UTILIZING THE RHIZOBIUM-SUBTERRANEAN CLOVER ASSOCIATION FOR RECLAMATION OF LIGNITE MINE SPOIL[1,3]

by

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Abstract. Field plots were established on freshly-levelled lignite mine spoil at Big Brown Mine near Fairfield, Texas, in the fall of 1986 to study the effects of fertilization and inoculation with *Rhizobium leguminosarum* biovar *trifolii* on the establishment and growth of subterranean clover (*Trifolium subterraneum* L.), to evaluate the survival of *Rhizobium* in mine spoil, and to examine the effect of clover presence on yields of subsequent coastal bermudagrass (*Cynodon dactylon* L.) crops. In 1987, clover yields were greatest in inoculated plots with added P and K. In 1988, greater subclover yields occurred in fertilized inoculated and uninoculated plots. Increased bermudagrass production was found when clover was grown prior to the grass crop. Low numbers of indigenous rhizobia were present at plot establishment and were effective with subterranean clover, but dry matter production was lower in uninoculated plots. All clover plots had from 10^4 to 10^5 rhizobia g $soil^{-1}$ at the end of the first and second clover growing seasons, and these populations remained sufficiently high throughout the experimental period to nodulate emerging clover in subsequent years.

ADDITIONAL KEY WORDS: *Trifolium subterraneum* L., *Cynodon dactylon* L., nitrogen fixation, mined land productivity.

Introduction

Reclamation of lignite mine spoils has become increasingly important in Texas, since it has been estimated that approximately one million acres of land will be disturbed by the mining of near-

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surface lignite deposits (Clarke and Baen, 1980). Coastal bermudagrass (Cynodon dactylon L.) monocultures are commonly used in reclamation of mine spoils in Texas, but the establishment and maintenance of this warm-season species requires large inputs of nitrogen fertilizer. Coastal bermudagrass grows from June through October, then becomes dormant during the winter and spring. The successful utilization of clovers in mine spoil reclamation has been reported in Texas as well as other regions (Hons et al., 1980; Jefferies et al., 1981). Their ability to meet their own nitrogen demands through the fixation of atmospheric nitrogen in a symbiotic association with Rhizobium, combined with the possibility of supplying nitrogen to neighboring nonleguminous species or subsequent crops, demonstrates their potential benefits in establishing a successful mine reclamation program. Major benefit could result from a reduction of nitrogen fertilizers.

Subterranean clover (Trifolium subterraneum L.), a cool-season, reseeding annual legume, has not been widely used in reclamation practices in Texas. This clover emerges in late November and actively grows from January through May. Subterranean clover will then set seed in May and is no longer present until the seed germinates the next season. Several characteristics which make this clover an excellent choice for revegetation of spoil include: dense ground coverage facilitating erosion and weed control, relatively easy establishment, tolerance of acidic soils, and dependable reseeding under continuous, close grazing (Evers, 1979; Evers and Shipe, 1980; Knight et al., 1982). Subterranean clover can be seeded into existing coastal bermudagrass sods, with the grass actively growing during the summer months and becoming dormant in the winter and spring when the clover emerges and establishes.

The presence of an effective nitrogen-fixing bacterial population (Rhizobium) is necessary for the successful establishment and maintenance of clovers in a reclamation program. The effectiveness of Rhizobium and its beneficial association with clovers can be influenced by many factors including pH, temperature, moisture, nutrient availability, and other physical, chemical, and biological parameters (Lowendorf, 1980). The failure of establishment and/or persistence of subterranean clover is frequently due to the lack of an effective population of Rhizobium (Knight et al., 1982). Specific strains of Rhizobium leguminosarum biovar trifolii are required for effective nodulation of subterranean clover and subsequent efficient nitrogen fixation. A major problem in the establishment of an effective Rhizobium leguminosarum biovar trifolii-subterranean clover relationship is the possible presence of ineffective indigenous strains of Rhizobium in the mine spoil. Indigenous strains have been found that will nodulate subterranean clover, but they often demonstrated little or no nitrogen-fixing ability in association with the clover (Holland, 1970; Mott, 1984; Mott and Zuberer, 1983).

Our major objectives in this study were to determine the effects of fertilization and inoculation with Rhizobium leguminosarum biovar trifolii on the establishment and growth of subterranean clover, to assess the effect of clover on performance of coastal bermudagrass, and to evaluate the survival of Rhizobium in mine spoil.

Materials and Methods

A field experiment was established in October, 1986, at the Big Brown Mine near Fairfield, Texas. Field plots of subterranean clover were established in a six-month-old coastal bermudagrass sod. 'Mt. Barker' subterranean clover was planted by hand broadcasting seed at a rate of 33.6 kg pure live seed (PLS) ha^{-1}, then raking the seed into the spoil. The seeds were inoculated with a commercial, mixed-strain inoculant (Nitragin Co.) prior to planting. Uninoculated clover plots and control plots containing only coastal bermudagrass were also established. Low numbers (0 to 10^2 rhizobia g $soil^{-1}$) of native rhizobia effective in association with subterranean clover were present in the mine spoil prior to plot establishment. Three fertilization treatments were also incorporated into the study. Plots were fertilized with 336 kg ha^{-1} of 17-17-17 or 0-17-17, corresponding to application rates of 57, 25, and 47 kg ha^{-1} of nitrogen, phosphorus, and potassium, respectively. Nonfertilized plots were also maintained. Individual plots were 2 m by 2 m and four replications of each treatment were included.

Clover plots were clipped twice in the spring of 1987 and 1988 to a height of 7.5 cm using a rear-bagging Snapper Hi-Vac mower. Coastal bermudagrass was not actively growing during this period. During the summer and fall of 1987 and 1988, coastal bermudagrass was cut to a height of 7.5 cm using the same mower. Subterranean clover was not present in the plots during this period. All plant samples were oven-dried at 70C for 4-5 days, and yield was determined on a dry weight basis. Clover and coastal bermudagrass samples were ground to pass through a 20 mesh screen and digested using the micro-Kjeldahl method (Nelson and Sommers, 1973). Total nitrogen and phosphorus were determined using a Technicon Autoanalyzer II Continuous Flow System (Technicon, 1977). Ground coverage (percentage area covered) was visually estimated periodically throughout the experimental period using a grid containing 100 squares measuring 20 cm by 20 cm over the entire plot (Brown, 1954).

Throughout the two years of the experiment, spoil samples were collected to a depth of 15 cm using a 2.5 cm diameter soil core probe. Samples were stored in sterile Zip-loc bags, and processed within 48 hours to determine the numbers of *Rhizobium leguminosarum* biovar *trifolii* in the spoil in the presence or absence of the host species. Populations of *Rhizobium* were estimated using a most probable number (MPN) plant infection technique (Mott, 1984; Weaver and Frederick, 1972). Serial dilutions of spoil material were inoculated into plastic growth pouches containing subterranean clover. After 4 to 5 weeks, roots were examined for the presence or absence of nodules. The MPN of *Rhizobium* capable of infecting subterranean clover was determined using a statistical table (Brockwell, 1963 in Vincent, 1970). The effectiveness of nodulated plants from the growth pouches was determined using the acetylene reduction (AR) assay (Hardy et al., 1968; Mott, 1984). This assay estimates nitrogen-fixing capacity by measuring ethylene production during incubation of plants with acetylene to measure nitrogenase activity. The MPN of effective *Rhizobium* was based on results of the AR assay. All statistical analyses in this study were performed using the Statistical Analysis System (SAS) (Helwig and Council, 1979).

Table 1. EFFECT OF INOCULATION AND FERTILIZATION ON SUBTERRANEAN CLOVER BIOMASS PRODUCTION FROM LIGNITE SPOIL PLOTS

	Harvest Date					
Treatment	3/87	4/87	Total-87	3/88	4/88	Total-88
			kg ha^{-1}			
Inoc - No Fert	98b*	346a	444c	1095b	1274a	2369c
Unin - No Fert	0b	222a	222c	976b	2052a	3028bc
Inoc - 0-17-17	1542a	914a	2456ab	1567ab	2116a	3683abc
Unin - 0-17-17	226b	679a	906bc	1592ab	2044a	3637abc
Inoc - 17-17-17	1742a	1060a	2802a	2471a	2109a	4580a
Unin - 17-17-17	410b	1114a	1524abc	1943ab	1988a	3931ab

*Means in each column followed by the same letter are not significantly different at p=.05 by Tukey's studentized range test.

Results and Discussion

Dry matter yields of subterranean clover in 1987 and 1988 are summarized in Table 1. At the first harvest in 1987, yields were greatest in the inoculated, fertilized plots. Yields were not significantly increased by the addition of N. Lowest yields were found in the inoculated and uninoculated plots where no fertilizer was added. At the second clipping, yields were not significantly different between any treatment but tended to be greater in all plots with added P and K. Total yields for 1987 were greater in the inoculated plots across all fertilizer treatments. Inoculated plots with P and K added had the highest yields, and addition of N did not significantly increase yields. In 1988, clover yields were more influenced by fertilizer addition than inoculation. At the first harvest, yields tended to be greater in the plots where N, P and K were added, although these yields were not significantly greater than those from plots receiving only P and K. Yields were not significantly different between any treatments at the second clipping. Total yields in 1988 tended to be greater in all plots receiving P and K. Approximately 900 kg ha^{-1} of extra dry matter resulted from inoculation with appropriate rhizobia in lieu of applying fertilizer N in 1987. Early season forage production was greatly improved through the use of inoculant on fertilized plots, with an average additional production of 1300 kg ha^{-1} (300% increase) of dry matter in the first clipping in 1987, even though low numbers of indigenous rhizobia were present in plots at the time of establishment. Average total yields in 1988 were nearly twice those obtained in 1987, probably due to the denser plant cover obtained with natural reseeding of the subterranean clover. By the end of the first growing season, ground coverage of all plots ranged from 80 to 95% of the plot area, and coverage achieved the same level in 1988 (data not shown).

Table 2 summarizes the dry matter production of coastal bermudagrass in 1987 and 1988. In 1987, no significant differences were found in yields from all treatments at the first clipping.

Table 2. EFFECT OF PREVIOUS CLOVER CROP AND FERTILIZATION ON COASTAL BERMUDAGRASS BIOMASS PRODUCTION FROM LIGNITE SPOIL PLOTS

	Harvest Date					
Treatment	6/87	10/87	Total-87	7/88	9/88	Total-88
			kg ha^{-1}			
Inoc - No Fert	1847a*	1454ab	3301a	2242bc	1544abc	3786abc
Unin - No Fert	1532a	1340ab	2872a	1872bc	1357abc	3229bc
Cont - No Fert	2377a	817b	3192a	1503c	986c	2489c
Inoc - 0-17-17	2148a	1623ab	3771a	3001ab	1604ab	4605ab
Unin - 0-17-17	1588a	1594ab	3181a	2646ab	1468abc	4114ab
Cont - 0-17-17	2466a	1089ab	3556a	1260c	1191abc	2451c
Inoc - 17-17-17	2484a	1851a	4336a	3468a	1746a	5214a
Unin - 17-17-17	2530a	1554ab	4084a	3373a	1579ab	4952a
Cont - 17-17-17	3059a	1156ab	4215a	2685ab	1158bc	3842abc

*Means in each column followed by the same letter are not significantly different at p=.05 by Tukey's studentized range test.

Yields tended to be higher at all fertilizer levels in the control plots where no clover had been grown. This may have been due to greater nutrient and moisture availability due to the absence of the clover. At the second clipping, yields tended to be lower in the control plots across all fertilizer treatments, with bermudagrass yields approximately 50% greater in the plots where clover had previously grown as compared to control plots at any given fertilizer level. Total dry matter production in 1987 was not significantly different among treatments. However, fertilized plots showed a trend toward greater yields, with 300 to 400 kg ha^{-1} of extra dry matter produced in plots fertilized with P and K as compared to unfertilized plots, while addition of N resulted in an additional increase of approximately 700 kg ha^{-1} of dry matter production. At the first clipping in 1988, bermudagrass yields tended to be greater on the clover plots where at least P and K were added, and lowest in the control plots (grass only) with no added N. Yields tended to be greater in the plots where clover had previously been present regardless of fertilizer treatment at the second clipping. Total dry matter production in 1988 tended to be greater in the plots fertilized with N, P and K. Yields tended to be lower in the control plots where no N was added. When no fertilizer was used, approximately 1000 kg ha^{-1} of additional dry matter were produced in the plots where clover had been grown as compared to control plots. When N, P and K were added, yield increases were about 1200 kg ha^{-1}. When only P and K were applied to the plots, an average of 1900 kg ha^{-1} of additional dry matter was produced from bermudagrass plots where clover had been present prior to the bermudagrass growing season. The use of clover with coastal bermudagrass presumably increased dry matter production through the transfer of N from the mineralization of the legume.

Table 3. EFFECT OF INOCULATION AND FERTILIZATION ON NITROGEN AND PHOSPHORUS HARVESTED IN SUBTERRANEAN CLOVER FROM LIGNITE SPOIL PLOTS

	Total Nitrogen		Total Phosphorus	
Treatment	1987	1988	1987	1988
		kg ha^{-1}		
Inoc - No Fert	13.1c*	50.5b	0.63c	2.74b
Unin - No Fert	6.4c	78.5ab	0.32c	5.10ab
Inoc - 0-17-17	84.4a	101.1ab	5.07a	6.29ab
Unin - 0-17-17	27.8bc	94.3ab	1.74bc	6.83a
Inoc - 17-17-17	91.7a	118.6a	5.87a	7.91a
Unin - 17-17-17	42.6b	98.5ab	2.78b	7.42a

*Means in each column followed by the same letter are not significantly different at p=.05 by Tukey's studentized range test.

Total nitrogen and phosphorus harvested in subterranean clover in 1987 were significantly greater in the inoculated plots receiving P and K (Table 3). About 60 kg ha^{-1} of N was gained through inoculation instead of adding fertilizer N, which would provide a substantial savings in fertilizer costs. An approximate 100% increase in total P harvested resulted from inoculation when P and K were applied. The response to inoculation was not observed in 1988, where total N and P yields were similar for both inoculated and uninoculated treatments when P and K were added (Table 3). The indigenous rhizobia were presumably able to form an effective relationship with the clover by the second year or inoculant rhizobia had been transported to the uninoculated plots. Table 4 shows the total N and P harvested in coastal bermudagrass in 1987 and 1988. In 1987, N and P production tended to be greater in all plots fertilized with N, P and K, and the inoculated plots with P and K added. The lower yields of the other treatments were probably due to the need for additional N. In 1988, total N and P in coastal bermudagrass tended to be greater in the plots where clover had previously grown at each fertilizer level. Increased uptake of N and P by the coastal bermudagrass was found when subterranean clover was seeded into the grass, with an approximate 100% increase in total N and P in the grass harvested from the clover plots as compared to the control plots at any fertilizer level.

The most probable numbers of Rhizobium in the lignite spoil plots throughout the experimental period are shown in Table 5. At the end of the first growing season (5/87), inoculated as well as uninoculated plots contained 10^4 to 10^5 rhizobia g $soil^{-1}$. Although numbers were similar in inoculated and uninoculated plots and the rhizobia in the uninoculated plots were shown to be effective in association with subterranean clover (data not shown), an efficient symbiotic relationship was not formed early by the rhizobia with the clover, as indicated by the yield data previously discussed. There were approximately 10^3 rhizobia g $soil^{-1}$ in all clover plots at the time of clover

Table 4. EFFECT OF PREVIOUS CLOVER CROP AND FERTILIZATION ON NITROGEN AND PHOSPHORUS HARVESTED IN COASTAL BERMUDAGRASS FROM LIGNITE SPOIL PLOTS

	Total Nitrogen		Total Phosphorus	
Treatment	1987	1988	1987	1988
	kg ha^{-1}			
Inoc - No Fert	27.4a*	34.4bcd	4.83a	5.85bcd
Unin - No Fert	21.8a	29.0cde	3.84a	5.38cd
Cont - No Fert	25.8a	19.6e	3.79a	3.14f
Inoc - 0-17-17	33.8a	42.8ab	6.54a	7.63ab
Unin - 0-17-17	27.2a	40.8abc	4.51a	6.86abc
Cont - 0-17-17	24.2a	17.8e	3.96a	3.47ef
Inoc - 17-17-17	39.9a	51.8a	7.17a	8.31a
Unin - 17-17-17	34.6a	48.3a	6.36a	8.26a
Cont - 17-17-17	29.7a	25.1de	5.02a	5.00de

*Means in each column followed by the same letter are not significantly different at p=.05 by Tukey's studentized range test.

Table 5. EFFECT OF INOCULATION AND FERTILIZATION ON MOST PROBABLE NUMBERS OF RHIZOBIUM IN LIGNITE SPOIL PLOTS

	Sampling Date					
Treatment	5/87	10/87	1/88	5/88	7/88	11/88
	log # g $soil^{-1}$					
Inoc - No Fert	4.39a*	3.01a	3.06ab	4.97ab	4.53ab	4.05abc
Unin - No Fert	5.03a	3.49a	3.24ab	5.34a	4.83a	4.52abc
Cont - No Fert	1.31b	0.63c	0.65c	2.56c	2.83bc	2.60abc
Inoc - 0-17-17	5.24a	2.96a	3.77a	5.52a	5.15a	4.79ab
Unin - 0-17-17	5.31a	3.33a	3.68a	6.21a	5.78a	4.78ab
Cont - 0-17-17	2.03b	1.08bc	1.86abc	2.91c	2.45c	1.86c
Inoc - 17-17-17	4.97a	2.56ab	3.65a	5.62a	5.25a	4.98a
Unin - 17-17-17	5.46a	3.44a	3.81a	5.87a	4.90a	4.67ab
Cont - 17-17-17	1.67b	0.58c	1.40bc	3.05bc	2.76bc	2.25bc

*Means in each column followed by the same letter are not significantly different at p=.05 by Tukey's studentized range test.

emergence in the second season (10/87), whereas rhizobia were virtually undetectable in the control (no clover) plots. Numbers of Rhizobium at the end of the second year (5/88) were similar to those found at the end of the first growing season. Populations declined somewhat throughout the rest of 1988. There were approximately 10^4 rhizobia g $soil^{-1}$ at the time of clover emergence at the beginning of the third year. The fluctuations in numbers of rhizobia were influenced by the presence or absence of the host species, with numbers decreasing in the summer months when subterranean clover is absent, and increasing again each year after clover emergence.

Summary

Inoculation with the proper strains of *Rhizobium* resulted in increased production of subterranean clover (75%), along with increased total N and P (100%), in the first clover growing season, even though low numbers of effective rhizobia were present in the mine spoil. Fertilization with P and K increased growth of clover in comparison with nonfertilized plots, while addition of N did not increase yields on fertilized plots. Coastal bermudagrass production was enhanced (75%) through the presence of subterranean clover prior to the bermudagrass growing season, probably due to the availability of additional N from the clover. Finally, sufficient numbers of *Rhizobium* were able to survive in the mine spoil, even when the host species was absent, to nodulate and form an effective symbiotic relationship with emerging subterranean clover in subsequent years.

Literature Cited

Brockwell, J. 1963. Accuracy of a plant-infection technique for counting populations of *Rhizobium trifolii*. Appl. Microbiol. 11:377-383.

Brown, D. 1954. Methods of Surveying and Measuring Vegetation. Bull. 42, Commonwealth Bureau of Pastures and Field Crops, Hurley, Burks. Bradley & Son, Ltd., The Crown Press, Reading, England.

Clarke, N. P. and S. R. Baen. 1980. Foreword. p.3. *In* L. R. Hossner (ed.) Reclamation of surface-mined lignite spoil in Texas. Texas Agric. Exp. Stn. Pub. RM-10.

Evers, G. W. 1979. Production and reseeding of cool season annual clovers in southeast Texas. Texas Agric. Exp. Stn. PR-3591.

Evers, G. W. and E. R. Shipe. 1980. Forage production of winter annual clovers, 1978-79. Texas Agric. Exp. Stn. PR-3683.

Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns. 1968. The acetylene-ethylene assay for N_2-fixation: laboratory and field evaluation. Plant Physiol. 43:1185-1207.
http://dx.doi.org/10.1104/pp.43.8.1185

Helwig, J. T. and K. A. Council. (ed.). 1979. SAS user's guide. SAS Institute Inc., Cary, North Carolina.

Holland, A. A. 1970. Competition between soil- and seed-borne *Rhizoium trifolii* in nodulation of introduced *Trifolium subterraneum*. Plant Soil 32:293-302.
http://dx.doi.org/10.1007/BF01372870

Hons, F. M., L. R. Hossner, and E. L. Whiteley. 1980. Reclamation and yield potential of various forages in surface-mined soil. p. 37-47. *In* L. R. Hossner (ed.) Reclamation of surface-mined lignite spoil in Texas. Texas Agric. Exp. Stn. Pub. RM-10.

Jefferies, R. A., A. D. Bradshaw, and P. D. Putwain. 1981. Growth, nitrogen accumulation and nitrogen transfer by legume species established on mine spoils. J. Appl. Ecol. 18:945-956.
http://dx.doi.org/10.2307/2402384

Knight, W. E., C. Hagedorn, V. H. Watson, and D. L. Friesner. 1982. Subterranean clover in the United States. Adv. Agron. 35:165-191.
http://dx.doi.org/10.1016/S0065-2113(08)60324-7

Lowendorf, H. S. 1980. Factors affecting survival of Rhizobium in soil. Adv. Microbiol. Ecol. 4:87-124.
http://dx.doi.org/10.1007/978-1-4615-8291-5_3

Mott, J. B. 1984. Evaluation of microbial populations, Rhizobium trifolii, and endomycorrhizal associations in reclamation of surface mine spoils in Texas. Ph. D. Dissertation. Dep. of Soil and Crop Sci., Texas A&M Univ., College Station, Texas.

Mott, J. B. and D. A. Zuberer. 1983. Ecology of R. trifolii and it's symbiosis with subterranean clover in reclamation of mixed overburden mine spoils. p. 11. Abstr. in 9th North Amer. Rhizobium Conf. 19-23 June, 1983. Ithaca, New York.

Nelson, D. W. and L. E. Sommers. 1973. Determination of total nitrogen in plant material. Agron. J. 65:109-112.
http://dx.doi.org/10.2134/agronj1973.00021962006500010033x

Technicon, 1977. Determination of nitrogen in BS digests. Technicon Industrial Method No. 334-74W/B. Technicon Industrial Systems, Tarrytown, NY.

Vincent, J. M. 1970. A manual for the practical study of root-nodule bacteria. Int. Biol. Programme Handbook No. 15. Blackwell Scientific Publ., Oxford.

Weaver, R. W. and L. R. Frederick. 1972. A new technique for most-probable-number counts of rhizobia. Plant Soil 36:219-222.
http://dx.doi.org/10.1007/BF01373474