

# Field-based Selection Method Creates Alfalfa Populations That Differ in Nitrate Nitrogen Uptake

JoAnn F. S. Lamb,\* Michael P. Russelle, and Diana M. Fenton

## ABSTRACT

Sustainable agricultural systems could benefit from alfalfa (*Medicago sativa* L.) germplasm that reduce N losses to the ground water and decrease N fertilizer inputs. With the goal of producing alfalfa populations with altered nitrate N uptake, our objectives were to develop, assess, and refine an inexpensive selection method using Br<sup>-</sup> as an analog for nitrate under field conditions. Two selection schemes using divergent herbage Br<sup>-</sup> concentration and/or divergent herbage Br<sup>-</sup> uptake, both in combination with high herbage N content, produced two Cycle 1 and four Cycle 2 alfalfa populations. We evaluated these populations for both Br<sup>-</sup> uptake and nitrate N uptake (estimated by N derived from fertilizer, Ndff [<sup>15</sup>N-labeled]) for one regrowth period in two locations in each of 2 yr. Weak associations between Br<sup>-</sup> uptake and Ndff were found during the establishment year, but strong correlations were found between Br<sup>-</sup> concentration and fraction of Ndff (fNdff) ( $r = 0.83$ ) and between Br<sup>-</sup> uptake and Ndff ( $r = 0.85$  to  $0.90$ ) when populations were evaluated during the first production year. Populations selected for high Br<sup>-</sup> concentration or uptake had greater Br<sup>-</sup> concentration and uptake, fNdff, and Ndff than populations selected for low Br<sup>-</sup>. Our method of selecting alfalfa plants for differences in herbage Br<sup>-</sup> concentration or uptake in combination with high herbage N content is the first to successfully produce alfalfa populations that differ in nitrate N uptake in a field environment.

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**Abbreviations:** DM, dry matter; fNdff, fraction of N derived from fertilizer; Ndff, N derived from fertilizer.

**N**EW ALFALFA (*Medicago sativa* L.) germplasm that reduce nitrate N losses to ground water and decrease fertilizer N requirements would be environmentally and economically beneficial and improve agricultural sustainability. About 50 yr ago, Stewart et al. (1968) suggested using alfalfa, a deeply rooted perennial, to remove residual nitrate from the soil profile. Apparent N uptake, measured as decreases in soil inorganic N under alfalfa in field conditions, totaled up to 300 kg nitrate N ha<sup>-1</sup> yr<sup>-1</sup> from below the rooting depths of most annual crops (Schertz and Miller, 1972; Mathers et al., 1975). Direct evidence of subsoil nitrate removal by alfalfa was demonstrated using <sup>15</sup>N-labeled nitrate (Blumenthal and Russelle, 1996). Total inorganic N uptake by alfalfa from fertilizer- or manure-contaminated sites ranged from 200 to 400 kg N ha<sup>-1</sup> (Russelle et al., 2001, 2007). Because inorganic N uptake usually reduces symbiotic N<sub>2</sub> fixation (Allos and Bartholomew, 1959; Lamb et al., 1995), potential nitrate N uptake may exceed 600 kg ha<sup>-1</sup> under high-yielding temperate conditions, such as those in the Argentine Pampas (Racca et al., 2001).

Alfalfa with greater capacity to remove nitrate would be useful for environmental protection and remediation, whereas alfalfa

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that is less competitive for nitrate should improve productivity and stability of mixed species plantings. Mixtures of alfalfa and grass are often grown to reduce the likelihood of livestock bloat, improve resilience of the stand (Sleugh et al., 2000), reduce weed encroachment (Spandl et al., 1999), and maintain quality of forage grasses when harvests are delayed (Berdahl et al., 2004).

Direct measurement of nitrate uptake in a  $N_2$ -fixing crop like alfalfa is best done by labeling the soil nitrate with  $^{15}N$ , but this approach is not economically feasible in a plant breeding program, where thousands of plants must be measured individually. In selecting alfalfa plants for differences in nitrate N uptake, a new field-based methodology is required that will reflect the contribution of nitrate N to total shoot N. Earlier efforts to select alfalfa for differential  $N_2$  fixation under greenhouse conditions did not translate successfully to the field (Teuber and Phillips, 1988; Heichel et al., 1989).

Bromide ( $Br^-$ ), a nonessential element for plant growth, has been used to trace nitrate N movement in soil-water systems, but  $Br^-$  uptake by corn (*Zea mays* L.), potato (*Solanum tuberosum* L.), perennial ryegrass (*Lolium perenne* L.), orchardgrass (*Dactylis glomerata* L.), and Kentucky bluegrass (*Poa pratensis* L.) plants has invalidated some of these studies (Onken et al., 1977; Owens et al., 1985; Jemison and Fox, 1991; Kung, 1990; Schnabel et al., 1995). Despite their different roles in the plant, the generally noncompetitive (Epstein, 1953) uptake of  $Br^-$  and nitrate N is correlated in corn and sorghum [*Sorghum bicolor* (L.) Moench] (Chao, 1966; Jemison and Fox, 1991). This suggested to us that  $Br^-$  could serve as an inexpensive tracer in plant selection programs for nitrate N in  $N_2$  fixing crops.

Magarian et al. (1998) showed that nitrate N/ $Br^-$  ratios in alfalfa herbage directly reflected the ratios of the nitrate N/ $Br^-$  in solutions applied across a wide range of tracer supply in the greenhouse. Individual plant analysis showed close agreement between  $Br^-$  and nitrate N uptake among plants in several different alfalfa germplasm. It also appeared that selection for nitrate N uptake using  $Br^-$  uptake would result in minimal error, compared to selecting with  $^{15}N$ -labeled nitrate directly. Under field conditions, strong correlations between  $Br^-$  uptake and nitrate N uptake ( $r^2 = 0.85$  to  $0.97$ ) were found among alfalfa cultivars and experimental germplasm (Magarian, 1996; Blumenthal et al., 1999).

Given the robust correlation between  $Br^-$  and nitrate uptake, our objectives were to develop, assess, and refine a field-based selection method using  $Br^-$  as an analog for nitrate to produce divergent alfalfa populations with altered nitrate N uptake.

## MATERIALS AND METHODS

### Selection Protocol and Plant Materials

We used two cycles of selection to develop alfalfa populations that differed in  $Br^-$  uptake and inorganic nitrate N uptake.

### First Cycle of Selection

The base or parent population from which selection was initiated was UMN 2899 MNVC93, a composite of 18 commercially available alfalfa cultivars, described by Magarian (1996). MNVC93 was seeded in a 1.8 by 12.6 m plot with 7.5-cm spacing between and within rows at the University of Minnesota Sand Plain Research Farm at Becker, MN (45°23' N, 93°53' W) on Hubbard–Mosford complex loamy sand soils (sandy, mixed, frigid, Entic and Typic Hapludolls, Soil Survey Staff) in the spring of 1993. Plants were maintained with typical harvest management for the area, pesticide applications as required, and 200 kg N ha<sup>-1</sup> of ammonium nitrate applied annually (in weekly increments of 10 kg N ha<sup>-1</sup>) to supplement mineralized soil N. On 1 July 1994, herbage was removed and twice weekly irrigation of CaBr<sub>2</sub> (0.5 mg Br<sup>-</sup> L<sup>-1</sup>) was applied through drip irrigation during the 4-wk herbage regrowth period. At each irrigation 5 cm of solution was applied (10 cm wk<sup>-1</sup>) to replace soil solution in the upper root zone. This loamy sand soil had an available water holding capacity of about 4.4 cm in the upper 60 cm, and a very low water holding capacity in the underlying sand and gravel. Recognizing that  $Br^-$  leaching is very likely under these conditions, our intention was to re-establish the  $Br^-$  concentration in the soil solution several times during regrowth, rather than to apply a particular rate of  $Br^-$  per unit land area.

Herbage was harvested from each plant at 10% bloom on 28 July 1994. Samples were oven dried at 60°C, weighed, and ground to pass a 1-mm mesh. Bromide concentrations were determined using a method modified from Magarian (1996). Representative subsamples (0.24 to 0.26 g) were weighed and placed in 15 by 150 mm screw-cap glass culture tubes. Finely ground activated charcoal (>2 g) and 25 mL deionized water were added to the tubes, which were shaken horizontally for 60 min. Extracts were filtered twice through Whatman 40 filter paper and filtrates were analyzed for  $Br^-$  concentration by flow injection analysis (Switala, 1990). This extraction and analytical method was well correlated with total  $Br^-$  analysis by X-ray fluorescence spectroscopy (Magarian et al., 1998).

Net  $Br^-$  concentration was calculated as the difference in  $Br^-$  concentration between samples from treated and nontreated areas of the plot. Total N percentage in each plant was estimated using near-infrared spectroscopy calibrated with a large population of samples as described by Sheaffer et al. (2000). Total N and  $Br^-$  contents were calculated as concentration × herbage dry matter (DM) yield of each plant. Plants for the first cycle of selection were selected for high herbage N content in combination with either high (UMN3028 HNHBrConcC1, referred to as HBrC1) or low (UMN3031 HNLBrConcC1, referred to as LBrC1) herbage  $Br^-$  concentration at a selection pressure of 5%. In this and other phases of selection and evaluation, we selected plants with high N content to help avoid inadvertent selection of undesirable plants (i.e., those with low DM yield or low  $N_2$  fixation plus inorganic nitrate N uptake).

### Second Cycle of Selection

HBrC1 was seeded in a 0.9 by 12 m plot and LBrC1 was seeded in a 0.9 by 4.5 m plot with 7.5-cm spacing between and within rows at the Sand Plain Research Farm at Becker in the spring of 1997, with management as described above. Plot size for LBrC1

was smaller because fewer seeds were available. On 4 June 1998, herbage was removed and twice weekly addition of 5 mg Br<sup>-</sup> L<sup>-1</sup> (KBr) and 28 mg N L<sup>-1</sup> [Ca(NO<sub>3</sub>)<sub>2</sub>] was applied using drip irrigation. Forage from individual plants was harvested at 10% bloom on 7 July 1998. Shoots were processed and assessed for Br<sup>-</sup> and N as described above.

In the second cycle of selection, two selection schemes for Br<sup>-</sup> were used: Br<sup>-</sup> concentration and Br<sup>-</sup> uptake. Two populations were created from HBrC1 by selecting for high N content in combination with either high herbage Br<sup>-</sup> concentration (UMN3198 HNHBrcC2, referred to as HBrC2) or high Br<sup>-</sup> uptake (UMN3197 HNHBrcUpC2, referred to as HBrUp2). Two populations also were created by selecting from LBrC1 for high N content in combination with either low Br<sup>-</sup> concentration (UMN3195 HNLBrcC2, referred to as LBrC2) or low Br<sup>-</sup> uptake (UMN3196, HNLBrcUpC2, referred to as LBrUp2). We selected for both Br<sup>-</sup> concentration and Br<sup>-</sup> uptake to test which of these two traits would lead to greater gain per cycle of selection for N uptake. All four Cycle 2 populations were created using a selection pressure of approximately 5%.

### Evaluation of Selected Populations

The six selected populations were seeded in early to mid-May 1999 in 12 replications of nine-plant plots in a randomized complete block design with 7.5-cm spacing between and within rows. These alfalfa populations were grown at the University of Minnesota Sand Plain Irrigation Research Farm, Becker, MN, on a Hubbard-Mosford complex loamy sand soils and at the Central Lakes Agriculture Center, Staples, MN (46°23' N, 94°48' W) on a Verndale sandy loam (coarse-loamy, mixed, superactive, frigid Typic Argiudoll) soil. Soils at both locations are moderately well to excessively well drained. Nutrients other than N were supplied in fertilizer based on University of Minnesota soil test recommendations (Rehm and Schmitt, 1989).

### Establishment Year Evaluation

Twice weekly tracer applications began after the first harvest in mid-July and continued for 28 to 33 d when the plants were about 14 wk old. Tracer concentrations were the same as used in Cycle 2 selections, but the nitrate source was labeled with 0.5 atom percent <sup>15</sup>N. Individual plants were harvested on 12 Aug. 1999 at Becker and 16 August at Staples, dried, and ground as described earlier. Tissue analyses also were the same, except that samples also were analyzed for <sup>15</sup>N/<sup>14</sup>N ratio by the Stable Isotope Facility at the University of California–Davis. Results from this analysis allowed us to calculate the proportion of N in the plant tissue that was derived from the <sup>15</sup>N-nitrate that we applied to the plots. The fraction of N derived from fertilizer (fNdff) was multiplied by the total N content to estimate the amount of N derived from fertilizer (Ndff) (Hauck, 1982). A subsequent herbage harvest was discarded in mid-October 1999 at both locations.

### First Production Year Evaluation

To minimize the effects of residual Br<sup>-</sup> and <sup>15</sup>N applied to the plants in mid-1999, we removed herbage twice at normal forage harvesting intervals before applying the tracers on 12 July 2000 at Staples and 4 Aug. 2000 at Becker. Protocols were the same as before, with sampling occurring on 10 August at Staples and

1 September at Becker, when plants were 66 and 70 wk old, respectively. Individual plants were harvested, processed, and analyzed as described above.

### Statistical Analysis

Data were analyzed as a randomized complete block with 12 replicates (PROC GLM, SAS Institute, 2003). In the analysis of variance, locations were considered random and harvest years and germplasm were considered fixed. Means for all traits evaluated for main effects and interactions of the two locations, two harvest years, and six alfalfa germplasms were compared (PROC GLM, SAS Institute, 2003). Mean herbage DM yield, Br<sup>-</sup> concentration and uptake, N concentration, fNdff, and Ndff for each germplasm within each selection scheme within each harvest year also were compared using least significant difference (Steel and Torrie, 1980). Regression analysis was conducted to investigate the relationship between Br<sup>-</sup> uptake and Ndff in both the establishment and first production years (PROC REG, SAS Institute, 2003). Correlation analysis was conducted to investigate relationships among DM yield, Br<sup>-</sup> and total N concentrations and uptake, fNdff, and Ndff (PROC CORR, SAS Institute, 2003). Significance was declared at *P* < 0.05 unless otherwise indicated.

## RESULTS

The main effects of years, locations, and entries, and many interactions among main effects were significant for yield and all Br<sup>-</sup> and N traits evaluated in our study. Several complex interactions were evident when all six entries were included in a single analysis of variance (results not shown). Most of these interactions for yield and some of the Br<sup>-</sup> and N traits were caused by Cycle 2 populations switching rank between locations within either the two high (HBrC2 and HBrUp2) or the two low (LBrC2 and LBrUp2) selections. Year by entry interactions from the overall analysis for yield and the N traits demonstrated that the entries responded differently for several of these traits in the establishment year compared to the first production year. This interaction has important implications for selection of alfalfa. We therefore present results of our study separately for each selection scheme in each year.

### Establishment Year

The analyses of variance for both selection schemes in the establishment year are shown in Table 1. For both schemes, herbage DM yield, Br<sup>-</sup> uptake, and total N concentration were greater at Staples, whereas Br<sup>-</sup> concentration, fNdff, and Ndff were greater at Becker (data not shown). A location by entry interaction for Br<sup>-</sup> concentration was found only for the Br<sup>-</sup> concentration selection scheme. At Becker, the Cycle 2 population, HBrC2, had greater Br<sup>-</sup> concentration (1.34 mg Br<sup>-</sup> g<sup>-1</sup> DM) than the Cycle 1 population, HBrC1 (1.25 mg Br<sup>-</sup> g<sup>-1</sup> DM), whereas these two populations did not differ at Staples (avg. 1.01 mg Br<sup>-</sup> g<sup>-1</sup> DM).

In both selection schemes, all populations for high Br<sup>-</sup> had greater Br<sup>-</sup> concentration and Br<sup>-</sup> uptake than those

selected for low Br<sup>-</sup> (Table 2). However, no differences among entries were found for fNdff in either selection scheme, nor were differences found for herbage DM yield and Ndff in the Br<sup>-</sup> concentration selection scheme. For the Br<sup>-</sup> uptake selection scheme, Cycle 1 populations for high and low Br<sup>-</sup> were similar in DM yield and Ndff, whereas the Cycle 2 populations differed (Table 2).

Correlations among DM yield, Br<sup>-</sup> concentration and uptake, total N concentration, fNdff, and Ndff from both selection schemes for the establishment year are shown in Table 3. Dry matter yield was moderately positively correlated with Br<sup>-</sup> uptake, negatively correlated with fNdff, and had little to no association with Br<sup>-</sup> or total N concentration, or with Ndff. Bromide concentration and Br<sup>-</sup> uptake were only moderately auto-correlated, whereas fNdff and Ndff were more strongly autocorrelated. Bromide concentration was positively correlated with fNdff and Ndff, but Br<sup>-</sup> uptake had only a moderate association with Ndff ( $r = 0.52$  to  $0.56$ ,  $P > 0.001$ ) and no association with fNdff. Our results differ from Magarian (1996), who reported a stronger association between Br<sup>-</sup> and nitrate N uptake among a smaller population of alfalfa entries during the establishment year ( $r = 0.67$  to  $0.85$ ,  $P > 0.01$ ).

### First Production Year

The analyses of variance for both selection schemes in the first production year are shown in Table 4. For both selection schemes, herbage DM yield was greater at Staples, but Br<sup>-</sup> concentration and uptake, total N concentration, fNdff, and Ndff, were greater at Becker (data not shown). A location by entry interaction for herbage DM yield occurred only in the Br<sup>-</sup> concentration selection scheme. Herbage DM yield differences between the two cycles of selection populations for high Br<sup>-</sup> concentration (HBrC1 < HBrC2) were greater at Staples (3.8 g plot<sup>-1</sup>) than at Becker (1.4 g plot<sup>-1</sup>).

**Table 1. Analyses of variance for dry matter (DM) yield, Br<sup>-</sup> concentration and uptake, total N concentration, fraction of N derived from fertilizer (fNdff), and N derived from fertilizer (Ndff) for the Br<sup>-</sup> concentration and the Br<sup>-</sup> uptake selection schemes in the establishment year.**

Selection scheme	Source	df	Mean squares					
			DM yield	Br <sup>-</sup> conc.	Br <sup>-</sup> uptake	N conc.	fNdff	Ndff
Br <sup>-</sup> concentration	Location (L)	1	646***	1.45***	57	1.26***	1.883***	12,005***
	Rep (L)	22	6	0.07***	27*	0.05	0.011	5,630***
	Entry (E)	3	10	0.19***	103***	0.13*	0.002	2,658
	L × E	3	10	0.05*	4	0.03	0.001	512
	Error	60	6	0.01	13	0.05	0.002	1,646
Br <sup>-</sup> uptake	Location (L)	1	508***	0.95***	67	0.916**	1.831***	144,542***
	Rep (L)	22	9	0.04***	20*	0.07	0.007***	3,317**
	Entry (E)	3	50***	0.22***	241***	0.05	0.003	14,720***
	L × E	3	5	0.01	12	0.01	0.004	1,020
	Error	60	6	0.01	11	0.05	0.002	1,419

\*Significant at the 0.05 probability level.

\*\*Significant at the 0.01 probability level.

\*\*\*Significant at the 0.001 probability level.

**Table 2. Entry means for dry matter (DM) yield, Br<sup>-</sup> concentration and uptake, total N concentration, fraction of N derived from fertilizer (fNdff), and N derived from fertilizer (Ndff) over two locations for the Br<sup>-</sup> concentration and Br<sup>-</sup> uptake selection schemes in the establishment year.**

Selection scheme	Entries	DM yield	Br <sup>-</sup> conc.	Br <sup>-</sup> uptake	N conc.	fNdff	Ndff
		g plot <sup>-1</sup>	mg g <sup>-1</sup>	mg plot <sup>-1</sup>	mg g <sup>-1</sup>	g g <sup>-1</sup>	mg plot <sup>-1</sup>
Br <sup>-</sup> concentration	HBrC2	16.8	1.15	19.0	3.45	0.391	213
	HBrC1	17.1	1.16	19.7	3.26	0.408	216
	LBrC1	15.7	1.03	15.9	3.35	0.397	197
	LBrC2	16.5	0.97	15.6	3.39	0.389	197
	LSD <sub>0.05</sub>	NS†	0.06	2.1	0.12	NS	NS
Br <sup>-</sup> uptake	HBrUp2	18.3	1.19	21.5	3.36	0.403	234
	HBrC1	17.1	1.16	19.7	3.26	0.408	216
	LBrC1	15.7	1.03	15.9	3.35	0.397	197
	LBrUp2	14.9	0.98	14.3	3.35	0.389	174
	LSD <sub>0.05</sub>	1.4	0.07	1.9	NS	NS	22

†NS, not significant.

**Table 3. Pearson correlations (r) for dry matter (DM) yield, Br<sup>-</sup> concentration and uptake, total N concentration, fraction of N derived from fertilizer (fNdff), and N derived from fertilizer (Ndff) from the establishment year among entries from the Br<sup>-</sup> concentration selection scheme (above the diagonal) and among entries from the Br<sup>-</sup> uptake selection scheme (below the diagonal).**

	DM yield	Br <sup>-</sup> conc.	Br <sup>-</sup> uptake	N conc.	fNdff	Ndff
DM yield	–	–0.37***	0.59***	0.25*	–0.70***	–0.16NS†
Br <sup>-</sup> conc.	–0.20NS	–	0.52***	–0.18NS	0.76***	0.79***
Br <sup>-</sup> uptake	0.68***	0.57***	–	0.07NS	0.01NS	0.56***
N conc.	0.09NS	–0.15NS	–0.01NS	–	–0.40***	–0.10NS
fNdff	–0.61***	0.69***	–0.01NS	–0.31**	–	0.77***
Ndff	–0.08NS	0.79***	0.52***	–0.10NS	0.79***	–

\*Significant at the 0.05 probability level.

\*\*Significant at the 0.01 probability level.

\*\*\*Significant at the 0.001 probability level.

†NS, not significant.

**Table 4. Analyses of variance for dry matter (DM) yield, Br<sup>-</sup> concentration and uptake, total N concentration, fraction of N derived from fertilizer (fNdff), and N derived from fertilizer (Ndff) for the Br<sup>-</sup> concentration and the Br<sup>-</sup> uptake selection schemes in the first production year.**

Selection scheme	Source	df	Mean squares					
			DM yield	Br <sup>-</sup> conc.	Br <sup>-</sup> uptake	N conc.	fNdff	Ndff
Br <sup>-</sup> concentration	Location (L)	1	293***	2.58***	383***	5.17***	0.520***	61,188***
	Rep (L)	22	17	0.01	15	0.12	0.006***	1,795*
	Entry (E)	3	89*	0.13***	166***	0.03	0.002*	6,215***
	L × E	3	73*	0.01	39	0.12	0.001	1,024
	Error	60	22	0.01	17	0.06	0.001	928
Br <sup>-</sup> uptake	Location (L)	1	176**	2.65***	601***	6.03***	0.575***	89,679***
	Rep (L)	22	20	0.01	13	0.07	0.005***	1,794*
	Entry (E)	3	179***	0.17***	335***	0.18*	0.003*	16,491***
	L × E	3	5	0.01	16	0.04	0.002	384
	Error	60	23	0.01	21	0.06	0.001	1,261

\*Significant at the 0.05 probability level.  
 \*\*Significant at the 0.01 probability level.  
 \*\*\*Significant at the 0.001 probability level.

For both selection schemes, the Cycle 2 populations selected for either high Br<sup>-</sup> concentrations or uptake (HBrC2 and HBrUp2) had greater in DM yield, Br<sup>-</sup> concentration and uptake, fNdff, and Ndff than the Cycle 2 populations selected for low Br<sup>-</sup> concentration or uptake (LBrC2 and LBrUp2) (Table 5). Populations from Cycle 1 were the same for both selection schemes, and HBrC1 had greater DM yield, Br<sup>-</sup> concentration and uptake, fNdff, and Ndff than LBrC1. For the Br<sup>-</sup> concentration selection scheme, essentially no gain from selection was observed for any of the traits when comparing the means of Cycle 1 to Cycle 2 high Br<sup>-</sup> concentration (HBrC1 to HBrC2), or Cycle 1 to Cycle 2 low Br<sup>-</sup> concentration (LBrC1 to LBrC2). The same results were found in the Br<sup>-</sup> uptake selection scheme when comparing

**Table 5. Entry means for dry matter (DM) yield, Br<sup>-</sup> concentration and uptake, total N concentration, fraction of N derived from fertilizer (fNdff), and N derived from fertilizer (Ndff) over two locations for the Br<sup>-</sup> concentration and Br<sup>-</sup> uptake selection schemes in the first production year.**

Selection scheme	Entries	DM yield	Br <sup>-</sup> conc.	Br <sup>-</sup> uptake	N conc.	fNdff	Ndff
		g plot <sup>-1</sup>	mg g <sup>-1</sup>	mg plot <sup>-1</sup>	mg g <sup>-1</sup>	g g <sup>-1</sup>	mg plot <sup>-1</sup>
Br <sup>-</sup> concentration	HBrC2	23.3	0.86	19.1	4.03	0.439	169
	HBrC1	21.7	0.82	17.6	3.97	0.442	161
	LBrC1	18.7	0.71	13.3	3.97	0.423	133
	LBrC2	20.3	0.70	14.2	3.95	0.422	142
	LSD <sub>0.05</sub>	2.7	0.04	2.4	NS <sup>†</sup>	0.015	18
Br <sup>-</sup> uptake	HBrUp2	25.0	0.87	21.2	4.10	0.438	189
	HBrC1	21.7	0.82	17.6	3.97	0.442	161
	LBrC1	18.7	0.71	13.3	3.97	0.423	133
	LBrUp2	19.5	0.67	13.0	3.88	0.416	132
	LSD <sub>0.05</sub>	2.8	0.05	2.6	0.14	0.017	21

<sup>†</sup>NS, not significant.

Cycle 1 low Br<sup>-</sup> concentration to Cycle 2 low Br<sup>-</sup> uptake (LBrC1 to LBrUp2). However, HBrUp2 had greater DM yield and Br<sup>-</sup> uptake and Ndff than HBrC1, although the concentration traits (Br<sup>-</sup> concentration and fNdff) were similar between these two entries.

Small, but noteworthy differences in DM yield were evident in the first production year between Cycle 1 populations selected for high and low Br<sup>-</sup> concentration (3 g plot<sup>-1</sup> between HBrC1 and LBrC1). In Cycle 2 using Br<sup>-</sup> concentration, the yield differential remained the same between HBrC2 and LBrC2, but the yield differential increased by 45% when we selected for high and low Br<sup>-</sup> uptake (5.5 g plot<sup>-1</sup>

difference between HBrUp2 and LBrUp2). It appears that in selecting for greater Br<sup>-</sup> uptake, we unintentionally selected larger plants or plants with greater yield potential. Because DM yield is a component in calculating nutrient uptake, both Br<sup>-</sup> and Ndff increased when DM yield increased.

Correlations among DM yield, Br<sup>-</sup> concentration and uptake, N concentration, fNdff, and Ndff from both selection schemes for the first production year differed from those found during the establishment year (Table 6). Dry matter yield was once again positively correlated with Br<sup>-</sup> uptake, but, unlike the establishment year, had a weak association with Ndff. Associations between DM yield and the concentration traits (Br<sup>-</sup> concentration and fNdff) were no longer evident in first production year stands. Bromide concentration and Br<sup>-</sup> uptake were autocorrelated, as were fNdff and Ndff. The positive association between Br<sup>-</sup> concentration and N uptake was essentially the same in both stand ages. Unlike the establishment year, Br<sup>-</sup> uptake was weakly associated with fNdff and Ndff in the first production year. The correlation of Br<sup>-</sup> concentration with fNdff ( $r = 0.83, P > 0.001$ ) and the association between Br<sup>-</sup> uptake and Ndff ( $r = 0.85$  to  $0.90, P > 0.001$ ) increased dramatically in the first production year compared to the establishment year.

## DISCUSSION

Because the relationship between Br<sup>-</sup> uptake and Ndff varied between the 2 yr of our study, we used regression analysis to compare entry responses for Br<sup>-</sup> uptake and Ndff in the establishment year (1999) and the first production year (2000)

(Fig. 1). For all six entries, there was a strong relationship between Br<sup>-</sup> uptake and Ndff in the first production year, but a poor relationship during the establishment year. Similar results were found when regression analyses were conducted separately for the Br<sup>-</sup> uptake and Br<sup>-</sup> concentration selection schemes. Regression equations for Br<sup>-</sup> uptake vs. Ndff were  $y = 4.5 + 104x$ ,  $r^2 = 0.84$  (Br<sup>-</sup> uptake scheme) and  $y = 6.3 + 95x$ ,  $r^2 = 0.74$  (Br<sup>-</sup> concentration scheme) in the first production year, and  $y = 5.9 + 40x$ ,  $r^2 = 0.27$  (Br<sup>-</sup> uptake scheme) and  $y = 7.8 + 46x$ ,  $r^2 = 0.33$  (Br<sup>-</sup> concentration scheme) in the establishment year.

Magarian (1996) found no differences between HBrC1 and LBrC1 under greenhouse conditions when the plants were 13 wk old. In the current study, these field-grown plants were 14 wk of age when they were sampled and assessed for nutrient concentration and uptake during the establishment year. Lamb et al. (2000) recommended that the minimum age for characterizing alfalfa plants for heritable root morphology traits should be 22 wk after planting in the field. Carlson (1925) also stated that the distinctive characteristics of alfalfa roots do not develop until after 3 to 4 mo of normal growth. Johnson et al. (1996) suggested that a change from a juvenile to mature growth stage for alfalfa root characteristics may occur at approximately 17 wk after planting. Differences in herbage yield demonstrated in the first and later production years among diverse alfalfa entries were not evident during the establishment year (Lamb et al., 2006). Rooting characteristics likely play a role in nutrient uptake and herbage yield because they are the point of interaction between the alfalfa plant and its soil environment. These alfalfa herbage yield and root morphologies studies demonstrate that a period of time is needed for alfalfa to develop into a mature plant with a developed root system. We speculate that during the establishment year, at least in climates typical of the U.S. Upper Midwest, alfalfa plants are not sufficiently developed to show an association between the complex traits involved in Br<sup>-</sup> and nitrate N uptake and concentration. We conclude that selection and evaluation of nitrate uptake and concentration in alfalfa should not be conducted during the establishment year.

Results from the first production year of our study demonstrated that we were successful in creating alfalfa populations that differed in nitrate N uptake (estimated by Ndff) using either Br<sup>-</sup> concentration or Br<sup>-</sup> uptake as the selection criterion. However, two aspects of our results were unexpected and led us to reexamine our methods and conclusions.

First, the greatest gain in Br<sup>-</sup> and nitrate N uptake occurred in the first cycle of selection, with little or no change in the means of the divergently selected Cycle 2 populations. Two possible reasons for the

**Table 6.** Pearson correlations (*r*) for dry matter (DM) yield, Br<sup>-</sup> concentration and uptake, total N concentration, fraction of N derived from fertilizer (fNdff), and N derived from fertilizer (Ndff) from the first production year among entries from the Br<sup>-</sup> concentration selection scheme (above the diagonal) and among entries from the Br<sup>-</sup> uptake selection scheme (below the diagonal).

	DM yield	Br <sup>-</sup> conc.	Br <sup>-</sup> uptake	N conc.	fNdff	Ndff
DM yield	–	0.16NS <sup>†</sup>	0.61***	-0.51***	-0.29**	0.39***
Br <sup>-</sup> conc.	0.01NS	–	0.67***	0.57***	0.83***	0.67***
Br <sup>-</sup> uptake	0.66***	0.74***	–	0.06NS	0.43***	0.85***
N conc.	-0.25*	0.67***	0.35***	–	0.63***	0.38***
fNdff	-0.20NS	0.83***	0.47***	0.65***	–	0.71***
Ndff	0.50***	0.75***	0.90***	0.55***	0.69***	–

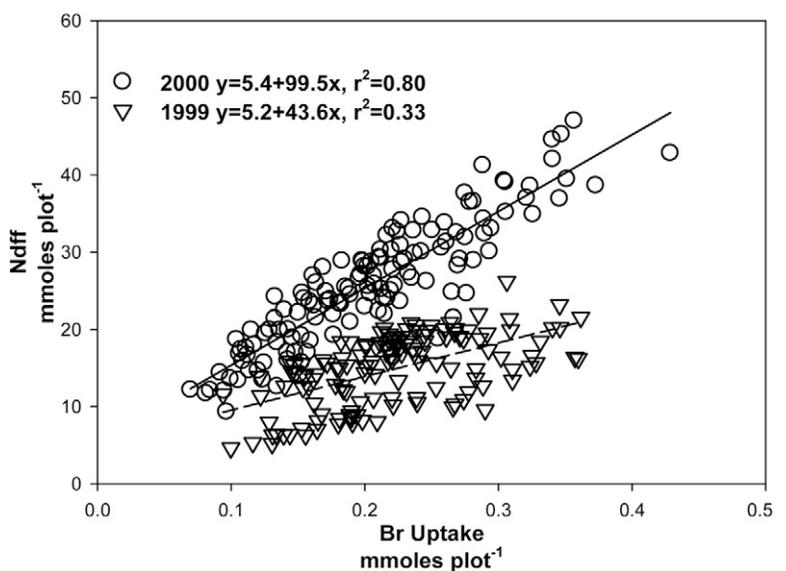
\*Significant at the 0.05 probability level.

\*\*Significant at the 0.01 probability level.

\*\*\*Significant at the 0.001 probability level.

<sup>†</sup>NS, not significant.

lack of gain for the second cycle are that (i) we reached the threshold minimum and maximum for nitrate N uptake in this N<sub>2</sub>-fixing legume in one cycle of selection; or (ii) our methods for Cycle 2 were ineffective to produce increased differences in the uptake traits for which we were selecting. For Cycle 1, approximately 4000 parent plants (UMN2899 MNVC93) were established and about 150 to 175 plants were selected for high Br<sup>-</sup> concentration (HBrC1) and a similar number were selected for low Br<sup>-</sup> concentration (LBrC1). We knew that the amounts of seed available for the second cycle of selection were less than ideal, but chose to conduct a second cycle of selection to test the feasibility of our selection method to create populations that differed in nitrate N uptake. Approximately 1000 Cycle 1 plants (HBrC1) were established for the second cycle of selection for high Br<sup>-</sup> concentration and Br<sup>-</sup> uptake, and 48



**Figure 1.** Regression analysis of Br<sup>-</sup> uptake versus N derived from fertilizer (Ndff) for all selected populations in the establishment year (1999) and first production year (2000).

plants each were selected and crossed to create HBrC2 and HBrUp2. Only 600 Cycle 1 plants were established for the second cycle of selection for low Br<sup>-</sup> concentration and Br<sup>-</sup> uptake, and 27 plants each were selected and crossed to create LBrC2 and LBrUp2. Effective population size for plant improvement in alfalfa has been debated by many researchers, and inbreeding depression can occur when population sizes get too small (Rumbaugh et al., 1988). Hill et al. (1969) suggested a minimum of 75 plants be recombined in each cycle of selection of an alfalfa recurrent phenotypic breeding program to avoid undesirable inbreeding and gene shifts. Our Cycle 2 population sizes were much smaller than this threshold, and this may have been a factor in the lack of improvement from Cycle 1 to Cycle 2 for the Br<sup>-</sup> and nitrate N concentration and uptake traits.

The second unexpected result was the DM yield differential between the high and low populations in each cycle of selection for both selection schemes. The yield differential between the high and low Br<sup>-</sup> populations remained the same for both cycles of selection in the Br<sup>-</sup> concentration selection scheme, but the differential between the high and low Cycle 2 populations increased compared to the first cycle in the Br<sup>-</sup> uptake scheme. These results suggested that using Br<sup>-</sup> uptake as the selection criterion inadvertently increased the differences in DM yield between the high and low Cycle 2 selections. Because DM yield is used to calculate Br<sup>-</sup> uptake and Ndff, the differential in these two traits between the high and low Cycle 2 populations increased as well. Magarian (1996) suggested that selection for Br<sup>-</sup> concentration in combination with high herbage yield and N content in alfalfa could result in differences in uptake that were mostly due to differences yield.

Regardless of the selection scheme, results could be interpreted to suggest that plants selected for high Br<sup>-</sup> were larger and had a greater capacity to take up the Br<sup>-</sup> and N tracers than the smaller plants selected for low Br<sup>-</sup>, implying that the differences in Br<sup>-</sup> concentration and uptake, fNdff, and Ndff were caused by differences in DM yield rather than any gain from selection using our method. This interpretation is refuted by the result that HBrC1, LBrC2, and LBrUp2 were similar in DM yield, but HBrC1 had greater Br<sup>-</sup> concentration, Br<sup>-</sup> uptake, fNdff, and Ndff than either of the Cycle 2 low Br<sup>-</sup> populations.

We were successful in developing a protocol that produced alfalfa populations that differed in nitrate N uptake (estimated by Ndff) using Br<sup>-</sup> concentration or uptake as the selection criterion. The efficiency of the field-based breeding program to increase nitrate N uptake in alfalfa likely would be improved by (i) monitoring selected plants for DM yield; (ii) using appropriate population sizes in each cycle of selection; and (iii) waiting until the first forage production year to select and evaluate plants for differences in Br<sup>-</sup> concentration or uptake.

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