JOURNAL OF PLANT NUTRITION, 25(4), 735–746 (2002)

BIOLOGICAL NITROGEN FIXATION AND HABITAT OF RUNNING BUFFALO CLOVER

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ABSTRACT

Running buffalo clover (RBC) [*Trifolium stoloniferum* (Muhl. ex Eat.)] is an endangered species whose survival is uncertain. An experiment was conducted on extant RBC sites to investigate biological nitrogen (N_2) fixation, associated plant species, and soil conditions under natural mountain settings. Isotope (^{15}N) dilution technique was used to calculate quantities of N fixation. Sites were sampled three times within a 2-yr period. Associated plant species were identified, and their dry matter contributions to the total system were assessed. Nitrogen fixation did not

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appear to contribute a significant portion of the plant N demand. Close cutting (3-cm height) did not seriously damage RBC stands, and RBC constituted between 11 and 44% of the stand throughout the sampling period. Clover forage quality was adequate for grazing animals with around 15% protein. Of the 37 different plant species identified in experimental plots, nettle (*Urtica dioica* L.) and deer tongue (*Panicum clandestinum* L.) were most closely associated with RBC based on significant correlation coefficients ($r = 0.90$ and 0.83, respectively). Native RBC was growing in relatively fertile soil with pH values above 5.5 and potassium (K) values above $96\,\mu g\,g^{-1}$. However, phosphorus (P) and magnesium (Mg) may be limiting growth since soil available values were below 2 and $80\,\mu g\,g^{-1}$, respectively. It appears that RBC is a non-N fixing legume whose cutting management may be similar to other forage clovers.

INTRODUCTION

Running buffalo clover (RBC) is a United States endangered plant species (1) that was historically reported growing within the disturbance trails associated with woodland bison (*Bison bison athabascae*), eastern elk (*Cervus elaphus canadensis*), and white tailed deer (*Odocoileus viginianus*) (2). The habitat ranged across the central United States, extending from Kansas to West Virginia (3). A lack of recent field observations and no recent botanical collections led Brooks (4) to conclude that RBC could possibly be extinct. However, Bartgis (2) found two extant populations in Fayette County, WV and subsequent populations have been found in the USA, primarily in Ohio (5), Kentucky (6), and Indiana (7).

RBC has many characteristics that could be useful as an agricultural forage plant if it recovers from near extinction. It is a perennial clover that is readily consumed by foraging animals, it produces hard seed that will germinate upon scarification or passage through an animal rumen, and it is easily cultivated (6). Its biological N_2 fixing capacity is not known since *Rhizobium sp.* have not been found that produce effective nodules on RBC (6). It has been reported to grow in moist, fertile soils with partial shade (6), and previous work has indicated that most frequently it was found in woodlands associated with water courses (4). Its growth and ecology in well drained, upland soils has not been reported.

Part of the recovery plan for RBC (7) calls for locating rhizobial strains (if any) that may be fixing nitrogen in RBC root nodules; determining competitive interactions between RBC and weedy associates; and determining nutrient limitations to RBC growth. The objectives of this experiment were to address these three issues to aid in the recovery of this species. The non-destructive

technique of ^{15}N labeling was used to detect biological nitrogen fixing associations with RBC. If significant N fixation occurred, selected endangered plants could be excavated and roots examined for presence of rhizobial infected nodules without destroying all RBC plants.

MATERIALS AND METHODS

Three field sites were selected in the Fernow Experimental Forests near Elkins, WV. All sites contained protected stands of RBC. Sites one and two were under a hardwood forest dominated by red oak (*Quercus rubra* L.), yellow poplar (*Liriodendron tulipefera* L.), and black cherry (*Prunus serotina* L.). RBC was growing on skid roads established ca. 1950. These roads have been used about once per decade in conjunction with ground-based logging systems for dragging tree-length logs to a landing. Site three was in a forest meadow near an old home site that is maintained by annual mowing. The area comprising all sites was $index_{50}$ 80 for northern red oak, which represents one of the most productive forest sites in the region (8). Distances between sites were around 1.6 km. Soil at all three sites was a Belmont soil series (fine-loamy, mixed, mesic Typic Hapludalfs). Four replications (plots) of 1 × 1 m in size per site were established about 30-m apart on Sites 1 and 2 and 15 m apart on Site 3. Each replication was selected from stands of RBC that appeared to have greater than 30% RBC plants. Elevations at Sites 1, 2, and 3 were 760, 737, and 836 m, respectively.

On 17 August 1993, all plants in each replication were cut by hand to a 3-cm height. The area was then sprayed with 75%, ^{15}N-enriched ammonium sulfate at a rate of 1 kg N ha^{-1} followed by spraying with 250-ml distilled deionized water to wash fertilizer N from the plant stubble. After cutting, plant species were separated, identified, dried to constant weight at 60°C, and weighed to determine dry matter. On 4 October 1993, plants were harvested again by the same method listed above, and six soil samples were taken 0.3 m from outside the plot to a depth of 15 cm with a soil probe (1.5 cm diameter). Soil samples were taken outside the plot area to avoid damaging RBC. Another harvest was taken on 19 July 1994 by the same methods.

On the last sampling date, RBC and rice cutgrass [*Leersia oryzoides* (L.) Sw.] (RCG) dry plant material were ground to pass a 1-mm screen and analyzed for $\%^{15}N$ using a mass spectrometer. Rice cutgrass was used as the control (non-N fixer) to determine N fixation in clover by isotope dilution (9). Plant material from the second sampling date was not analyzed because there was a very low dry matter yield at that time due to periodic drought in late summer. Three RBC and RCG plant samples at the first cutting were randomly selected for ^{15}N analysis to determine if it would be worth trying to detect N fixation by natural abundance technique. Since differences in $\%^{15}N$ were extremely small, remaining plants at

cutting one were not analyzed due to cost of analysis. Plant samples were analyzed for total %N (10) and $\%^{15}N$ using a mass spectrometer. Crude protein was calculated as 6.25 × % total N (10). Ratio of N from biological fixation to total N in RBC was determined according to the protocol defined by Weaver and Danso (9), as follows:

Ratio of N fixed in clover = 1 − ($\%^{15}N$ excess in clover/$\%^{15}N$ excess in grass). Soil samples were air dried, sieved through a 2-mm screen, and analyzed for pH (water) (11), Bray-1 P (12), ammonium acetate extractable K, calcium (Ca), and Mg (13), DTPA extractable manganese (Mn) (14), and KCl extractable aluminum (Al) (15).

Percent N fixed, protein concentration, soil mineral content, and total dry matter yield at each harvest were analyzed as a completely randomized design with independent variables of site and replication (16). Plant composition at each harvest was analyzed as a split plot design with site used as the split variable. Independent variables were site, plant, and replication. Plant composition data were analyzed using both untransformed and transformed (arc sin square root) calculations. However, the analysis results were similar regardless of transformation, and the figure representing the data in the text shows only non-transformed data with accompanying $LSD_{0.05}$. Also, for presentation purposes, if a plant species did not average more than 3% of the plant composition at each harvest, it was included in a miscellaneous (msc) category. Correlations between RBC and all other plant species were performed using site means for each harvest. If a species was only found in one harvest, it was not included in the correlation analysis.

RESULTS AND DISCUSSION

Calculated amounts of N in RBC from N fixation ranged from −4.1 to 17.6% (Table 1). However, these values may be within the experimental error with actual N fixation near zero because an ANOVA comparing $\%^{15}N$ of RBC with RCG at respective sites did not show statistically significant differences ($P = 0.10$). Even if the 17.6% were believed to be a true representation of N fixation and could be an ecological significant portion of N fixed in RBC, it would only represent less than 15 kg ha^{-1} N fixed at site 1. This quantity of N fixed is small compared to other forage clovers such as white clover (*Trifolium repens* L.) or red clover (*Trifolium pratense* L.), which may fix 128 and 154 kg N ha^{-1} yr^{-1} (17). Throughout the course of this study, leaves on all RBC plants appeared to be green and healthy, so we could not distinguish between plots that may or may not have contained symbiotic N fixing associations. Furthermore, at sampling, soil was carefully dug around some RBC roots and no root nodules were found. Therefore, we concluded there was little or no N fixation

Table 1. Percent N Fixation, ^{15}N Content, and Protein in Running Buffalo Clover and Rice Cutgrass at the 3rd Cutting

Site	%N Fixed in RBC	$\%^{15}N$ in RBC	$\%^{15}N$ in RCG	%Protein in RBC	%Protein in RCG
1	17.6	0.5255	0.5595	15.8	17.0
2	9.4	0.5640	0.5846	15.1	15.1
3	−4.1	0.4794	0.4749	15.4	13.2
Mean	7.6	0.5230	0.5397	15.4	15.5
LSD	ns[1]	ns	ns	ns	ns

[1]ns denotes non-significance at the 0.05 level of probability.

in RBC plots and decided not to excavate and destroy the endangered clover species in the hope of finding a few *Rhizobium sp.* that may fix small quantities of N.

Plant tissues were sufficiently labeled with ^{15}N (Table 1). Labeled N values of plants ranged from 0.47 to 0.56%; ambient atmospheric levels are 0.37% ^{15}N (18). For the isotope dilution estimations for N fixation to be valid, the primary assumption is that control and N fixing plants must both take up similar ratios of ^{14}N and ^{15}N from the soil (18). Ambient $\%^{15}N$ levels from native plant tissues before ^{15}N fertilizer was applied were 0.3789 ± 0.0012 SE for running buffalo clover and 0.3794 ± 0.0023 SE for rice cutgrass (data not shown), which indicates both plants may take up similar ratios of ^{14}N and ^{15}N from the soil and provides some evidence for validation of isotope dilution assumptions in our study.

These data corroborate observations made by Campbell et al. (6) that rhizobial infections on RBC roots were not found on clovers collected from Kentucky or West Virginia. Campbell et al. (6) inoculated RBC with various *Rhizobium trifolii* strains from buffalo clover (*Trifolium reflexum* L.) to try to obtain an effective association. Their efforts produced only a few small non-effective nodules, which indicates part of the associative genes needed for N fixation may be lacking in RBC plants.

Total dry yields were relatively low (average $499\,kg\,ha^{-1}$) and within the range expected from plants growing under shaded and unmanaged conditions (Table 2). In unfertilized and dense forested areas (19), forage yields have been reported to be between 250 and $750\,kg\,ha^{-1}$. The lower yields in the fall of 1993 were probably due to periodic draughty condition experienced in late summer of that year. For example, two weeks after the RBC harvest in August, only 1.4 cm rain fell, then for 10 continuous days in the middle of September, only 1.6 cm rain fell, and finally, one week prior to harvest in October, only 1.1 cm rain fell (20).

Of the total dry matter yield, running buffalo clover averaged 20, 44, and 11% of total forage mass at cuttings 1, 2, and 3, respectively (Fig. 1). It appears

Table 2. Total Dry Matter Yields (kg ha^{-1}) at Each Harvest in Running Buffalo Clover Sites

Site	Cut 1	Cut 2	Cut 3
1	516	35	359
2	574	20	390
3	598	26	830
Mean	564	28	499
LSD	ns[1]	ns	ns

[1]ns denotes non-significance at the 0.05 level of probability.

that the frequency of cutting had little effect on clover regrowth. Percent clover in spring of 1994 was similar to percent clover in the summer of 1993. Even with periodic drought after cutting, RBC persisted much better than the other associated plants and represented 44% of the herbaceous biomass.

RBC plots were species rich, and 37 sympatric species were identified (Table 3). To determine plants most closely associated with RBC, dry matter correlations among the various plant species were calculated (Table 4). Of the 37 total species, 19 were found growing in plots at two or more cuttings. Plants most closely associated with RBC were nettle (NT) ($r = 0.90$***) and deer tongue (DT) ($r = 0.83$***), both of which have been found with RBC in early reports dating from the 1700s and 1800s (6). NT averaged 9, 5, and 12% of the dry matter yields at cuttings 1, 2, and 3, respectively, while DT averaged 4, 2, and 4%, respectively, for the same cuttings. Even though NT and DT did not represent a large percentage of the total yield, it would appear that RBC, NT, and DT have similar growth habits. The type of associations between RBC and sympatric species (e.g. competitive or symbiotic) could not be determined from this experiment. RCG represented a large percentage of the dry yield at the first cutting (37%) and moderate percentage at the last cutting (average 12%), but did not regrow during the drought at the second cutting (Fig. 1). Consequently, its correlation with RBC was low and non-significant ($r = 0.43$). Sedge (SE) produced a large percentage of dry matter at cutting 2, site 3, and a moderate to low yield contribution at cuttings 1 and 3, so the correlation with SE was also low and non-significant ($r = -0.13$).

Forage quality of running buffalo clover as indicated by protein levels was moderate with an average of 15% crude protein (Table 1). For comparison, high N fixing white clover (*Trifolium repens* L.) can have as much as 28% crude protein in the immature stages of growth (21). These levels in RBC were similar to rice cutgrass, which had crude protein levels around 16% (Table 1). Because RBC had moderate levels of protein that were similar to RCG, this is further evidence for lack of symbiotic N_2 fixation in RBC.

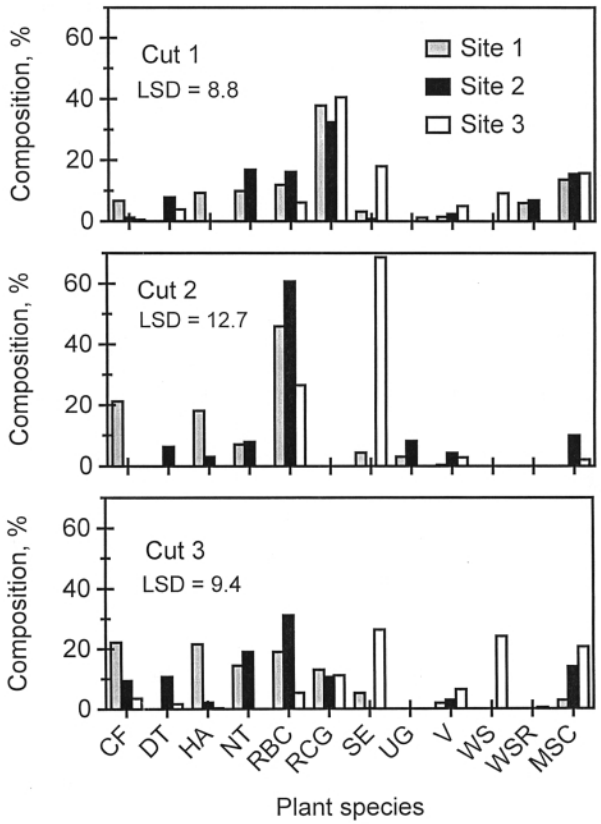

Figure 1. Dry matter contribution of plant species associated with RBC in three harvests. See Table 3 for definition of plant species symbols.

Analyses of soil shows pH was between 5.5 and 6.0 (Table 5). Although soil pH may not have been at optimum levels of 6 to 7 needed for RBC (22) it may have been adequate since soil Al and Mn levels should be non-toxic at pH above 5.5 (23,24). Phosphorus at 1.1 to 2.6 $\mu g\, g^{-1}$ was low and may limit forage production (25). This is not unexpected since Pritchett and Fisher (26) have indicated there are more reports of P deficiency than any other nutrient in forest regions. Potassium and Ca were adequate for plant growth at between 96 to 172 and 815 and 1449 $\mu g\, g^{-1}$, respectively (27). Aluminum was at medium to low levels ranging from 7 to 79 $\mu g\, g^{-1}$, while Mn levels were high, ranging from 62 to 107 $\mu g\, g^{-1}$ (23,28). However, neither Al nor Mn would have been expected to be toxic at the pH level found in this study. It appears there are nutrient imbalances

Table 3. Species Found in Running Buffalo Clover Sites

Symbol	Common Name	Scientific Name
A	Aster	*Aster sp* (L.)
BG	Bluegrass	*Poa sp* (L.)
BS	Bedstraw	*Galium asprellum* (Michx.)
CB	Creeping buttercup	*Ranunculus repens* (L.)
CF	Cinquefoil	*Potentilla canadensis* (L.)
CW	Clearweed	*Pilia pumula* (L.)
DSW	Dotted St. John wort	*Hypericum punctatum* (Lam.)
DT	Deertongue	*Panicum clandestinum* (L.)
F	Fern	*Unknown*
FB	Fleabane	*Erigeron Pulchellus* (Michx.)
GI	Ground ivy	*Glecoma hederacea* (Trevisan)
GR	Goldenrod	*Solidago arguta* (Ait.)
HA	Heal all	*Prunella vulgarus* (L.)
HP	Hog peanut	*Amphicarpa bracteata* ((L.) Fernald)
HW	Honewort	*Cryptotaenia canadensis* ((L.) DC.)
L	Lily	*Lilium sp* (L.)
MLF	Marginal leaf fern	*Dryopteris marginalis* (L.)
MM	Mountain mint	*Pycnantheum virginianum* (L.)
NT	Nettle	*Urtica dioica* (L.)
NS	Nightshade	*Circaea alpina* (L.)
P	Plaintain	*Plantain virginica* (L.)
R	Rush	*Junicus tenuis* (Willd.)
RBC	Running buffalo clover	*Trifolium stoloniferum* (Muhl. ex Eat.)
RCG	Rice cutgrass	*Leersia oryzoides* ((L.) Sw.)
S	Smartweed	*Polygonum pensylvanicum* (L.)
SE	Sedge	*Carex pensylvanica* (Lam.)
SSW	Small flower St. John wort	*Hypericum mutulum* (L.)
SG	Sourgrass	*Oxalis europaea* (Jord.)
SW	St. John wort	*Hypericum sp* (L.)
TBF	Tall bellflower	*Campanula americana* (L.)
TT	Yellow poplar seedling	*Liriodendron tulipefera* (L.)
UG	Unknown grass	*Poa sp* (L.)
V	Viola	*Viola sp* (L.)
WS	Wingstem	*Verbesina alternifolia* (L.)
WSC	Wild stonecap	*Sedum ternatum* (Michx.)
WSR	White snakeroot	*Eupatorium rugosum* (Houtt.)
WV	White verbane	*Verbena urticifolia* (L.)

Table 4. Dry Matter Correlations Between Running Buffalo Clover and Other Associated Plant Species

Plant Species	Correlation Coefficient	Plant Species	Correlation Coefficient
BS	0.21 ns[−1]	P	0.29 ns
CB	−0.15 ns	RCG	0.43 ns
CF	0.25 ns	SE	−0.13 ns
CW	0.21 ns	SG	0.03 ns
DT	0.83 ***	SW	−0.06 ns
GI	−0.12 ns	UG	−0.21 ns
HA	0.08 ns	V	0.16 ns
HP	0.01 ns	WS	−0.07 ns
HW	0.45 ns	WSR	0.44 ns
NT	0.90 ***		

[1]ns denotes non-significance and *** denotes significance at the 0.005 level of probability.

in mountain soils that are supporting RBC populations. Additions of P, Mg, and perhaps lime to soil may improve RBC growth in these areas.

Campbell et al. (6) have indicated that the decline in RBC may have been brought about by habitat destruction, poor seed dispersal due to ruminant decline in forests, competition from non-native plant species, reduced fire frequency, and lack of rhizobial infection. Jacobs and Bartgis (3) indicated there was circumstantial evidence linking the decline of RBC to the extermination of bison from the clover's range. We suggest that RBC may have lost biological nitrogen fixing capabilities due to close associations with buffalo, elk, deer, and other ruminant animals. As RBC plants and seeds were consumed by those

Table 5. Surface Soil Characteristics in Running Buffalo Clover Sites

Element	Site 1	Site 2	Site 3	Mean	Soil Test Value
pH[1]	5.8	5.5	6.0	5.8	adequate
P[2], μg g^{-1}	1.9 AB	2.6 A	1.1 B	1.9	very low
K[1], μg g^{-1}	114	96	172	128	adequate
Ca[1], μg g^{-1}	815	968	1449	1,077	adequate
Mg[1], μg g^{-1}	39	79	79	66	low
Al[1], μg g^{-1}	22	79	7	36	medium
Mn[2], μg g^{-1}	107 A	74 AB	62 B	81	high

[1]Differences between sites were not significantly different at the 0.05 level of probability.
[2]Means among sites followed by the same letter are not significantly different at $LSD_{0.05}$ level.

animals and viable seeds were excreted in the manure (6), there was a high enough N level in manure to support clover to complete its life cycle before another ruminate animal came along and consumed new seed. Also, established forested areas would have been expected to have organic matter in the surface soil that could have provided needed N and other essential nutrients for RBC growth. A hardwood forest may put back 43 kg N ha^{-1} annually in leaf litter (26), which could amount to a large quantity of available N over a long period of time. With high or moderate available soil N levels, N fixation may be inhibited (29). Ultimately, the rhizobial association may not have been necessary to the RBC, resulting in its natural selection against biological nitrogen fixation capability. Because of the lack of N fixing capabilities, RBC could not compete when habitat conditions changed across the eastern USA (6). During the 1700s and 1800s, large areas of land were cleared for crop and cattle production, and clovers capable of fixing high rates of N such as white clover were introduced resulting in excessive competition. Also, buffalo and elk were exterminated from the region. Those animals may have been the predominant means for seed dispersal and provided optimum nutrients in the manure for the RBC to complete its life cycle. Perhaps with the aid of genetics and newer technology, the full compliment of associative N_2 fixing genes could be infused back into RBC, which would improve the chances of survival of this species under natural conditions.

CONCLUSIONS

Our data indicated that RBC growing on mountain slopes in West Virginia does not obtain significant amounts of N from biological N_2 fixation. The potential for use as an agricultural crop in pasture areas appears to be limited because N fertilizer would have to be applied to meet the needs of the clover. However, the advantage of growing RBC in association with ruminant animals in a forest with high shade, low P and Mg fertility, and perhaps periodic draughty conditions in comparison to other forage species needs further investigation. Also, the possibility that the low P and Mg levels and perhaps other micronutrients (e.g. molybdenum) not analyzed in this study were limiting nodulation and N_2 fixation cannot be ruled out.

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