig. 2). The pattern was reversed when comparing the ¹⁵N enrichment method with the total N difference method:

the estimates for the two methods were similar at Lamberton ($P > 0.05$), but differed at Becker ($P < 0.001$) and Rosemount ($P < 0.05$), where estimates for the total N difference method were lower than for the ¹⁵N enrichment method.

Because of the low levels of N₂ fixation in the seeding year, Illinois bundleflower may benefit from supplemental N fertilization to improve establishment (Hojjati et al., 1978). Although Dovel et al. (1990) achieved good establishment of Illinois bundleflower in kleingrass without supplemental N, bundleflower grew very slowly in the establishment year. In Minnesota, the northern edge of its range, Illinois bundleflower may be more difficult to establish because significant growth in the first year does not occur until July (DeHaan et al., 2003). The use of supplemental N fertilizer sufficient to increase vigor and establishment, but not in amounts so that nodulation is suppressed (Streeter, 1988), may be advantageous at low-fertility sites like Becker.

Second Year

Accessions had similar herbage yield, aboveground N yield, and N₂ fixed estimated by the total N difference or ¹⁵N methods, or %Ndfa ($P > 0.05$) (Table 5). The lack of accession effects suggests that the first-year effects could have been due in part to factors such as seedling vigor and establishment, or perhaps in speed of nodulation. Second-year differences among the accessions noted by DeHaan et al. (2003), who grew Illinois bundleflower in space-planted nurseries, may have been masked in our experiments when plants were grown in dense stands.

Herbage yield was highest at Lamberton, where the three accessions averaged 8.3 Mg ha⁻¹ (Table 5). By comparison, DeHaan et al. (2003) extrapolated a biomass yield of 4.9 Mg ha⁻¹ for his best accession of Illinois bundleflower grown at a low planting density of 0.87 plants m⁻² and cut once in August in the second year. Like DeHaan et al. (2003), we observed the highest biomass yield at a location within the native range of the species and lower yield outside the native range at Becker and Rosemount. At Becker, the accessions averaged 4.6 Mg ha⁻¹, whereas at Rosemount, the accessions averaged only 3.0 Mg ha⁻¹. Wild senna yields varied from about 3.0 Mg ha⁻¹ at both Becker and Rosemount to 3.7 Mg ha⁻¹ at Lamberton.

A legume control with known yield potential was not included in this research. We are limited, therefore, to comparing data from Illinois bundleflower with pub-

Table 5. Herbage yield and aboveground N yield for second-year (2001) Illinois bundleflower.†

Accession	Herbage yield			Aboveground N yield		
	Becker	Rosemount	Lamberton	Becker	Rosemount	Lamberton
	Mg ha ⁻¹			kg ha ⁻¹		
PNL534	4.73a‡	2.89i	8.03p	97a	49i	179p
PNL539	4.36a	3.20i	8.61p	92a	46i	175p
PNL541	4.44a	2.89i	8.18p	97a	48i	186p
Location mean	4.54	2.99	8.27	95	48	180
Wild senna	2.91	3.07	3.68	28	35	38

† Values reported are least squares means of six replicate plots.

‡ Means followed by the same letter are not significantly different by the Tukey-Kramer approximate honestly significant difference ($P < 0.05$), with a for Becker means, i for Rosemount means, and p for Lamberton means.

lished data from other trials. Mean yields of established stands of alfalfa, red clover (*Trifolium pratense* L.), and birdsfoot trefoil (*Lotus corniculatus* L.) at Rosemount were 13.2, 10.5, and 7.4 Mg ha⁻¹, respectively (Minnesota Agricultural Experiment Station, 2002), compared with only 3.0 Mg ha⁻¹ for Illinois bundleflower in our trial. In the same report, alfalfa yielded 12.0 Mg ha⁻¹ at Lambertton, about 50% more than Illinois bundleflower in our experiment in 2002. Lower yield of Illinois bundleflower compared with these cool-season legumes is likely due to its shorter growing season, the undomesticated status of the accessions used, or to less-than-optimum N₂ fixation. Aboveground N yield of Illinois bundleflower was lowest at Rosemount, where the accessions averaged only 48 kg ha⁻¹, which was less than first-year N yield at that site. Nitrogen yields at Becker and Lambertton were about 100 and 400% greater, respectively, than at Rosemount. Nitrogen yield of wild senna was more consistent than Illinois bundleflower, with low accumulation (<40 kg N ha⁻¹) at all locations. On the basis of yields reported in the Minnesota cultivar trials cited above, and assuming a mean herbage N concentration of 26 g N kg⁻¹ dry mass, harvested herbage N of established alfalfa, red clover, and birdsfoot trefoil at Rosemount averaged 340, 270, and 190 kg N ha⁻¹, respectively, and alfalfa contained 310 kg N ha⁻¹ at Lambertton. Harvested N in Illinois bundleflower at the most productive site (Lambertton) was, therefore, about 40% lower than alfalfa and about equal to birdsfoot trefoil.

As in the first year, the two ¹⁵N methods differed in estimated %Ndfa ($P < 0.01$), with higher estimates of %Ndfa at enriched than at natural abundance levels of ¹⁵N. The location effect was significant ($P < 0.001$). Plants at the low-N Becker site were most dependent on fixed N₂, with %Ndfa ranging from 63 to 83% averaged across accessions. At Rosemount, %Ndfa of PNL534 and PNL541 was zero according to the ¹⁵N natural abundance method, whereas the ¹⁵N enrichment method estimated %Ndfa of 28 and 17% for these accessions, respectively. At Lambertton, %Ndfa across accessions was estimated at only 40% by the ¹⁵N natural abundance method, but 67 to 79% by the ¹⁵N enrichment and total N difference methods, respectively. In contrast to our results, Brandon et al. (1998), using the ¹⁵N natural abundance method, measured %Ndfa in inoculated second-year stands of the related species *Desmanthus virgatus* (L.) Willd. of 78, 48, and 26%, in an infertile sandy soil, a moderately fertile black earth, and a highly fertile clay soil, respectively. This response corresponds more closely to what is generally expected; that is, that legume reliance on symbiotic N₂ fixation declines with increasing N supply (Allos and Bartholomew, 1959). We conclude that factors other than soil N supply were important in our experiment.

The two ¹⁵N methods did not differ in estimating N₂ fixed at Becker and Rosemount ($P > 0.05$). The ¹⁵N methods estimated N₂ fixation of about 70 kg ha⁻¹ across accessions at Becker and <10 kg ha⁻¹ fixed at Rosemount. At Lambertton, estimates of N₂ fixed were different for the two methods ($P < 0.01$), with an estimate

only one-half as large with the ¹⁵N natural abundance method as with the ¹⁵N enrichment method.

Only 12 kg N ha⁻¹ was fixed at Rosemount according to the total N difference method, vs. 67 kg ha⁻¹ at Becker and 142 kg ha⁻¹ at Lambertton. The total N difference method produced higher estimates of N₂ fixed than did the ¹⁵N natural abundance method at Lambertton ($P < 0.001$), but the methods were similar at Becker ($P > 0.05$). In contrast, the total N difference method gave lower estimates of N₂ fixation than the ¹⁵N enrichment method at Becker ($P < 0.001$), but higher at Lambertton ($P < 0.001$), with no difference at Rosemount ($P > 0.05$). Except for the results at Rosemount, these estimates of N₂ fixation by Illinois bundleflower compare favorably to those of birdsfoot trefoil in the north-central USA, which generally have ranged from 50 to 205 kg N ha⁻¹, with a median of 90 to 100 kg N ha⁻¹ annually (Seguin et al., 2000). Under irrigated, high-yield conditions at Becker, others have estimated that established alfalfa fixed between 240 and 340 kg N ha⁻¹, depending on the method used to estimate N₂ fixation (Lamb et al., 1995).

Plants at Rosemount were visibly chlorotic in the second year. The relatively wet spring and dry summer in 2001 may have adversely affected N₂ fixation at Rosemount. Brandon and Date (1998) found that growth of *Desmanthus virgatus* was limited by S and Mo in a relatively fertile soil, and recommended a critical leaf S concentration of 2.0 g kg⁻¹. Sulfur concentrations in Illinois bundleflower leaves were much lower at Rosemount (1.8 g kg⁻¹) than at Becker (4.8 g kg⁻¹) or Lambertton (5.2 g kg⁻¹). Molybdenum concentrations were also lower in leaves from Rosemount (0.25 mg kg⁻¹) than in leaves from Becker (2.1 mg kg⁻¹) or Lambertton (3.0 mg kg⁻¹). These results are quite surprising, given the high levels of soil organic matter at Rosemount (Table 1). Sulfur and Mo fertilization research may help clarify whether these nutrients limit Illinois bundleflower growth.

The inoculant rhizobial strains accounting for almost all nodules at Rosemount and Becker may have been less than optimal in symbiotic efficiency considered. Dinitrogen fixation was highest at Lambertton, the only site within the native range of Illinois bundleflower. Over one-half of the nodules sampled were occupied by noninoculant indigenous strains at this site. The relative effectiveness of the inoculant and indigenous strains has not been characterized and is a promising direction for further investigations into the N₂-fixation capabilities of Illinois bundleflower.

Another factor that deserves investigation is the role of mycorrhizal fungi in enhancing uptake of nutrients and N₂ fixation in Illinois bundleflower. After conducting these studies, we learned that Illinois bundleflower is a host of *Gigaspora* spp. mycorrhizae (C. Picone, 2002, personal communication). If mycorrhizal inoculum present at Lambertton, where Illinois bundleflower is native, was not available at the other locations where the plant is introduced, differences in δ¹⁵N among locations might result. Infection by mycorrhizae generally leads to lower δ¹⁵N than noninfected plants (Handley and Scrimgeour, 1997), and would thus lead to higher esti-

mates for %Nd_{fa}. We did not inoculate with mycorrhizae in the experiment designed to determine ¹⁵N fractionation (Table 3). Inoculation with appropriate mycorrhizae also may improve access to nutrients that otherwise may limit plant growth.

Overall, %Nd_{fa} was lowest at Rosemount in both years. Little or no N₂ was fixed at Becker and Rosemount the first year, whereas 30 to 43 kg ha⁻¹ was fixed at Lambertson, depending on the measurement method. Dinitrogen fixation did not occur at Rosemount in the second year, whereas apparent N₂ fixation was 60 to 79 kg ha⁻¹ at Becker and 67 to 142 kg ha⁻¹ at Lambertson, depending on the measurement method.

Large differences in herbage yield among locations, but similar yields among accessions, indicate that conditions at one or two of the sites were suboptimal for Illinois bundleflower forage production. We expected to find larger differences among accessions in herbage yield, N yield, and apparent N₂ fixation. We confirmed that indigenous rhizobial strains reduced δ¹⁵N in herbage, compared with the inoculant strains. This implies that nodule occupancy should be determined when the natural abundance ¹⁵N method is used at locations that may differ in rhizobial strain presence and/or competitiveness. On the basis of our analysis of nodule occupancy, we recommend that additional rhizobial strains be sought for use as inoculum for this potential forage crop.

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