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Isolation of *Rhizobium leguminosarum* (biovar *trifolii*) Strains from Ethiopian Soils and Symbiotic Effectiveness on African Annual Clover Species

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Strains of *Rhizobium leguminosarum* (biovar *trifolii*) isolated from two Ethiopian soils or obtained from a commercial source were evaluated for symbiotic effectiveness on five African annual clover species. Numerous *Rhizobium trifolii* strains that exhibited varying levels of symbiotic effectiveness were isolated from both soils (a nitosol and a vertisol), and it was possible to identify strains that were highly effective for each clover species. The soil isolates were, as a group, superior to the strains from the commercial source. Several *R. trifolii* strains were found to be effective on more than one clover species, and there appeared to be at least two and possibly three distinct cross-inoculation effectiveness groups.

In their review of livestock farming practices in the African highlands, Haque and Jutzi (9) concluded that the use of forage legumes was the best method for utilizing biologically fixed nitrogen in African farming systems. The Forage Legume Agronomy Group at the International Livestock Center for Africa (ILCA) currently has over 8,000 germplasm accessions, including a large collection of African clovers (12, 13). Preliminary field evaluations in Ethiopia have reduced the number of these native *Trifolium* species and biotypes to a reasonable number of potential candidates for domestication and include the annual species *T. decorum*, *T. quartinianum*, *T. rueppellianum* Fres., *T. steudneri* Schwf., and *T. tembense* Fres (14, 15, 18). From ILCA field evaluations it was reported that the highest yields were obtained on soils subject to waterlogging (9) but that prolonged waterlogging during the growing season tended to reduce yields (13). Some species were particularly suited to high elevations and to sites with poor soil drainage, and with low (25 to 30 kg/ha) P fertilization rates, maximum dry matter yields ranged from 3.5 to 6.8 mg ha^{-1} (1). Further progress towards the domestication and utilization of such annual clovers may require that effective and competitive *R. trifolii* strains be found and used in inoculants. Such strains must effectively nodulate the host plant and provide adequate biological nitrogen fixation under African highland moisture and fertility constraints and survive low-moisture stress when the host plants have died. The objective of this research was to examine the *R. trifolii* populations from two Ethiopian soils and attempt to identify effective isolates for the five African annual clover species that have demonstrated agronomic potential.

MATERIALS AND METHODS

Seed. Seed samples (2 to 5 g) from accessions of five annual African *Trifolium* species were obtained from the ILCA Forage Germplasm Bank (12). The collection represents seeds from a range of environmental and soil conditions at the site of origin for each accession. The clovers were *T. decorum*, *T. quartinianum*, *T. rueppellianum*, *T. steudneri*, and *T. tembense*.

Soil. Two unfertilized Ethiopian soils were obtained for isolation of rhizobia and for greenhouse studies. Bulk samples (250 kg) were collected from the upper 20 cm of a nitosol and a vertisol; they were ground and sorted by hand to remove stones and plant material and were shipped to Virginia Polytechnic Institute and State University in sealed plastic drums. The nitosol (approximately equivalent to an ultisol of the U.S. Department of Agriculture Soil Taxonomy [19]) was collected at the Institute for Agricultural Research experiment station in Holetta. The vertisol was collected from the ILCA experiment station in Debre Zeit. This soil, designated as DZ/3/86, has been fully characterized by Kamara and Haque (16). Soil core samples were taken at each site from 8- and 22-cm depths for use in evaluation of physical characteristics. Prior to sterilization (U.S. Department of Agriculture-Animal and Plant Health Inspection Service regulations), subsamples of each bulk soil were removed for rhizobial isolation and unused portions were subsequently autoclaved.

Soil analyses. Samples of the bulk soils were air dried and ground to pass through a 2-mm-hole-size sieve before determining soil pH (1:1, soil to water ratio), Ca, Mg, and K (atomic absorption spectrophotometry after exchange with 1 N ammonium acetate), extractable Al ($BaCl_2$-triethanolamine titration), extractable P, K, Ca, Mg, Mn, Zn, and Cu (0.05 N HCl in 0.025 N H_2SO_4), organic matter by chromic acid digestion, and NO_3-N by an ion-specific electrode. All soil analysis determinations were performed according to Virginia Soil Testing and Plant Analysis Laboratory procedures (6).

Commercial *R. trifolii* strains. Eighteen *Rhizobium trifolii* strains, thought to be compatible with the selected African clover species, were obtained from The Nitragin Company, Inc., Milwaukee, Wis. These 18 cultures represented all those in the Nitragin collection that were originally obtained from Africa and might be effective on the annual clovers. Strains were streaked on yeast mannitol agar plates to check for purity and were stored on yeast mannitol agar slants at 4°C (29).

***Rhizobium* isolation from soil.** All accession lines of each clover species were planted in "cone-tainer" tubes filled

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with sterile sand-vermiculite potting mixture. Each tube was fertilized on a weekly basis with 5 ml of a weak (0.05%) solution of a 15-30-15 fertilizer supplemented with (liter^{-1}) 5 μg of B, 5 μg of Mn, 0.5 μg of Zn, 0.5 μg of Mo, and 0.2 μg of Cu. Separate suspensions of both nonsterile Ethiopian soils were made in a Waring blender by adding 10 g of soil to 100 ml of distilled water and mixing for 2 min. One week after planting, the clovers were separately inoculated with 5 ml of the appropriate soil suspension and each treatment was replicated four times. To ensure nodulation, the plants were reinoculated with fresh suspensions of the same soil two weeks later (22). The inoculated plants were grown in a greenhouse for 10 weeks with 12 h of supplemental light and an ambient photoperiod increasing from 12 to 14 h by the end of the period. The temperature in the greenhouse ranged from 12°C at night to 30°C during the day. After washing the roots free of soil, fresh nodules were carefully collected from one healthy plant for each species-soil suspension combination. Rhizobia were isolated by using a technique similar to that of Date and Halliday (5). Clean, fresh nodules were immersed in isopropyl alcohol for 30 s, rinsed three times in an excess of sterile distilled water, squashed in a droplet of water on yeast mannitol agar plates, and streaked for isolation. Plates were restreaked as necessary for isolation of colonies, and pure cultures were stored on yeast mannitol agar slants at 4°C (29).

Rhizobia isolated from the nitosol were collected from the roots of *T. decorum* 9437, *T. quartinianum* 7675, *T. rueppellianum* 6218, *T. steudneri* 9712, and *T. tembense* 9681. The accessions used for isolation of rhizobia from the vertisol were *T. decorum* 9447, *T. quartinianum* 8521, *T. rueppellianum* 6260, *T. steudneri* 7637, and *T. tembense* 9681. Over 200 nodules were collected, and pure strains were isolated. From this group, the most effective strains (35 total) were selected for use as inoculants in further evaluations. This subsample consisted of 3 to 4 strains from every soil-plant combination and was representative of the nodule sizes found on each root system.

***Rhizobium* strain-*Trifolium* species compatibility.** To enable the evaluation of a large number of rhizobia-plant combinations within a short period of time, a procedure with 100- by 15-mm plastic petri plates, which modified a similar technique developed by Gibson (8) and Bender and Rolfe (2), was used. Plates containing 20 ml of trace element-supplemented Jensen's seedling agar were prepared by melting a notch in the side of both the top and bottom of a plate with a hot steel spatula. Seedlings were placed in the plate so that the stem and leaves could grow through the notch and the roots could grow on the agar surface. Plants were inoculated with 1 ml of the appropriate *Rhizobium* strain (10^7 cells per ml), and they were wrapped with parafilm around the edges to hold the petri plate together. All 18 commercial strains and the 35 best soil isolates were individually evaluated on one accession of each clover species (10 plants per treatment). Petri plates were kept in a horizontal position overnight in an incubation box to allow the liquid of the inoculant to be absorbed into the agar. Groups of nine plates were then placed vertically, with notches aligned at the top, and completely wrapped in aluminum foil so that only the stem portions of the plants were exposed to light. Wrapped petri plates were randomly placed in two rows in clear plastic incubation boxes and transferred to the greenhouse. Incubation boxes were cleaned with antiseptic soap solution before use to minimize microbial contamination. High humidity was maintained in the boxes by placing water-saturated, sterilized paper towels between the rows of petri plates and replacing water as needed. Sunlight was supplemented with 12 h of incandescent lighting, providing 200 microeinsteins m^{-1} s^{-1}. The diurnal temperature range was 18 to 30°C.

TABLE 1. Chemical and physical analyses of Ethiopian soils[a]

Soil	pH	Exchangeable cations						
		P[b]	Ca[c]	Mg[c]	K[c]	Al[c]	CEC[c]	BS[d] (%)
Nitosol	6.4	24	15.6	5.9	2.3	0.15	37.8	62
Vertisol	6.1	16	21.8	7.2	1.0	0.10	38.4	78

[a] All analyses were performed according to Virginia Soil Testing and Plant Analysis Laboratory procedures (6).
[b] mg kg^{-1}.
[c] CEC, Cation exchange capacity (cmol 100 g^{-1}).
[d] BS, Base saturation.

Each plant was evaluated at 3 and 6 weeks for vigor, root growth, and nodulation. Plants were scored on a relative scale from 1 to 5, poorest to best, respectively. Acetylene reduction assays were performed on each plant by removing the parafilm seal and placing the entire leaf portion of the plant inside the petri plate with the roots. Using the petri plate as an incubation chamber, the plates were partially sealed with a parafilm strip, flushed with an excess volume of 10% acetylene gas delivered from a 50-ml syringe, and then completely sealed. Plates were placed under a fluorescent light source providing 200 microeinsteins m^{-1} s^{-1} and were incubated at 25°C; gas samples were removed after 1 h. Periodic analyses indicated that gas samples could be stored for up to 4 days in disposable 3-ml syringes, inserted into rubber stoppers, without apparent gas leakage. Analysis for ethylene concentration was performed on a Varian 3700 gas chromatograph equipped with a 1.8-m stainless steel column prepacked with GasChrom PICN. The column temperature was 50°C, and N_2 was used as the carrier gas. Nitrogen fixation rates were calculated from integrated peak areas by the method of Hardy and Holsten (10), assuming a ratio of 3 mol of C_2H_2 reduced per mol of N_2 fixed. Lastly, plant tops were removed and dried at 70°C for 48 h for determination of dry weight (biomass) accumulation. Persian clover (*T. resupinatum*), inoculated with Nitragin Co. strain 162U1, was included as a control for evaluation of the relative response of the clover-*Rhizobium* combinations.

Analysis of variance was performed on the dependent variables, which included nodulation, vigor, dry weight, and rate of biological nitrogen fixation for the 6-week rating by the General Linear Models procedure of SAS (24). Duncan's Multiple Range Test was used for mean separation when the overall F-test was significant at $P < 0.10$.

RESULTS

Soil characteristics. Both soils had nearly neutral pH values as well as high levels of cation exchange capacity and

TABLE 2. Chemical and physical analyses of Ethiopian soils[a]

Soil	Extractable cations							
	K[b]	Ca[b]	Mg[b]	Mn[b]	Zn[b]	Cu[b]	NO_3-N[b]	OM[c] (%)
Nitosol	380	2172	471	325	5.8	0.6	5	7.5
Vertisol	144	2340	487	143	1.6	0.6	3	2.8

[a] All analyses were performed according to Virginia Soil Testing and Plant Analysis Laboratory procedures (6).
[b] mg kg^{-1}.
[c] OM, Organic matter.

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TABLE 3. Chemical and physical analyses of Ethiopian soils[a]

Soil	Depth (cm)	Bulk density (g cm^{-3})	Sand (%)	Silt (%)	Clay (%)	Texture	K_{SAT}[b]
Nitosol	8	1.05	9.9	37.8	52.3	Clay	9.29
	24	1.08					7.33
Vertisol	8	1.26	1.1	35.6	63.3	Clay	2.03
	24	1.33					0.17

[a] All analyses were performed according to Virginia Soil Testing and Plant Analysis Laboratory procedures (6).
[b] 10^{-5} m s^{-1}.

percent base saturation (Tables 1 through 3). The high base saturation of the nitosol places this soil in the Food and Agricultural Organization classification of eutric nitosol and the U.S. Department of Agriculture soil taxonomy class of Paleudalf (7). The results of the cation exchange procedure for the vertisol were comparable to determinations made by ILCA soil scientists on the same soil (16). The dilute double-acid extraction procedure used in the Virginia Polytechnic Institute Soil Testing and Plant Analysis Laboratory (6) indicated that phosphorus was high in the nitosol and moderate in the vertisol. All other extractable nutrient levels fell within either the moderate to high or the sufficient categories, with the exception of the low levels of NO_3-N. The relatively high organic matter content of the nitosol (7.5%) provided a further indication of the fertility of this soil.

Both soils contained over 90% silt- and clay-size particles and were texturally classified as clay (Tables 1 through 3). The bulk density of the nitosol was nearly 1.1 g cm^{-3}, and hydraulic conductivity at saturation in the nitosol was nearly 10 times higher than that of the vertisol and did not decrease appreciably with depth. There was a 10-fold decrease in hydraulic conductivity in the vertisol with depth, and this typifies the poor drainage conditions usually associated with vertisols (7). The vertisol possessed a poor structure and was very sticky when wet and hard when dry. Although the nitosol had a high clay content, it was well structured and appeared to be well drained when collected from the field. Dudal (7) stated that limitations to the use of nitosols were more likely to be chemical, such as manganese toxicity at low pH levels, than physical.

Strain effectiveness. For the commercial *R. trifolii* strains, successful infection rates were low, as indicated by the mean value obtained for the nodule ratings (Table 4). A nodulation value of 2 represented an association in which several small white nodules, or only 1 to 2 small pink nodules, were formed. Significant ($P < 0.01$) and highly significant ($P < 0.001$) differences for nodulation ratings were detected for 4 of the 5 species, but no inoculant was rated higher than 4.05, the value obtained for Persian clover. For the strains isolated from soil, the mean values for nodulation were higher and significant differences within responses to inoculation were found for two species (Table 5). The average dry weight produced for each species was nearly three times greater for plants inoculated with the soil isolates than for plants inoculated with the commercial strains (Tables 4 and 5). Significant differences between mean weights in response to inoculant strain were not found for *T. decorum* or *T. tembense* inoculated with commercial strains or for *T. steudneri* inoculated with soil isolates.

Highly effective strains were not found among the commercially obtained rhizobia (Table 4). Evaluations of the strains isolated from the Ethiopian soils revealed higher rates of N fixation, and significant differences were observed between mean rates in response to different strains for three of the species (Table 5). In an attempt to incorporate the ability of the rhizobia to fix N into the selection of the best strain for each species, a variable, called productivity, was developed from the product of the values obtained for vigor, weight, and rate of N fixation. Relative differences between the mean productivity values of each inoculant strain were also found to be significant for three of the five clover species inoculated with the soil isolates.

Twelve strains were found to be effective on more than one species (Table 6). Sorting these cross-inoculation groups by the highest level of effectiveness revealed two distinct groups. The species *T. decorum*, *T. quartinianum*, and *T. rueppellianum* were in one effectiveness group, and *T. steudneri* and *T. tembense* were in the second. Arranging the *Trifolium* species into effectiveness groups based on the best five inoculant strains for each species revealed an additional group consisting of *T. quartinianum* and *T. steudneri*.

Nitrogen content. Plants from the most productive of the species-inoculant combinations (49 total) were selected for Kjeldahl digestion and determination of nitrogen concentration. The total amount of nitrogen fixed (milligrams $plant^{-1}$) was calculated as the product of N concentration and dry weight. The relationships of total N content to dry weight and to productivity are shown in Fig. 1 and 2, respectively. Total nitrogen content (total biological nitrogen fixation for

TABLE 4. Mean response of five African *Trifolium* species to 18 *R. trifolii* strains from a commercial source[a]

Species	Nodules[b]	Vigor[c]	Wt (mg $plant^{-1}$)	N fixed (nmol h^{-1} $plant^{-1}$)	Productivity[d]
T. decorum	2.10***[e]	2.63[f]	6.83[f]	0.0033*	0.059*
T. quartinianum	1.80***	3.46**	10.31***	0.0039***	0.139***
T. rueppellianum	1.92***	2.96[f]	7.94*	0.0026[f]	0.061[f]
T. steudneri	2.00**	2.90*	6.87*	0.0032***	0.073*
T. tembense	2.01[f]	2.52*	7.42[f]	0.0018[f]	0.034[f]
T. resupinatum	4.05	4.15	12.63	0.0134	0.702

[a] *T. resupinatum* was inoculated with the recommended commercial strain (16241).
[b] Nodulation rating: 1, no nodules; 2, all white nodules or 1 to 2 small red nodules; 3, 1 to 2 large red and/or 3 to 6 small red nodules; 4, 3 to 4 large red and/or 6 to 10 small red nodules; 5, >4 large red and/or >10 small red nodules.
[c] Vigor rating: 0, dead; 1, seedling growth only; 2, 2 to 3 leaflets with yellowing; 3, 2 to 3 green leaflets; 4, 3 to 4 leaflets and 1 to 2 primary leaves; 5, >4 leaflets and primary leaves with no yellowing.
[d] Productivity = vigor × weight × N fixed.
[e] *, **, and ***, Differences within species significant at the 0.05, 0.01, and 0.001 probability levels, respectively.
[f] Differences within species not significant.

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TABLE 5. Mean response of five African *Trifolium* species to 35 *R. trifolii* strains isolated from Ethiopian soils

Species	Nodules[a]	Vigor[b]	Wt (mg plant^{-1})	N fixed nmol hr^{-1} plant^{-1}	Productivity[c]
T. decorum	3.36**[d]	3.35***	28.34***	0.0150**	1.424***
T. quartinianum	4.15[e]	3.84[e]	42.12**	0.0146*	2.360*
T. rueppellianum	3.69[e]	3.32[e]	29.76*	0.0078[e]	0.771[e]
T. steudneri	2.85[e]	2.98[e]	22.93[e]	0.0096**	0.655*
T. tembense	2.93***	2.13***	17.95***	0.0063[e]	0.241[e]

[a] Nodulation rating: 1, no nodules; 2, all white nodules or 1 to 2 small red nodules; 3, 1 to 2 large red and/or 3 to 6 small red nodules; 4, 3 to 4 large red and/or 6 to 10 small red nodules; 5, >4 large red and/or >10 small red nodules.
[b] Vigor rating: 0, dead; 1, seedling growth only; 2, 2 to 3 leaflets with yellowing; 3, 2 to 3 green leaflets; 4, 3 to 4 leaflets and 1 to 2 primary leaves; 5, >4 leaflets and primary leaves with no yellowing.
[c] Productivity = vigor × weight × N fixed.
[d] *, **, and ***, Differences within species significant at the 0.05, 0.01 and 0.001 probability levels, respectively.
[e] Differences within species not significant.

plants grown in N-free media) was best expressed as a linear function of plant dry weight (Fig. 1) and could be predicted from dry weight with a high degree of precision (r^2 = 0.96). The STEPWISE regression method of SAS (24) identified the best relationship of the derived variable, productivity, to total N content as a linear function of productivity plus logarithmically transformed productivity values (Fig. 2). The lower correlation coefficient (r^2 = 0.63) and large confidence interval indicated the inferiority of this variable as a predictor of total N fixation for these symbiotic relationships. However, it does take into account the N fixation rate of the particular plant-rhizobium combination in the evaluation.

The best application of the petri dish method for growing plants appeared to be in the evaluation of N fixation ability by acetylene reduction (8), and in this study, the relative differences between the two strain collections were clearly evident. The combination of plant measurements into the productivity variable and comparison of the mean values of this term indicated the superiority of the Ethiopian strains as well. The usefulness of the productivity term is limited to assays where the relative differences between dry weights are small in comparison with the relative differences between N fixation rates.

DISCUSSION

Different species of *Trifolium* adapted to the same climate are frequently not effectively nodulated by the same strains of rhizobia. Vincent (28) noted that the rhizobia effective on red clover (*T. pratense* L.) and white clover (*T. repens* L.) were not effective on subclover (*T. subterranium* L.), crimson clover (*T. incarnatum* L.), and berseem clover (*T. alexandrinum* L.) and called this relationship "reciprocal incompatibility." For example, *Trifolium* species indigenous to the Mediterranean area are usually nodulated by rhizobia from other clovers that grow there (17), but Kura clover (*T. ambiguum* Bieb), which originated in the Caucasian region, is a notable exception (11, 23). This species is not only poorly nodulated by rhizobia isolated from other clovers but is specific in its association with rhizobia from other ecotypes of Kura clover (30).

Studies with the native clovers of sub-Saharan Africa show a variable response to *R. trifolii* isolated from and effective on European and American clovers (20, 21). African clovers formed no effective associations with rhizobia of European origin (25), while *Rhizobium* strains isolated from African soils were screened for N_2-fixing ability on African clovers and some effective strains were found (9). Subsequently, Scheffler and Louw (26) showed that some of the African clover rhizobia were also effective on strawberry

TABLE 6. Specificity groupings of five African annual *Trifolium* species

Species	*R. trifolii* strain											
	TdN5br	TqN4J	TqN5e	TqV4c	TrN5e	TrV3a	TrV5a	TsN3k	TsN5f	TsV4g	TtV3a	TtV5e
T. decorum	E[a]	M	—[b]	E[c]	—	E[a]	—	—	—	—	—	E[d]
T. quartinianum	I	E[a]	M	E	E	—	I	M	—	M	I	I
T. rueppellianum	E	—	M[a]	E	E[d]	—	E[a]	M	I	I	M	—
T. steudneri	—	—	M	M	—	—	—	E[a]	—	E[a]	—	M
T. tembense	—	—	M	—	I	—	M	E	E[a]	—	E[a]	M

[a] Strains selected as best for each species from productivity and dry weight values. Productivity = vigor × dry weight × rate N fixed.
[b] —, Nodulation rating <2.5; i.e., not able to form effective nodules.
[c] E, Effective association (upper 10% of those surveyed); M, moderately effective association (within upper 40%); I, ineffective (below 60th percentile), respectively, as evaluated from dry weight accumulation.
[d] Strains selected as best for each species from dry-weight values, not coincident with high-productivity values.

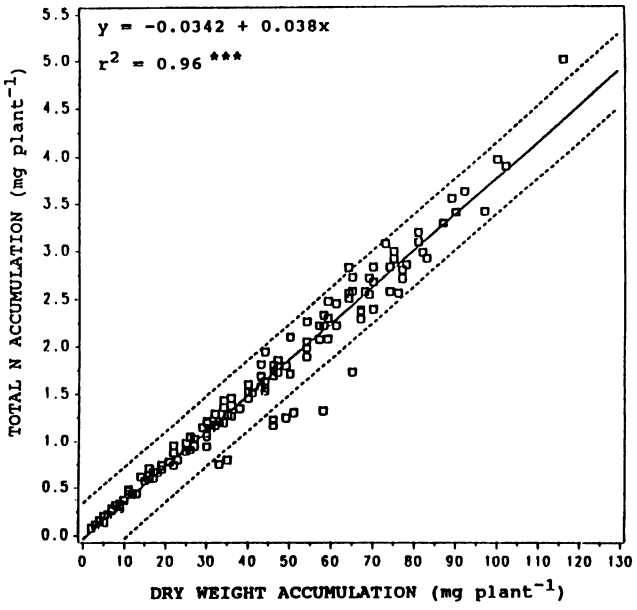

FIG. 1. Relationship of total biological nitrogen fixation accumulation to plant dry weights for all species. Dashed lines represent 95% confidence interval of best fit line (solid line).

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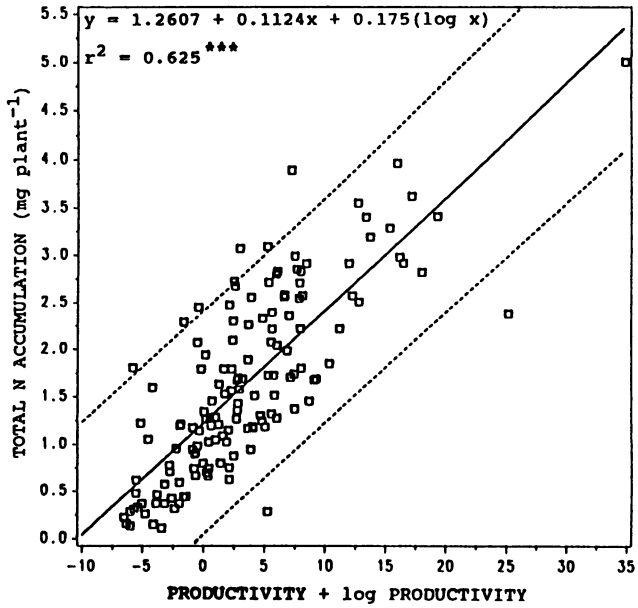

FIG. 2. Relationship of total biological nitrogen fixation accumulation to the derived productivity variable (plant vigor × dry weight × N fixed) for all species. Dashed lines represent 95% confidence interval of best fit line (solid line).

clover and subclover. However, these same strains were ineffective on red and white clovers.

The first investigations of the inoculation requirements of African trifoliums initially identified two subgroups within inoculation group S (21). In more recent lists of inoculation groups, Date (4) indicated four subgroups and Burton (3) designated the African clovers to five subgroups of group S. The cross-inoculation groups identified in this study (Table 6) were not clearly correlated with those reported by Burton (3). According to his classification, the subgroup C includes not only *T. steudneri* and *T. tembense* but *T. rueppellianum* as well. In the present study, *T. rueppellianum* did not have an effective *Rhizobium* strain in common with these two species. Subgroup D was reported to contain *T. rueppellianum* as well as *T. semipilosum* (Kenyan clover), and subgroup K contained *T. steudneri* along with several other clovers (3, 4). It is suggested here that the species *T. decorum* and *T. quartinianum* might be placed in subgroup D and that the association of *T. quartinianum* and *T. steudneri* falls in subgroup K. Verification of any of these groupings must be confirmed with testing of the associations of these rhizobia with the other clover species found in the respective subgroups (27). Furthermore, accurately placing the clover species *T. decorum* and *T. quartinianum* in any particular group will require additional cross-inoculation studies, since none have been reported to date.

The evaluation results of rhizobia and clover responses to controlled conditions in the greenhouse may be at variance with responses obtained when placed in natural field conditions in the African highlands. Verification of these results is required under the conditions of the target area prior to expanded use of these clover-rhizobia combinations. The following conclusions can be drawn from the results of this study: (i) strains of *Rhizobium* effective in combination with these five *Trifolium* species existed in two soils where the clovers were naturally adapted; (ii) the five *Trifolium* species evaluated fell within at least two, and possibly three, *Rhizobium* strain effectiveness subgroups; and (iii) the strains from the Ethiopian soils were much more effective as a group than the Nitragin strains were.

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