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NOVEL MICROBIAL TECHNOLOGIES FOR THE ENHANCEMENT OF PLANT GROWTH AND BIOCONTROL OF FUNGAL DISEASES IN CROPS

C. NEYRA, L. A. ATKINSON, O. OLUBAYI,
L. SADASIVAN, D. ZAUROV AND E. ZAPPI
Plant Science Department, 382 Foran Hall, Rutgers University,
New Brunswick, NJ 08903-0231, USA

Abstract : For many years our laboratory has been conducting research on beneficial bacteria with emphasis on two related approaches: 1) The use of Plant Growth Promoting Rhizobacteria (PGPR), primarily using *Azospirillum brasilense* to enhance shoot and root growth, improve seeding establishment and stand uniformity and eventually crop yields; or 2) identify rhizosphere bacteria with antifungal properties and with potential for the development of products acting as biocontrol agents against major fungal pathogens affecting crop productivity. Our research on the latter has focused on a *Bacilus licheniformis* strain (PRI-36a) isolated from the rhizosphere of perennial ryegrass. A USA patent application for this organism has been recently approved. (Neyra and Sadasivan, 1996). Bacterial inoculation of seeds (Seed coating; Solid matrix priming; Vacuum infiltrations, etc.) or soil (using liquid or solid formulations) has been practiced for many years (refer to the legume-rhizobium symbiosis literature) but quite often variable and inconsistent results have been obtained due to poor quality products or a lack of competitive ability against indigenous microbes. Nonetheless, through the many years of practice, and the associated research, we have learned a great deal about the different plant-microbe systems as well as the various biotic and abiotic factors controlling their interactions. The use of microbial technologies in agriculture is currently expanding quite rapidly with the identification of new bacterial strains which are more effective in promoting plant growth. Novel technologies have also been developed for the optimization of biomass production, product formulation and delivery systems. In principal, a high quality product using beneficial microbes must have a high cell titer, exhibit a prolonged shelf-life for storage and good survivability in the soil. In brief, new products formulated to deliver beneficial microbes have to meet the realities of the agricultural system under consideration. The economics of the market associated with the use of plant growth promoting microbes (more specifically biocontrol products) would support a more intensive effort in the commercial realization of these practices. For instance, 10 years ago (about 1987) all fungicides accounted for a $4.1 billion, herbicides a $8.6 billion, and insecticides a $6.1 billion dollar combined. Biological products accounted for less than 1% of that market, thus leaving an enormous window of opportunity for the development of new products with improved formulations and effectiveness of action. We will present in this report the most recent results obtained in our laboratory using either *Azospirillum brasilense* or the biocontrol agent identified as *Bacillus licheniformis* strain PRI-36a.

STRATEGIES FOR THE IDENTIFICATION AND USE OF PLANT BENEFICIAL MICROBES

The microflora surrounding the roots is quit diverse, including bacteria, fungi, yeast, algae, and actinomycetes. Some of these microorganisms may be deleterious to plants (more properly called pathogens) while others may be beneficial to plants promoting growth and crop productivities. Thus, the rhizosphere soil represent a good reservoir of microbes for the potential isolation of beneficial microbes. Specific protocols should be followed for the isolation of particular microbes. Studies using different soil types and defined plant species have indicated that the relative proportion of different microbes, as well as the concentration of particular microorganisms (species or strains) depends very much upon soil types and plant species. Thus, specific protocols must be followed to identify locally adapted beneficial microbes according to soil types, crops

under consideration, and even according to the agricultural practices predominant in a particular location.

Once a beneficial microorganism has been identified, strategies must be developed to help increase the concentration of the strain at the site of action (inoculation technologies). The initial step to augment the population depends on the type of fermentation system to increase the biomass. To obtain a relatively high biomass yield of a particular microbe solid or liquid fermentation systems can be used. In general, one can achieve a concentration of about 10^9 cells per milliliter of culture when using liquid fermentation. Solid fermentation, on the other hand, consists of a diluted liquid inoculum mixed with inert carriers like vermiculite or peat. Both of these carriers have been the preferred choice to date. Under proper conditions, the solid fermentation systems may reach 10^8 to 10^9 cells per gram of carrier about a weed of incubation.

Alternatively, one may use a concentrated cell inoculum containing about 10^9 cells per ml and mix it directly with the solid carrier to give almost immediately an inoculant product containing over 10^8 cells per gram. For instance, using a product formulated at 10^9 cells per gram of vermiculite or peat one could inoculate tomato seeds at a rate of 1.10 (w/w inoculant to seeds) providing 10^8 cells per gram of tomato seeds or about 5 x 10^5 (CFU: colony forming units) per seed which is about the optimum titer per plant for *Azospirillum brasilense*. Hadas and Okon (1987) reported large increases in root dry weight (50%), top dry weight (90%), leaf surface area (90%) and root length (35%) for tomato plants inoculated with 5 x 10^7 CFU/plant. Inoculated plants showed 10^4 to 10^5 CFU/root of one plant, indicating that colonization of tomato roots was satisfactory. In separate experiments, Bashan et al. (1989; 1991) successfully increased growth and yield of tomato, peppers, eggplants and cotton in experiments conducted under greenhouse conditions. These results and those of Hadas and Okon (1987) confirm that *Azospirillum brasilense* has a good potential for use in vegetable crops as a PGPR.

Other formulations useful to the application of beneficial microorganisms to seeds or plants make use of cross-linking organic polymers like alginate, carrageenan or polyacrylamide. These materials have been used extensively to experimentally immobilize plant, animal or microbial cells and even isolated enzymes (Fravel et al, 1985; Bashan, 1986; Papavizas et al, 1987; Mc Intyre and Press, 1991; Stormo and Crawford, 1992). The method leads to the formation of pelletized gels by mixing alginate with the microbial culture and then adding the mixture drop-wise into a solution of $CaCI_2$, which yields small beads of uniform size containing a high concentration of cells. The method has been used successfully to encapsulate *Azospirillum brasilense* strain Cd and the biocontrol agent *Bacillus licheniformis* PR1--36a (Bashan, 1986; Neyra, 1996) into small beads of about 1mm wet diameter and containing 10^8 to 10^9 CFU per gram of beads. The beads may be applied together with the seeds at sowing while keeping the same cell titer per gram of seeds as indicated above for tomato. Furthermore, seed encapsulation in a gelatinous pellet containing beneficial microbes provides an interesting variation of the method. Encapsulated tomato seeds showed above 90% germination rates that were similar to the non-encapsulated controls (Neyra, 1996), suggesting that O_2 diffusion was not limiting for embryo development (figure 1).

RESPONSE BY TURF AND FORAGE GRASSES TO *AZOSPIRILLUM*

The results presented here are part of a program to study the response of different turf and forage grass cultivars to inoculations with *Azospirillum brasilense* strain Cd. The inoculum was formulated to contain 5 x 10^8 colony forming units per gram of fine vermiculite, used as a solid support, with a 50% water holding capacity. The inoculant for all cultivars was provided as a seed coating with a final bacterial cell concentration per gram of seed of about 5 x 10^6 CFU. Table 1 shows the response of 10 different turf and forage grass cultivars to inoculation. Nine out of 10 cultivars included in this study showed a significant increase in dry weight for both shoots and

roots. The small increase observed in Kentucky bluegrass cv Baron was not statistically significant (Table 1).

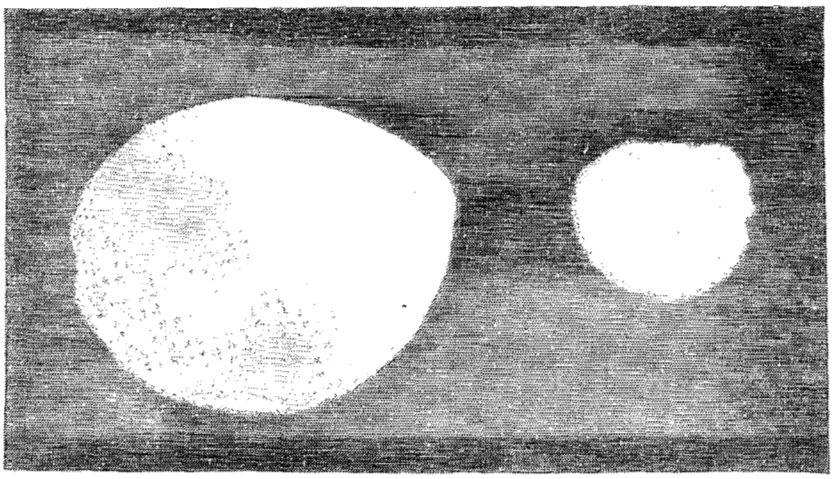

Figure 1. Illustration of shape and size of alginate- Ca Cl_2 wet microbeads immediately after formation (left hand side) and after dehydration (right hand side)

In a parallel study with 14 different cultivars there was a significant increase in total leaf N-content in response to the inoculation treatment (Table 2). These results also suggested that N-uptake and translocation was maintained in relation to the increased production of dry matter, as evidenced by the similarities in % N-content between the control and *Azospirillum* treatments (Table 2).

The results presented in Tables 1 and 2 are also in agreement with the contention that *Azospirillum* helps to enhance root growth and universal nutrient uptake (Lin et al, 1983; Okon, 1985; Hadas and Okon, 1987; Burdman et al, 1996). In addition to nutritional considerations, the growth promotion observed may be related to the demonstrated ability of *Azospirillum* to produce plant growth regulators like giberellins and indol acetic acid (Bottini et al, 1989; Fallik et al, 1989; Bar and Okon, 1995) (figure. 2).

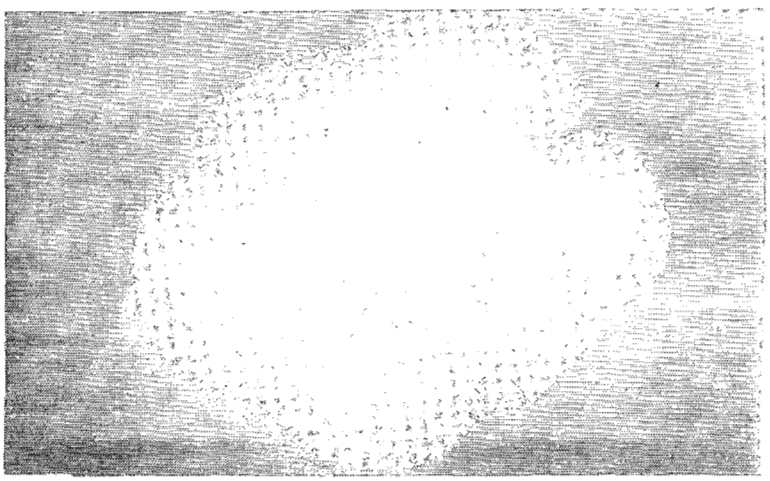

Figure 2. Colonization of nutrient by *Azospirillum brasilense* strain Cd entrapped in a wet microbead. The picture also illustrates the ability of viable bacteria to be released from an alginate-$CaCl_2$ microbead.

Table 1. Growth responses to inoculation with *Azospirillum brasilense* by several turfgrass

	Dry weights (mg/pot)			
	Shoots		Roots	
Plant Species	Control	Inoculated	Control	Inoculated
Tall Fescue « Cattle Club »	504	728*	594	672**
Bromegrass « Manchar »	580	791**	469	691**
Bromegrass « Regar »	727	948**	472	703*
Orchardgrass « Latar »	757	1044**	614	733**
Sheep Fescue « Covar »	119	179*	112	167*
Hard Fescue « Durar »	187	272*	169	259*
Arizona Fescue « Redondo »	182	235*	155	202*
Timothy « Climax »	268	378*	226	362*
Canby Bluegrass « Canbar »	264	378*	226	362*
Kentuchy Bluegrass « Baron »	315	346 NS	193	200 NS

Plants were grown under controlled encironmental conditions in a greenhouse. Azospirillum was provided as seed coating using a vermiculite formulation (10^8 CFU/g product) applied at a rate of 4g/100 seeds. Plants harvested at 32 DAS.
**, * Differences are significant at the 0.01 and 0.05 probability levels (LSD), respectively

Table 2. Leaf introgen increase in several grasses in response to inoculation with *Azospirillum brasilense*

	% Nitrogen		N-content (mg/pot)	
Plant Species	Control	Inoculated	Control	Inoculated
Tall fescue « Cattle Club »	2.94	3.12	14.82	22.71
Bromegrass « Manchar »	2.38	3.78	13.80	29.89
Bromegrass « Regar »	2.50	2.80	18.17	26.54
Orchardgrass « Latar »	2.09	2.54	15.82	26.51
Russian wildrye	2.043	2.46	19.73	23.27
Basin wildrye « Magnar »	2.29	2.74	18.36	26.05
Tall Wheatgrass « Alkar »	1.95	2.12	27.31	35.31
Wheatgrass « Newhy »	2.43	2.53	27.58	34.76
Intermediate wheatgrass	2.28	2.72	28.20	39.44
Western Wheatgrass	2.45	2.70	20.21	30.07
Bluebunch wheatgrass	2.55	2.78	20.27	29.24
Streambank wheatgrass	1.75	1.50	11.58	13.14
Thickspike Wheatgrass	2.08	2.18	30.05	37.86
Crested wheatgrass	2.26	2.61	23.23	34.21
Treatment Neans	2.31	2.61**	20.65	29.21**

Plants grown under contolled environmental conditions in a greenhouse. *Azospirillum* was provided as seed coating using a vermiculite formulation (10^8 CFU/g product) applied at a rate of 4g/100 seeds. Harvest at 32 DAS.
*, ** Significant differences at P = 0.05 and P = 0.01, respectively.

AZOSPIRILLUM-RHIZOBIUM INTERACTIONS AND PLANT RESPONSES TO CO-INOCULATION

Our laboratory has been working for several years on the development of novel inocultants for the delivery of plant beneficial bacteria and some of the results obtained throughout are presented here. One of the approaches for inoculant preparation is based on the physiological induction of massive cell aggregation, clumping and flocculation (Sadasivan and Neyra, 1985). The flocculent mass sinks to the bottom of the culture medium and is readily separable from the spent culture. Each milliliter of floc contains about 10^9 cells and a net yield of about 100 milliliters of floc is obtained per liter of broth culture. The separated floc comprises a fraction enriched in encysted cells with thick capsules and surrounded by a polysaccharide-rich network. All of these properties are responsible for the advantage exhibited by flocculated forms of azospirilla and/or rhizobia,which exhibit extended shelf-life, high cell titer, and increased adhesiveness. We have also been able to produce inoculants containing a mixture of both of cells, *Azospirillum* and *Rhizobium*, by coflocculation forming an intergeneric coaggregate. Their positive effect on growth and nodulation of the common bean (*Phaseolus vulgaris*) is reported in Table 3. The results showed that the use of flocculated *Rhizobium* (*R. et li* nov) was superior to the non-flocculated form in terms of nodule numbers and plant growth (Table 3). Notably, the addition of non-flocculated *Azospirillum* to the non-flocculated *Rhizobium* also resulted in a significant increase in nodulation and plant gowth. The highest nodulation efficiency however, was observed when coflocculated forms of *Rhizobium* and *Azospirillum* were used to inoculate *P. vulgaris* seeds. Altogether, the results indicated that the enhancement of nodulation efficiency in response to the triple interaction between Rhizobium, *Azospirillum* and flocculation resulted in a enhancement of shoot and root growth (Table 3). The effects of *Azospirillum* as a coinoculant with *R. etli* on root parameters in *Phaseolus vulgaris*_(common bean) plants are summarized in Table 4. The beneficial effects of using coaggregated forms of *Rhizobium* and *Azospirillum* were confirmed under field conditions for *Phaseolus vulgaris*_(Table 5) and *Medicago sativa*, also known as alfalfa (Table 6).

Table 3. Enhancement of nodulation efficiency and growth of *Phaseolus vulgaris* plants inoculated with flocculated or nonflocculated *Rhizobium etli* strain 2743 with or whithout *Azospirillum brasilense* strain Cd.*

Plant Species	Nodulation Efficiency	Dry weights (g/plant) Shoots	Roots
	(% of Control)**	(% of Control)**	
Non-Flocculated			
Rhizobium alone	100	100	100
Rhizobium + Azospirillum	182	142	158
Flocculated/Coflocculated			
Rhizobium alone	202	164	15
Rhizobium + Azospirillum	233	167	159

* Inocula was provided as seed coating
**Data presented as % of the nonflocculated Rhizobium control

Table 4. Effect of *Azospirillum brasilense* Cd coinoculated with *Rhizobium etli* on the length of tap roots, number of lateral roots and nodules/plant of a xenically grown *P. vulgaris* plants at 10 DAS.

Parameters	Control (minus *Azospirillum*)	Treatment (106 CFU/g soil of *Azospirillum* Cd)
Tap root length (cm:plant)	5.9 ± 0.4	10.0 ± 0.6*
Lateral roots (No/plant)	4.4 ± 1.1	8.9 ± 0.4*
Nodules (No./plant)	13.08 ± 1.5	24.0 ± 0.7*

* Neans are significantly different between treatment and control at P = 0.05 level of probability Values are means ± standard deviation for 5 replications.

Table 5. Responses of common bean plants (*P. vulgaris L*) to inoculation by seed coating with *Rhizobium etli* strain 2743 alone or mixed with *Azospirillum brasilense* (strains Cd and Sp 245) coflocculated with *Azospirillum lipoferum* (strain Br 17). Data represent the mean of four replications.

Seed Treatment	Above-ground dry matter g/100 plants	Seed yield g/100 plants	Estimated Economic Yield Kg seed/ha
Rhizobium etli+Azospirillum	1646*	802**	2165**
R. etli alone	1426	608	1642
Uninoculated control	1460	606	1628

*,** Indicate mean differences are statistically significant at P = 0.05 and 0.01 levels, respectively

Table 6. Dry matter yields of Alfalfa (*Medicago sativa L.*) plants in response to inoculation with different *Rhizobium meliloti* strains alone or mixed with *Azospirillum brasilense* (strains Cd and Sp 245) coflocculated with *Azospirillum lipoferum* Br 17. Data represent the cumulative means of four harvest times with four replications each.

Inoculation Treatments	Top Dry Matter yield (grams/meter) Control	+Azospirillum
Uninoculated	15.5	33.0**
R. meliloti 1577	15.2	41.9**
R. meliloti 101	29.6	66.1**
R. meliloti 164	35.2	34.3 NS

** Mean differences are statistically signeficant at P s 0.05
NS Non-significant differences.

The positive response to combined inoculations with *Azospirillum* and *Rhizobium* have already been reported for several legumes (Plazinski and Rolfe, 1985; Sarig et al, 1986; Burdman et al, 1996). As shown in Table 4, the beneficial effect of Azospirillum may be attributed primarily to an overall enhancement of nodulation and root growth. According to Burdman et al (1996) the nodulation promoting activity of *Azospirillum* could be explained, at least in part, by the

promotive effects of *Azospirillum* on the secretion of Nod gene induced signals by the legume roots and by an increase in the differentiation of root hairs. Enhancement of Nod gene expression could explain the greater infectivity of *Rhizobium* and earlier initiation of the processes leading to nodule formation. The enhancement of root hair production would help increase the number of infection sites for rhizobia as well as causing a general effect on the uptake of water and minerals as indicated above.

The usefulness of *Azospirillum spp.* as a plant growth promoting bacterium is not limited to grasses (Tables 1 and 2) or legumes (Tables 3, 4, 5 and 6); it also includes a diversity of other plants, particularly vegetables such as tomatoes, cucumbers, radishes, peppers, melons and others (Hadas and Okon, 1987; Bashan and Levanony, 1990; Levanono and Bashan, 1991). Because of the broad diversity of plant types acting as a host for *Azospirillum spp.*, it would appear unlikely that the various types of responses are a consequence of a highly specific mod of action. It would rather seem that a combination of physiological and biochemical phenomena may be operative on one or another plant host. Nonetheless, some general phenomena may be operative and could explain the observed effects of *Azospirillum spp.* These may include the general enhancement of root growth and proliferation of root hairs, the bacterial production of plant growth regulators like GA and IAA? and a generally improved capacity for the uptake of water and mineral nutrients by the plants. All of these phenomena occurring together could be responsible for the overall plant growth enhancement and the increase in crop yields.

The microbial partnership between *Azospirillum* and *Rhizobium* (Tables 4, 5 and 6) is presented here as a model system to illustrate the operation and benefits of using the flocculated morphotype as a delivery system for each cell type singly or in a coaggregated form. Our laboratory continues to search for bacterila partners capable of forming multigeneric aggregations for the production of multiple-purpose inoculants. These inoculant could consist of fungal antagonistic bacteria or pollutant-degrading bacteria, in addition to the plant growth promoting species like *Azospirillum*. We hope that a new generation of inoculant products, along these lines, will be forthcoming in the near future.

STRAIN OF *BACILLUS LICHENIFORMIS* AS A POTENTIAL BIOCONTROL AGENT

A novel *Bacillus licheniformis* strain (PR1-36a) was isolated from the rhizosphere of perennial rygrass *(Lolium perenne L)* grown in a local soil. The isolate showed a very broad spectrum of antigungal activity, including various economically important phytopathogenic fungi (Table 7). The pathogenic fungi susceptible to *B. Licheniformis* PR.1-36a are known to affect a number of economically important plants including vegetable crops, forage and cereal grasses, and coffee. The crude ethanolic extract (Et-OH) was obtained after acid precipitation of cell-free stationary phase cultures and yielded a diffusible antifungal principal that affected mycelial growth, causing malformation and swelling.

Bacillus licheniformis is as endospore-forming bacterium tolerant to the unfavorable environmental conditions of drought, high temperature, and low O_2; which makes strain PR1-36a a suitable candidate for use as a biocontrol agent against fungal pathogens in plants. The results using whole cells (Table 7) indicate that *B. Licheniformis* may be formulated using live cells either in a liquid or solid formulation. Our experience indicates that PR1-36a may use a variety of folid carriers like calys, peat, or vermiculite, and may even be formulated in the form of encapsulated alginate pellets by mixing the bacterial cells with 1% Na-alginate followed by a gellification in 0.25M $CaCl_2$ (Fravel et al, 1985; Papavizas et al, 1987; Bashan, 1986; Stormo and Crawford, 1992).

Seed priming is a technique that has been shown to increase the speed of germination and emergence of many vegetable crop seeds including lettuce, carrot, tomato and sweet corn. Seed priming and osmoconditioning are terms that describe a presowing hydration treatment to improve seedling establishment (Taylor et al, 1988). Preplant seed hydration can be achieved using a solid support such as clays in the solid matrix priming (Taylor et al, 1988) or a liquid support like the osmoticum polyethylene glycol (PEG-8000) in osmopriming. The idea is to provide sufficient water to initiate the germination process internally without reaching full or complete germination. The seeds are said to be preinduced to germinate. Following transfer of treated seeds to fully hydrated conditions they will normally show a faster and more uniform seeding emergence profile. Bio-priming, a combination of biological seed treatment and preplant hydration may also be quite useful to enhance stand uniformity due to improved seeding emergence. In this case, the early damping-off damage to the germinating seed is avoided, and the controlled hydration provides enhanced energy of germination. We have had mixed success in the use of seed priming as a delivery system for *Bacillus licheniformis* PR1-36a on sweet corn seeds. In Table 8 we show results of two experiments: (A) The seeds had only about 5% germinability due to fungal contamination and the percent emergence was greatly enhanced by using a solid matrix bio-priming (SMBP) technique, thus improving seed emergence from 50 to 97 percent. (B) The seeds of a different sweet corn cultivar exhibited slightly germination quality (about 70%), and germination or seedling emergence was increased only from 73 to 85 percent by osmopriming PEG-8000 at 10% and PR1-36a.

Table 7. Growth inhibition of fungal pathogens on PDA plates by whole cells and a crude ethanolic extract (Et-OH) from *Bacillus licheniformis* strain PR1-36a

	Inhibition level	
Fungal pathogen	Whole cells	Et-OH extract
Alternaria spp.	ND	VS
Aspergillus flavus	S	S
Aspergillus ochraceus		
Strain 15299	VS	ND
Strain 12704	VS	ND
Aspergillus niger	ND	S
Cercospora spp.	S	
Cladosporium spp.	S	
Colletotrichum spp.	S	
Curvularia spp.	S	
Diplodia maydis	ND	VS
Fusarium moniliforme	S	S
Fusarium oxysporum	ND	W
Fusarium roseum	S	S
Helminthosporium maydis	ND	VS
Magnaporthe poae NAVA5	VS	VS
Rhizoctonia solani AG-4	VS	VS

VS, very strong; S, strong; w, weak; ND, not done

Table 8. Growth inhibition of fungal pathogens on PDA plates by whole cells and a crude ethanolic extract (Et-OH) from *Bacillus licheniformis* strain PR1-36a

	Parameters		
	Seedlings Plants/pot	Emergence %	Plant height cm/plant
(A) Solid matrix bio-priming			
Control-no treatment	4.0	50	10.8
SMBP+PR1-36a	7.6	95	19.5
(B)Osmopriming (10% PEG-8000)			
Control	7.3	73	24.2
+PR1-36a	8.5	85	25.1

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