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Effects of Drought on the Survival of *Rhizobium leguminosarum* Biovar *trifolii* and the Nodulation of Subterranean Clover in an Acid Soil

Carmen Bueno
Utah State University

EFFECTS OF DROUGHT ON THE SURVIVAL OF *Rhizobium leguminosarum* BIOVAR *trifolii* AND THE NODULATION OF SUBTERRANEAN CLOVER IN AN ACID SOIL

by

Carmen Bueno

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Carmen Bueno

PLANT SCIENCE DEPARTMENT
Utah State University
Logan, Utah 84321
U. S. A.

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ABSTRACT

Effects of Drought on the Survival of Rhizobium leguminosarum biovar trifolii and the Nodulation of Subterranean Clover in an Acid Soil

by

Carmen Bueno, Master of Science

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Major Professor: Dr. William F. Campbell

Department: Plant Science

Twenty-nine Rhizobium leguminosarum biovar trifolii strains were tested for acidity tolerance in acidified liquid medium. Only 41% of the strains grew at pH 4.1. One acid-tolerant strain, USDA 2160, and one acid-sensitive strain, 162-X-103 from Nitragin Co., were inoculated on seeds of 'Nungarin', 'Seaton Park' and 'Clare' subclover cultivars. The inoculated and pelleted seeds were sown in potted Cluff soil with pH 5.7. Three desiccation levels were imposed by delaying watering for 0, 15 or 30 days. Four gravimetric soil water contents (6.0, 6.6, 10.5 and 12.5%) were maintained under a greenhouse line-source sprinkler system for 7 weeks. The desiccation treatments

were more detrimental to the survival of the acid-tolerant Rhizobium strain (USDA 2160) than they were for the acid-sensitive strain (162-X-103). Symbiotic effectiveness, measured as shoot dry weight, was higher with strain 162-X-103 than with strain USDA 2160 and was comparable to the N-fertilized control at the highest water level (12.5 %). At the lower water levels (6.0, 6.6 %) symbiotic N_2-fixation was more affected than N-uptake. The Rhizobium strains were able to survive and grow even at the lowest soil water level. The number of rhizobia in the soil and nodulation of the subclover plants had a correlation of 0.56.

(84 pages)

INTRODUCTION

Annual winter legumes are valuable plants in some semiarid rangelands ecosystems because of their ability to symbiotically fix nitrogen in association with Rhizobium species. In southwest Spain subterranean clover (Trifolium subterraneum L.) has been used to improve rangelands because of its productivity, nitrogen content, adaptation to the climatic conditions, and its capacity to reseed annually. Although subterranean clover originated from this area, improved cultivars from Australia have been used, but often do not yield well. In southwest Spain pastures are mostly situated in acid soils with pH 5 to 6 (Llano-Ponte et al. 1975). These soil pH levels are not limiting for the plant, but can be a constraint for the saprophytic and symbiotic performance of Rhizobium. Olea et al. (1986) found that fall precipitation was more highly correlated with subclover forage yield in this area than any other climatic and soil parameters studied.

Pastures are sown during September or early October after the first seasonal rainfall following a dry, hot summer. The soils usually have a low water potential at that time and no other effective precipitation may occur for several days. This desiccation period and the soil acidity may affect Rhizobium survival, seed germination and nodulation. It is important to evaluate the effect of

drought stress on the microsymbiont survival and infection of the host because nodulation failure can markedly reduce legume productivity.

LITERATURE REVIEW

The Macrosymbiont

Subterranean clover or subclover is the common name for Trifolium subterraneum L. and two other species, T. yanninicum Katzn. and Morley and T. brachycalycinum Katzn. and Morley. These species differ in morphological features and edaphic adaptation. Subclover originated in the Mediterranean region (McGuire 1985) and is an annual with winter growth, trifoliate leaves and prostrate habit. The inflorescence has 3 to 10 perfect papillonate flowers. After fertilization, a burr of calyces of abortive flowers develops and envelopes the seeds. The peduncles elongate toward the soil and bury the seed burrs. For this reason subclover can withstand grazing during the period of seed set.

Subclover was recognized as a plant of great potential in Australia in the early 1900s following its accidental introduction from Europe in grass seed lots (Morley 1961). In 1921 it was introduced into the United States through the Texas Agricultural Experimental Station (Knight et al. 1982). Because of its origin, subclover is adapted to a Mediterranean climate with hot, dry summers and mild, moist winters. It can, however, be found in very different habitats with annual precipitation from 350 to over 1100 mm (Morley 1961). It has been successfully introduced into

temperate and semiarid regions of the world, and reintroduction into Spain and Portugal in recent years has increased the productivity of many pasturelands.

The Microsymbiont

Subterranean clover increases the soil nitrogen content through its symbiosis with the nitrogen fixing bacterium Rhizobium leguminosarum biovar trifolii, formerly R. trifolii (Jordan 1984). Failure of establishment or persistence of subclover frequently is due to the lack of an effective microsymbiont. The bacteria must survive as saprophytes and be adapted to the soil's physical, chemical and biological conditions. They also must be present in sufficient number to effectively nodulate seedlings in the next growing season.

The susceptibility of rhizobial strains to different environmental conditions has been investigated by numerous authors. However, research dealing with two or more stresses occurring simultaneously, e.g., low and erratic rainfall and poor fertility due to acidity, is relatively sparse.

Acidity

Legumes and their associated rhizobia must be adapted to the soil conditions for nodulation to occur. Excellent reviews exist in the literature concerning the relationships of rhizobia and legumes under acid conditions (Blamey et al. 1987; Bushby 1982; Cooper et al. 1983;

Eaglesham and Ayanaba 1984; Freire 1984; Munns 1978). Low pH limits growth of some, but not all legumes. Munns (1968) showed that subclover can grow at pH 4.0 in culture solution when provided with the necessary nutrients. Kim et al. (1985) observed that growth of 11 subclover cultivars was vigorous at pH 4.0 in a flowing solution culture when an adequate nitrogen source was supplied. However, in a subsequent experiment where nitrogen supply was dependent on nodulation and nitrogen fixation, plant dry matter was severely depressed at pH 4.0 and 4.5. It was concluded that nodulation is more sensitive to low pH than plant growth. Similar results also were noted by Loneragan and Dowling (1958) in subclover cv. Bacchus Marsh where nodulation was less tolerant to low pH than was plant growth with adequate inorganic N. This reduction in nodulation can be mediated by a reduction in plant growth, the inability of the bacteria to grow and spread in the rhizosphere (Mulder and Van Veen 1960, Rice et al. 1977, Mulder et al. 1977), increased numbers of ineffective rhizobia (Holding and Lowe 1971) or by the prevention of the infection process by an acid-sensitive step which approximately coincides with the curling of root hairs (Munns 1968, Date 1981).

All root nodule bacterial strains studied by Vincent (1977) grew at a pH range of 5.5 and 7.0. Acid tolerance was greater among slow- than fast-growing bacteria. For example, Bradyrhizobium sp.(Lupinus) and B. japonicum were

more tolerant than R. leguminosarum biovars phaseoli, viceae and trifolii, and R. meliloti was the least tolerant to acidity. Pure culture studies showed that the critical low pH for growth varied between 4.0 and 6.0 (Graham and Parker 1964).

Thornton and Davey (1983b) demonstrated that strain sensitivity to soil acidity may be predicted with reasonable success in the laboratory. They found that acid-resistant effective strains of R. leguminosarum biovar trifolii can form effective symbiotic associations with clovers in acid soils. In some cases these associations approached 90 to 99% of the biomass yield of the nitrogen fertilized controls.

The effects of soil pH on Rhizobium-legume associations are difficult to study due to the interrelationships among pH, aluminum (Al) concentration in the soil solution, and the availability of essential nutrients. Soil acidity is accompanied by phosphorus (P) and calcium (Ca) deficiency and toxic levels of Al and manganese (Mn). These effects have been studied by Lowther and Loneragan (1968), who reported that decreasing Ca concentration from 246 uM to 4 uM progressively decreased both plant growth and nodule numbers in subterranean clover. Keyser and Munns (1979) showed that Al severely reduced the growth of free living rhizobia and that low P concentration inhibited growth of some strains. They found 10 strains of cowpea rhizobia tolerant to low pH (4.5), low

P (5-10 uM) and high Al (50 uM). Thornton and Davey (1983a) observed that a response to acidity did not imply a tolerance to Al in all cases. Rhizobial strains were capable of tolerating higher levels of Al if acidity was reduced. Liming the soil is one of the practices used to improve survival of inoculated rhizobia and nodulation in the field. Because low pH often has been implicated in legume inoculation failures, lime commonly has been used to cover the inoculated, pelleted seeds (Brockwell 1962; Loneragan et al. 1955). This coating with $CaCO_3$ can increase Rhizobium survival by changing the pH around the pellet and protecting against desiccation.

Recent results by Whelan and Alexander (1986) suggest that the failure of R. leguminosarum biovar trifolii to nodulate subclover growing in Keyser and Munns' (1979) medium at pH 4.5 was not a result of poor growth. Rhizobial numbers were similar at pH 4.5 and 5.2 after 3 days although not after 6 days. Instead, they suggested that an early stage of the infection process was impeded by acidity. Paulino et al. (1987) suggested that Al ions specifically affected nitrogenase activity and the Al effect was primarily responsible for the reduction in growth of peas (Pisum sativum).

The soil under subterranean clover pastures may become more acid with time (Williams 1980, Bromfield et al. 1983). This can result in a decline in productivity as observed throughout southeastern Australia, due to the failure of R.

leguminosarum biovar trifolii to survive in the soil (Coventry et al. 1985). Jones and Curnow (1986) studied this problem in acid soil and noted that nodulation was negatively correlated with the percentage of exchangeable Al and positively correlated with the percentage of exchangeable Ca.

Moisture Stress

Rhizobia do not tolerate hot, dry soil conditions (Marshall 1964). Few studies on the effect of water potential during saprophytic growth, which may be a prerequisite for satisfactory expression of the symbiosis, have been conducted. Mahler and Wollum (1981) investigated the effect of water potential on the survival of slow- and fast-growing rhizobia in seven soils with a broad range in soil texture. They observed that populations of B. japonicum serogroup isolate 122 increased from week to week for most textures and water potentials tested (-1.5, -0.5, -0.1 and -0.03 MPa). Population densities of serogroup 123 appeared to peak at 4 weeks, and then were maintained in three but reduced in four of the different soil textures used. R. leguminosarum serogroup isolate WA-02 showed a better colonization in soils of different textures over a wide range of water potentials than isolate WA-01. The data support the conclusion that slow-growing rhizobia withstand desiccation better than fast-growers.

The interactions among soil water potential, soil texture, soil temperature and soil pH, as suggested by

Mahler and Wollum (1981), may influence the saprophytic and symbiotic capacities of specific rhizobia. Marshall (1964) reported that rhizobia are more susceptible to desiccation in sandy soils and that amending these soils with certain clays improved the survival of the fast-growing bacteria. Bushby and Marshall (1977) evaluated changes in rhizobial populations inoculated in sterile soil that was rapidly dried. The rhizobia decreased rapidly in number. Bushby and Marshall (1977) proposed that differences in susceptibility among rhizobia were related to the amount of water retained by the cells during the process of drying.

Several authors have reported the biphasic nature of rhizobia survival as soils dry. An initial rapid decline in rhizobial numbers occurs during water loss by the soil, followed by a slower rate of death after all the free moisture has been removed. The remaining rhizobial populations are not more drought resistant because in following drying-wetting cycles the populations are reduced in each drying cycle (Pena-Cabriales and Alexander 1979).

In a study of desiccation tolerance of R. leguminosarum biovar trifolii in physically dissimilar sterile soils, Fuhrman et al. (1986) pointed out that soil type and differential desiccation rates affected rhizobial survival. The most consistent estimates of relative desiccation tolerance were obtained with the finer-textured Cecil soil (sandy clay loam), -500 MPa water potential desiccation regime and an incubation period of at least 21

days.

Reports of the effect of moisture deficits on the nodulation of legumes have been mainly observational. Worrall and Roughley (1976) observed that a reduction of soil moisture from 5.5 to 3.5% (-0.036 to -0.36 MPa) significantly decreased the number of infection threads and completely inhibited nodulation of T. subterraneum by R. leguminosarum biovar trifolii strain TA1, although the number of rhizobia in the rhizosphere was unaffected. They observed that root hairs were abnormally short and swollen at low moisture levels. Infection and nodulation were little affected between 5.5 and 9.5% moisture (-0.036 to -0.0089 MPa). Rewatering increased infection numbers in plants grown at 3.5% (-0.36 MPa), and nodules were formed at a rate equivalent to non-stressed plants.

Nitrogen fixation may be more sensitive than nodulation to moisture deficits (Sprent and Minchin 1983). Water deficit reduced the N content of T. repens more than it did plant growth (Engin and Sprent 1973). Sprent and Minchin (1983) suggest that there is an indirect effect of moisture stress on nitrogen fixation through the reduced availability of photoassimilates from the stressed host to the micro-symbiont.

Objectives

The objectives of the present study were to evaluate the effects of moisture stress in acidic soil on the

saprophytic survival of Rhizobium leguminosarum biovar trifolii strains, nodulation of T. subterraneum, and the effectiveness of their symbiosis under greenhouse conditions.

EXPERIMENT 1:

ACIDITY TOLERANCE OF *Rhizobium* STRAINS

Objective

The objective of this experiment was to determine the growth of *R. leguminosarum* biovar *trifolii* strains in an acidified liquid medium as a screening tool to select acid-tolerant strains.

Materials and Methods

Twenty-nine *Rhizobium leguminosarum* biovar *trifolii* strains were obtained from collections in the USA and Spain (Table 1) . The strains were collected initially from various locations having Mediterranean climates.

The liquid growth media was that of Keyser and Munns (1979) with the modifications of Thornton and Davey (1983a). The basal solution is composed as follows: mannitol, 10 g L^{-1}; Na-glutamate, 1.1 g L^{-1}; and yeast extract, 0.5 g L^{-1}; and the following salts in umol: $MgSO4$, 300; $CaCl_2$, 300; Ferric-EDTA, 10; KCl_2, 10; $MnCl_2$, 0.1; $ZnSO_4$, 0.4; $CuCl_2$, 0.1; $NaMoO_4$, 0.002; $CoNO3$, 0.002; KH_2PO_4, 1; K_2HPO_4, 1 and distilled water to make 1 L. The media was acidified by the addition of 1 N HCl prior to autoclaving to obtain a final pH of 6.8, 5.0, 4.5 or 4.1. A starter culture of each strain was grown in 20 ml yeast extract mannitol (YEM) at pH 6.8 (Vincent 1970) until a

Table 1. *Rhizobium leguminosarum* biovar *trifolii* cultures: source, origin and host from which they were isolated.

Accession number	Geographic origin	Host	Site characteristics
Donor: H. Keyser. USDA Agricultural Research Service. Beltsville, MD 20705			
USDA 2092	Unknown	*T. incarnatum*	
USDA 2130	"	*T. alexandrinum*	
USDA 2131	"	"	
USDA 2153	Morocco	*T.subterraneum*	
USDA 2154	California	"	
USDA 2155	"	"	
USDA 2156	"	"	
USDA 2157	"	"	
USDA 2158	"	"	
USDA 2159	"	"	
USDA 2160	"	"	
USDA 2161	"	"	
USDA 2162	Tunisia	"	
Donor: S. Smith. Nitragin Co.Inc. Milwaukee, WI 53209			
162-G-15	California	*T. hirtum*	
162-K-13	Alabama	*T. incarnatum*	
162-X-68	California	*T. subterraneum*	
162-X-103	Tunisia	"	
Donor: R. Orive. Experimental Station "La Rinconada". Sevilla (SPAIN)			
IST-1	Spain	*Trifolium* sp.	Acidic irrigated soil
IST-11	"	"	
IST-28	"	"	
IST-51	"	*T. subterraneum*	
IST-54	"	"	Acidic, dry soil
IST-61	"	*T. repens*	" "
IST-62	"	"	" "
IST-65	"	*T. subterraneum*	" "
IST-66	"	*T. fragiferum*	Alluvial, pH 7.4 irrigated soil
IST-69	"	"	" "
IST-71	"	*T. repens*	Acidic meadow
IST-73	"	*T. subterraneum*	" "

turbidity approximately corresponding to 3 x 10^8 cells/ml was attained. Aliquots of 0.5 ml of each strain suspension were inoculated in 72 mm x 100 mm tubes containing 4.0 ml of the acidified or neutral medium. The initial cell density in the tubes was about 6 x 10^7 cells ml^{-1} and no turbidity was observed. The tubes were incubated on a rotary shaker maintained at 25°C and operated at 120 rpm. Three tubes of each strain-acid level combination were used. Acid tolerance was assesed by bacterial growth measured as daily culture turbidity. This was quantified as optical density at 600 nm in a Varian DMS 100 spectrophotometer. The experiment was conducted with 3 tubes of each of the 29 rhizobial strains at each pH level in a complete randomized design. After seven days of incubation, all tubes were checked for contamination by the drop-plate method (Miles and Misra 1938).

Results

Based on pilot studies, the Thornton and Davey (1983a) medium proved suitable for growth of the bacterial strains tested. Days of culture, strains and acidity levels were highly significant (Table 2) and also the first order interactions. Day of culture and strain were significantly different at the lowest pH medium (Table 3). Twelve strains, representing 41.4% of those tested, showed an increase in optical density (Table 4) in Day 4. For each of these twelve strains, their growth period was preceded by a lag phase. Growth curves for representatives of

Table 2. Analysis of variance for optical density of rhizobial strains grown at four pH levels in Experiment 1.

Source	df	MS	Significance level
Day (D)	1	2.8061	* * *
Strain (S)	28	0.0413	* * *
Acidity level (A)	3	2.7248	* * *
DS	28	0.0088	* * *
DA	3	0.5626	* * *
SA	84	0.0167	* * *
DSA	84	0.0027	NS
Error	348	0.0018	_
Total	579		

NS Not Significant

* * * Significant at 0.01 probability level

Table 3. Analysis of variance for optical density of rhizobial strains grown at pH 4.1 for Experiment 1.

Source	df	MS	Significance level
Day (D)	5	0.3579	* * *
Strain (S)	28	0.2288	* * *
DS	139	0.0284	* * *
Error	346	0.0021	_

NS Not Significant

* * * Significant at 0.01 probability level

Table 4. Turbidity of cultures of rhizobial strains which grew at pH 4.1.

Strain	Days of culture growth.					
	1	2	4	5	6	7
	Optical density at 600 nm x 10^{-3}					
USDA 2154	94	88	332	513	608	638
USDA 2155	84	99	223	339	466	600
USDA 2156	71	59	104	184	281	316
USDA 2157	105	74	134	176	203	218
USDA 2158	46	56	220	266	377	465
USDA 2159	81	57	167	231	336	429
USDA 2160	97	48	302	373	466	483
162-X-68	51	60	213	301	357	511
162-K-13	41	32	94	147	183	267
IST-1	40	36	83	123	90	128
IST-71	19	31	218	275	452	579
IST-73	23	53	359	368	609	757

rhizobial strains that grew well (USDA 2160) and those that grew poorly (162-X-103) are presented in Figure 1. Upon termination of the experiment, the mean final pH was 4.3 for the medium in which the twelve rhizobial strains exhibited growth and 4.2 in those exhibiting little or no growth. All of the strains tested, even those that did not grow at the lowest pH, formed colonies with the drop-plate method indicating that the Rhizobium survived in the liquid medium. No contamination appeared in the cultures except in one plate of strain 162-G-15 at pH 4.5 and another plate of the strain 162-K-13 at pH 4.1.

Discussion

The lag period of rhizobia growth was not affected by acidity to the extent reported by Keyser and Munns (1979), possibly due to the high initial cell concentration (6×10^7 cells ml^{-1}). Keyser and Munns (1979) found that acidity affected rhizobia growth in broth culture (pH 4.5) by either increasing the lag time and/or decreasing the growth rate. In that study, acidity stopped growth of about 30% of the Rhizobium strains tested. Howieson (1985) stated that the Keyser and Munns medium had low buffer capacity. This was demonstrated in the present study by the rise of the pH due to rhizobial growth.

It is of interest that the Spanish rhizobial strains that grew in the acidified liquid medium included one that was isolated from an irrigated site with an acidic soil (IST-1) and two that were isolated from acidic soils from

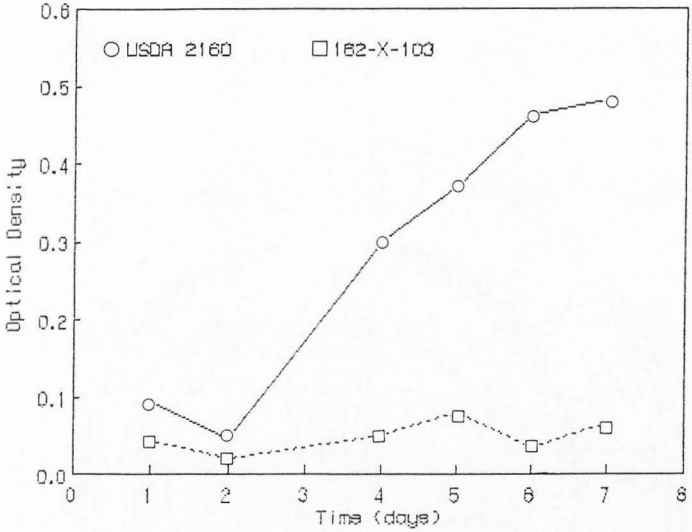

Figure 1. Optical density at 600 nm of two rhizobial strains growing for 7 days in liquid medium at pH 4.1. Each point is the mean of 3 cultures.

the humid region of Northwest Spain (IST-71 and IST-73). Strains collected from acidic soils at dry sites were not tolerant to low pH in the growth medium used in this study, possibly because they were not adapted to the liquid medium conditions. However, they may have been capable of growing in acidified agar media such as the one used by Ayanaba et al. (1983).

Cunningham and Munns (1984) observed a correlation between extracellular polysaccharide (EPS) production by Rhizobium and their acidity tolerance. Two Rhizobium strains used in this study, acid tolerant USDA 2160 and acid sensitive 162-X-103, were shown to have the same EPS production with "gummy or wet" colonies when plated in YEM-agar at pH 6.8. The difference in their acidity tolerance was consequently not due to a gross difference in EPS production. Because EPS causes turbidity, the spectrophotometric method used to quantify growth may have overestimated the growth rates of high EPS producing strains. The similarity in EPS production by the acid-tolerant and acid-sensitive strains indicated that the acidified liquid medium of Keyser and Munns (1979) and the spectrophotometric method to estimate bacterial growth were suitable means to differentiate acid-tolerant from acid-sensitive strains.

EXPERIMENT 2:

Rhizobium-SUBTERRANEAN CLOVER EFFECTIVENESS

Objective

The objective of this experiment was to determine the compatibility and symbiotic effectiveness of the host-*Rhizobium* association, as measured on seedlings in *in vitro* agar culture, in order to select the most compatible strains for the subsequent greenhouse experiments.

Materials and Methods

A total of seven *Rhizobium* strains were used in this experiment, these included: two *Rhizobium* strains that grew in the acidified liquid media from Experiment 1 and which were known to be effective with *T. subterraneum* (USDA 2156, USDA 2160); three Spanish strains (IST-1, IST-71 and IST-73); and two strains from the Nitragin Co. that did not grow in the acidified liquid media (162-G-15 and 162-X-103). These seven strains were grown in YEM broth at pH 6.8 under the same culture conditions as Experiment 1.

The subterranean clovers used were two *Trifolium subterraneum* cultivars, 'Nungarin' and 'Seaton Park', and one *T. brachycalycinum* Katzn and Morley cultivar, 'Clare', obtained from Dr. Leopoldo Olea, SIA Finca "La Orden" Badajoz 06080 (Spain). These cultivars were developed in Australia and tested and increased in southwest Spain. Their characteristics are indicated in Table 5.

Table 5. Edaphic adaptation and phenological characteristics of subclover cultivars when grown in southwest Spain.

Species/cultivar	pH adaptation	Days to flowering	Relative maturity
T. brachycalycinum	5.5 - 8.5		
cv. Clare		148	Mid-season
T. subterraneum	4.5 - 7.0		
cv. Nungarin		118	Very early
cv. Seaton Park		132	Early

Seeds from the three cultivars were surface sterilized by immersion in 0.02% mercuric chloride ($HgCl_2$) for 7 min and rinsed with sterile, distilled water six times. The seeds then were placed in water agar (0.75%) in Petri dishes at 25°C for germination.

After 48 h, germinated seeds were placed in 150 x 25 mm tubes containing 18 ml slopes of nitrogen-free Jensen's seedling agar (Vincent 1970). Tubes were closed with cotton plugs. Five-day-old seedlings were inoculated with 1 ml of the various Rhizobium suspensions. Each strain was inoculated onto eight seedlings of each cultivar in a complete randomized design. Controls of uninoculated seedlings supplied with 0.05% KNO_3 or without were also included. Tubes were placed in a shaded greenhouse. After 7 weeks of growth when the uninoculated treatment appeared N-deficient, the plants were observed for general appearance, removed from the culture medium and number of nodules were recorded. Plants were then dried for 24 h at 70 °C, and shoot and root dry weight were recorded.

Results

Symbiotic effectiveness of the rhizobial strains with each subclover cultivar was estimated using shoot dry weight. There were statistically significant differences among cultivars in mean shoot dry weight (Table 6) with Clare having the heaviest plant (0.0256 g/plant), Seaton Park being intermediate (0.0229 g/plant), and Nungarin having the lightest plants (0.0183 g/plant). This ranking

Table 6. Analysis of variance for shoot dry weight (g) of Experiment 2.

Source	df	MS	Significance level
Replication (R)	7	0.159×10^{-4}	NS
Cultivar (C)	2	0.408×10^{-3}	* * *
RC	14	0.659×10^{-5}	NS
Strain (S)	6	0.164×10^{-3}	* * *
RS	42	0.141×10^{-4}	NS
CS	12	0.128×10^{-4}	NS
Error	82	0.142×10^{-4}	-

NS Not Significant

* * * Significant at 0.01 probability level

for shoot dry weight was the same as that for seed size. The 7 strains produced significant differences in plant shoot dry weights, although the cultivar by strain interaction was not significant (Table 6). The effectiveness of three subclover cultivars inoculated with the 7 strains is shown in Table 7. None of the subclover plants inoculated with any of the rhizobial strains weighed more than the control to which N was added.

The number of nodules on the subclover plants was significantly different for cultivars, strains and the cultivar by strain interaction (Table 8). The number of nodules formed by each cultivar with the different strains differed significantly (Table 9) . The more effective strains produced fewer nodules than the ineffective strains IST-1, IST-71 and IST-73. These ineffective strains typically caused the formation of a rosary of white nodules, and the plants appeared N-deficient with small yellowish leaves.

Based on these results, the acid-tolerant strain, USDA 2160, and the acid-sensitive strain from the Nitragin Co., 162-X-103, were selected for use in further experiments.

Discussion

The effectiveness of a *Rhizobium* strain-legume association is influenced by the host. All of the cultivars used in this study became nodulated in an enclosed axenic medium when the inoculum concentration was high. However, nodulation success in a soil environment

Table 7. Shoot dry weight of subterranean clover cultivars inoculated with different Rhizobium strains and grown for 7 weeks. Each value represents the mean of 8 observations.

Inoculation Treatment	Subclover cultivars		
	Nungarin	Seaton Park	Clare
	Shoot dry weight (g/plant)		
USDA 2160	0.0191 b	0.0241 b	0.0263 ab
USDA 2156	0.0185 bc	0.0237 bc	0.0265 ab
162-X-103	0.0188 a	0.0209 bcd	0.0220 bc
162-G-15	0.0172 bcd	0.0192 d	0.0202 c
IST-1	0.0161 cd	0.0198 cd	0.0194 c
IST-71	0.0147 de	0.0177 d	0.0182 c
IST-73	0.0124 e	0.0186 d	0.0203 c
Uninoculated +N	0.0262 a	0.0285 a	0.0283 a
Uninoculated -N	0.0152 d	0.0214 bc	0.0262 ab

Means within columms followed by the same letter are not significantly different at the 0.05 probability level by LSD.

Table 8. Analysis of variance for number of nodules in Experiment 2.

Source	df	MS	Significance level
Replication (R)	7	48.94	NS
Cultivar (C)	2	256.30	* * *
RC	14	28.04	NS
Strain (S)	6	1155.67	* * *
RS	42	31.91	NS
CS	12	321.14	* * *
Error	82	33.92	-

NS Not Significant

* * * Significant at 0.01 probability level

Table 9. Nodulation of subterranean clover cultivars inoculated with *Rhizobium* strains and grown for 7 weeks. Each value represents the mean of 8 observations.

Rhizobium strain	Subclover cultivars		
	Nungarin	Seaton Park	Clare
	Number of nodules/plant		
USDA 2160	8 a	9 ab	7 ab
USDA 2156	9 a	7 ab	7 ab
162-X-103	10 a	6 a	4 a
162-G-15	10 a	6 a	4 a
IST-1	16 b	10 ab	14 b
IST-71	16 b	12 b	41 d
IST-73	20 b	20 c	23 c

Means within columms followed by the same letter are not significantly different at the 0.05 probability level by LSD.

may change as it can be modified by plant growth conditions (Demezas and Bottomley 1987).

The results of the present study substantiate the work of Robinson (1969) that showed that rhizobial strains are most effective with the host from which they were originally isolated. As can be observed in Table 4, the most effective strains were the USDA accessions 2160 and 2156 which were isolated from T. subterraneum. Strain 162-X-103 isolated from T. subterraneum also showed good effectiveness. Less effective strains were 162-G-15 isolated from T. hirtum, IST-1 isolated from an unspecified clover, IST-71 isolated from T. repens and IST-73 isolated from T. subterraneum.

The cultivar by strain interaction was significant for number of nodules, but was not significant for shoot weight. It could be that the experiment may have been terminated too early to notice any appreciable effect on shoot dry weight. If the experiment had been conducted over a longer period of time, the cultivar by strain interaction for shoot weight may have been significant. It would be expected that given enough time, the number of nodules would have an influence on shoot growth in a nitrogen-free environment.

EXPERIMENT 3:
EFFECTS OF DROUGHT ON NODULATION OF SUBTERRANEAN CLOVER BY *Rhizobium* IN POTTED ACID SOIL

Objective

The objective of this experiment was to determine whether moisture stress influences nodulation of *T. subterraneum* and the effectiveness of *Rhizobium* strains when preinoculated and pelleted seeds are planted in an acidic sandy loam soil under greenhouse conditions.

Materials and Methods

Soil Type. The study was conducted in an artificially mixed soil that resembled the soils of southwest Spain. The analytical description of that Spanish soil (Llano-Ponte et al. 1975) was: coarse sand, 32.4%; fine sand, 37.0%; silt, 18.2%; clay, 12.4%; and a pH (measured in 1:5 soil water suspension) of 5.5.

To make up the mixed soil, field soil was collected from a site located at a latitude 41° 38' 5''N and a longitude 111° 29' 23''W at 1000 m west and 600 m south of the northwest corner of the Red Spur Mountain Quadrangle map, Utah, 7.5 minute series. This field soil was a Cluff soil (Jalalian 1981) with a composition of 40% sand, 28% silt and 32% clay and the pH was 5.7 (measured in 0.01 M $CaCL_2$). Chemical properties determined by the Plant and

Soil Analysis Laboratory at Utah State University were as follows: P, 12.0 ppm; N, 2.4 ppm; Al, 0.84%; Mn, 24 ppm; Ca, 6.6 meq/100g; Mg, 1.0 meq/100g; and K, 2.5 meq/100g. The field soil was ground in a hammer mill to pass a 100 mm mesh screen and mixed with 30% w/w fine-ground silica to make a composition of 70% sand, 14% silt and 16% clay. This soil mixture was free of Rhizobium leguminosarum biovar trifolii as assessed by the lack of nodulation of subclover seedlings inoculated with soil suspensions and grown in enclosed seedling agar slopes. The soil was stored until used and then pasteurized with steam for 2 h. After drying, 622 g of soil were placed in cone-shaped pots 250 mm in length, 62 mm in diameter at the top and 55 mm in diameter at the bottom. The lower part was filled with 50 mm of pasteurized vermiculite to allow drainage and tamped down to achieve similar bulk density in each pot.

Seeds and Inoculum. Seeds of subterranean clover cultivars Nungarin, Seaton Park and Clare were surface sterilized by immersion in 0.02% $HgCl_2$ for 7 min, rinsed six times with sterile, distilled water, and then air dried.

Inocula of selected strains USDA 2160 (acid tolerant) and 163-X-103 (control, from the Nitragin Co.) were prepared from pure-culture suspensions in YEM at pH 6.8. After autoclaving, 10 g of finely ground charcoal was added as a carrier to 8 ml of the rhizobial suspension and allowed to mature for 10 days. The charcoal-based

inoculant (0.05 g) was mixed with 0.4 ml of gum arabic aqueous solution (40% w/v) in a polyethylene bag. Five grams of seeds were added and coated with the inoculant by gently shaking the bag until all the seeds appeared uniformly black. After inoculant coating, 2 g of $CaCO_3$ were added and the bag was again shaken until all seeds were covered. The pellets were placed on filter paper in Petri dishes and allowed to dry for five days. On the day of sowing the number of viable cells in the pellets was determined by serial dilution and plating on YEM-agar (Table 10). Uninoculated control pots were prepared with and without nitrogen fertilizer. The +N control fertilizer was a weekly application of 15 mg of N per pot as NH_4NO_3, starting the second week after sowing.

Desiccation Levels. Pots filled with dry soil were sown with five pelleted-inoculated or uninoculated seeds and then held in a dry room. Three desiccation levels were established by delaying transfer of the pots to the greenhouse and application of water until 0, 15 and 30 days after sowing.

Water Potential Regimes. The four water potential regimes applied were based on the relationship between the soil matric potential and water content as determined by the Plant and Soil Analysis Laboratory at Utah State University. The matric potential of the 7:3 Cluff soil:silica mixture for the four water content regimes were: 12.5%, -0.03 MPa; 10.5%, -0.1 MPa; 6.6%, -1.0 MPa;

Table 10. Number of viable rhizobial cells for each inoculum treatment in Experiment 3.

Cultivar	*Rhizobium* strain	
	USDA 2160	162-X-103
	Number of cells/pot ± SD	
Nungarin	$3.5 \times 10^4 \pm 1.3 \times 10^4$	$3.5 \times 10^4 \pm 1.2 \times 10^4$
Seaton Park	$8.0 \times 10^4 \pm 5.9 \times 10^4$	$1.7 \times 10^5 \pm 7.0 \times 10^4$
Clare	$2.0 \times 10^4 \pm 1.5 \times 10^4$	$8.5 \times 10^4 \pm 8.5 \times 10^4$

and 6%, -1.5 MPa (Fig.2). Initial water application at 0, 15 or 30 days was done gravimetrically by weighing the pot filled with soil and adding the calculated amount of water required to reach the desired soil matric potential. The pots were then placed on a ground bed under a line-source sprinkler system (Johnson at al. 1982). The system had one spray nozzle that moved along a fixed track that produced an irrigation pattern that was uniform along the length of the rectangular ground bed, but uniformly variable at right angles to the sprinkler track. Pots were spaced perpendicular to the sprinkler track in such a way that each pot received enough water to maintain the initial four soil water contents. The line-source sprinkler was programmed to provide two traverses every day and on very hot days it was set to make three traverses. The total amount of water that each water potential regime received during the experiment was approximately 222, 289, 348 and 522 mm for the 6.0, 6.6, 10.5, and 12.5 % moisture levels, respectively.

Plants grew for 7 weeks during the months of May, June and July in a shaded greenhouse. The experiment was a factorial experiment with a randomized block design with 288 pots and 1440 plants. Germination and seedling emergence were low in the low water level treatments. To have more uniform plants across water levels, the pots were reseeded with sterilized and pregerminated seedlings (as in Experiment 2) five days after the emergence of the first

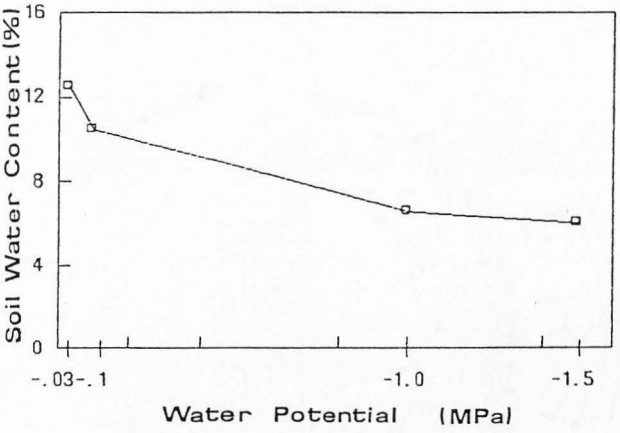

Figure 2. The relationship between soil water potential (MPa) and soil water content (%) for the 7:3 Cluff soil: silica mixture.

seedlings.

Results

Mean shoot dry weights of Rhizobium-inoculated and uninoculated plants were significantly different among treatments (Fig. 3). Plants that were inoculated with USDA 2160 and 162-X-103 Rhizobium strains did not differ significantly from each other, and plants inoculated with 162-X-103 did not differ from the +N control plants.

The inoculated and uninoculated plants in this experiment were subjected to an initial drought period by delaying irrigation for 0, 15 or 30 days. The desiccation periods produced significant differences in shoot dry weight with mean shoot dry weights decreasing as the desiccation period increased (Fig. 4). The interaction between desiccation period treatments with Rhizobium strains or controls was not significant, but there was a trend of decreasing shoot dry weights with successive desiccation periods (Fig. 5). Shoot dry weight increased significantly with increased soil water content(Fig. 6). The interaction between inoculation treatments and soil water contents was highly significant. Rhizobium-inoculated plants did not differ from the uninoculated -N control at the 6.0, 6.6 and 10.5% water contents (Fig. 7). At 10.5 and 12.5% soil water contents, the inoculated plants did not differ from the +N controls. At the highest water regime, the inoculated plants had significantly higher shoot dry weights than the uninoculated -N plants.

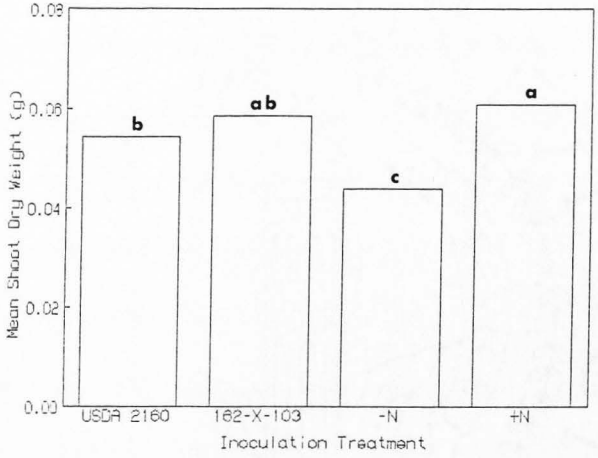

Figure 3. Mean shoot dry weight (g) of subclover cultivars over all the treatments inoculated with the strains USDA 2160 or 162-X-103 or uninoculated but with or without N added. The same letter on the top of the bars indicates no statistically significant differences at the 0.05 probability level by LSD. Values represent the mean of 349 observations.

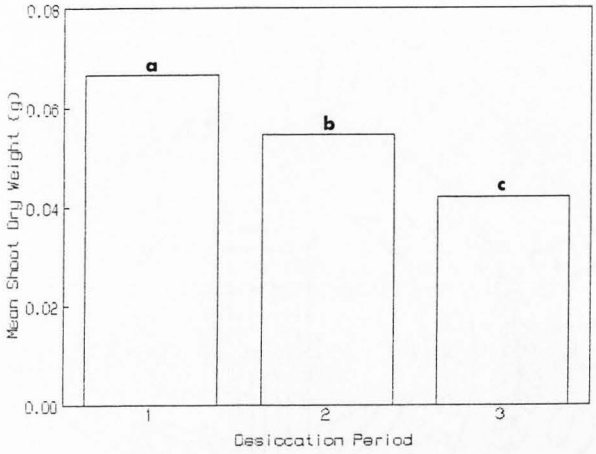

Figure 4. Mean shoot dry weight (g) of three subclover cultivars grown at four soil water contents in relation to desiccation periods (1 = 0 day; 2 = 15 days and 3 = 30 days). The same letter on the top of the bars indicates no statistically significant difference at 0.05 probability level by LSD. Values represent the mean of 465 observations.

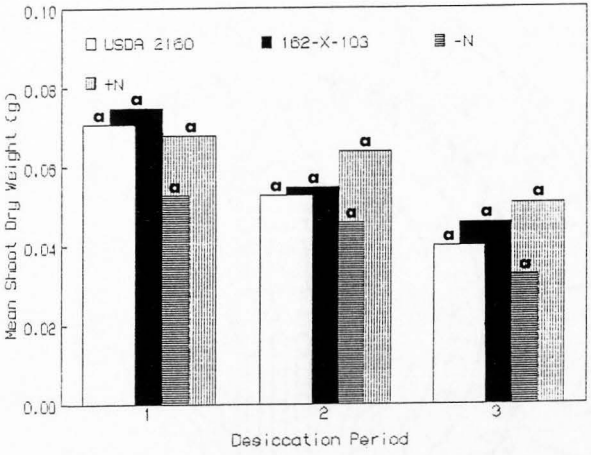

Figure 5. Mean shoot dry weight (g) of three subclover cultivars inoculated with strain USDA 2160 or 162-X-103 or uninoculated but with or without N added in relation to desiccation periods (1 = 0 day; 2 = 15 days and 3 = 30 days). The same letter on the top of the bars within the same desiccation period indicates no statistically significant differences at the 0.05 probability level by LSD. Values represent the mean of 117 observations.

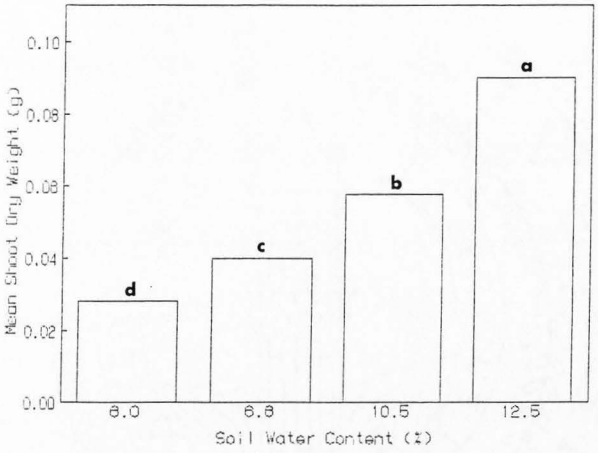

Figure 6. Mean shoot dry weight (g) of three subclover cultivars over all treatments in relation to the four soil water contents. The same letter on the top of the bars indicates no statistically significant differences at 0.05 probability level by LSD. Values represent the mean of 349 observations.

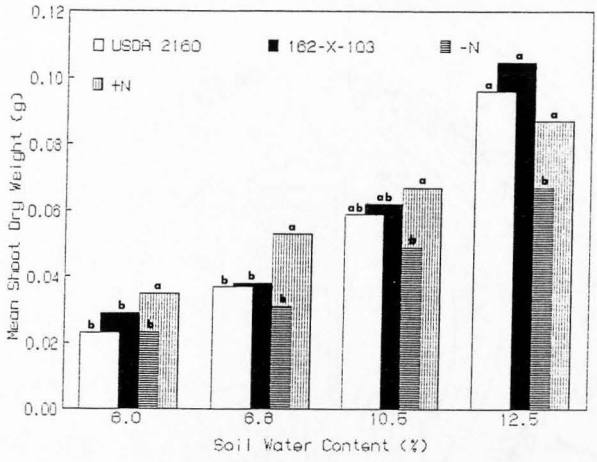

Figure 7. Mean shoot dry weight (g) of three subclover cultivars inoculated with strain USDA 2160 or 162-X-103 or uninoculated but with or without N added in relation to the soil water content. The same letter on the top of the bars within the same soil water content indicates no statistically significant differences at the 0.05 probability level by LSD. Values represent the mean of 87 observations.

There was no significant interaction between desiccation period treatments and moisture levels. However, there was a trend for reduced shoot dry weights with increasing desiccation periods for all four soil water contents (Table 11).

The subclover cultivars differed significantly in shoot dry weights (Fig. 8). Nungarin had lower shoot dry weight than Seaton Park and Clare with 0.0465, 0.0574 and 0.0596 g/plant, respectively. For Seaton Park the +N control plants yielded more than the Rhizobium-inoculated or uninoculated plants (Fig. 9). Inoculated and +N control plants of Clare did not differ in shoot dry weight but both treatments were significantly heavier than the -N control plants of that cultivar. For Nungarin no significant differences were observed among inoculation treatments.

The interaction between cultivars and water levels was significant. Except for the highest water level, dry weights of the subclover cultivars did not differ within the same water level (Table 12). At 12.5% soil moisture content Nungarin had a lower dry weight per plant than Seaton Park or Clare. However, the dry weights of all three cultivars increased with increasing soil water content. Higher level treatment interactions were not significant (Table 13).

After 15 and 30 days of desiccation, significantly fewer plants inoculated with strain USDA 2160 formed nodules than those inoculated with the strain 162-X-103 at

Table 11. Mean shoot dry weight combined over Rhizobium-inoculated and uninoculated treatments and subterranean clover cultivars in relation to soil water content· at three desiccation periods. Each value represents the mean of 120 observations.

Soil water content	Desiccation period (days)		
	0	15	30
	g/plant		
6.0 %	0.035 a	0.030 a	0.019 a
6.6 %	0.049 a	0.042 a	0.028 a
10.5 %	0.076 a	0.059 a	0.045 a
12.5 %	0.107 a	0.086 a	0.077 a

Means within a desiccation period followed by the same letter are not significantly different at the 0.05 probability level.

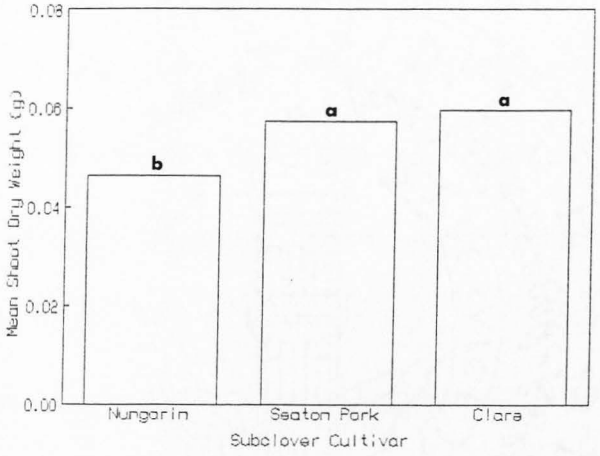

Figure 8. Mean shoot dry weight (g) of subclover plants over all treatments in relation to the three cultivars. The same letter on the top of the bars indicates no statistically significant differences at 0.05 probability level by LSD. Values represent the mean of 465 observations.

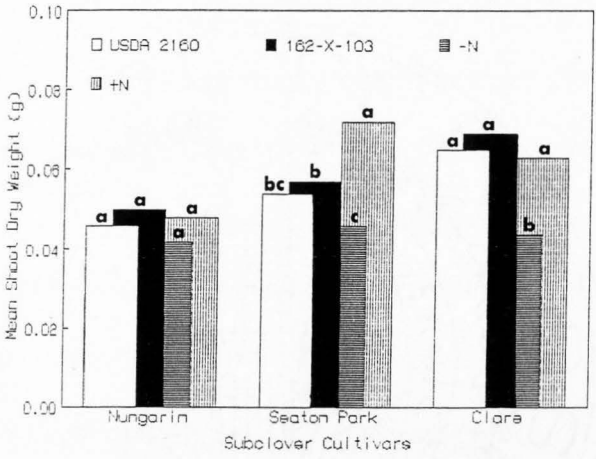

Figure 9. Mean shoot dry weight (g) of plants inoculated with strain USDA 2160 or 162-X-103 or uninoculated but with or without N added in relation to the subclover cultivars. The same letter on the top of the bars within the same cultivar indicates no statistically significant differences at the 0.05 probability level by LSD. Values represent the mean of 117 observations.

Table 12. Mean shoot dry weight combined over the Rhizobium-inoculated and uninoculated treatments and desiccation periods in relation to subterranean clover cultivars at four soil water contents. Each value represents the mean of 120 observations.

Soil water content	Subclover cultivars		
	Nungarin	Seaton Park	Clare
	g/plant		
6.0 %	0.024 a	0.031 a	0.030 a
6.6 %	0.036 a	0.043 a	0.040 a
10.5 %	0.053 a	0.059 a	0.068 a
12.5 %	0.074 b	0.096 a	0.100 a

Means within a soil water content followed by the same letter are not significantly different at the 0.05 probability level by LSD.

Table 13. Analysis of variance for shoot dry weight (g) for Experiment 3.

Source	df	MS	Significance level
Replication (R)	1	0.00034	NS
Desiccation (D)	2	0.0705	* *
RD (error a)	2	0.00179	-
Water level (W)	3	0.258	* * *
DW	6	0.00196	NS
RW + RDW (error b)	9	0.00196	-
Cultivar (C)	2	0.0233	* * *
Inoculation (I)	3	0.0197	* * *
CI	6	0.00434	* * *
DI	6	0.00215	NS
DC	4	0.00106	NS
WI	9	0.00480	* * *
WC	6	0.00261	*
DCI	12	0.00112	NS
DWC	12	0.00105	NS
DWI	18	0.00066	NS
WCI	18	0.00174	NS
DWCI	36	0.00144	NS
Error c	132	0.00126	-
Sampling error	1107	0.00056	-
Total	1394		

NS Not Significant
* Significant at 0.10 probability level
* * Significant at 0.05 probability level
* * * Significant at 0.01 probability level

6.0 and 6.6% soil water contents (Fig. 10). When the plants received water on the day of sowing, plants inoculated with USDA 2160 did not differ for percent nodulation with plants inoculated with 162-X-103.

A total of 32.5%, 21.5% and 17.6% of the plants of the uninoculated -N treatment became nodulated at the first, second and third desiccation periods, respectively. The uninoculated plants which formed nodules were mainly from treatments with 10.5 and 12.5 % soil water content and had higher shoot dry weight than the plants from the same pot which did not have nodules. The uninoculated plants that received NH_4NO_3 did not become nodulated.

Discussion

The relative symbiotic performance of the two rhizobial strains in acid soil was opposite to that expected on the basis of on their acid-sensitivity in liquid medium. At a soil pH of 5.7 the acid-sensitive Rhizobium strain 162-X-103 showed better symbiotic effectiveness than plants inoculated with the acid-tolerant USDA 2160. Possibly the Cluff soil was not sufficiently acid to affect the strains. Lindstrom and Myllyniemi (1987) selected acid-tolerant Rhizobium strains in Bromfield and Jones' medium on agar plates. Five strains were tested with red clover (T. pratense) in an acid clay mud (pH 4.1 and 5.2 adjusted by liming). They reported that at pH 5.2 there were no differences among the yields of the inoculated, uninoculated and +N control plants. No

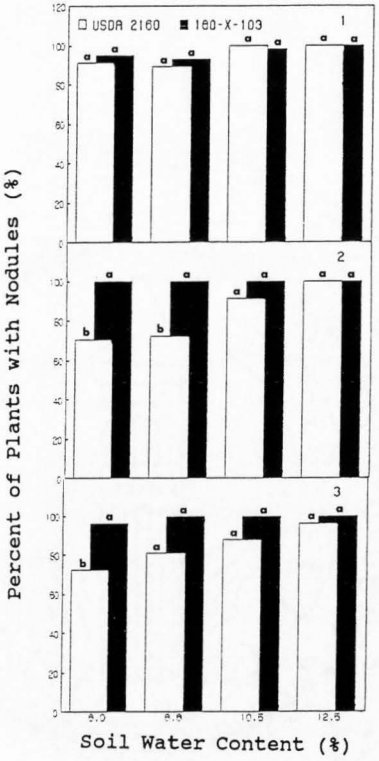

Figure 10. Percent of subclover plants inoculated with strain USDA 2160 or 162-X-103 which formed nodules in relation to the four soil water contents in three desiccation periods (1 = 0 day, 2 = 15 days and 3 = 30 days). The same letter on the top of the bars within the same soil water content indicates no statistically significant difference at the 0.05 probability level by LSD. Each value corresponds to a treatment total of 58 plants.

correlation was observed between the symbiotic properties of the strains at pH 4.1 and their growth on an acid laboratory medium. In the present study, with soil of higher pH (5.7), inoculated plants did not differ from the +N controls at the high soil water contents (10.5 and 12.5%), but they had different yields at low moisture levels (6.0 and 6.6%).

Results from Experiment 3 were different from the results of Experiment 2 where USDA 2160 had the highest symbiotic effectiveness with the subclover cultivars. This substantiates that growth conditions in nonsoil environments may enhance symbiotic performance, eg. host nodulation of one strain compared to another (Demezas and Bottomley 1987).

Nodules were formed at all soil water contents but after an initial desiccation period of 15 or 30 days, plants lacking nodules appeared at the low water levels. Only plants inoculated with strain USDA 2160 lacked nodules after those desiccation periods. Even if the host plant was infected by the microsymbiont, these nodules may have been shed after the imposition of drought.

Water stress also decreases symbiotic nitrogen fixation and growth of nodulated legumes (Sprent 1976). In the present study, plant growth was reduced at low soil water contents. However, growth of those plants in which nitrogen was being fixed was more susceptible to drought stress than in those to which N was added. This agrees

with results of De Jong and Phillips (1982) who subjected subclover (cultivar Woogenellup) plants to three cycles of water stress under two N regimes. They reported that the relative effect of water stress on growth under the two N regimes was similar, but N accumulation by symbiotic fixing clover was inhibited to a slightly greater extent than by NH_4NO_3-dependent plants. A possible reason for this is that biological N_2 fixation has a higher metabolic cost than does the utilization of combined N (Silsbury 1979). Nodulated plants in the low water treatment were fixing some nitrogen as they had higher dry weights and darker green color than the uninoculated -N plants. In the low water level regimes root growth was reduced. Similarily, in the study by Worrall and Roughley (1976), root and root-hair growth were inhibited by moisture stress in the subclover cultivar Mt. Barker.

Contamination from exogenous Rhizobium under greenhouse experimentation is difficult to avoid. Contamination in this experiment that caused nodulation of the uninoculated control plants may have been due to the air movement in the greenhouse, water splashing during irrigation, or from handling of the pots during set-up of the experiment. Although the exogenously inoculated plants that became nodulated had greater dry weight than non-nodulated plants, inoculated plants had significantly greater dry weights at 10.5 and 12.5% soil water contents than nonnodulated plants. At soil water contents of 6.0

and 6.6%, where shoot dry weights were not significantly different between the inoculated and uninoculated treatments, uninoculated plants did not form nodules. Consequently nodulation of uninoculated plants did not affect data interpretation.

EXPERIMENT 4:
RHIZOBIAL SURVIVAL

Objective

The objective of this experiment was to assess the colonization of an acidic soil by Rhizobium under different watering regimes by the indirect "plant infection" count method (Vincent 1970).

Materials and Methods

At harvest 11 g of soil from the top 10 cm of each pot containing inoculated plants were aseptically sampled (Vincent 1970) and mixed with 99 ml of sterilized, distilled water. A second 11 g sample was taken from the pots for determination of gravimetric soil water content. Bottles containing the first sample were shaken intermitently for a 30 min. period and then allowed to stand for 15-30 min. The supernate was removed by pipette and serially diluted in ten fold steps. Duplicate tubes containing five-day-old seedlings of cultivar Seaton Park growing in Jensen's seedling agar were inoculated with 1 ml of each dilution. Control tubes with sterilized and uninoculated seeds were included to check for contamination from the seeds.

After 6 weeks of growth in a shaded greenhouse, the presence or absence of nodules in each tube was recorded. This method assumes that a single Rhizobium cell added to

the test plant leads to a sufficient population in the root environment to cause nodulation. The number of tubes with nodulated plants was used to estimate the likely number of rhizobia in the initial suspension from the tables of Fisher and Yates (Vincent 1970). The data were expressed as rhizobial concentration in number of cells/g dry soil by correcting for the moisture content of the potted soil. Soil samples that did not show the presence of Rhizobium with the "plant infection" count method were recorded as having 0.6 cells/g dry soil, the lower limit of detection by this method.

Correlation between the logarithm of rhizobial concentration (cells/g soil and the number of plants which formed nodules from Experiment 3 was determined.

Results

The two rhizobial strains had significantly different ($P < 0.05$) population numbers with overall treatments means of 4.68×10^1 for strain USDA 2160 and 5.13×10^2 for strain 162-X-103. The water levels significantly affected the rhizobial populations (Fig.11). Numbers of rhizobia did not differ between the 6.0 and 6.6% soil water contents nor between the 10.5 and 12.5% soil water contents, but the two lower moisture regimes had significantly fewer rhizobia than did the two higher soil water levels. The interaction of desiccation periods with Rhizobium strain was significant. In soil that had undergone desiccation for 15 and 30 days, strain 162-X-103 was present in higher numbers

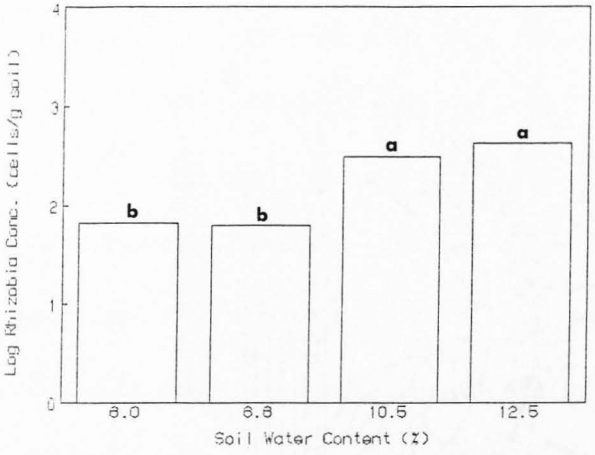

Figure 11. Logarithm of rhizobia concentration (cells/g soil) of two strains combined, in the potted soil from Experiment 3 in relation to soil water content. The same letter on the top of the bars indicates no statistically significant differences at the 0.05 probability level by LSD. Each value represents the mean of 38 observations.

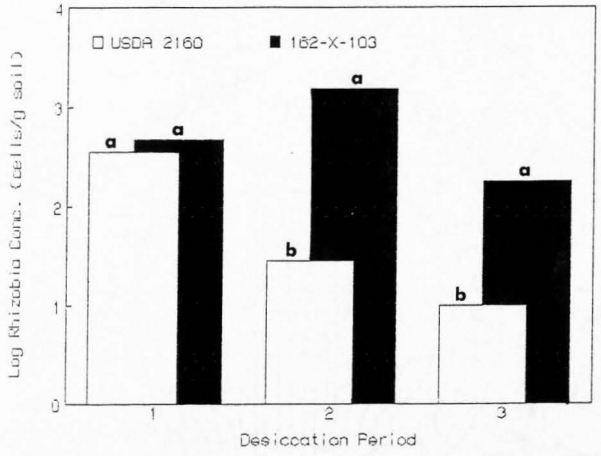

Figure 12. Logarithm of rhizobia concentration (cells/g soil) of two strains in the potted soil from Experiment 3 in relation to desiccation periods (1 = 0 day; 2 = 15 days and 3 = 30 days). The same letter on the top of the bars within the same desiccation period indicates no statistically significant differences at the 0.05 probability level by LSD. Each value represents the mean of 24 observations.

than strain USDA 2160 (Fig.12). However, the two rhizobial populations were equal when the pots received water on the day of sowing. There were no other significant differences in the first or higher order interactions (Table 14). Although the desiccation by water level by inoculation interaction was not significant, it is of interest to note that strain 162-X-103 maintained high population numbers at desiccation periods 2 and 3 while strain USDA 2160 numbers were reduced, especially at the low water levels (Fig. 13). There was a trend for rhizobial numbers of strain 162-X-103 to be higher at all the water levels compared to strain USDA 2160, particularly for cultivar Nungarin (Table 15). Strain USDA 2160 had higher numbers than strain 162-X-103 at the 12.5% soil water level with cultivar Clare.

The logarithm of rhizobia concentration (cells/g soil) and the plants which formed nodules had a correlation of 0.56 ($P = 0.01$).

Discussion

Both rhizobial strains had similar population numbers when the pots received water the day of sowing. After 15 or 30 days of dessication, the acid-sensitive strain 162-X-103 exhibited higher numbers than acid-tolerant strain USDA 2160. The desiccation period imposed on the Rhizobium-inoculated pelleted seeds presented a greater stress for strain USDA 2160 than for strain 162-X-103. Either strain USDA 2160 was not able to survive desiccation for 15 or 30 days, or it took more time to reach the population numbers

Table 14. Analysis of variance for log rhizobia concentration (cells/g soil) for Experiment 4.

Source	df	MS	Significance level
Replication (R)	1	1.73	NS
Desiccation (D)	2	12.38	NS
RD (error a)	2	1.15	-
Water level (W)	3	6.79	* * *
DW	6	0.77	NS
RW + RDW (error b)	9	0.68	-
Cultivar (C)	2	1.89	NS
Inoculation (I)	1	38.48	* * *
CI	2	3.74	NS
DI	2	18.16	* *
DC	4	1.29	NS
WI	3	1.06	NS
WC	6	1.78	NS
DCI	4	1.92	NS
DWC	12	0.65	NS
DWI	6	0.82	NS
WCI	6	2.01	NS
DWCI	12	0.75	NS
Error c	60	1.38	-
Total	143		

NS Not Significant
* * Significant at 0.05 probability level
* * * Significant at 0.01 probability level

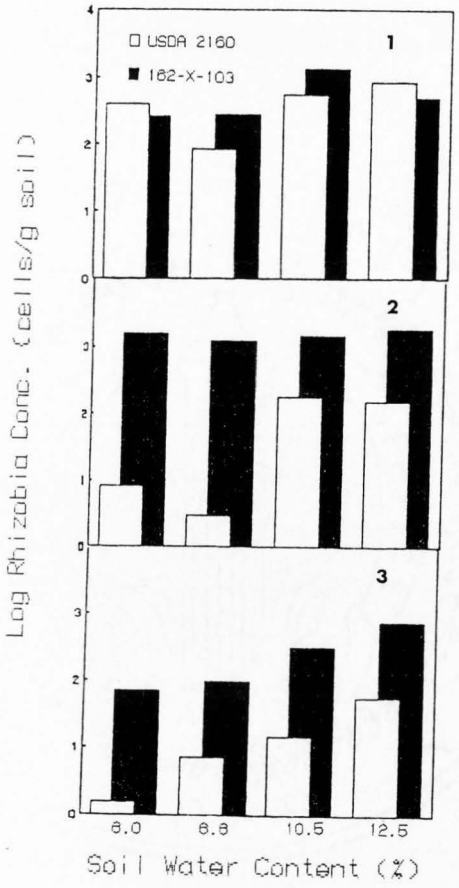

Figure 13. Logarithm of rhizobia concentration (cells/g soil) of two strains in the potted soil from Experiment 3 in relation to soil water content and desiccation periods (1 = 0 day; 2 = 15 days and 3 = 30 days). Each value represents the mean of 6 observations.

Table 15. Rhizobia concentration in the soil for the two strains combined over desiccation periods in relation to subterranean clover cultivars at four soil water contents. Each value represents the mean of 6 observations.

Soil water content	Strain	Subclover cultivars		
		Nungarin	Seaton Park	Clare
		cells/g dry soil		
6.0 %	USDA 2160	6.03	8.51	104.71
	162-X-103	177.83	85.11	1248.92
6.6 %	USDA 2160	13.80	5.89	22.90
	162-X-103	1000.00	190.55	177.83
10.5 %	USDA 2160	181.97	263.03	31.62
	162-X-103	1862.09	269.15	1258.93
12.5 %	USDA 2160	15.85	323.59	1584.89
	162-X-103	3630.78	501.19	398.11

of strain 162-X-103.

Both rhizobial strains were able to survive at low soil water contents when no desiccation treatment was imposed in this study. The ability of rhizobial strains to grow at low water potentials is in agreement with the results of Osa-Afiana and Alexander (1979) who observed that R. trifolii and B. japonicum survived in a silt loam soil in higher numbers at -0.8 MPa (10.0% soil water content) than at -0.15 MPa and higher soil water potentials. After 15 or 30 days of desiccation, strain 162-X-103 reached similar population numbers at all four soil water contents but USDA 2160 population numbers decreased, especially at the low water levels. This response for strain USDA 2160 may be due to rhizobial mortality during the desiccation periods and a subsequent growth at increasing water contents when the pots were watered.

The range of rhizobial numbers found in this Experiment 4 were equivalent to those reported in the literature. Gibson et al. (1975) found that R. leguminosarum biovar trifolii in pastures of different age, location, management and season ranged from $< 10^3$ to $> 10^6$ cells/g soil. Populations less than 10^3 cells/g soil were found in 89% of the sites studied by Coventry et al. (1985) in southeastern Australia. Rhizobial numbers were significantly correlated with pH. However, the numbers were $> 10^3$ cells/g soil when the sampling was made after

the first season of growth when inoculated subclover seed was sown. These rhizobial numbers were not related to the soil pH (Coventry et al. 1985).

Successful colonization of the soil by strain 162-X-103 at the low water levels could be due to the presence of germinating seeds and developing roots (Alexander 1984) because rhizobial growth may be mainly associated with root excretions (Pena-Cabriales and Alexander 1983). Survival of fast-growing Rhizobium also could be enhanced by the high content of fine soil particles. Clay can envelop bacteria and protect them from desiccation by forming a microsite within the soil. Bushby and Marshall (1977) found that greater quantities of water were retained at lower relative vapor pressures by desiccation-sensitive Rhizobium species (fast growers) than by the more resistant ones (slow growers). Foulds (1971) observed that the numbers of cells in natural populations of different Rhizobium species were not correlated with soil moisture content. Soil colonization by rhizobia may be improved by bacterial movement with percolating irrigation water. The use of non-sterile soil in this experiment and successful rhizobial colonization and growth implies that the rhizobial strains in this study were able to survive the predatory, parasitic and lytic organisms such as protozoa, Bdellovibrio and bacteriophage species that may be present in soil (Thornton and Davey 1984).

GENERAL DISCUSSION

Generally, studies of the effect of moisture stress on Rhizobium or legume growth are conducted with different kinds of soil, by letting the soil dry out with different rewatering cycles, or with non-soil medium. In our study different water potential regimes were maintained under non-sterile soil conditions. Plants grown in potted soil (Experiment 3) had greater dry weights than similar aged plants in Experiment 2 grown in Jensen's seedling agar medium containing complete minerals salts for plant growth less nitrogen. The difference in comparative symbiotic performance of the two rhizobial strains when tested in the soil or non-soil medium demostrates the value of test the strains symbiotic performance in a soil environment.

In this study, the reduction of subclover plant nodulation in soil at low water contents was accompanied, in most cases, by a decreased soil rhizobial population size. Rhizobial numbers may have been too low for efficient nodulation. In addition, when the soil water potential is low, rhizobia may not be able to reach root hairs because of discontinuities in waterfilled pathways which are necessary for their mobility. The process of infection and nodule formation may also be limited at low soil water potentials. Worrall and Roughley (1976) reported that in a sandy soil subclover nodulation was

inhibited at -0.36 MPa, whereas the number of rhizobia in the rhizosphere was unaffected by this water potential. The soil sampled from the pots in Experiment 4 included only the top 10 cm of soil. Consequently, numbers of rhizobia in the rhizosphere could have been greater. Some of the plants that became nodulated with strain USDA 2160 after a desiccation period of 15 or 30 days had more nodules on the lateral roots than on the crown or main root. This could mean that infection and nodulation took place later due to slower soil colonization by strain USDA 2160 than by strain 162-X-103. Because these plants may have been fixing nitrogen for a shorter period of time, they may have yielded less dry matter.

High plant density within the pots could influence Rhizobium growth. Lowendorf et al. (1981) reported that the density of two strains of R. meliloti was increased in soil at pH 6.4 in the presence of growing alfalfa. However, an acid-tolerant strain maintained greater numbers for a longer period by roots of growing alfalfa than an acid-sensitive strain in soil at pH 4.6.

Although in the present study rhizobial numbers were reduced from initial population sizes in the inoculum by most treatments, they were able to survive on pelleted seeds in sufficient quantity to effectively nodulate most of the seedlings at germination. Brockwell and Whalley (1970) demonstrated that R. meliloti associated with pelleted, inoculated Medicago seeds persisted in high

numbers for up to 200 days when in storage or mixed with dry soil. In a field experiment in California Jones et al. (1971) observed that pelleted, inoculated subclover seeds planted one month before natural rainfall produced an adequate stand, indicating that sufficient numbers of rhizobia had survived in the pelleted seeds until rain was received. However, Johnson et al. (1986) reported a failure of nodulation when inoculated subclover (cultivar Nangeela) was broadcast-seeded on top of the soil and only 4.1 cm of precipitation was received after seeding. Although Rhizobium survival may be improved in some clays, heavy-textured soils are generally harmful to N_2-fixation under water stress. The primary effect of water stress on nodules is to depress O_2 uptake (Sprent 1976) through a low O_2 difussion rate in heavy soils.

Failure of subclover nodulation observed in rangelands of southwest Spain could be due to poor Rhizobium survival caused more probably by desiccation rather than by soil acidity. It is recommended that when selecting rhizobial strains tolerant to a stress such as soil acidity for incorporation in seed pellets, the effect of desiccation on those strains be taken into account. It is also recommended that inoculated seeds be sown late in the fall when rainfall is more likely to occur.

CONCLUSIONS

Only the Spanish rhizobial accessions isolated from acidic soils in humid, natural rainfall or dry, irrigated sites grew in the acidified liquid medium. More studies are needed at soil pH levels below 5.0 to test the effects of acidity at soil water potentials below -0.1 MPa.

Two strains of R. leguminosarum biovar trifolii, acid tolerant USDA 2160 and acid sensitive 162-X-103, had similar production of extracellular polysaccharides (EPS) the acid-sensitive strain was more resistant to desiccation periods than the acid-tolerant strain.

Drought inhibited symbiotic N_2-fixation to a greater extent than N-uptake in a soil with pH 5.7.

Symbiotic performance varies between soil and non-soil environments. The effectiveness of nitrogen fixation was higher for strain USDA 2160 when tested in an enclosed medium than for strain 162-X-103. The opposite occured in a soil subjected to different moisture levels.

The subclover cultivars Nungarin, Seaton Park and Clare exhibited different responses in symbiotic performance in relation to soil water content. In Nungarin no significant differences were observed among inoculated plants or -N and +N controls. Seaton Park and Clare exhibited differences between -N control plants and inoculated and +N controls plants.

Levels of rhizobial strain 162-X-103 were unaffected by soil water contents in the range 6.0 to 12.5%

The failure of some plants to form nodules was associated with a low rhizobial population ($r = 0.56$).

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