Liquid Inoculant Development
Research Summary II - 1999

In the past year we have completed many projects that further the development and evaluation of liquid inoculants. First, we evaluated the performance of the G5 liquid inoculant (see the first research summary we sent to you for details on its development) in relation to other inoculant products. We then completed a long-term shelf life test of many different liquid formulations. We also performed several tests that address other aspects of the production and performance of liquid inoculants including evaluating glycerol based media and measuring the effects of aeration, pH control and other additives on cell growth and survival.

Performance of Liquid Inoculant Formulations.

Survival of Bradyrhizobium and Rhizobium after Application to Seed. We found earlier that we could correlate (r values ranging from 0.77 to 0.86) the survival of cells applied to seed and stored at 25 C and 66 % relative humidity with soybean nodulation and dry matter production in potted soils that were subjected to drought and temperature stress. We used this laboratory assay (see Appendix 3-1 and 3-6 for methods) to evaluate the capability of 17 commercial inoculant formulations to support rhizobia or bradyrhizobia survival after inoculation.

Table 1 (all tables and figures follow this text presentation) shows the results of this laboratory assay. Only two of the inoculants sent to us by collaborators (sample numbers 11 and 28) supported higher numbers of viable cells on seed 48 h after inoculation than the G5 liquid formulation with *B. japonicum* strain SEMIA 5019 (mean of four assays – most recent assay of G5 had 2.3 X 10^6 cells/seed survive). Sample 28 is a commercial liquid formulation producing consistently high survival rates performance. Several inoculants had no detectable or very low numbers of viable cells at the time of the assay and consequentially few cells survived 48 h after inoculation. Other inoculant formulations which had more than 10^9 viable cells at inoculation (numbers 1, 19, 25) supported populations on the seed 48 h after inoculation that were at least one log lower than the G5 formulation. Results of this assay show a wide variation in the ability of inoculant formulations to support cell survival on seed.

There was no correlation between cell survival in the laboratory assay and the response to inoculation in the field by the commercial inoculants relative to the inoculation response obtained with G5. Small sample size and other factors affecting the response to inoculation in the field (e.g. indigenous *B. japonicum* populations or yield potential) may have masked any relationship.

Results of this assay showed that the G5 formulation with SEMIA 5019 and other well formulated liquid inoculants can support cell survival after inoculation better than most of the commercial solid carrier formulations made available to us.

Response to Inoculation with the G5 Liquid Inoculant in Multi-Location Field Trials. Our goal was to determine whether the G5 liquid inoculant had field performance characteristics equivalent or better than local products being sold to farmers around the world. Collaborators from sixteen countries agreed to conduct trials (see Appendix 1 for details). A minimum set of critical treatments was included in all trials 1) uninoculated, 2) inoculated with G5, 3) inoculated with a local product. Some collaborators added other treatments in the trials including fertilizer N additions, multiple sites, cultivars and other strains in both the G5 and local product. Collaborators collected data for seed yield, nodule number and nodule weight and total seed N.

Table 2 shows the frequency distribution and relative response to inoculation by G5 compared to the uninoculated control and local inoculant products. The number of observations for the four performance indicators varies due to data collection problems at some sites. The complete data set from which Table 2 is derived can be found in Appendices 2-1 to 2-4. Many collaborators requested their field results remain anonymous so we coded collaborator and site designations. There are no field data included from trials where our tests indicted there were no bradyrhizobia in the local inoculant.

The G5 liquid inoculant increased crop seed yield above local products more than 68% of the time producing an average seed yield increase of 6%. Nodule number increased an average of 20% in 77% of the trials when G5 inoculant was compared to local inoculants. Increased nodule number indicates the G5 inoculant probably supported more viable cells between seed inoculation and the time roots emerged and colonization and infection by the inoculant began. That nodule number response to inoculation is relatively higher than measures of seed yield is not surprising. Many factors other than survival of cells in the inoculant and consequent root infection by the inoculant can attenuate the yield response.

Shelf Life of Liquid Inoculant Formulations. The quality of a legume inoculant at the farm is primarily determined by how many viable cells are in the inoculant and how well they survive after application to the seed. The most consistent feedback we received from inoculant producers was concern about the shelf life of liquid inoculants. It is a common perception that rhizobia and bradyrhizobia do not survive well in liquid media especially when stored without refrigeration. To address this concern we tested the long-term survival of several liquid media stored at 25 C for 180 days (see Materials and Methods in Appendix 3 for details of procedure). In addition, we evaluated whether long storage periods affected the ability of cells to survive after application to seed.

We evaluated the shelf life of a) G5 medium, b) a glycerol based medium, G6, (see below for discussion of this medium and Appendix 3 for formulation), c) G6 medium diluted with solutions of eight individual and combinations of additives that seem to promote cell survival after application to seed, d) a commercial liquid product, and d) a control consisting of washed cells suspended in water.

To simplify data presentation, Figures 1 and 2 only show results from a few treatments. After 180 days of storage the number of viable cells remained nearly constant for the G6 medium with 20 g polyvinylpyrrolidone (pvp) L^{-1}, yeast mannitol medium (YM) and the G5 medium (Figure 1). When glycerol, FeEDTA or gylcerol+FeEDTA+pvp was added to the G6 medium survival was poor (data not shown) and cell numbers declined to levels approaching the washed cells suspended in de-ionized water. The pH of the G6, G6 + pvp and the G5 media after 180 days in storage were 4.46, 3.77, and 6.66 respectively. The final pH of these stored media was similar to the pH at the end of cell growth in the fermentor.

Research has shown that carbon or nitrogen starvation at the beginning of the stationary growth phase creates stress resistance in rhizobia cells (Morita, 1993; Thorne and Williams, 1997). It may be that when glycerol is added in the stationary growth phase as we did, the stress resistance was not generated. The G6 and the G5 medium survived better than the commercial liquid product tested even though the original number of cells in the G6 medium was less. Without pvp, the G6 medium performed poorly. The population declined to less than 1.0% of the original population.

Both the G5 and G6 + pvp liquid media enhanced survival of cells after inoculation onto seed (Figure 2). There was 10 times the number of cells surviving on the seed compared to YM medium and 100 times the number compared to G6 without additives or G6 with additives other than pvp (not all data shown). The better performance of the YM medium compared to G6 with no additives is surprising since the main difference between the two media is the carbon source. Several papers have shown that the production of exo-polysaccharides protects cells from desiccation (Ophir and Gutnick, 1994; Roberson and Firestone, 1992). It is well known that significant polysaccharide production occurs in YM medium. Our observations suggest little polysaccharide production takes place in glycerol based media. It may be that the performance differences between the G6 and G5 media are related to differences in polysaccharide production. This could be a fruitful area of investigation if the effect of pvp and polysaccharides on cell survival is synergistic.

Glycerol Based Media.

Comments from many recipients of our first report indicated interest in a medium that could produce high numbers of cells but was less expensive to formulate than the G5 medium. The main objections to the G5 medium revolved around the need for arabinose and mannitol. We evaluated several media for *B. japonicum* and found that formulas relying on glycerol as a carbon source met these requirements.

Glycerol based media are not new. Lorda and Balatti (1996) describe the growth characteristics of *B. japonicum* in glycerol based liquid media under various environmental conditions. They showed more rapid growth in a “balanced” medium that used 10 mL of glycerol as a substitute for mannitol. Populations in the glycerol sometimes reached densities in excess of 1×10^{10} cells mL^{-1}. We conducted several

experiments with a glycerol-based medium to evaluate cell production with different strains and under different conditions. Results that follow are based on 1.5 L batch cultures grown in 2.0 L glass fermentors that are aerated with filtered sterilized air delivered to the fermentors by a oil-less air pump (see Appendix 3-3 for details).

Growth of *B. japonicum* in G6, G5 and YM Media. Data in Figure 3 compares the growth of *B. japonicum* strain SEMIA 5019 in several media (see Appendix 3-4 for formulation of G5 and G6). Media temperatures ranged between 22 C and 24 C and aeration was approximately 2.0 L air L^{-1} media min^{-1}. Cell production was higher in the G5 and G6 media (both contain glycerol) compared to the YM medium. Adding $CaCO_3$ to the G6 medium to buffer the pH had an adverse effect on growth. In several other tests cell populations in the G6 medium reached 3 X 10^{10} cells mL^{-1}. From our experience, however, the populations reached in Figure 3 are more the norm in media containing glycerol.

Growth of Inoculant Strains in G6 Medium. We evaluated several inoculant quality strains for their ability to grow in the G6 medium (Figure 4). All *B. japonicum* strains tested grew well in this medium reaching populations greater than 10^9 cells mL^{-1} five days after inoculation. The SEMIA 5019 and SEMIA 587 strains reached slightly higher populations than the other *B. japonicum* strains. There was a rapid decline in pH as the cultures approached the stationary phase of growth. The pH of all cultures but SEMIA 587 declined to 4.0 and the rate of pH decline in SEMIA 5019 culture was slightly less than the non-SEMIA strains.

The Effect of Additional Nutrients and pH Buffering on Cell Production in Glycerol Based Media. We evaluated whether buffering the culture against pH decline and adding additional nutrients would increase cell production. Figure 5 shows the growth of SEMIA 5019 in G6 medium with various supplements of K_2HPO_4, yeast extract and glycerol. Cultures were grown at 27-28 C and aerated with 1.0 L air L^{-1} medium min^{-1}. The highest cell production (1 X 10^{10} cells mL^{-1}) was recorded in the G6 medium and G6 plus 6 mL glycerol L^{-1} medium. The final pH of these treatments declined to 3.3 compared to a pH of 7.0 or above in cultures buffered with K_2HPO_4. In comparison, the buffered cultures reached cell pop of 5-6 X 10^9 cells mL^{-1}, a half log lower than the non-buffered cultures. Adding extra yeast extract to broth also buffered the medium but did not increase cell production.

It appears pH has little effect on *B. japonicum* growth and survival in the liquid media we tested. Several strains, which were grown in G6 media, all reached high cell numbers despite large declines in pH as they entered stationary phase (Figure 4). When pH decline was minimized by yeast extract or K_2HPO_4 there was no positive effect on cell production. Lastly, cell survival rates were the same after 180 days of storage in the G6 + pvp (pH = 3.77) or the G5 media (pH = 6.62).

Other Considerations for Producing Liquid Inoculants.

Feedback from several producers led us to address some issues related to mass culture of cells for commercial production. Among these issues were flow rates in aerated cultures, problems with foam production in aerated cultures, packaging requirements and adding cell protective compounds to culture after growth. Following is a description of how we addressed some of the issues.

Controlling Foam Production and Aeration of Culture Media. Many producers, especially in India, mass culture large quantities of cells on rotary shakers. Others force air through the culture. The later method of aerating the culture can create substantial amounts of foam, especially when compounds such as pvp, gums or large amounts of yeast extract are added to the growth medium. Excess foam clogs air exit filters and can be a source of contamination. There are several approaches the problem 1) add cell-protecting compounds after the cells are harvested for packaging, 2) reducing air flow or eliminating it entirely with shake cultures, and 3) adding anti-foam agents to the culture.

Ef**fect of Anti-Foam Agents on Cell Growth**. Mike Sadowsky at the U of Minnesota tested some food grade anti-foam agents for their effect on the growth of SEMIA 5019. Results in Table 3 indicate all the anti-foam agents tested had a fairly large effect on cell production. It may be that other strains are not as sensitive as SEMIA 5019 or there are other agents that are less inhibitory but further tests will be required if this method is used to reduce foaming associated with certain liquid media.

Air Flow Rate. We grew SEMIA 5019 in the G5 media (contains 20 g L^{-1} pvp) under six air-flow rates ranging from 0.050 to 1.000 L air L^{-1} media min^{-1}. Cultures were grown at temperatures between 29.7 C and 30.7 C. There was a negative correlation between air-flow rate and fermentor temperature (r = 0.93).

There was a significant (r = 0.91) positive linear correlation between the flow rate and cell number reached after eight days of growth (Figure 6). Cell numbers in the 1.000 L air L^{-1} media min^{-1} treatment reached 1.04 X 10^{10} cells mL^{-1} compared to only 1.97 X 10^{9} cells mL^{-1} with 0.050 L air L^{-1} media min^{-1}. Cultures growing with the three highest air-flow rates reached a stationary growth phase after three days. Cell numbers continued to increase in the lowest air flow rate until the experiment was ended at nine days from inoculation. We cannot determine from this test whether this extremely low flow rate would have reached numbers equivalent to the other treatments with additional time.

It appears that there is a only a small response to greater aeration and mixing in the fermentor when air flow rates are above 0.25 L air L^{-1} media min^{-1}. The effect of flow rates below 0.25 L air L^{-1} media min^{-1} was attenuated with time. Regardless of flow rate, however, all the cultures eventually produced copious amounts of foam that filled the fermentor head space as cell numbers exceeded 10^{9} mL^{-1}.

Cell Growth in Fermentors with Forced Air and in Flasks with Rotary Shaking. Many small and medium sized inoculant production facilities rely on shake cultures for mass culturing cells. The method requires less equipment investment and is more reliable We compared growth rates in cultures grown in G5 and G6 media with forced air (1.0 L air L^{-1} media min^{-1}) in 2.0 L fermentors and in 250 mL flasks placed on a rotary shaker (200 rpm; 2.54 cm orbit). Figure 7 shows that slightly higher cell numbers were attained in the flasks agitated on a rotary shaker than when aerated with forced air.

The Effect of Gum Additives to G6 Medium on Cell Survival. Several producers asked us to evaluate other inexpensive and commonly available materials that increase shelf life and performance of liquid inoculants. We conducted a preliminary screen of several plant and bacterial derived gums to determine whether any had rapid toxic affects on survival in liquid media and whether any could enhance survival of cells after application to seed. The gums were added to a culture of SEMIA 5019 grown in G6 medium by diluting the culture 1:1 (v:v) with a sterile solution containing 8.0 g L^{-1} of each gum (see Appendix 3-5 for details). We added the gums after growing the SEMIA cells to avoid adding the effect of the culture additives on cell growth as a variable. After nine days of storage at 25 C the diluted culture was applied to soybean seed in our standard seed survival assay.

None of the gums tested had any acutely toxic effects on cell survival in he liquid media over the nine-day storage period (Table 4). All but one gum supported populations greater than 10^9 cells mL^{-1} and sustained viable populations several times higher than the water control. The level of cell survival after application to seed had a wider range between the gum treatments than in storage. All gum treatments performed better than the water control. Results show that some plant and bacterial derived gums may be valuable additives to liquid inoculants and provide an alternative to pvp. Additional and more rigorous tests should be performed on these materials to determine optimum amounts to provide cell protection.

References

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Roberson and Firestone, 1992. Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. App. Environ. Micro. 58(4) p. 1284-1291)

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Table 1 Survival of Bradyrhizobium and Rhizobium in various inoculant formulations after application to seed.

Sample Number	Initial Bradyrhizobia/ Rhizobia *Viable cells/g Inoculant*	Contaminants *cfu/g*	Bradyrhzobium/ Rhizobium Applied to seed *Cells/seed*[a]	Bradyrhizobium/ Rhizobium Surviving after 48h[d] *cells/seed*	Decline in viable Bradyrhizobium/ Rhizobium *log/seed*	Notes
1	1.35×10^9	2.5×10^8	6.75×10^5	5.0×10^4	1.13	General bacterial contaminants
3	No bradyrhizobia detected by plate count, obtained a few nodules on plants w/ 0.5g inoculant/plant	$>1 \times 10^9$	NA[8]	Too many contaminants to count B. japonicum	NA	Fast growing contamiants
2	1.85×10^9	1.5×10^{10}	9.25×10^5	2.6×10^5	0.58	General bacterial contaminants
8	2.50×10^7	4.0×10^8	1.25×10^4	Too many contaminants to count the bradyrhizobia	NA	General bacterial contaminants
9	1.03×10^8	1.3×10^9	5.15×10^4	7.0×10^2	1.87	General bacterial contaminants
10	3.65×10^8	3.0×10^6	1.82×10^5	7.55×10^4	0.38	Very few contaminants
11	4.90×10^9	2.0×10^6	2.45×10^6	7.35×10^5	0.52	Very few contaminants
14	2.00×10^7	1.0×10^7	1.0×10^4	Too many contaminants to count the bradyrhizobia	NA	Very fast growing contaminants
19	1.20×10^9	1.8×10^9	6.0×10^5	2.1×10^4	1.46	Contaminants yellow bacterium
20Gm[e]	1.50×10^8	4.1×10^9	7.5×10^4	2.5×10^4	0.48	Many types of contaminants; 60 ul gum arabic with inoculant on seed
20 Vu	2.20×10^9 *B. sp*	3.5×10^9	1.1×10^6	2.5×10^5	0.64	Many types of contaminants; used 60 ul gum arabic with inoculant on seed
20 Pv	2.00×10^8 *R. leg*. bv. *Phaseoli*	9.6×10^9	1.0×10^5	Too many contaminants to count the rhizobia	NA	Many types of contaminants; used 60 ul gum arabic with inoculant on seed
23 a	No bradyrhizobia detected by plate count or by inoculation of soybean with 0.5g inoculant	1.2×10^8	NA	NA	NA	Very fast growing contaminants
23 b	No bradyrhizobia detected by plate count or by inoculation of soybean with 0.5g inoculant	8.0×10^7	NA	NA	NA	Very fast growing contaminants
25	1.19×10^9	5.0×10^7	5.95×10^5	3.0×10^4	1.30	General bacterial contaminants

Table 1 (cont.) Survival of Bradyrhizobium and Rhizobium in various inoculant formulations after application to seed.

Sample Number	Initial Bradyrhizobia/ Rhizobia *Viable cells/g Inoculant*	Contaminants *cfu/g*	Bradyrhzobium/ Rhizobium Applied to seed *Cells/seed*[a]	Bradyrhizobium/ Rhizobium Surviving after 48h[d] *cells/seed*	Decline in viable Bradyrhizobium/ Rhizobium *log/seed*	Notes
26	No bradyrhizobia detected by plate count or by inoculation of soybean with 0.5g inoculant	> 1 X 10^9	NA	NA	NA	Very fast growing contaminants
28	4.15X10^9/ml	None	2.07 X 10^6	6.0 X10^5	0.54	Commercial liquid inoculant
G5[c]	3.65 X 10^9/ml	None	1.83 X 10^6	2.85 X 10^5	0.69	Liquid inoculant

[a] Inoculants applied at standard rate of 0.5 mg per seed; [b] Not Applicable; [c] Data for G5 media the mean of four assays – results of most recent assay: surviving cells/seed = 2.23 X 10^6/seed, decline in viability after 48 h log decline = 0.06/seed. [d] Inoculated seed stored for 48 h at 25 C over a saturated solution of $CuCl_2$ (r.h. of 68%). [e] Gm = G. max; Vu = V. unguiculata, Pv = P. vulgaris

Table 2. Field Performance Summary of the G5 Inoculant Formulation Compared to Uninoculated Control and Local Inoculant Products

	Response of G5 inoculant above:			
	Control		Local Inoculant	
Response Indicator (n)	Relative frequency	Percent Increase	Relative frequency	Percent Increase
			%	
Seed Yield[1](n=37)	97	90	68	6
Total Seed N (26)	96	112	50	3
Nodule no. (n=39)	97	>1000	77	20
Nodule wt. (n=42)	97	>1000	71	14

[1] Tabulated results do not include results of sites 1a – 1d (see Appendices 2-1 to 2-4) since flawed genetic compatibility between SEMIA 5019 (USDA 74 sero-group) in the G5 formula and the local cultivar generated rhizobiatoxins causing chlorosis in early growth.

Table 3. Reduction in cell growth by *B. japonicum* SEMIA 5019 in the presence of common antifoam agents

Antifoam	Method of foam reduction	% maximum growth compared to no antifoam control
PPG Industries – Muza DF 204	Undisclosed	5.4
Osi Specialties – SAG 471	Silicone	5.4
Sigma – Antifoam 204	Undisclosed	3.1
General Electric Silicone AF72	Silicone	36.6

Data generated by Mike Sadowsky, U of Minnesota, Biological Process Technology Institute with support from SM-CRSP

Table 4. Effect of bacterial and plant gums on survival of *B japonicum* strain SEMIA 5019 in liquid media and 48 h after application to soybean seed

Inoculant medium	Cells surviving after nine days storage at 25 C*	Cells surviving 48 h after seed inoculation
	cells mL^{-1}	*Cells* $seed^{-1}$
G5	5.6×10^9	1.9×10^5
*G6 diluted (1:1) with:***		
Water	3.5×10^8	2.5×10^4
Xanthan gum	1.3×10^9	1.4×10^5
Carageenum	2.3×10^9	2.4×10^5
Locust bean gum	1.2×10^9	1.1×10^5
Karaya gum	1.4×10^9	6.0×10^4
Gum Ghatti	1.2×10^9	1.1×10^5
Tragacanth gum	1.0×10^9	6.5×10^4
Guar gum	9.2×10^8	7.0×10^4

* Initial populations of SEMIA 5019 in G5 and G6 media (cells mL^{-1}): G5, 8.2×10^9, G6 (after 1:1 dilution), 4.42×10^9. * *G6 culture of SEMIA 5019 diluted 1:1 (v:v) with a solution containing 8.0 g L^{-1} of the indicated material. Fifty mL of each liquid media was stored in 125 mL Erlenmeyer flasks stopped with foam plugs. Seed survival assay followed standard procedures (see Appendix 3-5)

Figure 1

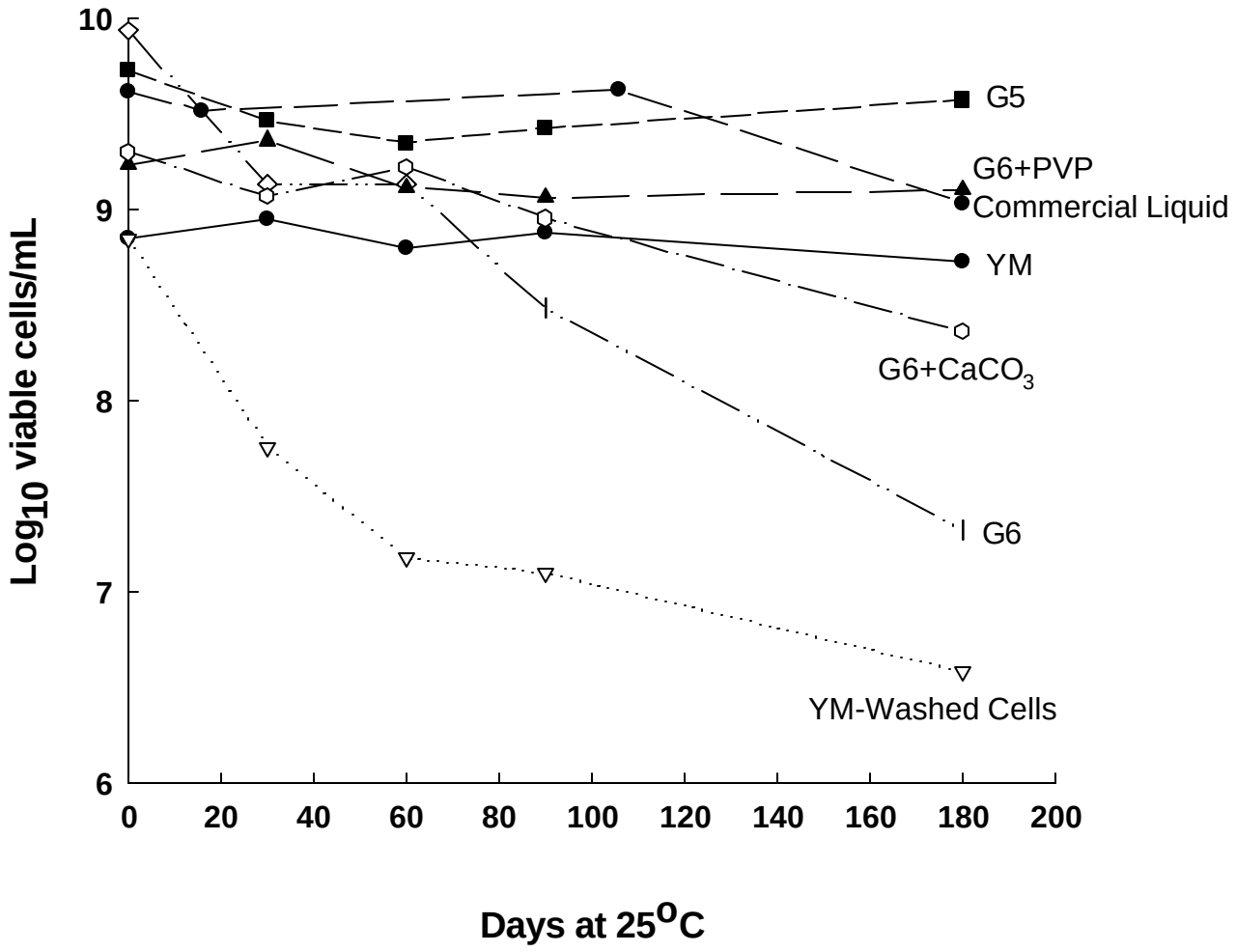

Fig. 1. Survival of *B. japonicum* SEMIA 5019 in liquid media stored at 25°C.

Figure 2

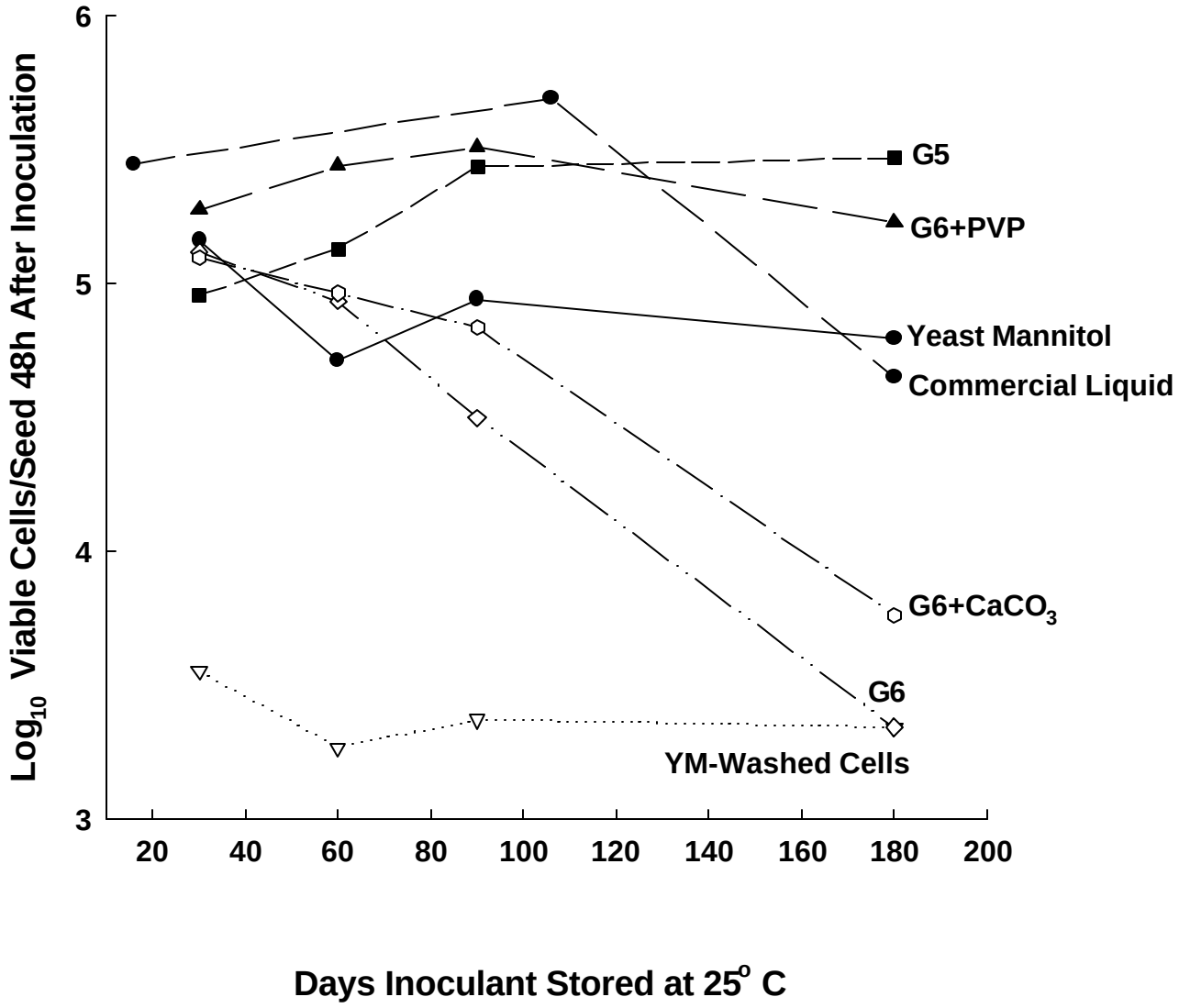

Fig. 2. Survival of *B. japonicum* SEMIA 5019 on seed 48h after inoculation.

Figure 3

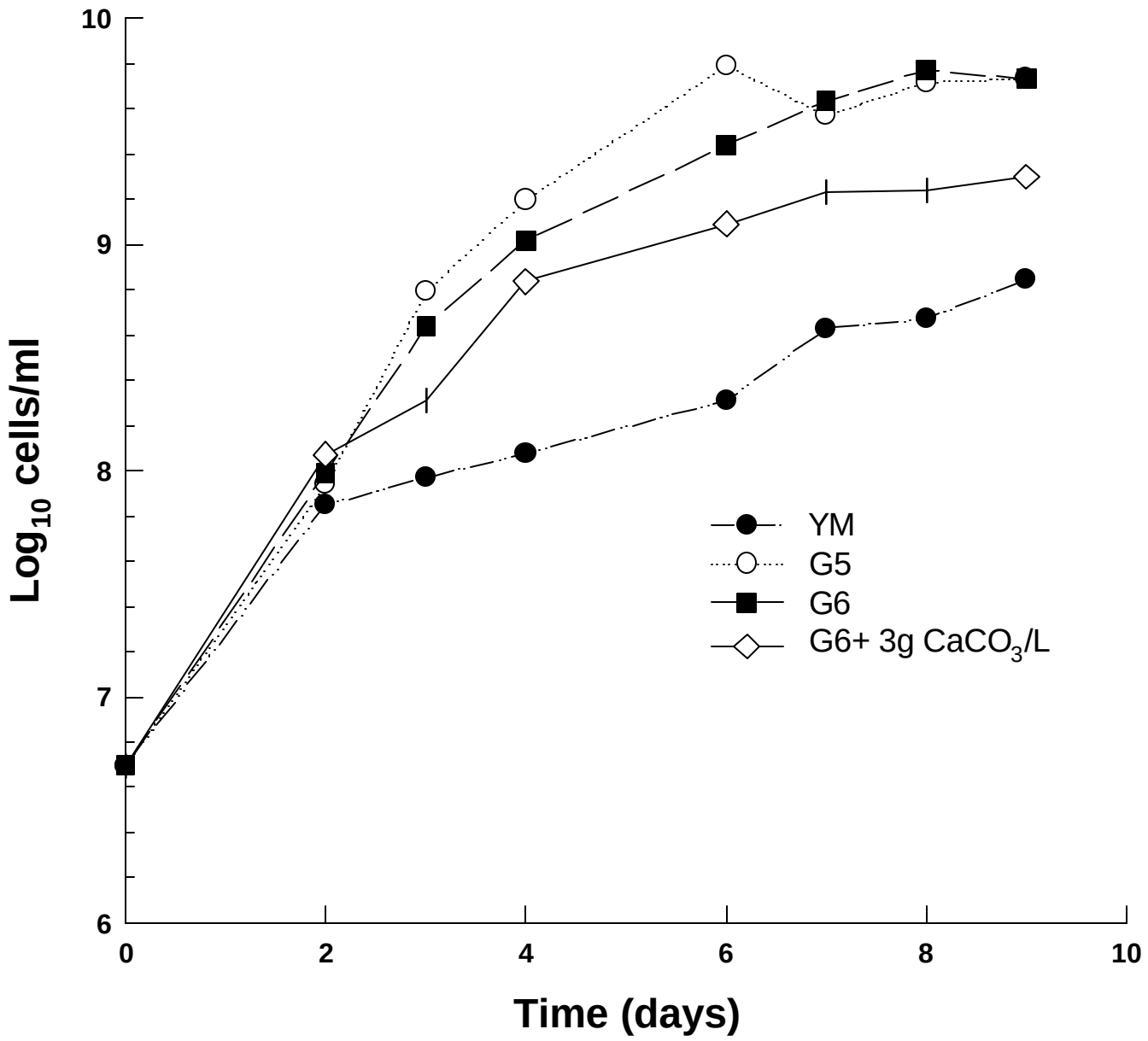

Fig. 3. Growth of Semia 5019 in different liquid media.

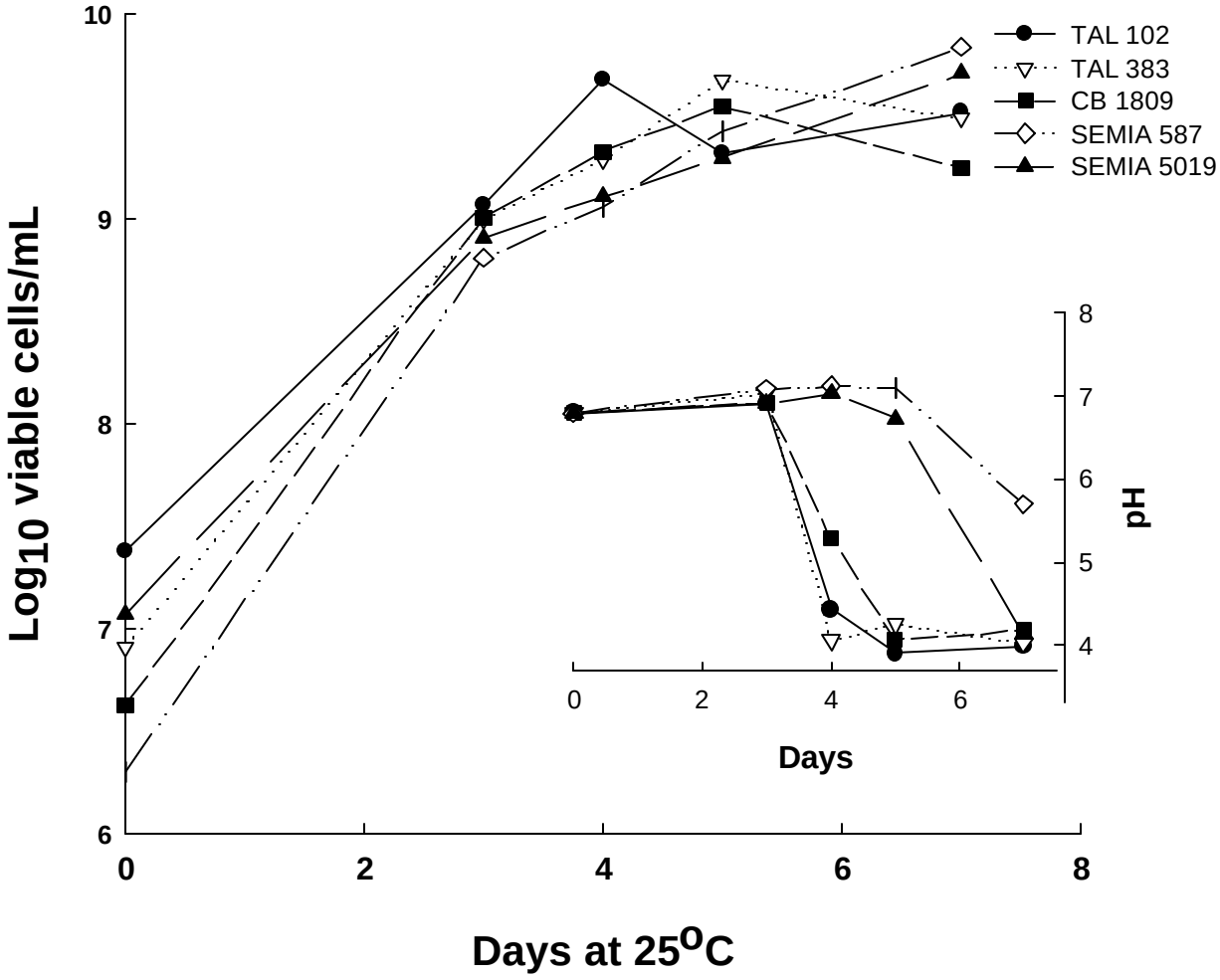

Fig. 4. Growth of Inoculant Quality Strains of *B. japonicum* in G6 Medium.

Figure 5

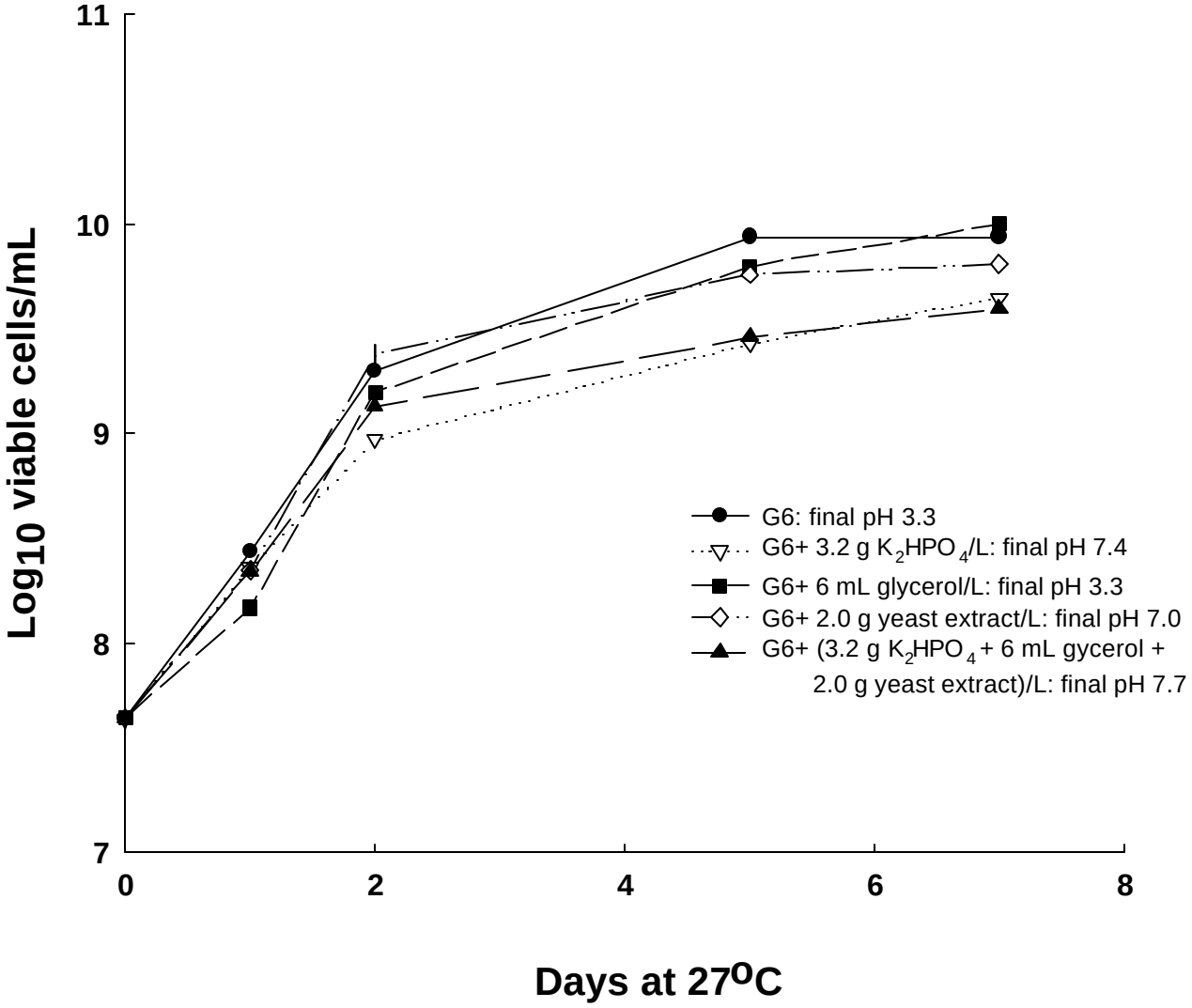

Fig. 5. Growth of SEMIA 5019 in Different G6 Media With Aeration (provided continously by pump at 1.0 L air/L culture/min).

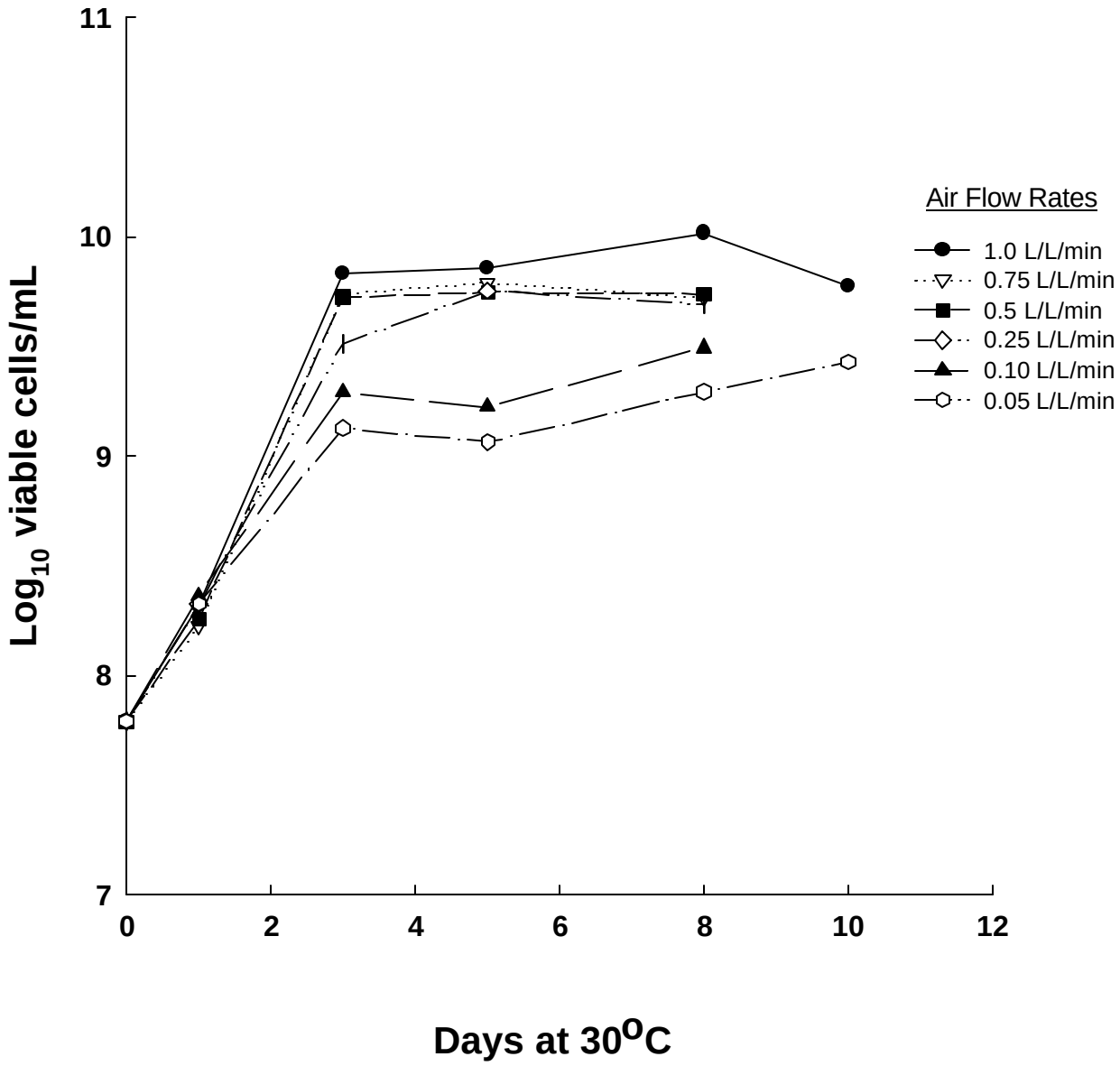

Fig. 6. Growth of SEMIA 5019 in G5 Medium With Different Air Flow Rates.

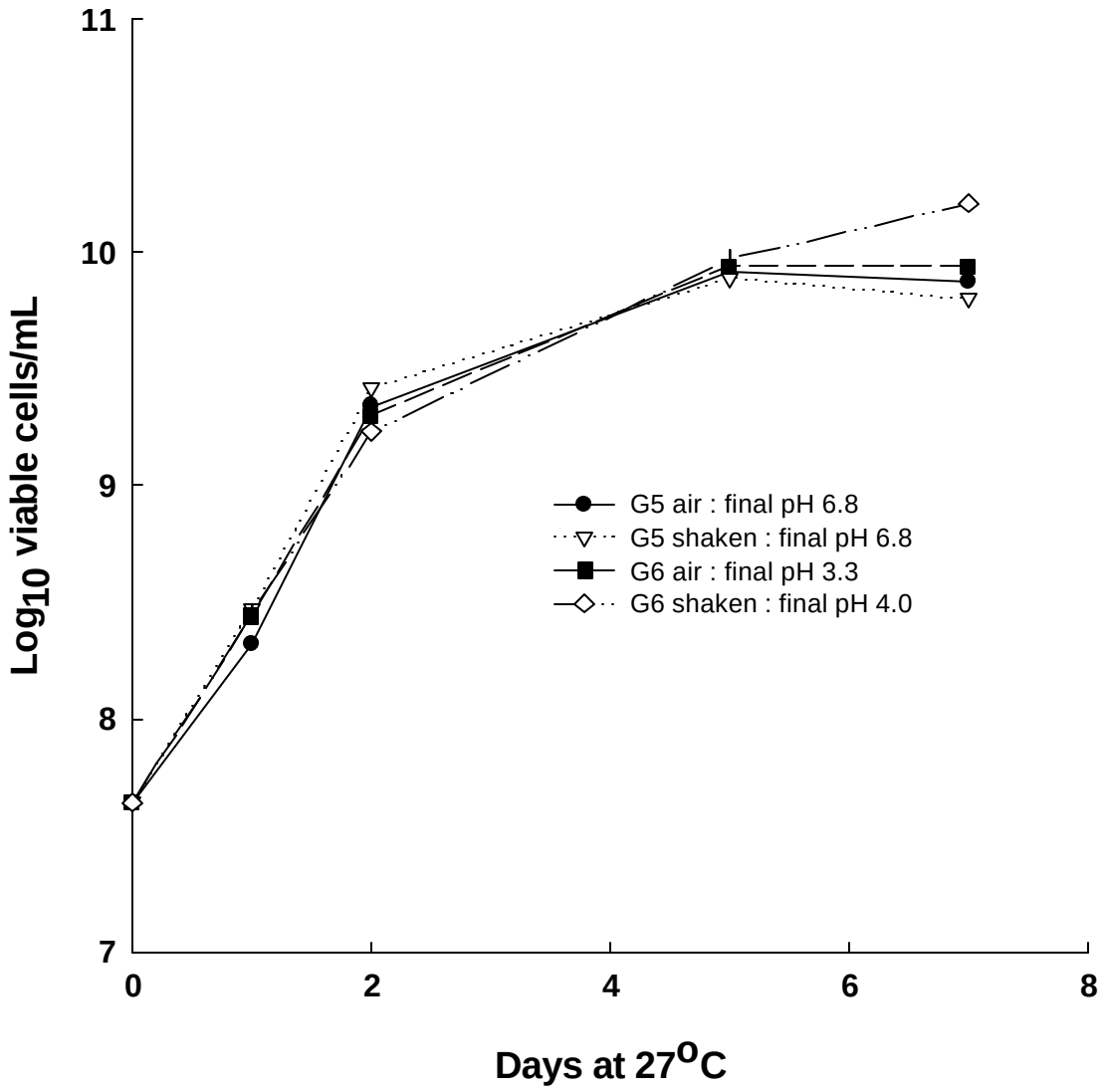

Fig. 7. Growth of *B. japoniucm* strain SEMIA 5019 in aerated and in shaken media.

Air = 1.0L air/L culture/min continously provided by a pump.
Shaken = culture continously shaken at 200 RPM in an incubator-shaker with a gyrotory, 1inch circular orbit.

Appendix 1. List of Collaborators

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Seed Yield Response to Inoculation with G5 Liquid Inoculant Formulation and Local Inoculant Products

Site	Control	NifTAL Liquid	Local Inoculant	Response to NifTAL Inoculant above: Control		Response to NifTAL Inoculant above: Local Inoc.		Relative Response above: Control	Relative Response above: Local Inoc.	Remarks
	kg/ha	*kg/ha*	*kg/ha*	*kg/ha*		*kg/ha*		*%*	*%*	
1a	810	1405	1465	595	*	-60		73	-4	Cultivar used in sites 1a-1d had
1b	1403	1632	1655	229		-23		16	-1	nodulation problems with SEMIA
1c	743	1141	1276	398	*	-135		54	-11	used in the G5 formula. This
1d	740	1820	1893	1080	*	-73		146	-4	is a chorosis producing symbiosis.
2b	1340	1800	2040	460	*	-240		34	-12	
2c	540	1260	1300	720	*	-40		133	-3	Experiments at sites 2a-2h
2d	960	1540	1260	580	*	280	*	60	22	conducted on two cultivars and
2e	470	2800	2120	2330	*	680	*	496	32	with two different strains in
2f	730	2040	1790	1310	*	250		179	14	liquid and local inoculant carrier.
2g	1080	1910	1930	830	*	-20		77	-1	Sites with ' indicate USDA 110
2h	1130	2030	1940	900	*	90		80	5	was used; other sites used SEMIA
2b'	1340	2370	2200	1030	*	170		77	8	
2c'	540	1240	1580	700	*	-340	*	130	-22	
2d'	960	1330	1240	370	*	90		39	7	
2e'	470	2510	2510	2040		0		434	0	
2f'	730	2080	2040	1350	*	40		185	2	
2g'	1080	2140	1900	1060	*	240		98	13	
2h'	1130	2130	2080	1000	*	50		88	2	
8a	1726	1946	1905	220		41		13	2	
9a	1360	1466	1589	106		-123		8	-8	
9b	2499	2380	2311	-119		69		-5	3	
10	2451	3088	3051	637		37		26	1	
11										
14	2102	2172	2224	70		-52		3	-2	
14'	2102	2641	2224	539		417		26	19	14' used G5 as a in furrow application
15a	2719	3964	4362	1245		-398		46	-9	No Stats Available-Steam sterilzed peat
17a	944	1210	1063	266	*	147		28	14	
17a'	944	1210	892	266	*	318	*	28	36	
18a	526	1352	1179	826	*	173	*	157	15	
18b	1152	2329	2187	1177	*	142		102	6	
18c	887	2019	1950	1132	*	69		128	4	
18d	466	1059	1066	593	*	-7		127	-1	

Site numbers indicate individual collaborators, site alphas indicate different locations and ' indicates multiple local inoculant products or cultivars within a location.

Seed Yield Response to Inoculation with G5 Liquid Inoculant Formulation and Local Inoculant Products

Site	Control	NifTAL Liquid	Local Inoculant	Response to NifTAL Inoculant above: Control	Response to NifTAL Inoculant above: Local Inoc.	Relative Response above: Control	Relative Response above: Local Inoc.	Remarks
18e	1855	2533	2533	678	0	37	0	
19a	929	1095	954	166	141	18	15	
19b	874	1132	1076	258	56	30	5	
19c	1496	1788	1933	292	-145	20	-8	
20a	238	666	409	428 *	257 *	180	63	
20b	1665	3119	3057	1454 *	62	87	2	
20c	1821	3692	3033	1871 *	659	103	22	
21a	2735	4182	4232	1447	-50	53	-1	
21b	3580	4292	4155	712	137	20	3	
21c	2298	3142	2607	844	535	37	21	
Mean	**1306**	**2089**	**2005**	**783**	**84**	**90**	**6**	

Site numbers indicate individual collaborators, site alphas indicate different locations and ' indicates multiple local inoculant products or cultivars within a location.

Nodule Number Response to Inoculation with G5 Liquid Inoculant Formulation and Local Inoculant Products

Site	Control	NifTAL Liquid	Local Inoculant	Response to NifTAL Inoculant above: Control	Local Inoc.	Relative Response above: Control	Local Inoc.	Remarks
	no/ha (x106)	*no/ha (x106)*	*no/ha (x106)*	*no/ha (x106) 6)*	*no/ha (x106)*	*%*	*%*	
2a	2.200	10.200	7.700	8.000 *	2.500 *	364	32	Sites 2a-2h gave nodule/plant
2b	1.700	8.700	7.400	7.000 *	1.300	412	18	data. Assuming 400,000 plants
2c	0.000	5.900	4.900	5.900 *	1.000 *	not defined	20	in harvest area.
2d	0.200	5.600	2.900	5.400 *	2.700 *	2700	93	Experiments at sites 2a-2h
2e	0.032	4.232	2.632	4.200 *	1.600 *	13125	61	conducted on two cultivars and
2f	0.468	4.600	2.932	4.132 *	1.668 *	883	57	with two different strains in
2g	1.500	5.400	6.400	3.900 *	-1.000	260	-16	liquid and local inoculant carrier.
2h	2.100	4.100	7.000	2.000	-2.900 *	95	-41	Sites with " indicate USDA 110
2a'	2.200	9.500	6.500	7.300 *	3.000 *	332	46	was used; other sites used
2b'	1.700	6.600	6.200	4.900 *	0.400	288	6	SEMIA.
2c'	0.000	5.200	4.500	5.200 *	0.700	not defined	16	
2d'	0.200	4.600	3.300	4.400 *	1.300 *	2200	39	
2e'	0.032	4.632	4.032	4.600 *	0.600	14375	15	
2f'	0.468	4.900	3.368	4.432 *	1.532 *	947	45	
2g'	1.500	5.800	4.700	4.300 *	1.100	287	23	
2h'	2.100	7.700	4.400	5.600 *	3.300 *	267	75	
8a	5.330	6.710	5.360	1.380	1.350	26	25	
9a	4.230	5.610	3.430	1.380	2.180 *	33	64	
9b	3.030	2.470	2.870	-0.560	-0.400	-18	-14	
10	11.297	12.565	13.655	1.268	-1.090	11	-8	
15a	0.000	5.347	6.319	5.347	-0.972	not defined	-15	No Stats-sterle Australian peat
17b	0.363	6.000	6.220	5.637 *	-0.220	1551	-4	
17c	0.303	5.960	7.670	5.657 *	-1.710	1865	-22	
17b'	0.363	6.000	5.630	5.637 *	0.370	1553	7	
17c'	0.303	5.960	6.530	5.657 *	-0.570	1867	-9	
18a	0.000	16.400	15.400	16.400 *	1.000	16399900	6	
18b	0.000	20.200	17.800	20.200 *	2.400	20199900	13	
18c	0.000	17.800	16.400	17.800 *	1.400	17799900	9	
18d	0.000	20.600	18.150	20.600 *	2.450	20599900	13	
18e	0.000	22.200	19.750	22.200 *	2.450	22199900	12	
19a	3.940	5.590	5.410	1.650	0.180	42	3	
19b	0.220	8.670	7.220	8.450 *	1.450	3841	20	
19c	0.250	6.600	5.320	6.350 *	1.280	2540	24	

Site number indicates individual collaborator. Site alpha indicates multiple locations, ' indicates second cultivar or second local inoculant product at a site

Nodule Number Response to Inoculation with G5 Liquid Inoculant Formulation and Local Inoculant Products

Site	Control	NifTAL Liquid	Local Inoculant	Response to NifTAL Inoculant above: Control		Local Inoc.	Relative Response above: Control	Local Inoc.	Remarks
	no/ha (x106)	*no/ha (x106)*	*no/ha (x106)*	*no/ha (x106)*	*6)*	*no/ha (x106)*	*%*	*%*	
20a	0.000	2.560	1.690	2.560	*	0.870	2559900	51	
20b	0.000	12.130	11.810	12.130	*	0.320	12129900	3	
20c	0.000	8.380	4.190	8.380	*	4.190	* 8379900	100	
21a	0.000	1.410	2.330	1.410	*	-0.920	14099900	-39	
21b	1.560	5.310	4.170	3.750		1.140	240	27	
21c	2.100	14.570	14.050	12.470	*	0.520	594	4	
26	2.780	15.540	2.710	12.760	*	12.830	* 459	473	
26'	2.780	15.540	23.970	12.760	*	-8.430	* 459	-35	Local- sterile Canadian peat w/5019
Mean	**1.348**	**8.483**	**7.486**	**7.135**		**0.997**	**3537387**	**29.264**	

Site number indicates individual collaborator. Site alpha indicates multiple locations, ' indicates second cultivar or second local inoculant product at a site

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Nodule Weight Response to Inoculation with G5 Liquid Inoculant Formulation and Local Inoculant Products

Site	Control	NifTAL Liquid	Local Inoculant	Response to NifTAL Inoculant above: Control		Response to NifTAL Inoculant above: Local Inoc.		Relative Response above: Control	Relative Response above: Local Inoc.	Remarks
	kg/ha	*kg/ha*	*kg/ha*	*kg/ha*		*kg/ha*		*%*	*%*	
1a	25	62	87	36	*	-26		144	-29	Cultivar used in sites 1a-1d had
1b	56	71	72	16	*	-1		28	-2	nodulation problems with SEMIA
1c	12	127	113	115	*	14		957	13	used in the G5 formula. This
1d	26	79	79	53	*	0		200	0	is a chorosis producing symbiosis.
2a	12	39	51	28	*	-12		238	-23	
2b	6	34	27	29	*	7		506	27	
2c	0	89	82	89	*	7		not defined	9	Experiments at sites 2a-2h
2d	0	88	59	87	*	29	*	25942	49	conducted on two cultivars and
2e	0	24	41	24	*	-17	*	5463	-42	with two different strains in
2f	10	47	35	37	*	12		389	33	liquid and local inoculant carrier.
2g	25	96	85	71	*	11		287	13	Sites with " indicate USDA 110
2h	30	68	70	38	*	-2		127	-3	was used; other sites used SEMIA
2a'	12	36	53	24	*	-18	*	206	-33	
2b'	6	36	29	30	*	7		534	25	
2c'	0	85	79	85	*	6		not defined	7	
2d'	0	59	56	59	*	3		17430	4	
2e'	0	51	46	51	*	6		11775	12	
2f'	10	30	33	21	*	-3		217	-9	
2g'	25	70	57	45	*	13		183	22	
2h'	30	46	59	16		-13		53	-22	
8a	37	52	42	16	*	10	*	42	24	
9a	40	51	42	11		9		28	21	
9b	27	26	25	-1		1		-4	4	
10	70	82	79	11		3		16	4	
14	3	5	15	2		-10	*	59	-64	
14'	3	6	15	2		-10		62	-64	14' used G5 as an in furrow applicatio
17b	5	80	70	74	*	9		1402	13	
17c	13	134	94	122	*	40		957	43	
17b'	5	80	93	74	*	-14		1402	-15	
17c'	13	134	99	122	*	36		957	36	

Site numbers indicate individual collaborators. Site alpha indicates differeent locations or cultivars and ' indicates second local inoculant products.

Nodule Weight Response to Inoculation with G5 Liquid Inoculant Formulation and Local Inoculant Products

Site	Control	NifTAL Liquid	Local Inoculant	Response to NifTAL Inoculant above: Control		Response to NifTAL Inoculant above: Local Inoc.		Relative Response above: Control	Relative Response above: Local Inoc.	Remarks
	kg/ha	*kg/ha*	*kg/ha*	*kg/ha*		*kg/ha*		*%*	*%*	
18a	0	106	94	106	*	12		105999900	13	
18b	0	120	107	119	*	13		119499900	12	
18c	0	114	99	113	*	15	*	113499900	15	
18d	0	108	94	108	*	14		107999900	15	
18e	0	152	132	152	*	20		151999900	15	
19a	21	29	27	8		1		38	4	
19b	1	46	36	44	*	9		3037	26	
19c	0	32	28	32	*	4		10699900	16	
20a	0	25	15	25	*	10	*	25299900	66	
20b	0	157	74	157	*	83	*	156899900	111	
20c	0	205	179	205	*	26		204599900	14	
21a	0	1	2	1		-1		1409900	-39	
21b	2	5	4	4		1		240	27	
21c	2	15	14	12		1		594	4	
26	15	82	19	66	*	62		429	323	
26'	15	82	113	67	*	-31		447	-27	Local is Sterile Canadian peat
Mean	**12**	**69**	**61**	**57**		**7**		**22681441**	**14**	

Site numbers indicate individual collaborators. Site alpha indicates differeent locations or cultivars and ' indicates second local inoculant products.

Seed Nitrogen Response to Inoculation with G5 Liquid Inoculant Formulation and Local Inoculant Products

Site	Control	NifTAL Liquid Inoculant	Local Inoculant	Response to NifTAL Inoculant above: Control		Response to NifTAL Inoculant above: Local Inoc.		Relative Response above: Control	Relative Response above: Local Inoc.
	kgN/ha	*kgN/ha*	*kgN/ha*	*kgN/ha*		*kgN/ha*		*%*	*%*
1a	46.8	86.9	87.0	40	*	0		86	0
1b	68.7	78.0	78.0	9	*	0		14	0
1c	41.9	59.9	67.9	18	*	-8		43	-12
1d	39.4	93.5	101.5	54	*	-8	*	137	-8
2b	76.1	104.2	108.5	28	*	-4		37	-4
2c	23.5	167.9	136.4	144	*	32	*	615	23
2d	36.6	122.0	98.9	85	*	23	*	234	23
2e	26.1	60.5	67.6	34	*	-7		132	-11
2f	40.7	78.7	55.3	38	*	23	*	93	42
2g	63.8	127.8	118.5	64	*	9		100	8
2h	72.9	127.0	122.6	54	*	4		74	4
2b'	76.1	149.0	142.0	73	*	7		96	5
2c'	23.5	129.3	136.9	106	*	-8		451	-6
2d'	36.6	125.1	127.9	89	*	-3		242	-2
2e'	26.1	61.9	78.8	36	*	-17	*	137	-21
2f'	40.7	59.0	65.5	18	*	-7		45	-10
2g'	63.8	119.1	121.2	55	*	-2		87	-2
2h'	72.9	134.9	144.8	62	*	-10		85	-7
8a	87.9	95.6	93.6	8		2		9	2
9a	80.9	87.2	90.1	6		-3		8	-3
9b	149.5	141.2	136.4	-8		5		-6	4
17a	44.7	65.2	57.8	21	*	7		46	13
17a'	44.7	65.2	57.6	21	*	8		46	13
19a	66.2	81.3	72.7	15		9		23	12
19b	52.5	79.9	72.4	27		8		52	10
19c	101.7	124.5	134.2	23		-10		22	-7
Mean	**58**	**101**	**99**	**43**		**2**		**112**	**3**

Remarks

Cultivar used in sites 1a-1d had nodulation problems with SEMIA used in the G5 formula. This is a chorosis producing symbiosis.

Experiments at sites 2a-2h conducted on two cultivars and with two different strains in liquid and local inoculant carrier. Sites with " indicate USDA 110 was used; other sites used SEMIA

Site alphas indicate different locations or two cultivars tested at the same site; ' indicates two different local products compared to G5 liquid inoculant at a site.

Appendix 3-1

Laboratory Assay for Testing Inoculant Survival on Seed: Materials and Methods

BACKGROUND:

To evaluate formulations or their components, a rapid laboratory assay was needed that provided an indication of how well an inoculant will perform after application to seed. We evaluated survival of *B. japonicum* after seed inoculation with various formulations (strains, additives, commercial formulations etc.) under standard laboratory conditions (68% relative humidity and 26° C). We then correlated survival in the laboratory assay with performance in potted soil in the greenhouse. A stress period was imposed on the inoculant by leaving the soil dry for 48 h after planting. The soil at seed level consistently reached maximums of 45-48° C during this period. Table 1 shows that survival of *B. japonicum* on seed under laboratory conditions was a reasonable indicator of inoculant performance on soybean planted in dry soil at high temperatures.

Table 1. Correlation between number of *B. japonicum* cells surviving on soybean seed in the laboratory at 26° C and 68% R.H. and nodulation and growth of soybean planted into dry potted soil.

Time from inoculation (h)	Nodule Dry Weight	Shoot Dry Weight
	R	
0	0.72*	0 .70
2	0.76*	0.70
4	0.92***	0.84**
24	0.83**	0.76*
48	0.86**	0.77*

*** P≤.01; ** .05≥P>.01; *.1≥ P >.05; Pots irrigated 48 h after planting; Pots reached maximum temperatures of 48°C for 2 d before irrigation and 38 C for next 7 d; harvest 33 DAP

PROCEDURE:

A liquid culture of *Bradyrhizobium japonicum* SEMIA 5019 in late log phase was used to inoculate seed. Williams soybean seed were inoculated in batches of 120 seeds with 60 µL liquid inoculant or 0.06 g peat. Peat inoculated seeds were pre-coated w/ 75 µL gum arabic sticker solution (40 g gum arabic in 100 mL H_2O, heated in microwave). Seeds were coated in sterile 50 mL beakers with a glass stir rod. Immediately following inoculation seeds were poured into Petri dishes which were then placed in sealed containers above saturated $CuCl_2.2H_2O$ solution in a 26° C incubator, yielding 68% RH and a seed moisture content of 0.13 g H_2O/g seed. At 48h after inoculation, seed were removed from the incubator and poured into a 250 flask containing 120 mL sterile diluent (0.85% NaCl, 0.01% Tween 80). The flask was shaken for 5 min on a Burrell wrist action shaker set at maximum speed, followed by 5 min of ultrasound exposure in a Bronson ultrasonic cleaner containing water up to the level of diluent.

Three steps of serial, ten-fold dilutions in saline diluent were prepared, with 100 µL aliquots from each of the 10^{-1} to 10^{-3} dilutions spread on G6 agar plates. The plates were incubated at 28°C for 5-7 days, and plates with 30-300 colonies were chosen for counting.

Notes:

1. Samples should be replicated, and duplicate plate counts made from a given dilution level.
2. YM, AG or other suitable media can be substituted for G6 in the inoculant broth or agar media. In our experience the G6 medium supports the most rapid growth.

Appendix 3-2

Laboratory Assay for Testing Shelf Life of Liquid Inoculants: Materials and Methods

Bradyrhizobia strain. SEMIA 5019 was used in all media. The sources for inoculation into media were early stationary phase cultures (population densities $>10^9$ cells/mL) of YM, G5, G6 or G6+3g $CaCO_3$/L. Samples from these sources were used undiluted or diluted, as described below.

Treatments. Different media were evaluated for ability to sustain high densities of viable cells over a 180 day period at a constant temperature of 25°C. The media tested were the following:

1. YM undiluted.
2. YM cells washed (shaking + centrifugation) 3 times in saline, final re-suspension in distilled water.
3. G5 undiluted.
4. G6 undiluted.
5. G6 diluted 1:5 in 25g PVP/L.
6. G6 diluted 1:5 in 15mL glycerol/L
7. G6 diluted 1:5 in 400uM Fe-EDTA
8. G6 diluted 1:5 in 25g PVP/L + 15mL glycerol/L + 400uM Fe-EDTA.
9. G6+3g $CaCO_3$/L undiluted.
10. Commercial liquid undiluted.

Experimental units consisted of 100 mL volume in a 250 mL flask fitted with a foam stopper (for air exchange). Dilutions were prepared by addition of 20 mL of the inocula source to 80 mL of the sterile diluent. All diluents were prepared with deionized water. Treatments consisted of five replicates.

Storage Conditions. Treatments were kept in a gravity convection incubator at a constant temperature of 25°C. Humidity was not controlled.

Sampling. Two replicates of each treatment were sampled for viable population density and survival on seed (see Appendix 3-1) for 48h at 30, 60, 90 and 180 days after placement in the incubator, using G6 agar medium. The commercial liquid inoculant treatment was added after initiation of the experiment, and was sampled at 16, 106 and 180 days after incubation.

Appendix 3-2
Description of Aerated Fermentor Vessels.

The fermentor set up consisted of a 2.0 L boro-silicate screw top bottle (Corning brand) containing 1.5 L of liquid media. Each 2.0 L bottle was connected to the same style 250 mL bottle by silicone tubing. The second bottle was a reservoir for any foam generated by the culture that filled the head space of the 2.0 L bottle.

The centers of the both bottles' plastic screw caps were drilled out. An 8.0 mm thick silicone gasket with two holes was fit to the inside of each bottle cap. One hole in the silicone gasket of the 2.0 L bottle was fit with a 7 mm i.d. polypropylene air sparger tube that extended to 2 cm of the bottom, of the bottle. A flexible silicone tube connected the sparger tube to a 0.2 uM Millipore filter that sterilized air entering the 2.0 L vessel. The other hole in the gasket of the 2.0 L bottle was fit with a 7 mm i.d. silicone tube (flush with the gasket surface) that also connected to the gasket of the 250 mL bottle. Air exited the 250 mL bottle through another silicone tube that terminated in a 50 mL plastic syringe body filled with two foam plugs.

The fermentor assemblies were connected to an air delivery manifold via a brass needle valve to control air flow. Air flow was monitored at the air outlet of each assembly with a glass tube air flow meter. Back pressure in the air manifold was maintained at 2.0 psi. Assemblies were placed on a heat mat with a the thermocouple of the controller placed into a dummy vessel that was also aerated. Temperatures were monitored with a thermocouple pressed against the glass of the 2.0 L vessel with a 10 mm thick piece of latex rubber. Temperatures measured this way were within 0.2 C of temperatures measured by immersing the thermocouple into the liquid.

Each 2.0 L vessel had an 18 mm dia. hole drilled into the side and near the bottom of the vessel to accommodate a tapered (17 mm dia at the small end) red rubber septum. The septum port was used for sterile sampling of the media.

Appendix 3-4
Formulation of several liquid media.(g L^{-1} unless otherwise stated)

	YMB *	G1	G2	G4	G5	G6+ pvp
Mannitol	1.0	10.0	1.0	1.0	1.0	
K_2HPO_4	0.5	0.5	0.5	0.5	0.5	0.8
$MgSO_4$ $7H_2$	0.2	0.2	0.2	0.2	0.2	0.5
NaCl	0.1	0.1	0.1	0.1	0.1	0.1
Yeast extract	0.5	1.0	1.0	1.0	1.0	2.0
Glucose		1.0	1.0	1.0	1.0	
Arabinose			1.0	0.5	0.5	
PVP-40**		20.0	20.0	20.0	20.0	20.0
Trehalose		10 mM	2mM			
FeEDTA		200µM	200µM	400µM	200µM	
$CaCl_2$						0.14
$FeCl_3$						8.4mg***
NH_4Cl						0.6
NaOH						0.16****
Glycerol		1.0 mL	1.0 mL	4.0 mL	4.0 mL	12.0 mL

* Controls in this work used YMB media modified from Vincent, 1970 by incorporating 1 g/L mannitol rather than 10 g/L. ** PVP av. mol. wt. = 40,000, K value (viscosity) = 26-35. Add the pvp last and shake or agitate media viigorously to dis solve. PVP can cause foaming when forced air is used to aerate culture. ***Add 8.4 mL of a stock solution containing 1.0 g $FeCl_3$ L^{-1}. **** Add 4.0 mL of a 1.0 M NaOH stock solution (40 g NaOH L^{-1}) . The final pH will be approximately 6.8

Appendix 3-5

Laboratory Assay for Evaluation of Gums to Increase Efficacy of Liquid Inoculants: Materials and Methods

Bradyrhizobia strain. SEMIA 5019 was used in all media. The source for inoculation into the gum media was an early stationary phase culture in G6 medium. An early stationary phase culture in G5 medium that was used only as a control for comparison with the gum media inoculated with the G6 culture.

Treatments. Different natural gums were evaluated for their ability to promoted short term suvival of inoculant and survival on seed after 48h. The treatments included:

1. G5 undiluted
2. G6 diluted 1:1 in deionized water
3. G6 diluted 1:1 in xanthan gum
4. G6 diluted 1:1 in guar gum
5. G6 diluted 1:1 in locust gum
6. G6 diluted 1:1 in karaya gum
7. G6 diluted 1:1 in ghatti gum
8. G6 diluted 1:1 in tragacanth gum
9. G6 diluted 1:1 in carrageenum gum

Experimental units consisted of 50 mL volume in a 100 mL flask fitted with a foam stopper. dilutions were prepared by addition of 25 mL of the G6 inocula into 25 ml of gum diluent. All gums were prepared by mixing 8g/L deionized water and sterilized by autoclaving – hence, final concentration of all gums was 4g/L. Treatments were prepared in duplicate.

Storage conditions. Treatments were kept in a gravity convection incubator at a constant temperature of 25^{o}C. Humidity was not controlled.

Sampling. Both replicates were sampled at 9 days after incubation for viable population density and survival on seed (see Appendix 3-1) for 48h. G6 agar medium was used for these determinations.

Appendix 3-6

Quality Control of Collaborators' Inoculants: Materials and Methods

1. INOCULANT POPULATION DENSITY

Upon receipt, collaborator's inoculants were placed in a refrigerator (4C) and stored there until sampled. Inoculants, which were friable, were mixed thoroughly by hand; the surface of the bag was wiped with ethanol, and sliced open with a flame-sterilized scalpel. A 10.0g sample was obtained with a flame-sterilized spatula and placed on sterilized weigh paper on a scale. The scale was on a clean tabletop exposed to the ambient air of the laboratory. Our experience with this procedure makes us confident that very few if any contaminants are introduced during this brief exposure to non-sterile air.

The sample was then transferred with the aid of a sterile-paper funnel to a flask containing 90 ml sterile saline (0. 85 % NaCl). The flask was attached to a wrist action shaker and run at maximum speed for 20 minutes.

In a laminar flow transfer hood, a serial, seven-step, 10-fold dilution series was prepared (the shaken flask sample = 1/10 dilution step) in sterile 9 ml saline in test tubes. Transfers were done with 1.0 ml sterile pipette tips followed by mixing on a tube mixer (vortex mixer), and each tip was wetted and exhausted three times before the 1 ml aliquot for transfer was removed to the next tube.

Dilution steps four through seven were sampled and transferred to yeast-mannitol agar plates (Somasegaran and Hoben) by placing a 1.0 mL aliquot at the center of the plate, followed by thorough spreading with a flame-sterilized glass rod (prepared in the shape of the letter "L"). Duplicate samples were plated from each step. After two hours, the plates were inverted, placed in a plastic bag to prevent dehydration and placed in a 27 C incubator. Plates were checked daily for at least seven days for presence of bradyrhizobia and contaminants. In some cases the presence of fast-growing contaminants limited the choice of dilution steps from which bradyrhizobia could be counted. Where possible, plates with 30-300 putative bradyrhizobia colonies were counted. Putative bradyrhizobia colonies on the highest dilution plates were selected for authentication by inoculation of soybean plants.

2. INOCULANT SURVIVAL ON SEED

Williams soybean seed ('Belts95", USDA, Beltsville MD) were inoculated in batches of 120 seeds with 0.06 g peat. The inoculant sample was obtained as described above. In a laminar flow transfer hood, seeds were pre-coated with 60 uL of sterile deionized water. Seeds were coated in sterile 50 mL beakers with a flame sterilized glass stir rod.

The inoculated seeds were placed in sterile Petri dishes in sealed containers above saturated $CuCl_2.2H_20$ solution in a 25 C incubator, yielding 68% RH and a seed moisture content of 0. 13 g H20/g seed. At 48 h after inoculation, the petri dishes were opened in the hood and the entire contents transferred to a flask containing 120 mL sterile saline. The flask was placed on a wrist action shaker and run at full speed for 5 minutes, followed by bath sonication for 5 mm. A three step, 10-fold serial dilution, sampling to plates, incubation and counting of all three steps in duplicate was carried out as described above.

3. AUHENTICATION OF INOCULANTS

To confirm the putative plate counts of *Bradyrhizobium* or *Rhizobium* collaborators' inoculants were tested for authenticity by production of nodules on soybean plants. We used 0.5 g of pure inoculant and single colonies picked from the highest available dilution plates used in the population density assay.

Seed of the appropriate legume host were imbibed with deionized water. Imbibed seed were placed in sterile vermiculite for germination. After 48 h, seed with adequate radicals were selected and placed individually in growth pouches supplied with 30 ml N-free nutrient solution (see Somasegaran and Hoben). Pouches were placed in a growth room equipped with high-pressure sodium grow lights, alternately water with N-free solution and sterile water.

A 10 gram sample of inoculant was placed in 90 ml sterile deionized water. A five mL sample of this 1/10 dilution was inoculated onto four replicate soybean seedlings in pouches. Two to four single colonies from plates of each inoculant were removed with a sterile cotton-tip swab and dispersed into 1.0 mL sterile water, which was then inoculated onto a single soybean seedling.

Plants were evaluated daily after 10 days for the presence of nodules for at least 14 days. Colonies from SEMIA 5019 were used as a positive control, and uninoculated plants served as negative controls, with four replicates each. All positive controls were nodulated, while none of the uninoculated plants had nodules.