Viability of *Bradyrhizobium japanicum* on Soybean Seeds Enhanced by Magnetite Nanoparticles during Desiccation

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Abstract—The aim of this study was to investigate whether magnetite nanoparticles affect the viability of *Bradyrhizobium japanicum* cells residing on the surface of soybean seeds during desiccation. Different concentrations of nanoparticles suspended in liquid medium, mixed with and adhering to *Bradyrhizobium japanicum*, were investigated at two temperatures, using both soybean seeds and glass beads as surrogates. Statistical design was a complete randomized block (CRB) in a factorial 6×2×2×6 experimental arrangement with four replications. The most important variable was the viability of *Bradyrhizobium* on the surface of the seeds. The nanoparticles increased *Bradyrhizobium* viability and inoculated seeds stored at low temperature had greater viability when nanoparticles had been added. At the optimum nanoparticle concentration, 50% bacterium viability on the seeds was retained after 5 days at 4ºC. Possible explanations for the observed effects are proposed.

Keywords—*Bradyrhizobium japanicum*, magnetite nanoparticles, soybean seed, viability.

I. INTRODUCTION

BRADYRHIZOBIUM *japanicum* are Gram-negative bacteria living in symbiotic association with certain plants and enhancing their nitrogen fixation rate. With the trend toward the use of liquid inoculants for treating seed prior to sale, the issue of survival of *Bradyrhizobium* cells on seeds has gained importance. One major impediment to artificial inoculation is the rapid death of the bacteria when applied to seeds or to the soil [7],[15]-[16]. Desiccation of seeds has been the assumed cause of death of *Bradyrhizobium* cells on seeds, and many laboratory studies of desiccation stress and resistance in diverse strains of *Bradyrhizobium* have been reported [18]. In many of these studies, coating the seeds with various materials (e.g., clay, peat, sugars, Gum Arabic) supposedly to enhance bacterial association has been tested [16], [18]-[19], but only relatively small and/or inconsistent improvements have been obtained [15]-[16].

In the only study directly comparable with this report, the death of *Bradyrhizobium* sp. applied to lupin seeds in a peat inoculant was reported to be 95% after a few hours and 99% after 22 h [15]. The present report extends the previous observations to other strategies for increasing the viability of *Bradyrhizobium* cells on seed, namely the use of magnetite nanoparticles. Because of their vast specific surface area, they have the potential to intervene in surface interaction either directly at the seed-bacterium interface or indirectly by modulating the local environment.

II. MATERIAL AND METHOD

A. Bacterial survival on seeds

Seeds of the soybean *Glycine max* L. Merr (cultivar L.504) were obtained from the Safi Abad Agriculture Research Centre, Dezful, Iran. Bacterial culture was conducted as previously described [7]-[17]. At the stationary phase (with a population of 10^9 ml^{-1} of the viable cells) 250±1 μl of the medium mixed as previously described [7]. This was achieved with different concentrations of magnetite nanoparticles were applied to each soybean seed or glass bead (diameter 4 or 5 mm). Seeds and glass beads were grouped into two sets and each set was allocated six sterilized 250 ml flasks, each flask containing 20 seeds or glass beads. To each flask 125 μl of 40% Gum Arabic was added. The flask was gently shaken by hand for 1 min. Seeds were then tipped out, dried on clean paper for 3-5 min and then placed in a fresh flask and inoculated with 20 μl of liquid medium containing 10^8 or 10^9 *B. japanicum* ml^{-1} and nanoparticles), again slowly shaken by hand for 1 min and then again tipped out and dried on clean paper for 3-5 min at room temperature. When the seeds and glass beads appeared dry they were placed in new flasks and kept at 25 ºC and 4 ºC for 9 days, and repeated for each concentration of magnetite nanoparticles. Treatments are defined in Table 1. Statistical design was a complete randomized block (CRB) in a factorial 6×2×2×6 experimental arrangement with four replications, and the statistical calculations were facilitated by MSTAT-C software.

B. Preparation of magnetite nanoparticles

A 0.5 M solution of sodium hydroxide (about 125 ml) was poured into a three-necked flask under nitrogen gas with vigorous stirring at 65 ºC. 12.5 ml of an equimolar mixture of iron (II) and iron (III) (counterion = Cl$^-$) (each 0.9 M) was prepared in deaerated distilled water and further purged with nitrogen gas for 30 min. Then the iron solution was added dropwise to the sodium hydroxide during 30 min while stirring vigorously. The suspensions were separated and purified by centrifuging at 20,000 *g* and resuspending three times in water

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and then HCl. The particles were finally dried in a vacuum oven at 70-80 ºC.

TABLE I
DEFINITION OF TREATMENTS

Factor	Designation	Unit	Values
1	Desiccation time (D)	h	0, 24, 48, 72, 96, 216
2	Temperature (T)	ºC	4, 25
3	(Surrogate) Seeds (SG)	-	Soybean seeds, glass Beads
4	Nanoparticle concentration (N_P)	µg ml^{-1}	0, 20, 40, 60, 80, 100

C. Determination of viability

In this paper, viability is defined as N_I, the number of viable cells ml^{-1}. The viability was first measured immediately after inoculation of the SG with liquid media. 20 seeds or glass beads were removed from each flask and divided into four subsamples (i.e., 5 seeds or glass beads) in test tubes containing distilled water (10 ml). After vortexing for 2 min, one ml from each tube was removed and six times sequentially fivefold diluted into new test tubes. To count the bacteria, twice 100 µl of each of these six tubes were removed and spread on two 9 cm Petri dishes containing YMA solid medium incorporating Congo Red[†] to ensure and indicate the absence of invasive foreign strains and Brilliant Green to suppress fungal growth[‡] during incubation. The Petri dishes were immediately transferred to an incubator and kept at 28-30 ºC for 6-8 days. Counting the numbers of colony forming units N_f on the surfaces of the Petri dishes was started after 5-6 days [17]. Similar determinations were carried out at the different times after inoculation.

III. RESULTS

A. Effect of desiccation duration

According to the analysis of variance (Table 2), desiccation significantly decreased the viability. Viability was initially greater on seeds than on glass beads, latterly less. 50% of the initial population of *Bradyrhizobium* cells on the seeds was lost after 9 days and on the glass beads after a slightly longer interval (Fig. 1). During desiccation, viability of *Bradyrhizobium* decreased more slowly at 4 ºC than at 25 ºC. 50% of the *Bradyrhizobium* cells was lost after 6 days at 25 ºC, while at 4 ºC this loss was delayed by 2–3 days (Fig. 1).

† Congo Red is an indicator to detect *Bradyrhizobium* among other agrobacteria. It inhibits contaminants like penicillin-susceptible stains, hence colonies of rhizobium stand out as white, translucent, glistening and elevated [17].
‡ Brilliant Green also inhibits gram-positive bacteria [17].

TABLE II
ANALYSIS OF VARIANCE OF THE VIABILITY

Sources of Variation	df [†]	Mean square variation of N_I
Replication (R)	3	
Desiccation time (D)	5	343.5 [ab]
Temperature (T)	1	30.4 [ab]
Seed / Glass beads (SG)	1	2.01 [n.s.]
Nanoparticle concentration (N_P)	5	20.33 [ab]
D × T	5	5.35 [a]
D × SG	5	1.02 [n.s.]
D × N_P	25	4.03 [ab]
T × SG	1	12.64 [a]
T × N_P	5	5.36 [a]
SG × N_P	5	6.10 [a]
D × T × SG	5	1.70 [n.s.]
D × T × N_P	25	2.4 [a]
T × SG × N_P	5	2.3 [n.s.]
D × T × SG × N_P	25	0.57 [n.s.]
R × D × T × SG × N_P	429	2.4

† number of degrees of freedom; a and b F test indicates significance at $P < 0.05$ and $P < 0.01$, respectively; n.s. indicates that there is no significant difference.
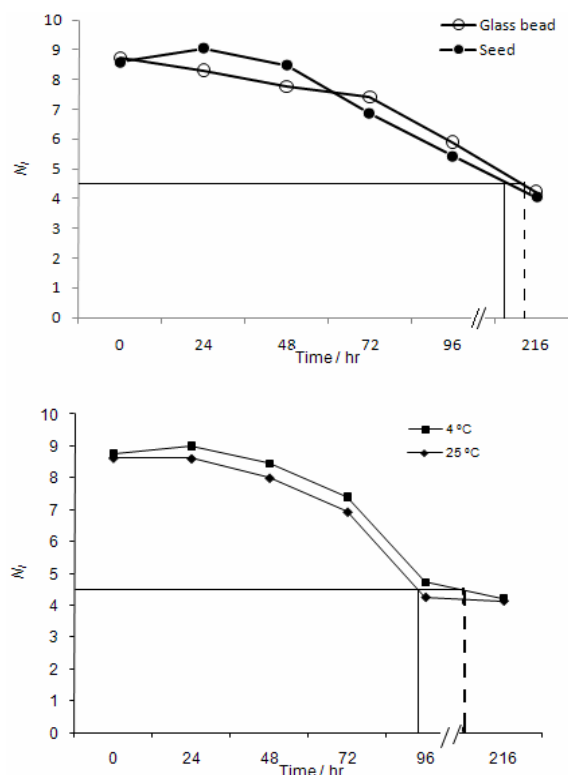

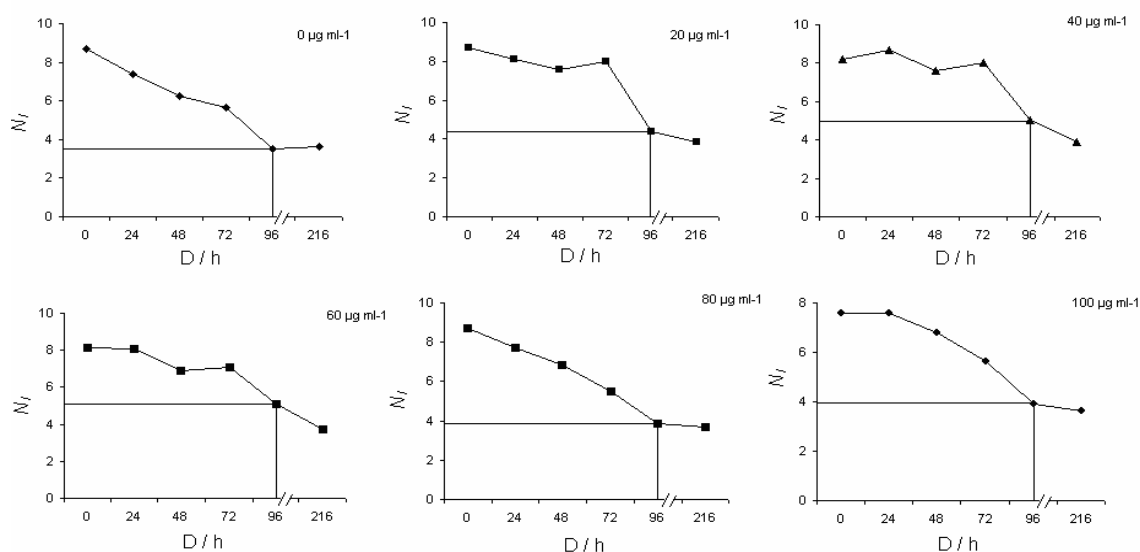Fig. 1 Effects of temperature and SG on the viability index for increasing desiccation times

Fig. 2 Effects of nanoparticles on *B. japanicum* viability during desiccation

The results indicated that the combined effects of desiccation times and nanoparticles had a significant effect on the cell viability at $P < 0.05$ (Table I). Comparison of different concentrations of nanoparticles at different desiccation times illustrated that the 20 and 40 μg ml^{-1} concentrations of nanoparticles could almost preserve the primary population of bacterial cells for 4 days (Fig. 2).

B. Effect of temperature

According to the analysis of variance (Table 2) the viability was significantly altered by temperature at $P < 0.01$, and a greater number of viable cells was obtained at 4 °C (Fig. 3). Cooling to 4 °C significantly increased viability on SG; persistence of bacteria on the seeds was greater than on the glass beads (Fig. 4). The combined effects of temperature and nanoparticles resulted in significantly increased cell viability at $P < 0.05$ (Table II and Fig. 5).

C. Effect of SG

The results indicated that the third factor (seeds, glass beads) had no significant effect on *Bradyrhizobium* viability (Table II and Fig. 6), but in combination with temperature *Bradyrhizobium japanicum* could be significantly preserved on seeds at 4 °C with $P < 0.05$ (Table II), whereas at 25 °C viability was lower (Fig. 4). Likewise, combined effects of SG and nanoparticles significantly increased viability at $P < 0.05$ (Table II). Overall, viability was greater on glass beads at low concentrations of the nanoparticles and on the seeds at high concentrations (Fig. 7).
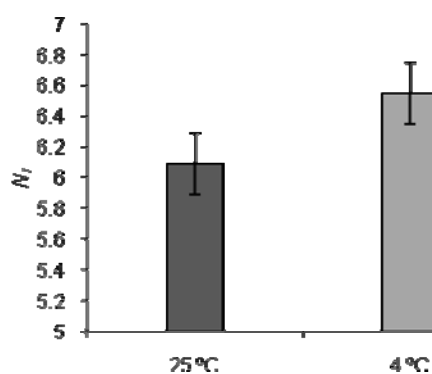

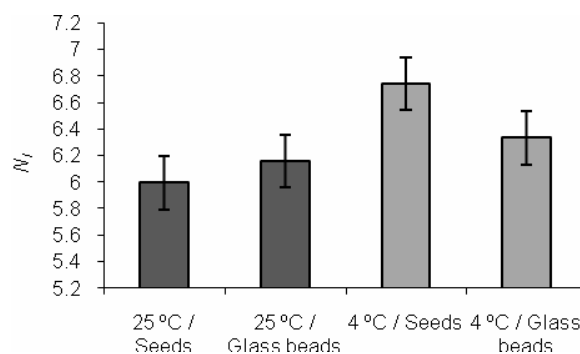Fig. 3 Effect of temperature on the viability of *B. japanicum*

Fig. 4 Combined effects of temperature and SG on *B. japanicum* viability
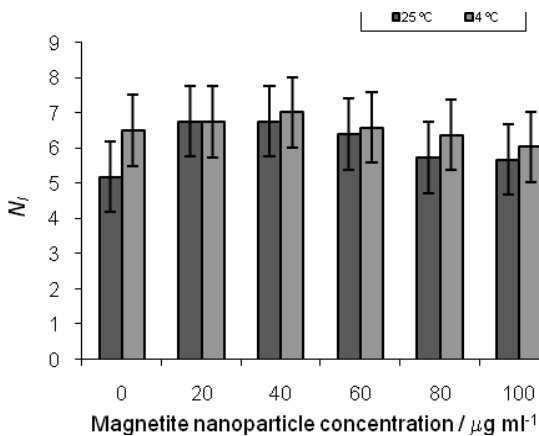
Fig. 5 Combined effects of temperature and nanoparticles on *B. japanicum* viability
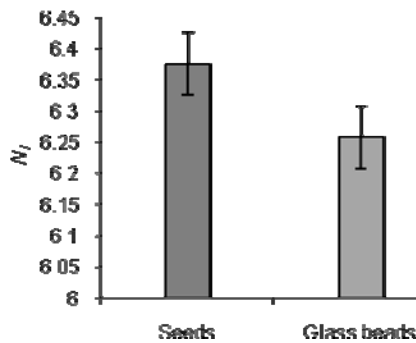

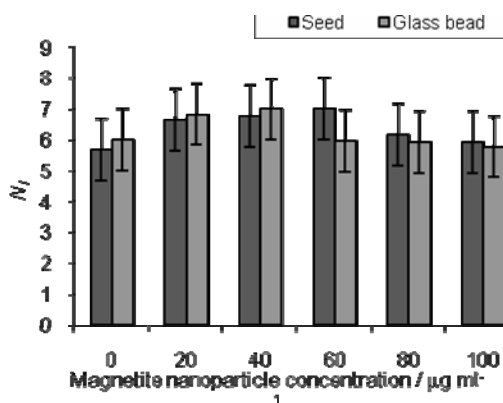Fig. 6 Effect of SG on the viability of *B. japanicum*

Fig. 7 Combined effects of SG and nanoparticles on *B. japanicum* viability

§ Previous studies have shown that temperature as an environmental factor can induce a physiological response, e.g., the production of heat shock proteins and chaperones, or the accumulation of compounds that decrease the membrane midpoint transition temperature, such as trehalose [6, 21, 22].
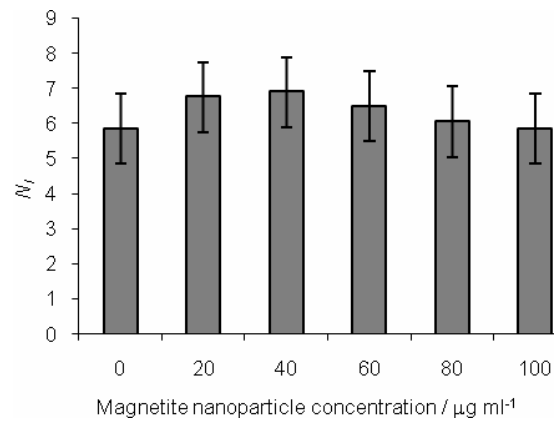

Fig. 8 Effect of nanoparticles on the viability of *B. japanicum*

D. Effect of magnetite nanoparticles

According to the analysis of variance (Table II) viability in the presence of nanoparticles significantly increased ($P < 0.01$). Likewise the results illustrated that the concentrations 40 and 20 μg ml^{-1} of nanoparticles gave the greatest N_l, about 8×10^6 and 6×10^6 viable cells ml^{-1} respectively (Fig. 8). *Bradyrhizobium* viability under the triple effects of desiccation, temperature and nanoparticles only became significant at $P < 0.05$ (Table II).

III. DISCUSSION

Although, several studies of desiccation stress on the *B. japanicum* have been conducted, the mechanisms underlying bacterial death have not been clarified; alteration of membrane composition [3] and membrane permeability, and alteration of the concentration of salt and trehalose in the medium have been suggested as underlying potential mechanisms [19].

According to our results several factors affect the viability of *Bradyrhizobium* on seeds. In particular, adding nanoparticles to the medium indicated that they could increase cell viability under desiccation. The following possible explanations seem worth considerating:

1. Nanoparticles induce secretion of some materials from the bacterium which enable it to protect itself against environmental stress. Previous work has revealed the favorable effect of the disaccharide trehalose and secreted lipopolysaccharides in combating dehydration and, hence, desiccation stresses. Trehalose has indeed many functions when the bacterial cell is under stress conditions, for instance trehalose acts as an energy reserve; as a stabilizer of proteins and membranes, protecting them from dehydration (desiccation), heat or cold, and damage by oxygen radicals; as a phase-sensing compound; as a growth regulator; and as a structural component of the bacterial cell wall [6], [21]-[22].

2. Nanoparticles bind the cell wall through by hydrogen bonding between its oxygen or Fe and polar residues in the protein and lipopolysaccharide molecules in the cell wall.

3. Nanoparticles scavenge free radicals, binding with reactive oxygen species (ROS: O_2^-, H_2O_2, and $HO^.$ produced by the cell metabolism during growth).

4. Preserving a moist layer on the seeds is already known to be important for the survival of bacteria [18]-[19]. According to our results the adsorption of water molecules at the surface of magnetite nanoparticles has been suggested. The surface of nanoparticles becomes covered by hydroxyl groups coordinated to the underlying Fe atoms upon water adsorption, and this absorption is followed by further adsorption of water molecules hydrogen-bonded to the surface hydroxyl (OH) groups forming a highly density structure in the subsequent layers.

5. During desiccation, decreasing water content causes the accumulation inside the bacterium of salts and other compounds that lead to osmotic and salt stress, but could increase the secreted solutes (e.g., disaccharide trehalose) and increase osmolytes in the bacterial cytosol, either obtained by uptake from the environment (exogenous) or through fresh biosynthesis (endogenous) promoted by adding nanoparticles [12], which therefore indirectly contributes to cell turgor by the sharp increase in the level of osmolytes [21]. The combination of nanoparticles and desiccation significantly increased cell viability (Table 2), although direct comparison of these results to other in the literature is difficult because of different experimental methods [19],[4], [11]-[14].

6. Temperature is involved in survival under desiccation through triggering membrane phase changes during drying or rewetting, leading to the loss of membrane integrity [21]. Survival is sensitive to fluctuations of storage temperature by more than 4-7 °C, because the membrane is more sentive to temperature changes when the cell wall is water-poor [10]-[21]. During rehydration the reverse transition (gel → liquid crystal) occurs but defects may thereby be introduced, leading leakage.

We propose that nanoparticles added to the medium adsorb on the membrane and preventthe phase transition. Our low temperature results (Table II) suggest that the nanoparticles control desiccation effects, and hence offer a route to achieving preservation of cell viability on the surface of seeds. This result is consistent with the fact that the low temperature can enhance the cells viability [2], [5], [9], [13], [21]§.

§ Previous studies have shown that temperature as an environmental factor can induce a physiological response, e.g., the production of heat shock proteins and chaperones, or the accumulation of compounds that decrease the membrane midpoint transition temperature, such as trehalose [6, 21, 22].

IV. CONCLUSION

Viability of *Bradyrhizobium japanicum* is favourably affected by magnetite nanoparticles. Although all concentrations of nanoparticles could increase cell viability during desiccation and at low temperature, at concentrations of 80 and 100 µg ml^{-1} the effects were almost the same as without nanoparticles. Also this study suggest that storage temperatures 4-7°C could preserve almost 50% of the bacterial cells for 4-5 days more than at ambient temperature (25 °C). We reasonably propose that the magnetite nanoparticles increase the tolerance of *Bradyrhizobium japanicum* to desiccation by protecting both the bacterial membranes and structural proteins, by creating a water reservoir on the inoculated seeds.

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