# Research Summary I of Liquid Inoculant Formulations

## Developing a Laboratory Assay for Testing Inoculant Formulations.

To evaluate formulations or their components a rapid laboratory test is needed that provides 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 – see details in Appendix A-2). We then correlated survival in the laboratory test 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. We have not yet conducted trials to evaluate inoculant performance in the field.

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

## Effect of Amendments on *B. japonicum* Growth in Liquid Media and Survival after Application to Seed.

**Reasoning behind Selection of Test Compounds**. Several compounds were tested for their ability to promote survival of *B. japonicum* after inoculation. Selections were made to counter various stresses the cell may encounter after application to seed. We have not yet examined the interactions between compounds on cell survival. Synergistic interactions may be a highly productive area of research to improve the performance of liquid formulations.

1. Polyvinylpyrrolidone – PVP may bind toxic compounds present in seed exudates that are mobilized during inoculation and seed germination. It has a high water-binding capacity and appears to slow drying of the inoculant after application. Its sticky consistency may enhance cell and inoculant adherence to seed.

2. FeEDTA – One report in the literature hypothesized that seed-released compounds bind iron in the yeast extract, making it unavailable to cells. Supplementary iron may, therefore, replace Fe bound by exudate toxicity.

3. Glycerol – Glycerol is a carbon source for rhizobia. Glycerol has a high water-binding capacity and may protect cells from the effects of desiccation by slowing the drying rate. Its flow characteristics appear to promote more rapid and even coating of seeds.

4. Trehalose – This compound is widely reported to enhance cell tolerance to desiccation and to osmotic and temperature stress. Trehalose acts by stabilizing both enzymes and cell membranes, and is a compatible osmoticum as well. Trehalose is readily manufactured by *Bradyrhizobium* given the correct conditions (see below). We found that additions of trehalose to the growth media had no effect on survival of *B. japonicum* applied to seed.

5. Glucose – Glucose promotes the production of the storage product glycogen. In the absence of glycogen, osmotically stressed cells are reported to release their supplies of trehalose, an undesirable outcome. Glucose also enhances exopolysaccharide production, which could protect cells during the rapid drying they experience at inoculation.

6. Mannitol – Mannitol has many potential roles in a liquid inoculant. It is a carbon source for all strains of *Bradyrhizobium*, and in conjunction with arabinose it is reported to support the greatest production of trehalose in *B. japonicum* TAL 102. Mannitol is reported to protect cells during the rapid drying they experience at inoculation both as a solute and in enhancing polysaccharide production. When the mannitol was removed from the media survival of *B. japonicum* was adversely affected.

**Effects of Amendments on *B. japonicum* Growth in YMB Culture Media**. Figure 1 shows the effect that three additives to YMB broth have on growth of *B. japonicum*. It is important to ensure that potential additives that protect cells do not impair growth. Only 8.0 mL/L glycerol had a large effect, slowing growth and reducing the final cell number. In this case, maximum cell density was reached when 4 mL/L was added to the YMB media (data not shown).

**Effects of Amendments on Survival of *B. japonicum* After Application to Seed. FeEDTA** (Fig. 2), **PVP K30** (Fig. 3), and **glycerol** (Fig. 4) affected survival of *B. japonicum* on soybean seed. PVP and FeEDTA appear to enhance survival of *B. japonicum*, and the promotion is concentration-dependent. The data for glycerol is less clear.

There was a significant ($r^2$ >.93) linear relationship between FeEDTA concentration and the proportion of viable cells remaining on soybean seed at 2 h and 48 h after inoculation. While high concentrations of FeEDTA seem to promote survival on seed, we have some evidence that concentrations exceeding 400 $\mu$M reduce survival in the inoculant formulation. This sensitivity in the media may be strain dependent.

Similarly, increasing amounts of PVP K30 in the media increased survival on the seed by 100-fold 48 hours after inoculation. In a parallel experiment we showed water extracts of soybean seed inhibited growth of *B. japonicum* (TAL 102) on agar plates unless the inoculant contained 20 g/L PVP (see Appendix A-4 for details of the assay).

**Differences Between Strain Survival on Seed**

Several *B. japonicum* were grown in a fourth generation formulation (G4 media – see Appendix A-1 for composition) and surviving cells measured at inoculation (where the inoculant was removed from seed within 1 min of application) and 4 h and 48 h later. At 48 h from inoculation, the number of viable cells per seed for the liquid formulation ranged from log = 2.4 to log = 5.0. The peat inoculant (TAL 102 in gamma irradiated Australian peat) supported survival better than any strain in the liquid formulation.

Two factors determined the number of viable cells remaining on seed 48 h after inoculation: 1) differences in cell numbers in the inoculant, 2) the rate of cell death. The most important factor is the rate of cell death. Populations in the liquid inoculant formulations ranged from $2.8 \times 10^8$ to $2.4 \times 10^9$ $mL^{-1}$. Cell number in the inoculant explained only 57% of the variance between strains for the number of cells surviving at initial inoculation (t=0). By 4 h and 48 h after inoculation the number of cells in the inoculant explained only 15% and 14% of the differences in viable cells remaining on the seed.

The rate with which viable cells declined differed between strains and formulation. The rate of cell death in Australian peat (with gum arabic as a sticker) was less than in the liquid formulations. Despite the same number of cells on the seed as peat at T=0, the Commercial 5 liquid formulation exhibited very rapid cell death. Some strains such as 532C, SEMIA 587 and SEMIA 5019 in the G4 media also survived very poorly compared to other strains.

When the same set of strains was tested in G5 media results were very different. Strains that performed poorly in G4 media (e.g. SEMIA 5019, SEMIA 587) survived better than the others when grown in G5 media. It appears there is a large interaction between strain and media components in determining cell numbers in the inoculant and their degree of survival after inoculation onto seed.

Selecting strains for survival on seed may be a very productive way of increasing performance of liquid inoculant. Research to improve survival characteristics of liquid inoculants must, however, tailor the formulation to the individual strain.

**Physiological Pre-conditioning of Cells to Tolerate Stress**.

Several research papers have shown that when bacteria are either starved for carbon or exposed to oxidative stress ($H_2O_2$ for example) they become more tolerant to many harmful environmental conditions (low pH, desiccation, salinity, temperature etc.). There is evidence that cells exposed to stress generate signal molecules that induce stress response genes in other cells. It may be possible, therefore, to produce inoculant with cells that are pre-conditioned to better tolerate the stresses they encounter after application to seed.

We tested whether inoculant age influenced cell survival after seed coating. It was hypothesized that with age, cells may run out of carbon or other metabolites that will pre-condition them to tolerate environmental stress.

The G2 media enhanced survival in the culture compared to the YMB media (Figure 6A) over a 40 day period. The G2 media also promoted even greater relative survival of cells than YMB after their application to seed (Figures 6B, 6C, and 6D). Aging the culture, however, did not enhance survival of

cells after application to seed. The regression of cell survival in the G2 media after inoculation on culture age is essentially horizontal.

Our attempts to pre-condition cells of *B. japonicum* with $H_2O_2$ met with mixed success. There was a fine line between applying enough oxidative stress to elicit cell survival mechanisms and killing the cells.

**Laboratory Performance of Commercial Liquid Inoculant Formulations**.

We evaluated several commercial formulations for their ability to support survival of *B. japonicum* inoculated onto soybean seed. Figure 7 compares the number of viable cells remaining on seed over a 30-day period for four commercial liquid formulations, gamma irradiated Australian peat or cells suspended in water.

Results demonstrate the large variation in performance that is due to formulation and the apparent advantage of high quality peat carriers over existing liquid formulations. The initial inoculation level of this batch of peat inoculant was almost one log lower than Commercial 1 formulation. Despite this poor start, the peat inoculant still supported as many cells on the seed within a few days after inoculation as Commercial 1.

**Component Costs of Prototype Formulations**.

We have attempted to improve inoculant performance and at the same time reduce costs by evaluating action levels of various compounds. Costs stated below would be much less if bulk chemicals were substituted for the reagent grade chemicals quoted below.

The cost of peat that is finely ground, sterilized and neutralized usually falls in the range of $3.00 - $8.00/kg. When YMB broth is added at the rate of 66% of the dry weight of the peat, the total materials cost would be $2.41 – $6.46/kg finished inoculant. Even though costs of the liquid formulations presented below are derived from prices for small quantities of reagent grade chemicals, they cost substantially less than processed peat carrier.

Table 2. Material costs for media.

| Compound | Unit Cost* | YMB** | G2 | G5 |
|---|---|---|---|---|
| | $/kg | $U.S./L | | |
| Mannitol | 40.75 | 0.41 | 0.04 | 0.04 |
| Glycerol | 37.50 | 0.00 | 0.04 | 0.15 |
| Arabinose | 495.00 | 0.00 | 0.50 | 0.25 |
| Glucose | 11.05 | 0.00 | 0.01 | 0.01 |
| Trehalose | 628.50 | 0.00 | 0.48 | 0.00 |
| $K_2HPO_4$ | 65.00 | 0.03 | 0.03 | 0.03 |
| $MgSO_4.7H_2O$ | 42.70 | 0.01 | 0.01 | 0.01 |
| NaCl | 12.10 | <0.01 | <0.01 | <0.01 |
| Yeast Extract | 127.90 | 0.64 | 0.13 | 0.13 |
| FeEDTA | 36.54 | 0.00 | <0.01 | <0.01 |
| PVP K30 | 26.14 | 0.00 | 0.52 | 0.52 |
| | | | | |
| Total | | 1.09 | 1.76 | 1.15 |

*Costs are based on current quotes from 1998 Sigma Catalogue. ** YMB from Vincent, 1970 (A manual for the practical study of root nodule bacteria. IPB Handbook)

## Appendix.

**A-1. Composition of Selected Formulations**

| | YMB* | G1 | G2 | G4 | G5 |
|---|---|---|---|---|---|
| **Mannitol** | 1.0 | 10.0 | 1.0 | 1.0 | 1.0 |
| **$K_2HPO_4$** | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| **$MgSO_4$ $7H_2O$** | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| **NaCl** | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| **Yeast extract** | 0.5 | 1.0 | 1.0 | 1.0 | 1.0 |
| **Glucose** | | 1.0 | 1.0 | 1.0 | 1.0 |
| **Arabinose** | | | 1.0 | 0.5 | 0.5 |
| **PVP K30** | | 20.0 | 20.0 | 20.0 | 20.0 |
| **Trehalose** | | 10 mM | 2mM | 0 | 0 |
| **FeEDTA** | | 200µM | 200µM | 400µM | 200µM |
| **Glycerol** | | 1.0 mL | 1.0 mL | 4.0 mL | 4.0 mL |

* Controls in this work used YMB media modified from Vincent, 1970 by incorporating 1 g/L mannitol rather than 10 g/L.

**A-2. Materials and Methods - Laboratory Survival Studies**

A stock culture of *Bradyrhizobium japonicum* TAL 102 in late log phase was used to inoculate all liquid carriers, unless otherwise noted. One mL stock culture (in YMB) was placed into a sterile 125 mL flask containing 50 mL media, and inocula were grown in a shaking incubator at 30 C at 220 rpm to stationary phase. Inoculant culture counts were obtained the day of inoculation. The $10^{-6}$ to $10^{-7}$ dilutions were spread plated (100 µL) on AG plates.

Williams soybean seed ("Belts95", USDA, Beltsville MD) were inoculated in batches of 30 seeds (approximately 5 g) with 15 µL liquid inoculant (3 mL/kg) or 0.015 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. Tests of different strains were inoculated at a higher rate (25 µL liquid inoculant; 0.025 g peat) to correspond to the inoculation rate used in the parallel greenhouse experiment.

Immediately following inoculation, samples of 10 seeds each were assayed to determine the number of viable bacterial cells initially inoculated onto seed. Each 10 seed sample was placed into a 25 mL flask containing 10 mL diluent (0.85% NaCl, 0.01% Tween 80), vortexed for 1 min., bath sonicated for 5 min., and vortexed for 1 min. Sonication increased cell recovery by 4-5 fold. The $10^{-2}$ to $10^{-4}$ dilutions were spread plated (100 µL) on AG media petri dishes.

The remaining seeds were held in Petri dishes 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. Ten seed samples were assayed as above at 2 or 4 h and at 48 h after inoculation.

**A - 3. Materials and Methods - Greenhouse Pot Tests**

**Plant culture.** All experiments were conducted in soil from the Kula Agricultural Park, Maui, Hawaii. The soil, a Torroxic Haplustall, had a pH of 7.5, organic C of 1.3% organic N of 0.3%, and clay, silt and sand fractions of 42%, 43% and 14% respectively. Soil was mined to a depth of 25 cm, passed through a screen with mesh dimensions of 0.64 X 0.64 cm. Liquid stocks of $KH_2PO_4$ and $MgSO_4.7H_2O$ were sprayed onto the air-dried soil (15 g $H_2O$/g soil) as it rotated in a concrete mixer. These stocks provided 128 mg K, 100 mg P, 20 mg Mg, 27 mg S per kg soil. A liquid micro-nutrient mix (Hawaiian Horticultural Mix – Monterrey Chemical) was also sprayed onto the soil at the rate of 0.5 mL/kg soil to provide 7.5 mg Fe, 2.5 mg Zn, 2.3 mg Mn, 1.75 mg B, 0.75 mg Mg, 0.2 mg Mo and 0.15 mg Cu per kg soil.

Plastic pots (3.75 L) were filled with 2.6 kg air-dried soil. A circular depression (6.4 cm dia.) was made to a depth of 0.5 cm in the soil with the cross section of a PVC pipe. Four inoculated seeds were planted in this depression. Seeds were immediately covered with an additional 350 g of soil and the soil was pressed onto the seed to uniformly cover with 2.5 cm of soil. Pots were planted by block. The entire planting procedure took between 20 – 60 min. Thermometers were placed into the several pots at a depth of 2.5 cm. Soil temperatures were recorded at 1400-1430 h and at 0630-0700 h.

Except for control treatments, which were watered immediately at planting, the soil remained dry for 48 h when irrigation was applied to all pots simultaneously via a drip irrigation system. A drip irrigation emitter was placed at the center of each pot and 30 ml of de-ionized water was applied to each pot followed by 50 mL at 12 min intervals until 380 mL had been applied. This watering schedule simulated a 1.5 cm/h rainfall event. Pots were brought up to 36% moisture over a period of days and then maintained at 36-37% moisture until harvest at 30-33 days after planting.

Shoots were cut at soil level, dried at 70 C and weighed. Nodules were removed from roots, counted, dried at 70 C and weighed. Uninoculated controls remained non-nodulated.

**Seed Inoculation.** Manufacture of the inoculant followed procedures Appendix A-2. Williams soybean seed ("Belts95", USDA, Beltsville MD) was inoculated in lots of 48 g (approximately 250 seeds) with 240 µL liquid inoculant or 0.24 g peat. Peat inoculated seeds were pre-coated w/ 720 µL gum arabic sticker solution (40 g gum arabic in 100 mL $H_2O$, melted in microwave). Seeds were coated in sterile 250 mL beakers with a glass stir rod and approximately half the seed removed to a sterile petri-dish for enumeration and storage at 68% R.H. and 26 C. The remaining half was placed into an insulated box with ice and removed to the greenhouse. Inoculated seed were kept in the chilled box except when used for planting.

**A - 4. Materials and Methods - Seed Exudate Assay**

**Extraction of seed exudates**. Seed exudates were extracted from 100 unsterilized Williams soybean seed ("Belts97", USDA, Beltsville MD) in a sterile flask with 100 mL sterile deionized $H_2O$ and aeration on a platform shaker at 200 rpm. After extraction for four hours the seeds were rinsed with sufficient sterile $H_2O$ to return the volume of extract to 100 mL. The extract was filter sterilized (0.2

µM) and 10 mL aliquots placed into sterile plastic tubes, frozen at –20 C for 2h, and lyophilized over 42 h on a Super Modulyo freezer dryer (Edwards). Extracts were held in a desiccator in a freezer until use.

**Growth of bacterial cells**. *B. japonicum* TAL 102 were grown to stationary phase in 250 mL flasks on a 200 rpm shaker at 25 C in 50 mL of either YMB or YMB with PVP (polyvinylpyrrolidone) K30 at 20 g/L.

Inoculant cultures were serially diluted to $10^{-3}$ in the appropriate media and one mL of the $10^{-3}$ dilution (equivalent to the number of cells inoculated onto 10 seeds) was placed into a tube containing the lyophilized seed exudate derived from 10 seeds and into an empty tube for the control. The cultures were vortexed well to mix and held on a 200 rpm shaker at room temperature for 24 h. Serial dilution was resumed and the $10^{-5}$ to $10^{-7}$ dilutions were spread plated (100 µL) on AG plates.

## A- 5. Selected Bibliography.


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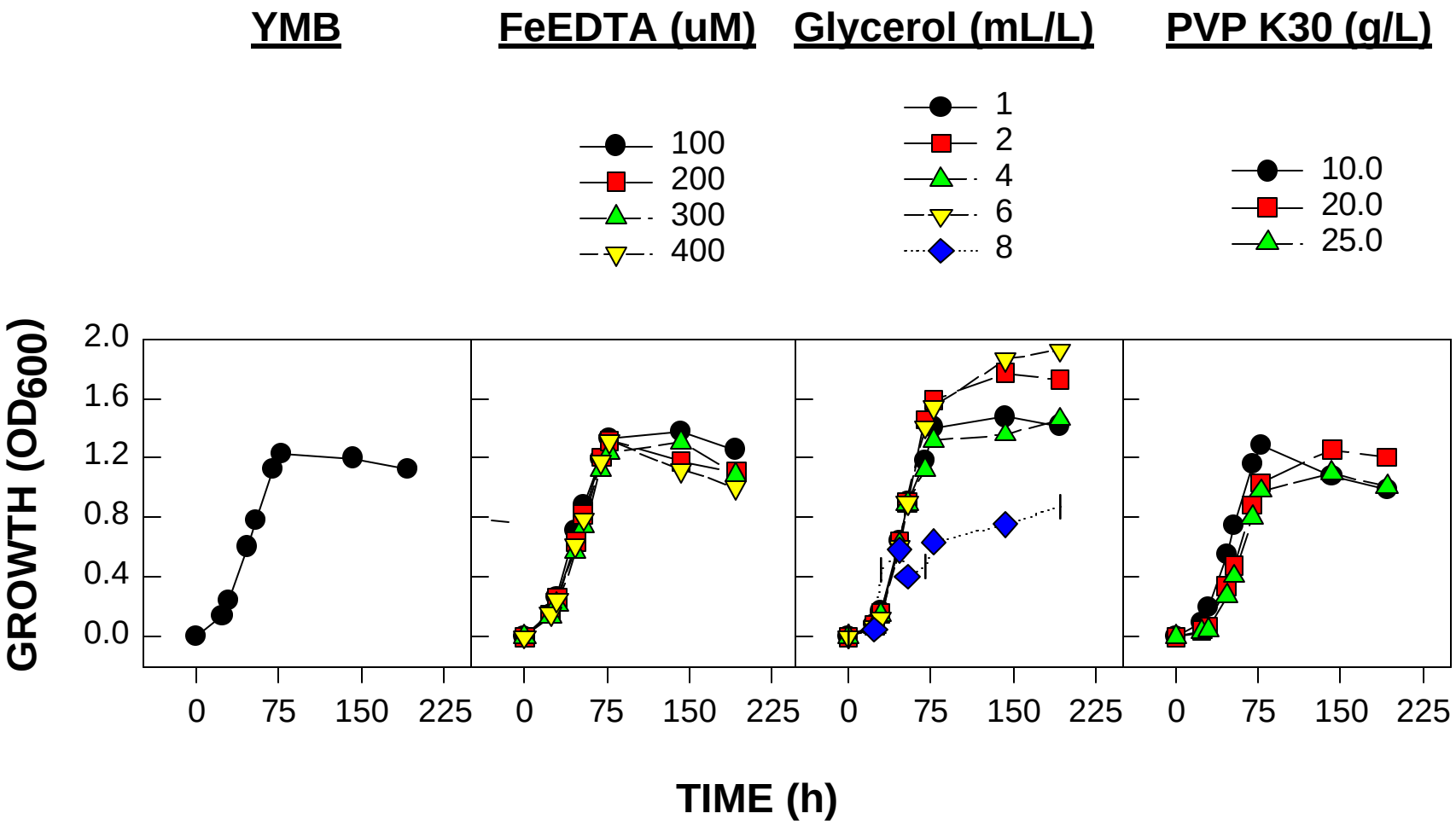
# Fig. 1. Growth rate of *B. japonicum* in YMB amended with different levels of protective compounds



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# Fig. 2. Survival of *B. japonicum* on seed when 100 to 1000 uM FeEDTA is added to YMB media

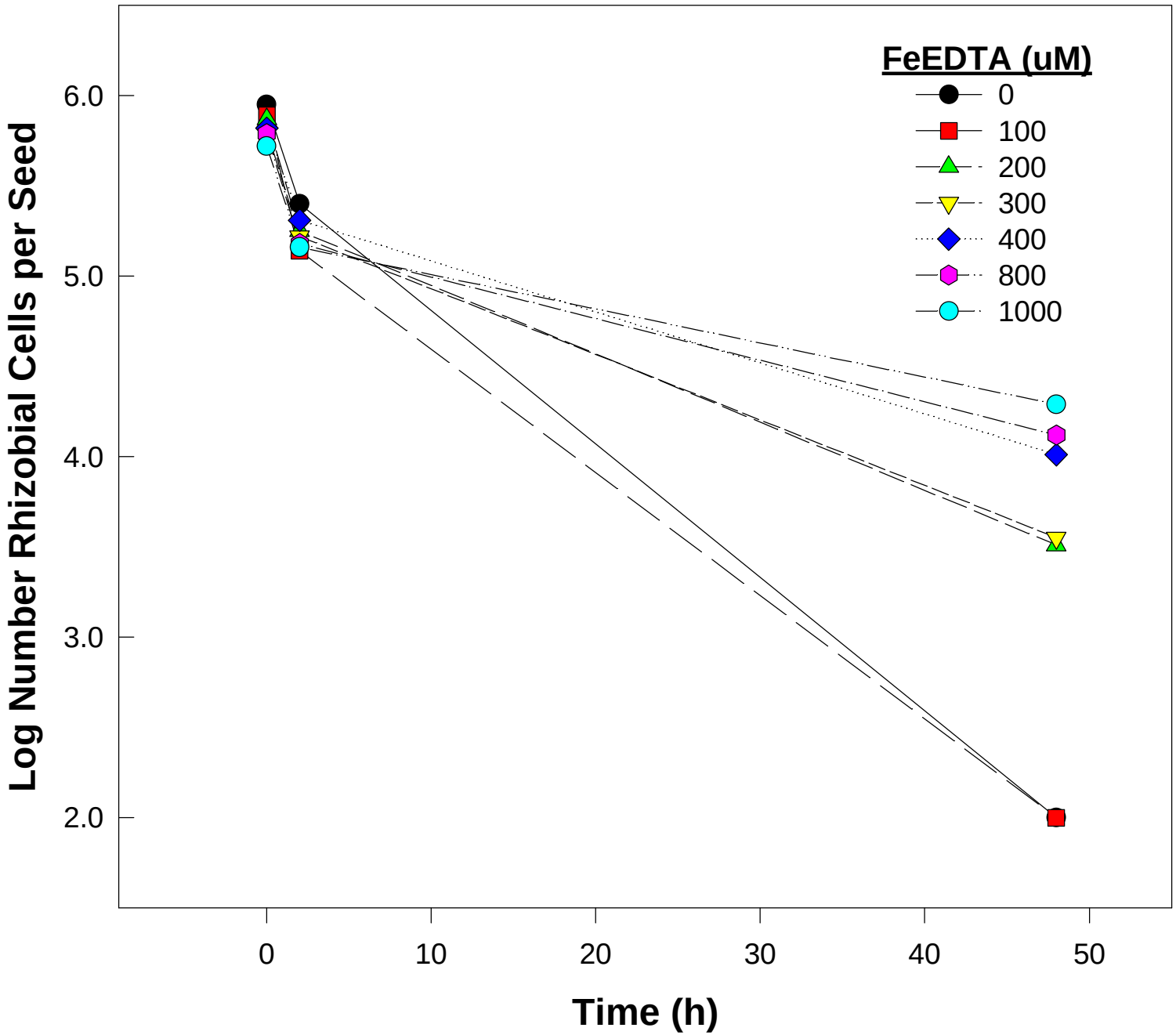
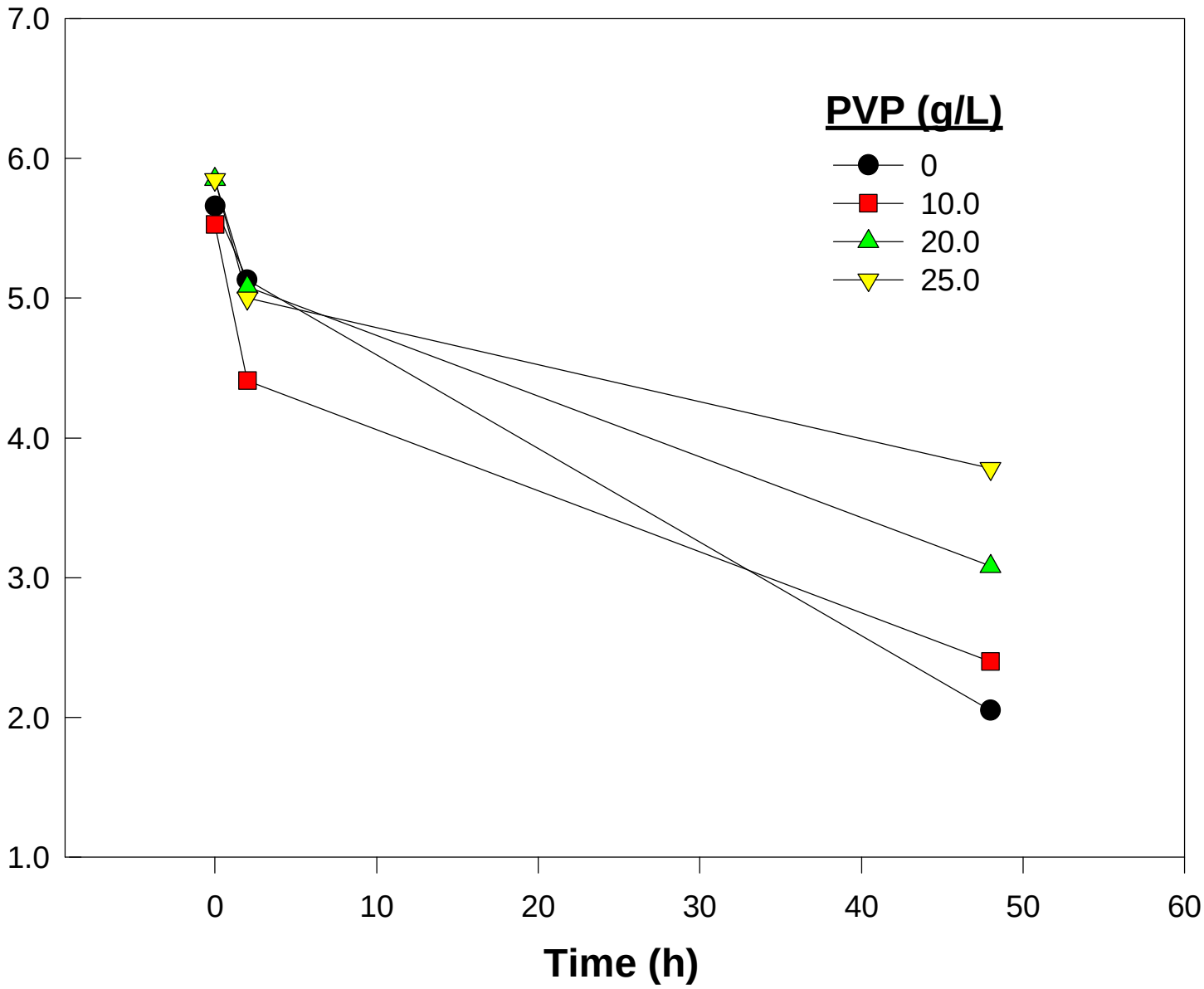
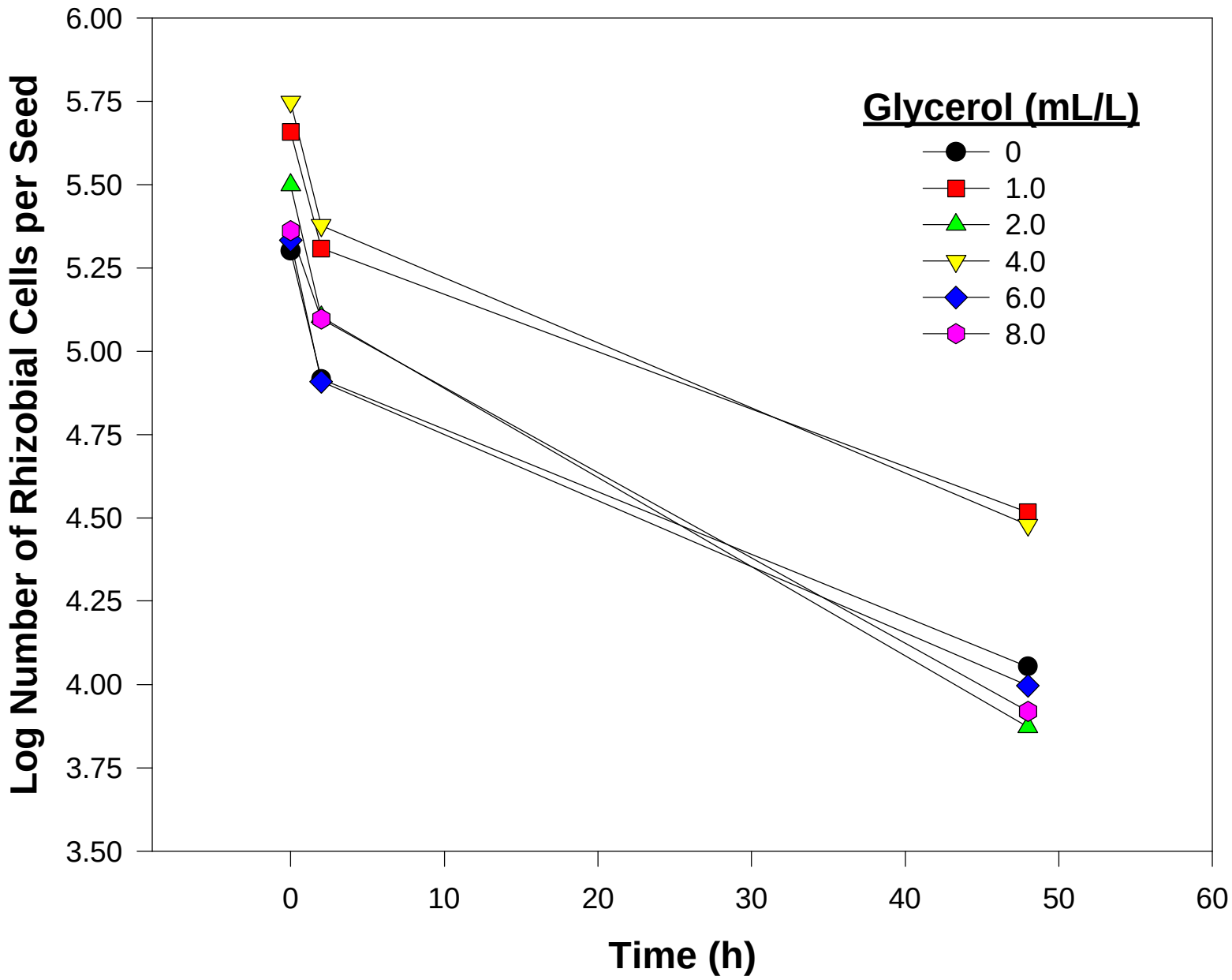


**Fig. 3. Survival of *B. japonicum* on seed when 0 to 25 g/L PVP K30 is added to YMB media**

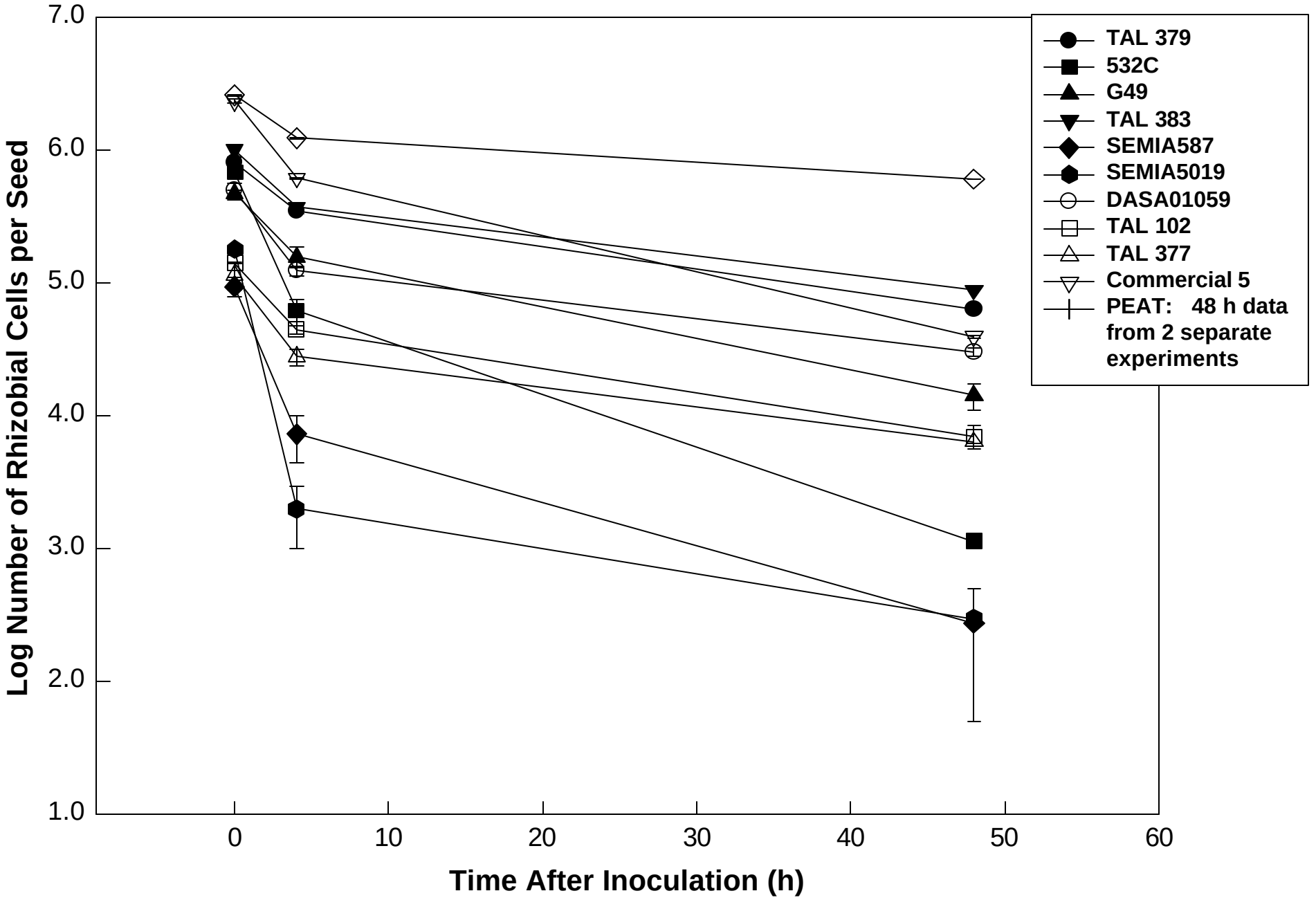


# Fig. 4. Survival of *B. japonicum* on seed in YMB containing from 0-8 mL/L glycerol



GH3SEED2.spv

Fig. 5. Effect of *Bradyrhizobium japonicum* strain on survival on seeds. All strains were tested in NifTAL's G4 inoculant, with the exceptions of peat and Commercial 5 product.



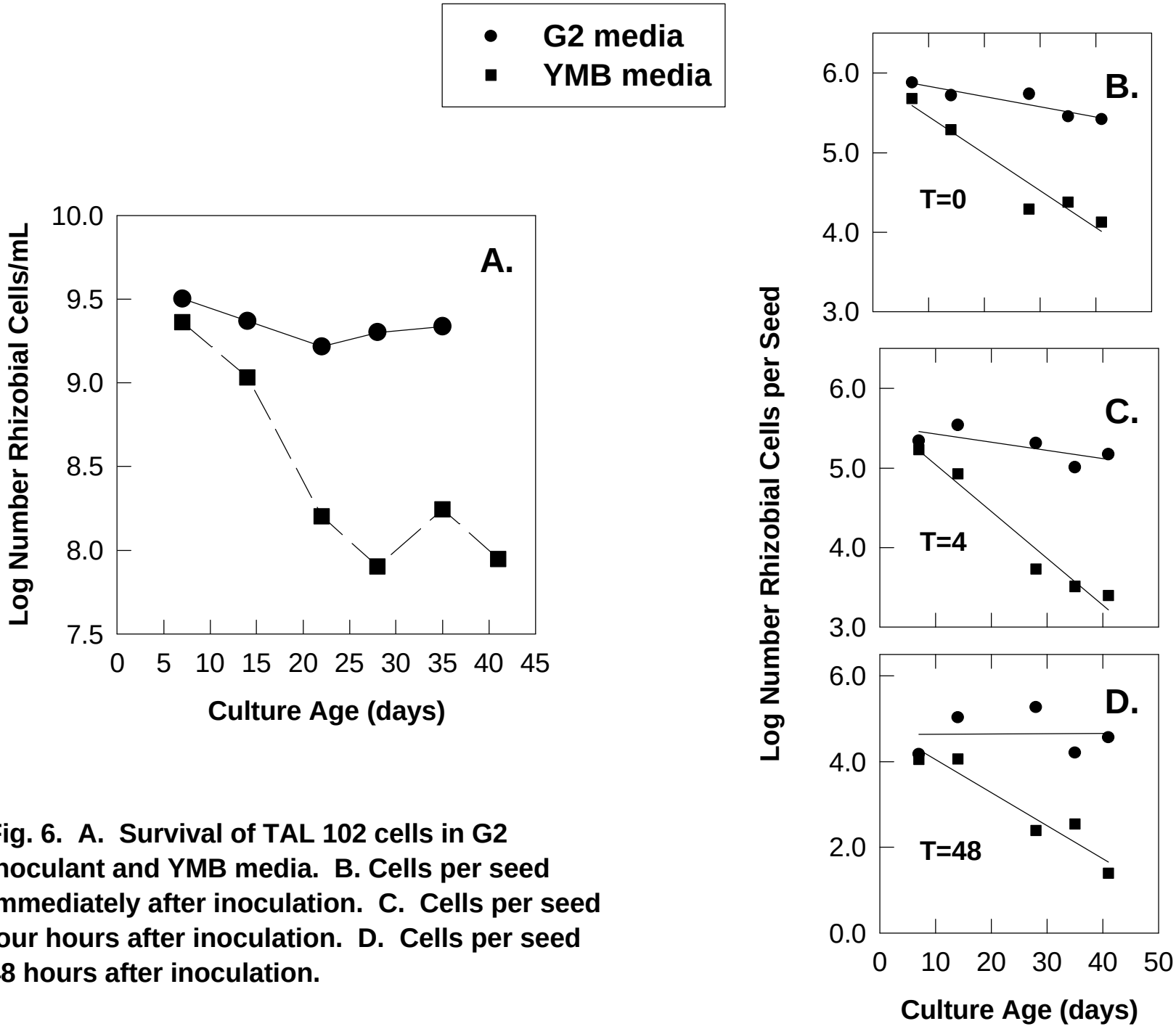



**Fig. 6. A. Survival of TAL 102 cells in G2 inoculant and YMB media. B. Cells per seed immediately after inoculation. C. Cells per seed four hours after inoculation. D. Cells per seed 48 hours after inoculation.**

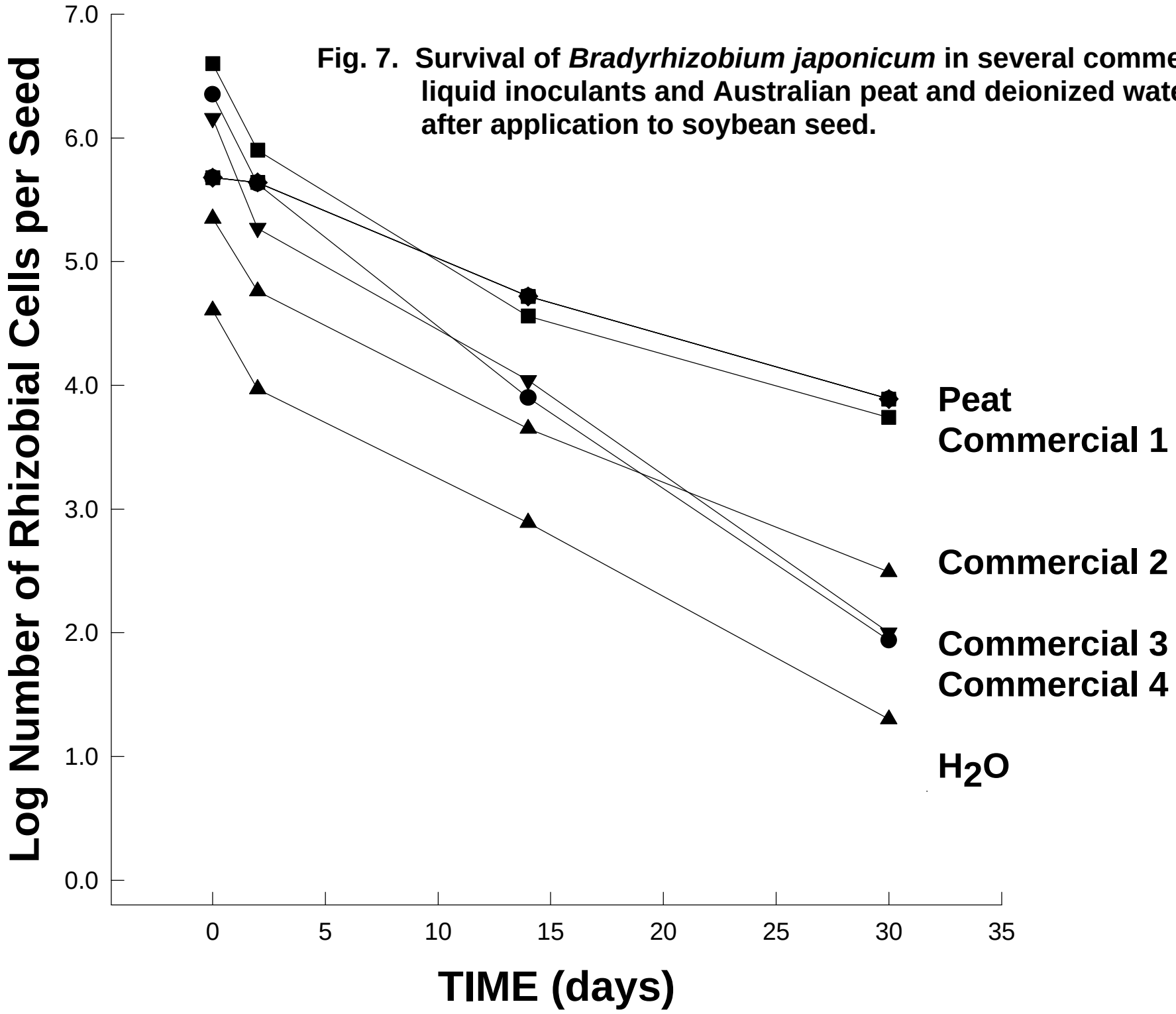


Fig. 7. Survival of *Bradyrhizobium japonicum* in several commercial liquid inoculants and Australian peat and deionized water after application to soybean seed.
Log Number of Rhizobial Cells per Seed
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
5
10
15
20
25
30
35
TIME (days)
Peat
Commercial 1
Commercial 2
Commercial 3
Commercial 4
$H_2O$