

## Pea Protein Alginate Encapsulated *Bacillus subtilis* B26, a Plant Biostimulant, Provides Controlled Release and Increased Storage Survival

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### Abstract

Plant growth promoting bacteria (PGPB) represent a wide variety of soil and endophytic bacteria that have the ability to promote growth or to protect against stress of its host plant. Encapsulation of PGPB, using vegetable proteins instead of animal or petroleum-derived polymers, is a new technology to crop production and protection. Pea protein isolates (PPI) alginate capsules were synthesized and used for the protection and delivery of *Bacillus subtilis* B26 as plant inoculum for agricultural applications. The capsules provided a protective site to B26 strain allowing good survival, between 45 to 50%, after 112 days of incubation at different temperatures. The loaded microcapsules sustained a large population of bacteria, up to 8.3 LogCFUg<sup>-1</sup> of soil after 3 weeks post application. The soil concentration stabilized to 7.3 Log CFUg<sup>-1</sup> after 8 weeks post application. Capsules loaded with B26, once incorporated in the soil, successfully colonized test plants, and cell numbers inside plant tissues were sustained at 3.5 and 3.3 Log 7.3 CFUg<sup>-1</sup> in the root and the shoot respectively. These results indicate that PPI-alginate technology represents a good choice for commercial application of *B. subtilis* B26 in agriculture.

**Keywords:** *Bacillus subtilis*; Bio-inoculant; (PPI)-Alginate capsules; *Brachypodium*; Timothy

### Introduction

Plant growth promoting bacteria (PGPB) represent a wide variety of soil and endophytic bacteria which, when grown in association with a host plant, result in stimulation of growth of their host. Growth promotion by PGPB can be either through phytostimulation, biofertilization, bioremediation, plant stress control or biocontrol of plant pathogens [1,2]. The immediate response of plants to PGPB varies considerably depending on the bacteria, plant species, soil type, inoculant density and environmental conditions, resulting in a progressive decline of the inoculated bacterial density and a failure to elicit plant response. Therefore, a major goal of inoculant formulation is to provide suitable microenvironment for survival in the soil [3]. Formulation of inoculant carrier is the industrial art of converting a promising laboratory bacterium to a commercial field product [4]. Immobilization of microbial cells into a polymeric matrix has proved to be advantageous over direct soil inoculation. The main goals of encapsulation of PGPB are to protect them from harsh environment, to reduce microbial competition, and to release them gradually to facilitate the colonization of plant roots [5,6]. Encapsulation of living cells in polymeric gels is a well-established technology suitable for a wide range of applications [4,7]. Several studies thus far have used alginate matrix as the encapsulating material since it forms microbeads instantaneously in the presence of polyvalent cations such as calcium [6,8]. Alginate beads have several positive attributes: (i) they are capable of entrapping a sufficient number of bacteria [9]; (ii) they protect the cells by providing a pre-defined and constant microenvironment thus allowing the cells to survive and maintain metabolic activity for extended period of time, and (iii) they provide a controlled release of microorganisms as well as serve as energy source for the microorganisms [10]. Natural polysaccharides and proteins such as starch, malt dextrin, gum Arabic, pectin, chitosan, alginate and legume proteins are widely used for encapsulation of bacterial cells [11,12]. Vegetable polymers present a number of positive attributes including biocompatibility, biodegradability, decent amphiphilic ability, water solubility and emulsifying and foaming capacities [12]. Recently, microcapsules based on Pea Proteins Isolate (PPI) and alginate, were

developed for probiotic use [11,13,14] and this entrapment technology has provided probiotic protection and a nutrient base [11]. To the best of our knowledge, PPI-alginate biopolymer has never been used on plant growth promoting bacteria for agricultural applications.

We previously demonstrated that *Bacillus subtilis* strain B26, is a growth enhancer of switchgrass (*Panicum virgatum* L.) and *Brachypodium distachion* seedlings [15,16]. The strain's ability to migrate from the roots to aerial parts of the plant and thus behaving as a competent endophyte [17] was also demonstrated. *B. subtilis* B26 conferred resistance against drought stress in the model grass plant *Brachypodium*, which is linked to the upregulation of expression of several drought-responsive genes, the modulation of the DNA methylation process, and the increase in total soluble sugars and starch [16]. These qualities suggest that the endophytic ability of this strain is a biological requirement for survival in nature and it has a strong potential as bio-stimulant for biomass, enhancement of grasses, and enhancement of plant's defense against abiotic stress such as drought stress.

This study was undertaken as proof-of-concept to demonstrate the usefulness of extrusion-based PPI-alginate capsules for the protection and delivery of *B. subtilis* B26 as soil inoculum. The effect on storage, performance and the efficacy of bead encapsulated strain B26 as compared to free cell inoculum was assessed using two plant species

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known to be positively affected by B26 isolate [16].

## Materials and Methods

### Maintenance and preparation of *B. subtilis* B26 inoculum

The *B. subtilis* strain B26, previously isolated from switchgrass and fully characterized [15], was maintained on Luria Broth (LB) (1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl) with glycerol (25% final volume) and stored at -80°C. *B. subtilis* B26 was revived on LBA (1.5% Agar) plates. The inoculum was prepared by placing a single colony of *B. subtilis* B26 in 250 mL of LB and incubated for 18 h at 37°C until an OD600 of 0.7, representing colony forming units (CFU) of  $3.38 \times 10^8$ /mL was reached on a shaker at 250 rpm to the mid-log phase, pelleted by centrifugation, washed and suspended in sterile distilled water.

### Preparation of alginate capsules and microencapsulation

Pea protein isolates (PPI) (Propulse N™) containing 81.73% protein, <10.3% sugars, <0.7% starch, <3.4% moisture, <0.5% fat, <4.0% ash was obtained from Nutri-Pea Ltd. (Portage la Prairie, MB, Canada). Alginate acid sodium salt with low viscosity (viscosity of 1% aq. solution: <300cps) was purchased from MP biomedical (Solon, OH, USA). Calcium chloride (CaCl<sub>2</sub>) dehydrate, hydrochloric acid (HCl) and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium hydroxide was purchased from EMD (Damstadt, Germany). Tryptone, Yeast Extract, NaCl, and Agar were purchased from Difco (Franklin Lakes, NJ, USA). Ammonium sulphate, Potassium phosphate dibasic trihydrate, monopotassium phosphate, trisodium citrate and magnesium sulfate heptahydrate were purchased from Sigma-Aldrich (Co., St. Louis, MO). Glycerol was purchased from ThermoFisher Scientific (Waltham, MA). The qPCR SYBRII master mix and the ROX were purchased from Agilent Technologies (Morrisville, NC, USA) while BSA was purchased from Sigma-Aldrich (Oakville, On, Canada). The PPI-alginate microcapsules were prepared via extrusion technology [11]. The distilled water and the glassware were sterilized by autoclaving before the encapsulation process. The 3.6% (w/v) protein solution was prepared by dissolving pea protein isolated powder in 0.05 M NaOH solution, and heated to 80°C for 30 min to denature and dissolve the proteins. The solution was cooled to room temperature in a cold water bath and then neutralized to pH 7 with 1M HCl. The solution was reheated to 80°C and the alginate powder was added to produce a final concentration of 0.9% (w/v). The solution was maintained at 80°C under magnetic stir for 30 min to dissolve the alginate powder. The solution eventually cooled down to room temperature. The bacteria suspension was subsequently added to the solution at a bacteria-to-polymer ratio of 1:10 (v/v), a similar ratio used for PPI-alginate microcapsules [11]. The bacteria-loaded microcapsules were formed via extrusion of the bacteria-polymer solution through a 26 G needle into a 0.05 M CaCl<sub>2</sub> solution. The resulting microcapsules were allowed to harden in the CaCl<sub>2</sub> solution for 30 minutes before they were collected and rinsed with sterilized water. Finally the microcapsules were flash-frozen with liquid nitrogen and then lyophilized using a vacuum freeze-dryer (7670520, Labconco Co., Kansas City, USA) for 48 h.

### Examination of microcapsules by scanning electron microscopy

The morphological properties of the microcapsules loaded or not loaded with *B. subtilis* B26 were observed by scanning electron microscopy (SEM) (Quanta Feg 450, Fei, Hillsboro, USA) at an

accelerated voltage of 10.0 kiloVolts. The microcapsules were coated with platinum beads (Leica Microsystems EM ACE600 High Resolution Coater, Nubloch, DL) before observation. The microcapsules were lightly crushed to expose the inside of the spheres.

### Survival and viable bacterial counts from microcapsules

Freeze-dried microcapsules (0.1 g) were suspended in 9.9 mL of sterile modified phosphate buffer [18] consisting of ammonium sulphate 0.15 M, potassium phosphate dibasic trihydrate 0.8 M, monopotassium phosphate 0.44 M, trisodium citrate 40 mM and magnesium sulfate heptahydrate 8 mM. The microcapsule and buffer (pH 7) were incubated for 1 hr at room temperature and shaken at 250 rpm. Bacteria entrapped in the microcapsules were serially diluted and enumerated by the plate count method [14,16]. The *B. subtilis* B26 inoculum broth used for every batch of microcapsules was also serially diluted to provide the actual amount of CFU/mL in the microencapsulation process. Three technical replicates (three plates) were used to estimate the amount of viable cells for each of the four biological replicates performed in the experiment. The survival rate estimated as % of the bacteria was calculated by applying the equation:

$$\text{Encapsulation yield} = (N_{\text{FD}} \times M_{\text{FD}}) / (N_{\text{E}} \times 10) \quad (1)$$

Where  $N_{\text{FD}}$  (CFU/g) is the concentration of viable cells after freeze drying while  $M_{\text{FD}}$  (g) is the mass of freeze dried microspheres.  $N_{\text{E}}$  (CFU/ml) represents the concentration of viable cells input in the microspheres of the liquid culture and 10 (ml) are the volume of culture used.

### Recovery and viability of bacteria from microcapsules stored at different temperatures

In order to investigate the shelf life of encapsulated bacteria under various storage conditions, they were transferred into 50 mL falcon tubes (Fisher Scientific, Fair Lawn, NJ) and covered with aluminum foil to prevent from light degradation. The tubes were incubated at three different temperatures: (1) ambient temperature of  $22 \pm 1^\circ\text{C}$ ; (2)  $4 \pm 1^\circ\text{C}$ ; (3)  $-15 \pm 1^\circ\text{C}$  [19]. Freeze-dried free cells (non-microencapsulated *B. subtilis* B26) served as control treatment and were subjected to the same incubation temperatures. An aliquot of 0.1 mL of cell suspension was transferred into a 1.5 mL centrifuge tube (Eppendorf, Hamburg, DK). The tubes were centrifuged using a micro-centrifuge at 8000 rpm for 10 min and the supernatant was removed. The pelleted bacterial cells were freeze-dried for 48 h and stored under the same three temperatures as the microencapsulated bacteria (microcapsules). A minimum of three biological replicates was performed. The experiment was repeated twice. Encapsulated bacteria (0.1 g) were withdrawn every 7 days for the first 56 days and after 112 days at each storage temperature. The viability of bacteria, estimated as CFU, was confirmed by serial dilution plating on LBA plates incubated at 37°C for 14 hrs. The viability of the free non-microencapsulated bacteria (control treatment) was confirmed by rehydrating the bacterial pellets with 900 µL of modified phosphate buffer and shaking for 1 hour following the same condition as described above. Freeze-dried bacteria were tested for viability every 14 days for 56 days.

### Recolonization capacity of microcapsules

**Growth conditions:** To determine whether the entrapped B26 in microcapsules have the ability to recolonize plants, the model plant *Brachypodium distachyon* from the inbred line Bd21 [20] and timothy (*Phleum pratense* L.) cultivar Novio were used. Seeds were surface sterilized by sequentially immersing them in solutions of 70% ethanol

for 30 seconds and 1.3% solution of sodium hypochlorite for 4 minutes before rinsing them three times in sterile water [21]. Pots of 10 × 10 cm (ITML, Brantford, On, Canada) filled with sterilized Agro Mix® G6 (Fafard et frères, Qc, Canada) containing ten seeds per pot were used. Prior to use, the pots were surface sterilized for 12 hr in 0.1% NaOCl and rinsed with distilled water. The Agro Mix® G6 was previously autoclaved for 3 hr at 121°C on three constitutive days. Plants were stratified at 4°C for 7 days after which they were placed in a climatically controlled chamber (Conviron, Winnipeg, Mb, Canada) under a 16-hr photoperiod with a light intensity of 150 µmoles/m<sup>2</sup>/s and a day/night temperature regime of 25°C/23°C. Plants were watered three times per week with sterile distilled water and fertilized every 14 days with 100 mL of a solution of 2 g/L of N-P-K fertilizer 20-20-20 (Plantprod, Laval, Qc, Canada) per pot. Plants were thinned to five seedlings per pot after 3 weeks of growth and the experiment was kept going for another 5 weeks. The experiment was repeated twice in different growth chambers to control any confounding growth chamber effect.

**Inoculation:** Two different inoculation methods were evaluated. The first consisted of incorporating the loaded microcapsules in the top 3 cm of the potting mix prior to seeding (pre-planting method). The second consisted of spreading the loaded microcapsules on the surface of the potting mix and around the crowns of 21-day-old *Brachypodium* and timothy plants (post-planting method). For both methods, the amount of microcapsules was adjusted to provide 5 millions CFU per pot. Microcapsules containing no bacteria were used as control.

**Microbiological and molecular monitoring of *B. subtilis* B26:** Soil samples from the top 3 cm were collected every two weeks after 1 Week Post Application (WPA) and at harvest time (corresponding to 8 WPA), to evaluate the concentration of B26 in the soil. Special effort was made to remove the microcapsules from the samples in order to obtain true bacterial counts in the soil. One gram of soil, from inoculated and control plants, was diluted in 9 mL of phosphate buffer, shaken for 30 min, incubated at 95°C for 5 minutes, serially diluted in phosphate buffer and plated on LBA [22]. Plates were incubated at 37°C for 24 hr. CFU counts were determined per gram of soil fresh weight. Four biological replications per inoculation method were performed each time. A biological replicate consisted of two pots per plant species, each containing 5 seedlings. To ensure that B26 successfully and systemically colonized timothy and *Brachypodium*, bacteria cell numbers and DNA copy numbers were determined in the root and shoot tissues of the inoculated and the control plants. Surface sterilized tissue samples (200 mg) were homogenized, serially diluted in modified phosphate buffer and plated on LBA, following established procedures [15]. Plates were incubated at 37°C for 24 hr. Colony forming units (CFUs) were determined and calculated to CFU per gram of fresh weight of tissue. There were three biological replicates containing five plants for each treatment.

For DNA extraction, surface sterilized *Brachypodium* and timothy tissues were reduced to powder in liquid nitrogen, and genomic DNA was extracted from 200 mg of powdered tissue using the CTAB method [23]. Genomic DNA from *B. subtilis* B26 colonies was extracted by direct colony PCR [24]. Briefly, single colonies were mixed with sterile distilled water, incubated at 95°C followed by centrifugation. The supernatant was used as template DNA in conventional PCR assays. *B. subtilis* B26 amplicons from strain specific primers [15] were purified, cloned and used to build a standard curve for qPCR assays. The qPCR assay was performed exactly as previously described [16].

**Statistical analysis:** One-way ANOVA was performed using the

JMP 10.0 software (SAS Institute, Cary, NC, USA). The significance of the effect of the treatments was determined via Tukey HSD with a magnitude of the F-value ( $p < 0.05$ ). In the case of repeated experiment trials results were tested using Levene's test for equality of variance ( $p < 0.05$ ) and pooled if permitted.

## Results and Discussion

### Morphology and characteristics of the microcapsules

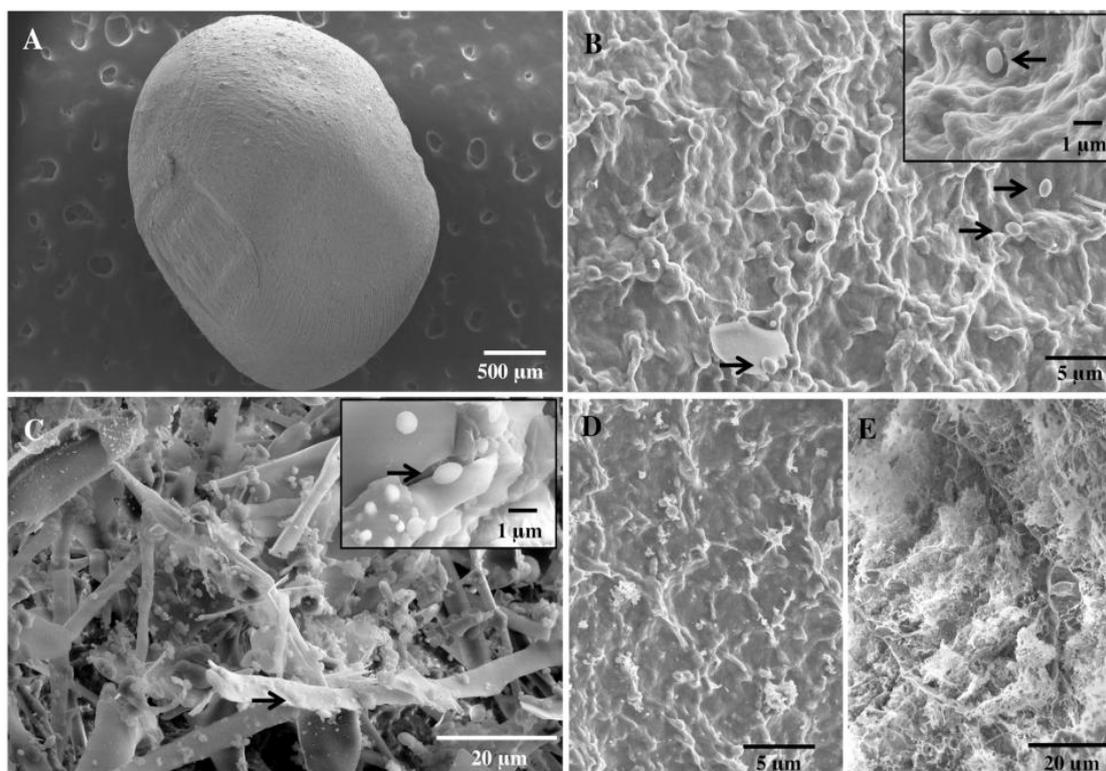
The external and internal microstructure of the freeze-dried microcapsules were observed using SEM (Figure 1). The microcapsules had an average weight of 2.4 mg with spherical forms having a diameter ranging between 2.5 and 3.5 mm, and had a relatively smooth surface (Figure 1A). At higher magnification (3600 ×), the outer topography of the microcapsules with bacteria (Figure 1B) and without bacteria (Figure 1D) contained ridges with cavities. A closer examination of the cavities showed that bacterial cells in the form of endospores were dislodged in the cavities and were also found on the ridges (Figure 1B inset). The internal layer of the microcapsules had pore-like microstructures with a diameter of 2 µm (Figure 1C). These structures contained bacterial endospores (Figure 1C and inset) as compared to microcapsules not loaded with bacteria (Figure 1E). It is estimated that each microcapsule contains about  $2.27 \times 10^5$  CFU (i.e.,  $1.20 \times 10^8$  CFU/g of capsule) of *B. subtilis* B26.

### Survival of *B. subtilis* B26

The successful survival of the encapsulated microbial cells depends on the choice of the polymer. In this study we have opted to use calcium alginate hydrogel that is gelled by ionic bonds and swells and dissolves in a solution containing phosphates, a chelating agent present in the soil [6]. Although several biomaterials are available, the alginate is preferred because the capsules are formed instantaneously by one-step gelling with cell loss reduced to a minimum [25] and the microbial cell density could be maintained as high as high as  $10^{10}$ - $10^{11}$  CFU.g<sup>-1</sup> of capsule [8]. In our study, we achieved a concentration of 8.08 Log CFU.g<sup>-1</sup> of bacteria in freeze-dried microcapsules and a survival rate of 14%; a concentration close to what was achieved under similar conditions (8.28 Log CFU.g<sup>-1</sup>) with *Lactococcus casei* [14]. Direct comparison of cell levels and survival rates in the alginate formulation enriched with PPI with others [8] could be misleading due to several factors including different conditions for alginate preparation and its enrichment with a nutrient base, bacterial free cells pelleted or in liquid form, and storage conditions of wet or dry capsules [8]. For example, the enrichment of alginate capsules with humic acid and glycerol to the alginate formulation increased the stability and survival rates (up to 25%) of microbial cells [26]. It was also reported that the addition of skim milk and clay improved encapsulated bacterial survival [6]. Another factor that could lead to a better survival rate is the use of spore-producing bacteria as they are more resistant to harsh environmental condition than vegetative cells [27].

### Effect of storage temperatures on the survival of *B. subtilis* under free and encapsulated state

Little is known about the effect of temperature on the storage survival of PGPB inside alginate microcapsules supplemented with natural protein. Calcium alginate alone (not in the form of microcapsule) was found to maintain the survival rate of the plant growth promoting bacteria *Pseudomonas striata* and *Bacillus polymyxa* at higher temperatures [28] over an extended period of time. The literature showed that encapsulation would increase the probiotic



**Figure 1:** Scanning Electron Microscopy micrographs of PPI-alginate microcapsules loaded or not with *Bacillus subtilis* B26. (1A) Surface of an intact microcapsule. (1B) Surface of an enriched microcapsule loaded with *B. subtilis* B26 showing the distribution of B26 cells. Inset. B26 endospore lodged in a cavity of the gel matrix. (1C) Inside a crushed loaded microcapsule. Inset. Arrow pointing to an endospore cell of B26. (1D) Surface of a microcapsule not loaded with B26. (1E) Inside a crushed non-loaded microcapsule.

bacteria survival rate during storage, especially at higher temperatures [14,29].

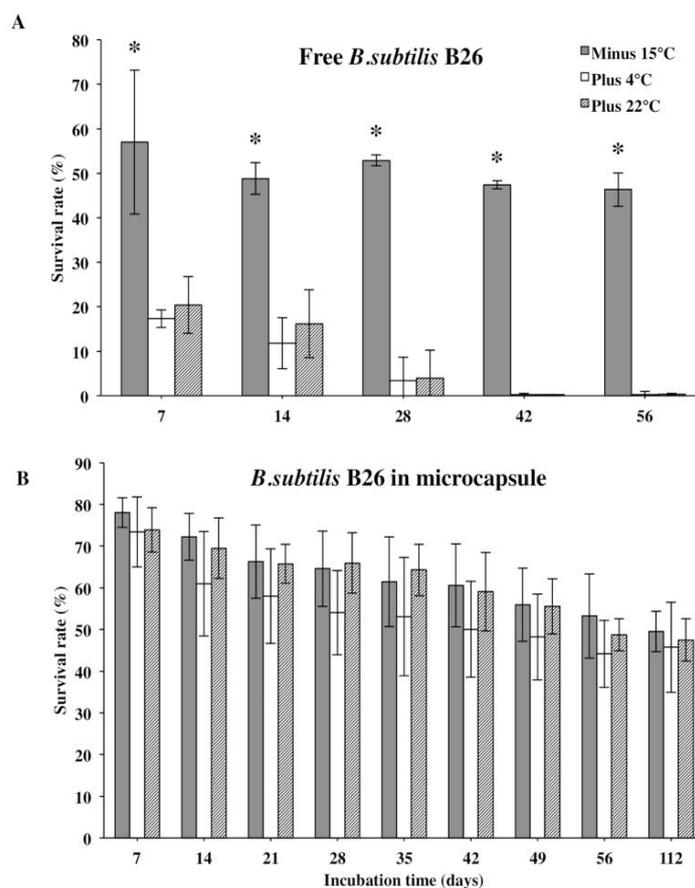
This lack of information prompted us to investigate this important factor. In order to assess the effect of storage temperature on the survival ability of *B. subtilis* B26 under free and encapsulated states, three storage temperature regimes were investigated: -15, +4 and +22°C representing the most likely storage temperature conditions available to farmers. The survival rate of free cells of B26 was evaluated every 14 days for 56 days (Figure 2A) and every 7 days for 112 days (Figure 2B) for the encapsulated cells. Survival rates of encapsulated cells over an extended period of incubation (112 days) were significantly higher and similar at all storage temperatures than those of free cells (Figure 2A and 2B). In contrast, the free cells showed significant differences in survival rates by 28 days of incubation (Figure 2A and 2B). The PPI-alginate matrix supported bacterial densities with stable survival rates even after almost 4 months of incubation, thus indicating that the encapsulation of bacterial cells pre-empted the detrimental effect of warmer temperatures. This is in accordance with other studies. The encapsulated cells had an increase in survival rates by 21, 56 and 53% at the respective temperatures of -15, +4 and +22°C as compared to those of free cells after 7 days of incubation (Figure 2B). At 56 days of incubation, the survival rates of encapsulated cells were 7, 44 and 48% greater than those reported for free cells at the same incubation temperatures (Figure 2A and 2B). Of interest, the survival rates of encapsulated cells at +22°C and -15°C were not significantly different. These results indicate that encapsulation protects the cells from sub-optimal storage temperatures. The porous nature of the alginate-gel

matrix as observed in SEM micrographs protected the cells against mechanical stress, facilitated the survival for prolonged storage periods and incubation temperatures and helped in cell release from the beads. Also additional supplementation of the alginate gel with PPI is presumed to enhance stability, provides protection for cell membranes against detrimental dehydration process during the freeze-drying operation [8,30], and nutrition in the form of protein, sugar, starch, lipid, and ash to the encapsulated cells [12]. Taken together, all the above attributes make this formulation a stable and protective microenvironment for bacterial cells.

The storage temperature affects greatly the survival rate of free cell. In this experiment, the high temperature quickly reduced the amount of viable cells, while the cold temperature reduced the biological activity, including enzymatic activity, spore germination and respiration rate [31]. Thus, a better survival rate was to be expected under such conditions. A report on *Lactobacillus* showed similar results [32]. The microcapsule, by protecting the cells membranes during the dehydration process, might have reduced cells susceptibility to these adverse conditions.

### Survival of microcapsule entrapped cells upon introduction into the soil

Incorporation of the alginate capsules in the soil prior to planting achieved the highest concentration of bacteria in the soil reaching 8.26 and 8.30 Log CFUg<sup>-1</sup> of soil fresh weight by 3 WPA of timothy and *Brachypodium*, respectively (Figure 3A). Cell numbers gradually decreased to 8.09 and 8.06 Log CFUg<sup>-1</sup> and stabilized at 7.29 and 7.32



**Figure 2:** Percent survival of *Bacillus subtilis* B26 free cells or encapsulated and incubated under different temperature conditions. (2A) Free cells of B26 (2B) Encapsulated B26 \* Represents a statistically significant difference  $p < 0.05$ .

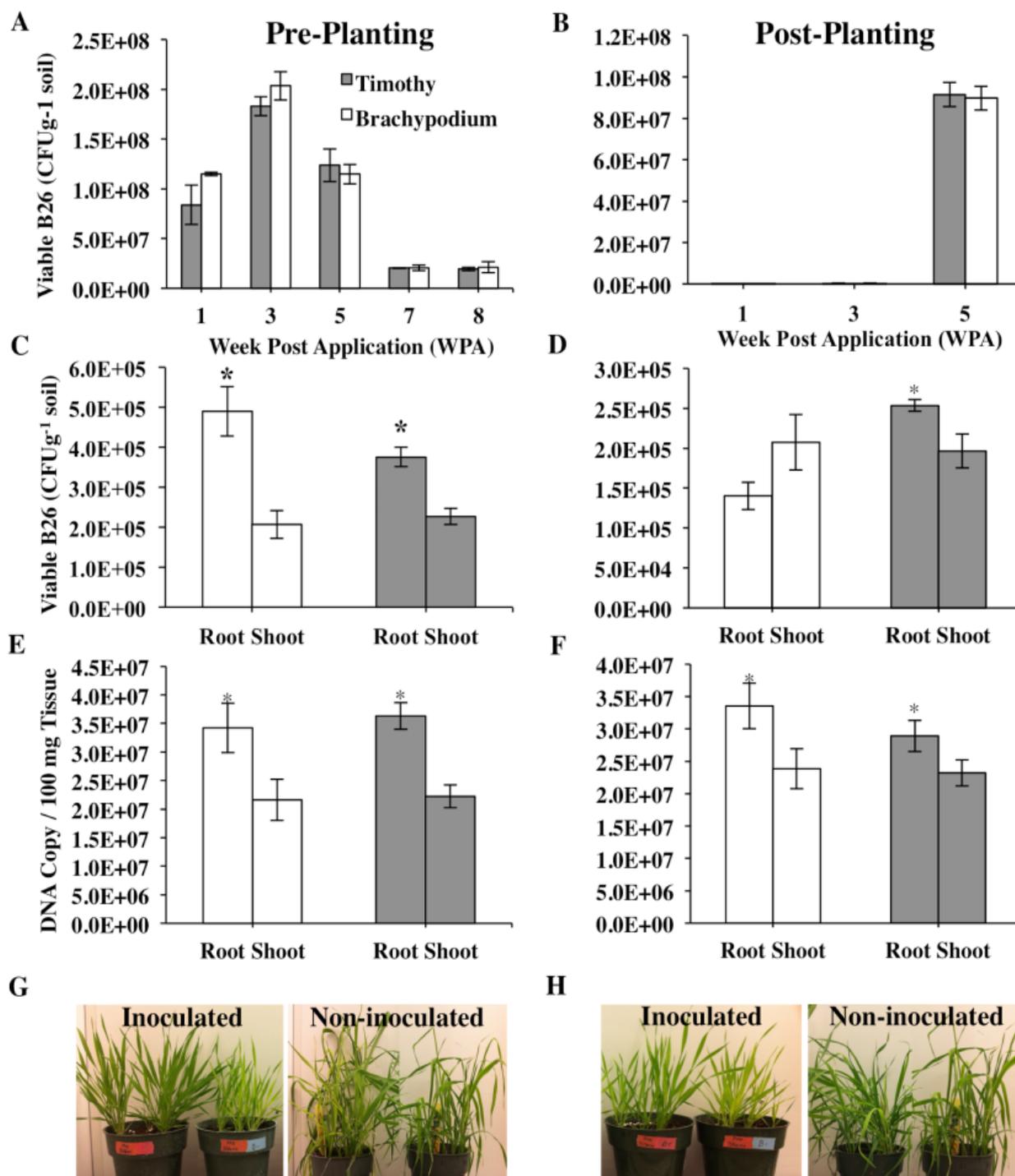
Log CFUg<sup>-1</sup> soil by 8 WPA for timothy and *Brachypodium*, respectively (Figure 3A). Lower cell numbers (7.95 and 7.96 Log CFUg<sup>-1</sup> soil) were observed when microcapsules were applied to the surface of the soil and around the base of both plants at 5 WPA (Figure 3B). However, it is worth mentioning that the concentration of bacteria in the post planting treatment (Figure 3B) may have not peaked by 5 WPA. Nevertheless, bacterial population levels in the soil in both treatments were maintained at threshold levels of 7 logs, a level exceeded to what has been previously reported for this strain (3 Logs) [16] and for other bacterial strains [33] when used as free cells in soil drench treatments. It is not surprising that initial high population densities of free bacterial cells in the soil or on the root decline over time [34]. Thus, encapsulation enables slow and controlled release from the immobilized alginate gel matrix upon inoculation into the soil and facilitates in establishing stable bacterial populations [26]. Additionally, the microcapsules in this study were formed with PPI, which contains sugars, starch, lipid, ash in addition to protein thus providing extra nutrient and energy source for bacterial growth that may also contributed to release and sustain large population of bacteria over a long period of time.

Under our conditions, the results indicated that the application of the microcapsules prior to seeding leads to better colonization and spread of the bacteria in the soil. This is corroborated by the fact that

alginate microcapsules are gelled by ionic bond and these bond are known to swell and dissolve in solution containing chelating agents such as phosphate [6]. This is partly the reason that this technology is best considered for agriculture applications [6-8,35]. It is therefore expected that the incorporated microcapsules (pre-treatment) would degrade and release their bacteria content faster than the non-incorporated microcapsules (post treatment).

#### Plant tissue colonization capacity of the recovered bacteria

The ultimate test for encapsulated bacteria with plant growth promoting abilities is its ability to colonize its target plants [5,6,16]. The cell number (Figures 3C and 3D) and DNA copies of *B. subtilis* B26 (Figures 3E and 3F) were internally estimated in root and shoot tissues of *Brachypodium* and timothy at the end of the experiment. Overall, viable cell numbers as well as DNA copy numbers of B26 were similar and significantly higher inside roots than shoots of both test plants (Figures 3C-3F) applying both methods. The higher viable numbers in roots than shoots is in agreement to previous results in which soil drenching of free cells of B26 was applied to soil of *Brachypodium* [16] and timothy (unpublished data; Gagne-Bourque and Jabaji). This is an expected behaviour of endophytes [17]. Furthermore, our results clearly indicated that the nature of the interaction between the endophyte and its host is more important than the concentration of the



**Figure 3:** Survival of *Bacillus subtilis* B26 in the soil and plant tissues following the release of inoculum from PPI-alginate microcapsules, and timothy. (3A) Viable cell estimates (CFU) of B26 released from microcapsules incorporated into the soil before seeding (Pre-treatment). (3B) Viable cell estimates (CFU) of B26 released from microcapsules broadcasted on soil surface (Post-treatment). (3C) Viable cell estimates (CFU) of B26 root and shoots of *Brachypodium* and timothy at harvest from the Pre-treatment. (3D) Viable cell estimates (CFU) of B26 in root and shoot of *Brachypodium* and timothy at harvest from the Post-treatment. (3E) DNA copy number of B26 in root and shoot of *Brachypodium* and timothy at harvest from the Pre-treatment. (3F) DNA copy number of B26 in root and shoot of *Brachypodium* and timothy at harvest from the Post-treatment. (3G) Phenotypic response of 56-days-old *Brachypodium* and timothy as a result of incorporation of loaded microcapsules or sterile microcapsules into the soil (Pre-treatment). (3H) Phenotypic response of 56-days-old *Brachypodium* and timothy as a result of broadcast of loaded microcapsules or sterile microcapsules into the soil (Post-treatment). \*Represents a statistically significant difference  $p < 0.05$ .

bacteria present in the soil [36,37] and that the microcapsules did not change the endophytic nature of the B26.

The growth promoting effect of *B.subtilis* B26 was not the subject of this study as it is reported elsewhere [16]. In this study, the goal was to investigate as proof-of-principle the potential of PPI-alginate microcapsules to efficiently maintain cell viability during storage and to evaluate their potential as an inoculation carrier. Phenotypic observations (Figures 3G,3H) demonstrated a growth promoting effect brought by the presence of encapsulated B26 with a more pronounced effect when microcapsules were incorporated in the top layer of the soil before seeding. This may likely be due to the longer exposure of plants to the microcapsules. These observations are similar in part with previously reported results on B26 [16] that showed an increase in plant biomass. Taken together, the encapsulation of *B. subtilis* strain B26 did not appear to alter its growth promoting behaviour.

## Conclusion

The use of PPI-alginate microcapsules represents a good encapsulation method for *B. subtilis* B26. Encapsulated cells introduced into the soil are protected from adverse soil effects over an extended period of time. The capsules appeared to provide a protective site to the immobilized cells and the ability to release and sustain large population of bacteria over a long period of time. Similar to previously published study [16], the internal B26 concentrations in different plant tissues and phenotypic observations of inoculated plants with loaded microcapsules confirmed that the encapsulation process did not interfere with the endophytic nature nor the plant growth promoting ability of the isolate. Thus, for these reasons we believe that PPI-alginate represent a good choice for commercial application of *B. subtilis* B26.

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