Growth Temperature Tolerance and Survival of Peanut *Rhizobia* in Peat Inoculants[1]

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ABSTRACT

A study was conducted to evaluate the temperature tolerance and survival of 18 strains of peanut *rhizobia* at elevated temperatures in peat. Tolerance was expressed as optimum and maximum survival temperatures, respectively, and was based on growth response to increasing temperature, from 27-48°C in a polytemp block. Survival in peat was judged by following numbers of survivors from 0 to 70 d at 29°C and 41°C constant temperature, and 41/29°C (8/16h) fluctuating temperature as expressed as colony-forming units on agar plates. Six of the 18 strains were classified as high-temperature tolerant. All strains tested survived well at 29°C; however, considerable variation in survival occurred at either 41°C or 41/29°C. Only two strains (NC 92 and TAL 425) survived well enough for 70 d in the 41/29°C regime to be considered candidates for wide-spread utilization in inoculants. Although NC 71, was not an outstanding survivor at 41/29°C, it nevertheless survived very well at a constant 41°C. Its performance in this latter situation suggests that NC 71 also be given further consideration as a suitable inoculant strain when high temperature may be encountered.

COMPENDIO

Se llevó a cabo un estudio para evaluar la tolerancia y sobrevivencia de 18 cepas de *rhizobia* de maní a temperaturas elevadas en un medio de turba. La tolerancia fue expresada con base en temperaturas óptimas y de máxima sobrevivencia respectivamente, y se basó en la respuesta en crecimiento a temperaturas incrementales desde 27 hasta 48°C, a temperatura constante. La sobrevivencia en turba se evaluó con base en números consecutivos de sobrevivientes desde 0 hasta 70 días en 29 y 41°C a temperatura constante y a 41/29°C (8/16 horas) temperatura fluctuante, expresadas como unidades de colonias en agar. Seis de las 18 cepas fueron clasificadas como tolerantes a altas temperaturas. Todas las cepas sobrevivieron bien en 29°C, sin embargo, se observó una variación considerable en la sobrevivencia en 41°C o en 41°/29°C. Solamente dos cepas (NC 92 y TAL 425) sobrevivieron suficientemente bien durante 70 días en el régimen de 41°/29°C para ser consideradas para inoculaciones. NC 71 no sobrevivió tan bien en 41°/29°C pero si a 41°C constantes. Esto sugiere que a NC 71 debe dársele más consideración como inoculante cuando se trate de altas temperaturas.

INTRODUCTION

Grain legumes are important to many people because they provide substantial amounts of protein for human nutrition. In addition the legumes participate in biological nitrogen (N) fixation by reducing atmospheric N into a plant-usable form in association with *rhizobia*. This relationship is specific because each species generally nodulates a certain legume host plant. An adequate yield of grain legumes is often promoted by seed inoculation with specific *rhizobia* (11). However, the success of inoculation is frequently dependent on the availability of high-quality rhizobial inoculants.

Several factors contribute to the quality of the inoculant, including production techniques, utilization of superior *rhizobia* strains and the availability of suitable carrier material (11). However, even good inoculants often fail to provide satisfactory nodulation.

Hot and dry conditions are particularly harsh on the survival of *rhizobia* in prepared inoculants (2). Nowhere are these conditions as evident as in some tropical and subtropical regions. In these areas, legumes are grown under environmental conditions were temperatures may be extremely high (6). For example, poor nodulation and N fixation were observed for cowpea (*Vigna sinensis*) growing in hot, black earth soils in northern New South Wales, Australia, where soil temperatures frequently exceeded 38°C for much of the day (1). In addition, nodule formation was most sensitive to high temperatures immediately after sowing. Presumably temperature affected the saprophytic survival of the *rhizobia* or their ability to bind to roots.

High temperatures may also affect inoculant *rhizobia* during storage. To overcome adverse conditions during storage and handling, inoculants should be produced using carrier materials with properties which promote a longer rhizobial shelf life. Conversely, using high temperature-tolerant *rhizobia* may extend shelf-life and enhance the potential for subsequent effective nodulation.

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High-temperature tolerance exists among the soybean *rhizobia*. In a survey of some 50 strains of *Bradyrhizobium japonicum* it was found that approximately eight strains could be considered high-temperature tolerant based on evaluations of optimum growth (OT) and maximum survival (MST) temperatures (3). In addition, high-temperature tolerant strains nodulated better and fixed more N when exposed to high temperatures as compared to strains considered to be temperature neutral or intolerant (4, 5). In another study, high-temperature tolerant strains of *B. japonicum* survived longer and in greater numbers in soils exposed to a continuous temperature of 32°C than high temperature-intolerant strains exposed to similar conditions. No comparable studies have been conducted for peanut *rhizobia* (*Bradyrhizobium* spp.). A study was therefore conducted to investigate: 1) the growth temperature tolerance of some peanut *rhizobia*, and 2) the survival of peanut *rhizobia* in inoculants exposed to elevated temperatures.

MATERIALS AND METHODS

Growth temperature determinations: A total of 18 different strains were tested for their temperature characteristics (Table 1). Included were strains NC92, CB 756, and 32H1 which have been widely used as effective symbionts for a variety of host plants. While these strains are commonly used, strain selection still remains a local or regional option and takes into account such factors as compatibility with the host, soil conditions and management objectives. The procedures outlined by Munevar and Wollum (3) were followed without modification for growth temperature determinations. A total of 19 temperature treatments were imposed with a Poly-Temp apparatus (Lab-Line Instruments, Inc., Melrose Park, IL). A starter culture of each strain was grown to the early stationary phase in 250 ml Erlenmeyer flasks containing 50 ml of yeast extract-mannitol medium (YEM). The medium was composed of: K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; mannitol, 10 g; yeast extract, 0.5 g; $CaCO_3$, 0.001 g; per liter of distilled water. After adjustment to pH 7.0, all media were autoclaved at a pressure of 15 lb and 121°C for 30 min. For solid media 20 g agar L^{-1} were added to the broth prior to sterilization.

Fresh broth was inoculated with a volume of starter culture equivalent to 1% of the volume of fresh broth. After mixing, 4 ml aliquots were taken to fill sterile, matched 18 x 150 mm test tubes. Bacterial growth was monitored by measuring the optical density (OD) of the cultures at 600 nm at the following incubation times (h): 0, 12, 24, 48, 72 and 96. To maximize aeration, each tube was vigorously vortexed every 3 h during the day and twice during the night. There was no effect of temperature on the OD of non-inoculated tubes incubated at the different temperatures.

Survival temperature: After 96 h incubation in the Poly-Temp apparatus, cultures exhibiting no change in OD during the previous 96 h (remaining at the same OD as 0 h reading) were transferred to an incubator maintained at 28°C ± 1.5°C. The OD was determined as previously described at (d): 1, 2, 4, and 6 after the initiation of the survival test. Cultures were mixed as before, but only every 12 h. A bacterial culture was considered to have survived the temperature treatment when its OD increased at any time during the six-day period after removal from the Poly-Temp apparatus.

Survival in Peat: Cultures used to inoculate peat were grown in 50 ml quantities of YEM contained in 250 ml Erlenmeyer flasks on a rotary shaker (200 rpm) to the late log or early stationary phase of growth (generally about 5 to 7 d). Cells were concentrated by centrifugation at 8000 rpm, for 30 min and washed in sterile 0.85% saline (NaCl) prior to being resuspended in sterile saline. The concentration of the cells in the saline was adjusted so that on delivery the peat would contain about 10^8 cell/g and have a moisture content of about 45% (dw basis).

A standard milled peat was provided by the Nitragin Company. The peat material was neutralized by adding 2.5 g of $CaCO_3$ to 100 g of the peat and 10 ml water. After mixing, the material was incubated for at least seven days at ambient temperature with daily mixing. During the equilibration period the acidity decreased from pH 4.5 to 6.3. Subsequently, inoculated peat samples were incubated for an additional 7 d period prior to imposition of temperature treatments.

After equilibration, samples were stored under three different temperature regimes for a maximum of 70 d. The treatments were: a constant 29°C or 41°C, and repeated cycles of 41°C for 8 h and 29°C for 16 h. Populations were determined by plate counts on (d): 0 (day that temperature treatments were imposed), 15, 30, 50 and 70. Groups of four strains were evaluated at separate times.

After storage, samples were decimally diluted in sterile distilled water to a dilution of 10^{-7}. Then 0.1 ml aliquots were spread on YEM plates amended with cyclohexamide at a concentration of 0.002% to suppress extraneous fungi. Populations were estimated from plates exhibiting 30 to 300 colonies per plate. For enumeration, plates were incubated at 28°C, for four to seven days. Data were corrected for moisture content of the peat and transformed to the logarithmic (base 10) number. Data were analyzed by analysis of variance procedures to determine statistical significance and the least significant difference (LSD) procedure to separate means (9)

RESULTS

Temperature-characteristics: The OT and MST for 18 different strains of peanut *rhizobia* varied considerably (Table 1). The OT for these strains ranged from 30.0-38.7°C, while the MST from 38.7-44.3°C. Those strains that possessed a high OT tended to have the highest MST and those with the lowest OT tended to have the lowest MST. However, strain TAL 425, which had the highest OT, did not have the highest MST. Likewise, TAL 420, which had a moderate OT, ranked with three other strains as having the highest MST.

Table 1. Temperature characteristics for strains of peanut *rhizobia*

Strain number*	Optimum temp. (OT)	Maximum survival temp. (MST)
	°C	
TAL 425	38.7	42.8
NC 92	37.4	44.3
NC 7-1	37.4	44.3
THA 205	36.1	44.3
NC 70-1	36.1	40.0
TAL 420	34.9	44.3
CB 756	34.9	41.3
176A22	34.9	41.3
NC 6	34.9	38.7
SM5-2	34.9	38.7
32 H1	33.7	40.0
NC 56	33.7	38.7
176A34	33.7	38.7
NC 123	32.4	41.3
8B4	32.4	38.7
NC 146-1	31.4	41.3
3G4B5	31.4	38.7
3G4B21	30.0	40.0

* All strains were provided by Dr. G.H. Elkan, Department of Microbiology, North Carolina State University, Raleigh, NC 27695, USA. [Origin of strains designated by its prefix or identification number.] NC = North Carolina; THA = Thailand; 3G4B21, 3G4B5 = USDA, Beltsville; 176A34 = Nitragin Company; TAL = NifTAL; and CB 756, 32H1, 8B4 = CSRO, Australia

Survival in peat: Survival of peanut *rhizobia* in the peat carriers varied with strain and temperature regime (Tables 2, 3 and 4). Those strains exposed to a continuous 29°C survived better than those exposed to either fluctuating or continuous 41°C temperature regimes. In general, effects of high-temperature storage were more pronounced after 30 d than earlier in the storage period.

In the first group of strains tested, CB756 survived as well as the other strains even though the OT and MST for this strain were less than those of the other strains (Table 2). There was an increase in rhizobial numbers during the first 15 d for CB756 and THA205, but not for the other two strains. In addition, survival during storage was comparable when strains were maintained under a fluctuating or high-temperature regime, except for NC71 which survived very well at a continous 41°C. Strain CB756, with the lowest MST of this group, survived as well as the other strains with higher MSTs.

Results of the second group of strains differed from that of the first (Table 3). The starting number in the inoculant was less by about a factor of 10. In addition, the survival of *rhizobia* was better under a fluctuating temperature regime than under the continuous high-temperature regime. Strain TAL425, which had the highest OT and MST did not survive any better than strains NC146-1 and 3G4B21, which had somewhat lower OTs and MSTs. Strain 32H1 survived as well as the others in this group at 29°C and the fluctuating temperatures although survival of 32H1 at a continuous 41°C was poorer than the other strains, even though all four strains had comparable MSTs

Among the strains in the last group, no single strain stood out as a particularly good survivor at either the elevated temperature of 41°C or with the fluctuating temperature regime, although NC56 was better than the others tested in the group of four strains (Table 4). All strains survived reasonably well at the 29°C temperature compared to the other strains in previous tests. However, populations of three of the four strains tested were below 10^5 at the highest temperature tested at the end of 70 d storage, whereas only one of the other eight tested were below 10^5 at the end of 70 d storage.

DISCUSSION

Peanut *rhizobia* can be characterized according to their temperature tolerances. Generally those with the highest OT had the highest MST. This observation is similar to that made for soybean *rhizobia* (3). Likewise, those strains with the highest MSTs generally exhibited better survival than those with lower MSTs at the higher temperatures.

Table 2. Effect of temperature and storage time on the survival of four peanut *rhizobia* strains.

	Strain NC92			Strain NC71			Strain THA205			Strain CB756		
	29	F[a]	41	29	F	41	29	F	41	29	F	41
Time	Temperature, °C											
days	Log_{10} cfu · g $carrier^{-1}_{DW}$											
0	7.90	7.90	7.90	8.17	8.17	8.17	8.38	8.38	8.38	7.69	7.69	7.69
15	7.41	7.50	7.49	7.51	7.02	7.15	8.43	7.30	7.26	8.36	7.18	7.23
30	7.29	7.00	6.25	6.89	7.20	6.71	7.01	6.81	6.92	7.12	6.92	7.30
50	7.40	6.72	6.81	6.81	5.81	6.99	7.18	5.91	6.78	7.22	7.30	7.02
70	7.23	6.08	6.17	7.26	5.79	6.88	7.22	5.92	6.27	7.02	5.94	5.93

$LSD_{.01} = 0.26$; $LSD_{0.05} = 0.20$

F[a] = Fluctuating temperature, 8 h at 41°C and 16 h at 29°C

All of the strains tested had reasonably good storage characteristics assuming a maximum temperature of 29°C. A portion of the death of *rhizobia* at 29°C could be attributable to the non-sterile nature of the peat. This was also observed by Roughley and Vincent (7), who found the survival of *rhizobia* was higher in peat which had been sterilized prior to the addition of the *rhizobia* than the case where *rhizobia* were added to non-sterile peat. However, the 10^7 value maintained under this condition is considered an acceptable level for a quality inoculant, even under extremely adverse storage or field conditions (8). Maintenance of a high population in the inoculant would be important in any situation, particularly in Third World countries where refrigeration is not normally available for storage of inoculants for legumes.

Three of the strains tested (NC71, THA205 and 8B4) were affected more by the fluctuating temperature regime than by the continuously high storage temperature. This behavior limits the suitability of these strains as inoculant *rhizobia*. Fluctuating temperatures are common if refrigeration is not available between manufacture and use.

The constant high-temperature treatment of 41°C had a harmful effect on all 12 strains. However, the different response of each strain is comparable to the results obtained by Roughley (8) and Van Schreven (10) working with other *rhizobia*. Strains 8B4 and 3G4B5 were affected to a greater degree than the other strains. This was expected because these strains were among the least temperature-tolerant strains tested. Also strains 3G4B21 and NC146-1, which could be considered high-temperature intolerant, were also among the poorest survivors after high-temperature storage.

Although populations declined at the higher temperatures, some of the inoculants maintained a suf-

Table 3. Effect of temperature and storage time on the survival of four peanut *rhizobia* strains.

	Strain TAL425			Strain 32H1			Strain NC146-1			Strain 3G4B21		
	29	F	41	29	F	41	29	F	41	29	F[a]	41
Time	Temperature, °C											
days	log_{10} cfu · g $carrier^{-1}_{DW}$											
0	7.60	7.60	7.60	6.66	6.66	6.66	7.01	7.01	7.01	6.88	6.88	6.88
15	7.66	7.74	7.35	7.25	7.35	6.78	7.50	7.61	7.26	7.05	6.70	6.49
30	7.59	7.20	6.67	7.35	6.82	6.14	7.53	6.92	6.50	7.02	6.86	6.72
50	7.75	6.78	5.56	7.55	6.58	6.63	7.64	6.57	6.51	7.06	6.49	5.99
70	7.55	6.23	5.50	7.52	5.80	4.94	7.29	5.74	5.53	6.94	5.96	5.57

[$LSD_{0.01} = 0.26$; $LSD_{0.05} = 0.20$]

F[a] = Flctuating temperature, 8 h at 41°C and 16 h at 29°C.

Table 4. Effect of temperature and storage time on the survival of four peanut *rhizobia* strains.

	Strain NC56			Strain 176A34			Strain 8B4			Strain 3G4B5		
	29	F	41	29	F	41	29	F	41	29	F[a]	41
Time	Temperature, °C											
days	$\log_{10}$ cfu · g carrier$^{-1}_{DW}$											
0	8.32	8.32	8.32	6.83	6.83	6.83	8.09	8.09	8.09	7.67	7.67	7.67
15	8.12	7.90	5.89	7.73	6.60	4.75	7.87	7.12	5.59	7.54	6.67	5.17
30	8.07	7.49	6.58	7.81	6.20	5.52	7.86	6.34	4.84	7.50	6.00	4.82
50	7.85	6.59	4.81	7.55	4.80	3.82	7.60	5.19	4.55	7.27	4.99	4.49
70	7.73	6.06	5.55	7.50	6.84	4.53	7.49	4.83	4.51	7.02	5.10	4.56
[$LSD_{0.01}$ = 0.27; $LSD_{0.05}$ = 0.20]												

F[a] = Fluctuating temperature regime, 8 h at 41°C and 16 h at 29°C.

ficient number of *rhizobia* during a major portion of the storage period to be useful as inoculants. Strains CB756, NC92, NC71, and THA205 survived at a satisfactory level for 30-50 d. Even after 70 d inoculants with some of these strains could be satisfactorily used, when environmental conditions at planting are not severe or when the level of indigenous *rhizobia* is low.

Results of this study suggest that organisms could be selected for suitability for inoculant storage at high temperatures based on their OT and MST. However, tests should be conducted after storage to assure that the most temperature-tolerant strains are also efficient symbionts and that temperature-stress cells have not lost their ability to nodulate after high temperature storage. The use of high-temperature tolerant rhizobia also increases the probability of survival under adverse conditions in the field and subsequent enhancement of legume production under difficult conditions.

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