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Article *in* Journal of Applied Microbiology · November 2013

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Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Development of alginate-based aggregate inoculants of *Methylobacterium* sp. and *Azospirillum brasilense* tested under *in vitro* conditions to promote plant growth

M.M. Joe[1,2], V.S. Saravanan[3], M.R. Islam[4] and T. Sa[1]

1 Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Korea
2 Department of Microbiology, School of Life Sciences, Vels University, Chennai, India,
3 Department of Microbiology, Indira Gandhi College of Arts and Science, Kathirkamam, Pondicherry, India
4 Department of Biological Sciences, Inha University, Incheon, Korea

Keywords
ACC deaminase, *Azospirillum brasilense*, biofilm, co-aggregation, *Methylobacterium oryzae*, Plant growth-promoting bacteria, water stress.

Correspondence
Manoharan Melvin Joe and Tongmin Sa, Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea.
E-mails: tomsa@chungbuk.ac.kr; mel_vin@sify.com

2014/1112: received 6 June 2013, revised 4 October 2013 and accepted 30 October 2013

doi:10.1111/jam.12384

Abstract

Aim: To develop co-aggregated bacterial inoculant comprising of *Methylobacterium oryzae* CBMB20/*Methylobacterium suomiense* CBMB120 strains with *Azospirillum brasilense* (CW903) strain and testing their efficiency as inoculants for plant growth promotion (PGP).

Methods and Results: Biofilm formation and co-aggregation efficiency was studied between *A. brasilense* CW903 and methylobacterial strains *M. oryzae* CBMB20 and *M. suomiense* CBMB120. Survival and release of these co-aggregated bacterial strains entrapped in alginate beads were assessed. PGP attributes of the co-aggregated bacterial inoculant were tested in tomato plants under water-stressed conditions. Results suggest that the biofilm formation efficiency of the CBMB20 and CBMB120 strains increased by 15 and 34%, respectively, when co-cultivated with CW903. Co-aggregation with CW903 enhanced the survivability of CBMB20 strain in alginate beads. Water stress index score showed least stress index in plants inoculated with CW903 and CBMB20 strains maintained as a co-aggregated inoculant.

Conclusions: This study reports the development of co-aggregated cell inoculants containing *M. oryzae* CBMB20 and *A. brasilense* CW903 strains conferred better shelf life and stress abatement in inoculated tomato plants.

Significance and Impact of the Study: These findings could be extended to other PGP bacterial species to develop multigeneric bioinoculants with multiple benefits for various crops.

Introduction

Genus *Methylobacterium*, also called as pink-pigmented facultative methylotrophic bacteria (PPFM) dominate the phyllosphere microbial population of numerous plants species (Idris *et al.* 2004). Additionally, members of this genus were isolated from buds, roots and plant rhizosphere region (Corpe and Basil 1982; Pirttilà *et al.* 2005). Diverse *Methylobacterium* spp. fall under the category of plant growth-promoting bacteria (PGPB) because they benefit plants either by the production of indole acetic acid (IAA), cytokinins and vitamin B12 or through the production of growth modulating enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Madhaiyan *et al.* 2007, 2010).

On the other hand, the species *Azospirillum brasilense* is the most widely commercialized bioinoculant in several countries including Argentina, Mexico, India, Italy, France and Korea (Hartmann and Bashan 2009), resulting from variable mechanisms of growth promotion (Bashan and de-Bashan 2010). Production of ACC deaminase by bacteria is regarded as a PGP trait to ameliorate stress response, because ACC is an immediate precursor for stress ethylene production (Glick *et al.* 1998). A few

Azospirillum lipoferum strains such as AZm5 4B, CRT1, CN1, N4 and TW3 were found to be positive for ACC deaminase (AcdS) activity (Blaha *et al.* 2006; Prigent-Combaret *et al.* 2008; Esquivel-Cote *et al.* 2010) ; however, to our knowledge, ACC deaminase activity was previously not reported in any of the *A. brasilense* strains.

Previously, *A. brasilense* was inoculated as single culture to promote the plant growth (Bashan and Holguin 1997); however, to improve their PGP abilities, they were co-inoculated with other PGPB, and this technique had generated a considerable attention in the field of microbial inoculants production, mainly due to its high success and profitability (Bashan and Holguin 1997). This co-inoculation can be achieved by co-aggregation; defined as clumping when the different cell types are mixed together as demonstrated by Cisar *et al.* (1979). Based on this idea, Neyra *et al.* (1997) proposed the co-flocculation [co-aggregation] technique, in which several bacteria having PGP activity can be co-flocculated with flocculating bacteria such as *A. brasilense* sp. to produce inoculants with bacteria possessing different PGP attributes. They further stated that the flocculated cultures had enhanced shelf life and better survivability in soil and seed surfaces. Recently, Trejo *et al.* (2012) reported the formation of the microbial complex between microalgae *Chlorella sorokiniana* and the plant growth-promoting bacterium *A. brasilense* within beads comprising micro-colony aggregates of both species. In an earlier study, de-Bashan *et al.* (2011) observed a similar type of interaction between *Azospirillum* and root less single cell-microalgae and they demonstrated that interaction of *A. brasilense* with roots of higher plants occurs through fibrils and sheath material. The aggregation ability in *Methylobacterium* strains having PGP activity was demonstrated in our previous study (Joe *et al.* 2013).

Several studies proposed the drought stress alleviation potential of *A. brasilense* and other PGPB (Bashan and de-Bashan 2010; Kim *et al.* 2012). El-Komy *et al.* (2003) reported that inoculation with *A. brasilense* alleviated the drought stress on wheat plants, mainly by improving their water uptake (Bashan and de-Bashan 2010) or through changes in the fatty acid profiles of major root phospholipids (Pereyra *et al.* 2006). Furthermore, in one of our earlier study (Joe *et al.* 2012), in comparison with nonflocculated cells, flocculated *A. brasilense* MTCC125 cells improved maize growth and yield under water deficit conditions, especially, the alginate-entrapped flocculated cells were better in their survival when compared to disinfected soil-based carrier.

The use of synthetic carriers including alginate for agricultural inoculants production is a concept proposed for two decades and was preferred over peat-based inoculants due to the advantages like nontoxic nature, biodegradability and slow release of micro-organisms embedded in them into the soil environment (Bashan 1986b).

Hypothetically, if a PGPB with ACC deaminase activity (here *Methylobacterium oryzae* CBMB20) was used as a co-aggregate partner with *A. brasilense*, it may enhance the efficiency of *A. brasilense* CW903 strains under water-stressed condition because the plant stress ethylene levels may be effectively reduced by ACC deaminase. To test this hypothesis, ACC deaminase-positive *Methylobacterium* CBMB20 and *A. brasilense* CW903 co-aggregated cell-based inoculants were prepared. *Methylobacterium suomiense* CBMB120, isolated from rice rhizosphere, which successfully colonized the rice and tomato plants on inoculation studies found to possess plant growth-promoting (PGP) properties like IAA and cytokinins production, with an exception for negative in ACC deaminase activity (Madhaiyan *et al.* 2006; Poonguzhali *et al.* 2008), was included in the present study for comparative purposes.

In the present work, biofilm formation and co-aggregation efficiency among the two *Methylobacterium* strains with *A. brasilense* CW903 was evaluated; further, the survival and release of co-aggregated cells from the alginate beads were also assessed. Lastly, the PGP potential of the inoculants was investigated in tomato plants under drought-stressed conditions.

Materials and methods

Bacterial strains and growth conditions

The details of the bacterial strains used in this study are listed in Table 1. They were grown under high C/N fructose minimal growth medium to promote flocculation in *A. brasilense* CW903 as described by Burdman *et al.* (1998). The high C/N medium contained (g l^{-1}) D-fructose (6·67), $MgSO_4$ (0·2), NaCl (0·1), $CaCl_2$ (0·02), K_2HPO_4 (6·0), KH_2PO_4 (4·0), yeast extract (0·1), NH_4Cl (0·214) and microelements as described by Okon *et al.* (1977). The components of the media was adjusted to a pH of 7·0, and the cultures were inoculated in separate flasks and incubated on a shaking incubator (Model: VS-8480SF; Vision Scientific, Daejeon, Korea) at 150 rpm and maintained at 28 ± 2°C for 72 h. This medium was specifically used to promote flocculation by *Azospirillum*.

Initial screening in microtitre plate for biofilm formation

Biofilm formation assay was performed with high C/N fructose minimal medium as per the protocol of Djordjevic *et al.* (2002) with required modifications in 96 well PVC microtitre plate (Nunc 96-well plates, Thermo Scientific, Roskilde, Denmark). The optical density (OD) level of the crystal violet present in the de-staining solution was

Table 1 Bacterial strains used in the present study and their PGPR traits

Strain	GenBank Acc. No.	PGPR traits	References
Methylobacterium oryzae CBMB20/CBMB20-*gfp*1*	AY683045	N_2 fixation, indole acetic acid (IAA) production and ACC deaminase activity	Madhaiyan *et al.* (2006), Lee *et al.* (2011)
Methylobacterium suomiense CBMB120/CBMB120-*gfp*29*	AY683047	IAA, cytokinin production	Madhaiyan *et al.* (2006), Poonguzhali *et al.* (2008)
Azospirillum brasilense CW903/CW903-pLA†	AY518780	N_2 fixation and IAA production	Kim *et al.* (2005)

*CBMB120-*gfp*29 and CBMB20-*gfp*1 through triparental mating using *E. coli* S17-1 (pFAJ1820 - *Tn5gusA-gfp*) [Poonguzhali *et al.* (2008); Lee *et al.* (2011)].
†Conjugation with *E. coli* PLA-*lacZ* (Kim *et al.* 2005).

measured at 595 nm (EZ Read-400, Biochrom, Cambridge, England). The microtitre plate biofilm assay was performed three times for all strains, and for these values, the averages, standard deviation and standard error were calculated using Box and Whiskers plot.

Estimation of co-aggregation

Visual scoring assay

The degree of co-aggregation by the strains was monitored by a visual assay described by Cisar *et al.* (1979). The scoring is as follows, a score ranging from 0 to 3+ was assigned to–0: no visible aggregates in the cell suspension; 1+ : small uniform co-aggregates in suspension; 2+: definite co-aggregates seen but the suspension remained turbid; 3+: large co-aggregates that settled rapidly, leaving a clear supernatant.

Co-aggregation and auto-aggregation assays

For co-aggregation assay, bacteria were grown in high C/N fructose minimal medium as described above and the cells were harvested by centrifugation at 5000 ***g*** for 15 min, washed twice and re-suspended in phosphate-buffered saline (PBS) (0·1 mol l^{-1}, pH 6·8) to give viable counts of *c.* 10^8 CFU ml^{-1}. Equal volumes (2 ml) of each bacterial strain's cell suspension were mixed together in pairs by vortexing for 10 s. Control tubes were maintained with 4 ml of bacterial suspension for each individual strains. The absorbance (A) at 600 nm of the suspensions was measured after mixing the strains and after 24 h of incubation at a temperature of 28 ± 2°C. The percentage of co-aggregation was calculated using the equation of Handley *et al.* (1987)

$$\text{Co} - \text{aggregation } (\%) = \frac{\left(\frac{Ax+Ay}{2}\right) - A(x+y)}{Ax + Ay/2} \times 100$$

where Ax and Ay represent the absorbance of the two strains in the control tubes and A $(x + y)$ the absorbance of the mixture of two strains after a time period of 24 h. x denotes *A. brasilense* CW903 and y represents either *M. oryzae* CBMB20 or *M. suomiense* CBMB120 strain.

Auto-aggregation assays were performed according to Del Re *et al.* (2000) with certain modifications to compare auto-aggregation potential of the strains with their co-aggregation efficiency. Cell suspensions (4 ml) were mixed by vortexing for 10 s and auto-aggregation was determined during 24 h of incubation at room temperature. After 24 h, 0·1 ml of the upper suspension was transferred to another tube with 3·9 ml of PBS buffer and the absorbance (A) was measured at 600 nm. The auto-aggregation percentage is expressed as: Auto-aggregation (%) = 1 - $\frac{A_t}{A_0} \times 100$ where A_t represents the absorbance at 24 h and A_0 the absorbance at $t = 0$.

Estimation of bacterial population under co-aggregated conditions

The total bacterial population of co-aggregated cells was determined by plating in Luria Bertani (LB) plates. *A. brasilense* CW903 population was determined in N-free malate medium supplemented with X-gal (30 μg ml^{-1}). Alternatively, the GFP-tagged *Methylobacterium* strain population was also determined in ammonium mineral salts (AMS) medium described by Whittenbury *et al.* (1970) supplemented with supplemented with filter-sterilized cycloheximide (10 μg ml^{-1}) and methanol (0·5% v/v) at 28 °C.

Scanning electron microscope

The samples of bacterial cells were washed with 0·1 mol l^{-1}, phosphate buffer maintained at pH 7·2 and fixed by suspending in 2·5% glutaraldehyde solution overnight. The sample was then fixed with 0·1% osmium tetroxide and dehydrated in a series (30, 50, 60, 70, 80, 90 and 95%) of ethanol for 30 min each. Finally, the pellet was suspended in isoamylacetate for

20 min and then air-dried for 36 h in a clean bench, coated with gold–palladium for 60 s in a Pelco-3 sputter coater and visualized using a Hitachi S-2500C Scanning Electron Microscope with GEMINI column (Hitachi Co., Tokyo, Japan).

Preparation of alginate bead-based inoculant

A slightly modified approach developed by Joe *et al.* (2012) based on the earlier work of Bashan *et al.* (2002) was adopted to develop alginate bead-based inoculants. The wet alginate beads were placed in sterile Petri dishes, dried aseptically initially in clean bench for 48 h and then in oven maintained at a temperature of about 40°C; the contents were then sealed in sterile Petri dishes and stored at 30°C. The survival of bacterial population was estimated as described earlier.

Determination of residual bacterial population

The residual bacterial population can be described as the cumulative bacterial release from microcapsules into disinfected soil (Moistened and autoclaved at 121°C at 15 psi, cooled and again moistened (30% by weight) with sterile phosphate buffer (pH 7·2) at various time intervals. Disinfected soil containing alginate-entrapped bacteria were serially diluted in sterile saline solution and the amount of viable bacteria was determined as described earlier. Experiments were carried out at 28 ± 2°C for determination of residual bacterial population.

Growth in pectin or carboxyl methyl cellulose amended media

Liquid medium containing 1% apple pectin or CMC, 0·03% $(NH_4)_2SO_4$, 0·6% K_2HPO_4, 0·20% KH_2PO_4 and 0·01% MgSO4·7H_2O, pH 6·0, was autoclaved for 15 min at 121°C. After cooling at room temperature, the medium was inoculated with 1·0 ml (0·5 ml of each strain for mixed culture) of bacterial suspension and the cultures were grown in 200 ml Erlenmeyer flasks with 50 ml of medium in a rotary shaker (150 rpm) at 30°C for 72 h. The bacterial populations were determined as described earlier.

Preparation of pectic enzyme for the assay

After determination of the bacterial populations, the cultures were harvested by centrifugation at 23 000 ***g*** at 3°C for 2 h. The supernatant solution was used for enzyme activity assay after it had been dialysed twice for 24 h at 1°C against 50 volumes of distilled water.

Pectin lyase activity

The substrate solution containing 0·5% apple pectin (dissolved in 0·05 mol l^{-1} Tris–Hcl buffer, pH 8·0) and 0·5 ml pectic enzyme as described earlier was incubated at 30°C for 60 min. Pectin lyase (PL) activity was determined according to the method of Manachini *et al.* (1988) by measuring the increase in absorbance of the unsaturated oligogalacturonates at 235 nm. One unit (U) of enzyme activity was defined as the amount that caused an increase in A235 equal to 0·555 absorbance units per minute (Albersheim 1966).

Pectin methyl esterase activity

Pectin methyl esterase (PME) activity was carried out according to the method described by Hagerman and Austin (1986). Five millilitre of 0·5% solution of apple pectin (prepared in 0·15 mol l^{-1} NaCl) was added to 1 ml of 0·01% solution of bromophenol blue, pH 7·5 and 0·5 ml of pectic enzyme as described earlier (adjusted to pH 7·5 with con NaOH). The decrease in absorbance was measured at 620 nm and one U of PME was defined as the amount of enzyme that released 1 μ mole of carboxyl group per minute.

Endoglucanase activity

Endoglucanase activity was determined according to the method described by Nitisinprasert and Temmes (1991). Reaction mixture containing 1 ml of pectic enzyme with 1 ml of 1% CMC in carbonate buffer was maintained at pH 9·5 and incubated at 50°C for 10 min. The amount of reducing sugars released was determined by phenol–sulfuric acid method using glucose as a standard. One U of enzyme activity was defined as the amount of enzyme producing 1 μ mole of reducing sugars in 1 min under the assay conditions.

Testing of PGP activity under water stress in growth chamber conditions

In vitro *plant growth studies*

Surface-sterilized tomato seeds (*Lycopersicon esculentum* L. cv. Mairoku, Sokata Korea, Seocho-dong, Seoul, Korea) were sown in plastic pots (top diameter, 5 cm; bottom diameter, 2·5 cm; height, 5 cm) filled with *c.* 90 g of disinfected soil. The amount of nutrients were expressed in terms of kg^{-1} of the soil, which includes 2·9 g organic matter, 9·98 mg nitrogen in the form of NO_3^- and 2·74 mg of N in form of NH_4^+, 81·4 mg P in form of P_2O_5, 7·39 pH, 0·21 EC and 0·01% salinity and the soil belong to sandy loam textural class and inceptisol group.

Surface-disinfected tomato seeds were sown in plastic pots as described above and maintained under conditions as described below. The treatments were maintained devoid of any fertilizers and irrigation with distilled water. Three days after germination of the seeds, the beads (with a population concentration of 8 log CFU g^{-1}) were placed in close proximity to root zone of the germinated seedlings. As the beads were of not uniform in size and the weight per bead also varied between 0·002 and 0·003 g, the population varied considerably. So, the inoculum size was defined either as CFU g^{-1} dry weight or as CFU per 100 mg dry weight (an average of 4–5 beads). After bacterial culture inoculation, the pots were arranged in a completely randomized design and were maintained in a growth chamber operating at a temperature of 25°C, relative humidity of 70% and light intensity of 90–110 μmol m^{-2} s^{-1}. The parameters observed in terms of plant growth includes, stress ethylene levels, peroxidase activity and malondialdehyde content.

Water stress induction and determination of soil moisture content

Two weeks after germination, the pots were irrigated to field capacity (FC) and then the irrigation was withheld. The moisture content of the soil was determined by gravimetric method according to Black (1965) and the data are given in Fig. 4a.

Assay of peroxidase activity

Peroxidase was assayed by the method of Kumar and Khan (1982). Assay mixture for Peroxidase activity contained 2 ml of 0·1mol l^{-1} phosphate buffer (pH 6·8), 1 ml of 0·01 mol l^{-1} pyrogallol, 1 ml of 0·005 mol l^{-1} H_2O_2 and 0·5 ml of plant extract. The solution was incubated for 5 min at 25°C after which the reaction was terminated by adding 1 ml of 2·5N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a reagent blank prepared by adding the extract after the addition of 2·5N H_2SO_4 at zero time. One unit of the enzyme activity corresponded to an amount of enzyme that changes the absorbance by 0·1 min^{-1} mg^{-1}. The activity was expressed in U mg^{-1} protein. Protein in the enzyme extract was measured using the Lowry method (Lowry *et al.* 1951), with bovine serum albumin as standard.

Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content according to the method of Duan *et al.* (2011). A sample containing 0·5 g of plant material was mixed with 5 ml of 5% trichloroacetic acid and centrifuged at 12 000 ***g*** for 25 min. Two millilitres of the supernatant was mixed with 2 ml of 0·67% thiobarbituric acid solution and heated for 30 min at 100°C. After cooling, the precipitate was removed by centrifugation. The absorbance of the sample was measured at 450, 532 and 600 nm using a blank containing all the reagents. The MDA content of the sample was calculated using the formula,

$$C/\mu\text{mol/l} = 6 \cdot 45\ (A_{532} - A_{600}) - 0 \cdot 56A_{450}$$

Ethylene emission in tomato plants under water stress and dry weight estimation

Ethylene emissions from tomato plants were measured following the protocol of Madhaiyan *et al.* (2007) with required modifications. Tomato plants from different treatments were uprooted and washed using distilled water to remove soil from roots and were placed inside 120-ml narrow-neck McCartney bottles. The bottles were kept open for 30 min to let the air to escape and then sealed using a rubber septum and kept for 4 h. One millilitre sample of the headspace air of each bottle was injected into a gas chromatograph (dsCHROM 6200, Donam Instruments Inc., Sungnam-City, Kyungki-Co, Korea) packed with a Poropak Q column maintained at 70°C and equipped with a flame ionization detector. The amount of ethylene emission was expressed as p mol ethylene g^{-1} fresh weight h^{-1} by comparing with the standard curve generated using pure ethylene (Praxair Korea Co., Ltd., Kangnam-Ku, Seoul, Korea). The plant dry weight was determined by drying the plant samples to a constant weight in an oven maintained at 50°C and expressed in mg $plant^{-1}$.

Statistical analysis

Results were statistically analysed in one- or two-way ANOVA using STATISTICA 8.0 Software (Statsoft Inc., Tulsa, OK, USA). To quantify and to evaluate the sources of variation and critical differences (CD), values were calculated at P level of 0·05%.

Results

Biofilm formation, co-aggregation assay and enumeration of bacterial population

Methylobacterium strains CBMB20 and CBMB120 in combination with *A. brasilense* strain CW903 was screened for biofilm formation based on the adherence to polystyrene microtitre plates (Fig. 1a). The biofilm formation by *Methylobacterium* CBMB20 and CBMB120 strains, when cultivated under mixed culture conditions, along with *A. brasilense* CW903 increased by 13·8 and 26·3%, respectively, compared to biofilm formed by individual strains.

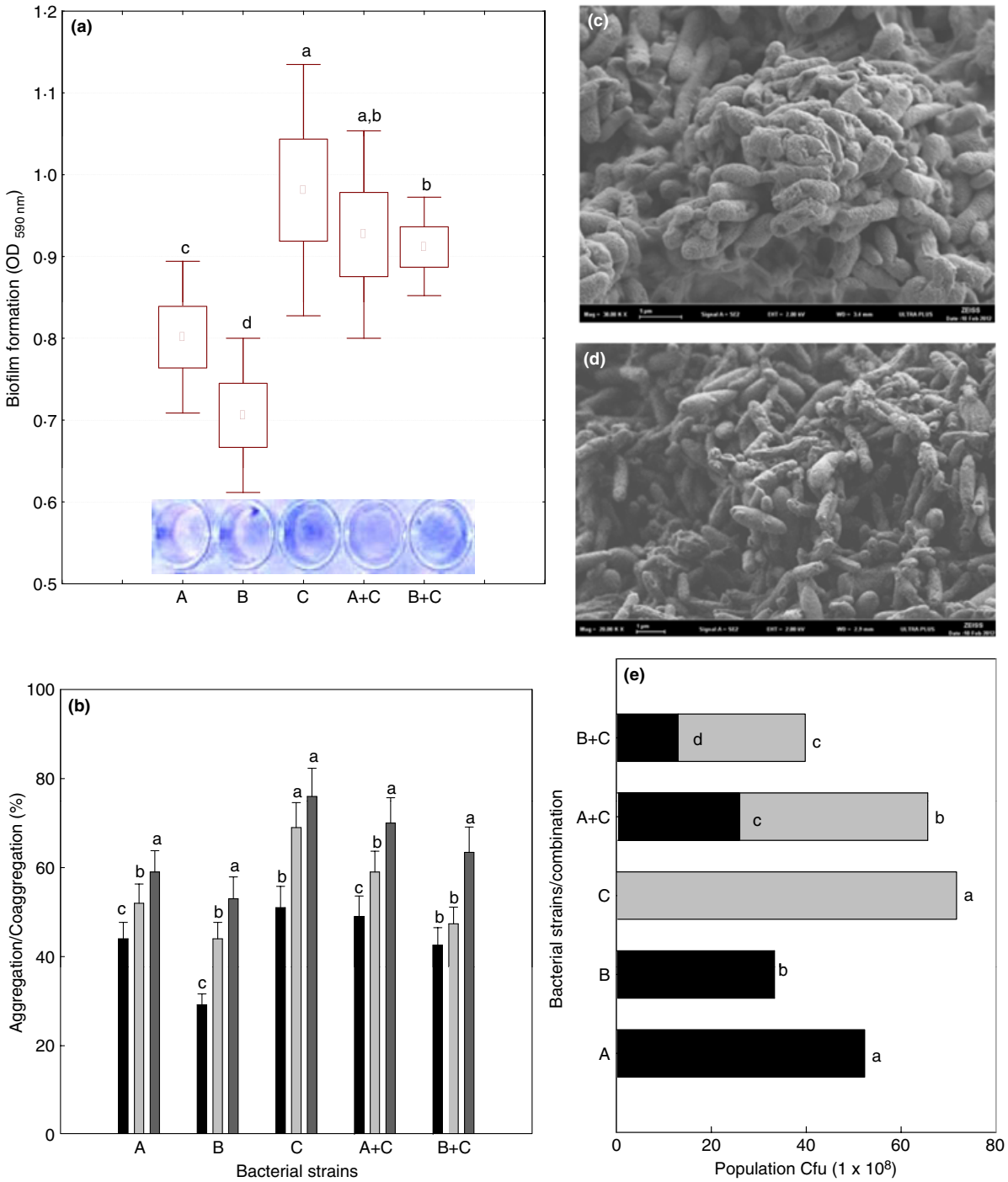


Figure 1 Autoaggregation/Coaggregation of *Methylobacterium oryzae* (CBMB20) and *Methylobacterium suomiense* (CBMB120) with *Azospirillum brasilense* (CW903). The experiments were conducted using single strain (A-CBMB20; B-CBMB120; C-CW903) and mixed strains (A + C–CBMB20 and CW903; B + C-CBMB120 and CW903) (a) Box plot analysis showing biofilm formation in microtitre plates under single strain and mixed culture conditions. The insert is the photograph showing the biofilm formation in microtiter plate. (▯) Mean; (▭) Mean+SE and (▭) Mean+SD (b) Autoaggregation/coaggregation under single strain and mixed culture conditions in the presence of calcium chloride and aluminum sulphate. Scanning electron microscope (SEM) view of CBMB20 + CW903 strains. (■) Control; (▩) calcium chloride and (■) aluminium sulphate (c) and CBMB120 + CW903 strains (d) under coaggregated conditions. (e) Population expressed in CFU ml^{-1} of wet weight of *Methylobacterium* and *A. brasilense* strains under autoaggregated/coaggregated conditions. Values are a mean of six replications ± SD. Different letters after values indicate that there is a significant difference at a *P* value of 005, according to Tukey's test. (■) *Methylobacterium* and (▩) *A. brasilense*.

Based on a visual scoring assay, it was observed that both *Methylobacterium* strains scored a higher score when co-aggregated with *A. brasilense* CW903. The combination of *M. oryzae* CBMB20 with *A. brasilense* CW903 was found to exhibit the highest co-aggregation score of 4+. The other *Methylobacterium* strain CBMB120 recorded a score of +3 under co-aggregated conditions with *A. brasilense* CW903 (Data not shown).

The exact co-aggregation percentages of *Methylobacterium* strains were worked out based on the spectrophotometric assay. Similar to the visual scoring assay, the percentage aggregation of both *Methylobacterium* strains increased under co-aggregated conditions (Fig. 1b). The methylobacterial strains CBMB20 and CBMB120 under co-aggregated conditions with *A. brasilense* CW903 recorded an aggregation percentage of 49·0 and 42·6%, respectively. Addition of aluminium sulpfate increased co-aggregation of *Methylobacterium* strain CBMB20 by 43% and CBMB120 strain by 49%.

Scanning electron microscope analysis of two *Methylobacterium* strains CBMB20 and CBMB120 co-aggregated with *A. brasilense* CW903 is depicted in Fig. 1c,d. *Methylobacterium* strains CBMB20 and CBMB120 recorded a population of $16{\cdot}0 \times 10^8$ and $13{\cdot}0 \times 10^8$ under co-aggregated conditions (Fig. 1e). *Azospirillum brasilense* CW903 recorded a population of $39{\cdot}6 \times 10^8$ and $26{\cdot}8 \times 10^8$ under co-aggregated conditions with CBMB20 and CMBM120 strains.

Survivability and cell release from alginate beads

The strains CBMB20 and CBMB120 recorded a population of 9·3 and 9·4 log CFU g^{-1} of bead under co-aggregated condition with *A. brasilense* CW903 (Fig. 2a). Log reduction in bacterial population after 1 year of storage at room temperature was highest ($-3{\cdot}2$ log CFU g^{-1}) in the combination of CBMB120 and CW903 strains (Fig. 2b). On the other hand, least population reduction ($-1{\cdot}6$ log CFU g^{-1}) was observed in the combination of CBMB20 and CW903 strains.

The cell release of CBMB20 and CBMB120 strains from alginate beads under co-aggregated conditions with CW903 was evaluated, and the results are presented in Fig. 2. (c,d and e). No significant reductions in bacterial populations were noticed under co-aggregated conditions when compared to the pure cultures of CBMB20 strain (Fig. 2c). The bacterial population of CBMB20 under co-aggregated condition was 5·1 log CFU g^{-1} in soil. Significant reduction in bacterial population up to 20%, compared with pure culture was observed with CBMB120 strain when co-aggregated with *A. brasilense* CW903 (Fig. 2d). No significant differences in bacterial population compared with pure culture were observed for CW903 strain when co-aggregated with either CBMB20 or CBMB120 strain (Fig. 2e). View of *Methylobacterium* CBMB20 and *A. brasilense* CW903 co-aggregated cells entrapped in alginate is given in Fig. 2 (f,g). Colony morphology of CBMB20 and CW903 strains in LB broth with x-gal if provided in Fig. 2 (h) and colony morphology of CW903 strain in Nitrogen free malate medium is provided in Fig. 2(i).

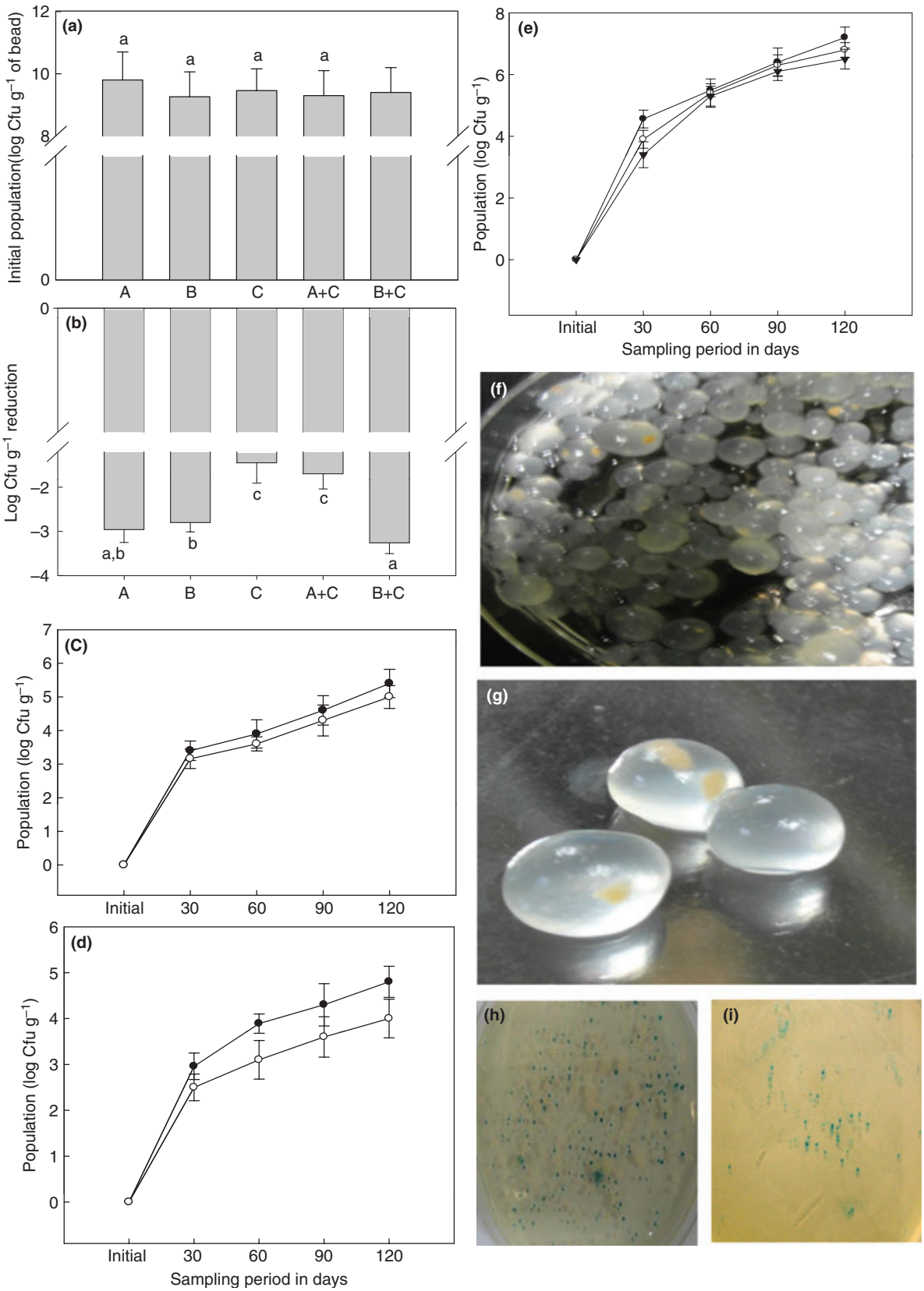
Screening for plant colonization competency traits

Growth, hydrolytic enzyme production and aggregation of *Methylobacterium* strains CBMB20 and CBMB120 strains in pectin and CMC under single and mixed culture conditions with *A. brasilense* CW903 were studied and the results are summarized in Fig. 3 (a–c).

Under mixed culture conditions, along with *A. brasilense* CW903, the total bacterial population was found to be high in pectin than CMC (Fig. 3a). In pectin, total viable population of 7·71 log CFU ml^{-1} and 7·59 log CFU ml^{-1} was observed for CBMB20 and CBMB120 strains, respectively, under mixed culture conditions with *A. brasilense* CW903 (Fig. 3a). In CMC, under *A. brasilense* CW903 mixed culture conditions, a total viable count of 5·4 log and 4·5 log CFU ml^{-1} were recorded for CBMB20 and CBMB120 strains.

In the presence of pectin or CMC, the hydrolytic enzyme production efficiency of CBMB20 and CBMB120 strains under single and mixed culture conditions with CW903 was evaluated and the results are presented in Fig. 3b. Highest PL activity of 980·39 units mg pro-

Figure 2 Survival and cell release from alginate bead entrapped *Azospirillum brasilense* (CW903) and *Methylobacterium* (CBMB20, CBMB120) coaggregated strains grown as single strain (A-CBMB20; B-CBMB120; C-CW903) and as mixed cultures (A + C–CBMB20 and CW903; B + C-CBMB120 and CW903) (a) Population (log CFU g^{-1}) of bacterial cultures under single strain alone and coaggregated conditions (b) Log reduction in bacterial populations maintained at a 30°C temperature after one year of storage. c,d and (e) show the cell release in soil from the formulation prepared with single strain alone and under coaggregated (Co-ag) conditions in soil. (f) Photograph of alginate entrapped CBMB20 and CW903 coaggregated cells (g) Close up view of CBMB20 and CW903 alginate bead entrapped coaggregated cells of (h) Colonies of CW903 and CBMB20 coaggregated cells without antibiotic and x-gal supplementation in Luria Bertani (LB) agar (i) Colonies of CW903 in LB agar plates supplemented with kanamycin (25 μg ml^{-1}) and X-gal (30 μg ml^{-1}). Values are a mean of six replications ± SD. Different letters after values indicate that there is a significant difference at a *P* value of 005, according to Tukey's test.(a) (▣) Bacterial population. (b) (▣) population reduction; (c) (–•–) CBMB20 and (–○–) CBMB20 (coag CW903). (d) (–•–) CBMB120 and (–○–) CBMB120 (coag CW903). (e) (–•–) CW903; (–○–) CW903 (coag CBMB20) and (–▾–) CW903 (coag CBMB120).


(a)
Initial population(log Cfu g−1 of bead)
12
10
8
0
a
a
a
a
a
A
B
C
A+C
B+C
(b)
Log Cfu g−1 reduction
0
−2
−3
−4
a,b
b
c
c
a
A
B
C
A+C
B+C
(C)
Population (log Cfu g−1)
7
6
5
4
3
2
1
0
Initial
30
60
90
120
(d)
Population (log Cfu g−1)
6
5
4
3
2
1
0
Initial
30
60
90
120
Sampling period in days
(e)
Population (log Cfu g−1)
8
6
4
2
0
Initial
30
60
90
120
Sampling period in days
(f)
(g)
(h)
(i)

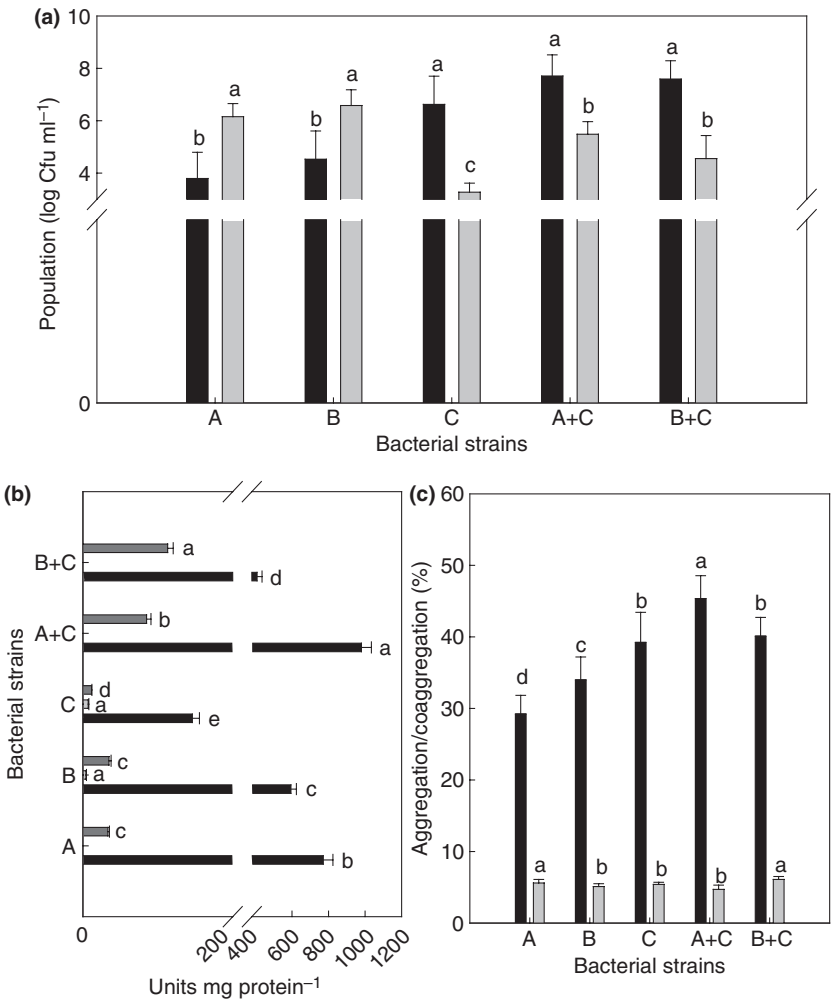


Figure 3 Growth and hydrolytic enzyme production of *Methylobacterium oryzae* (CBMB20) and *Methylobacterium suomiense* (CBMB120) with *Azospirillum brasilense* (CW903), in which culture were grown in minimal salts medium supplemented with Pectin or CMC and experiments were conducted using single strain (A-CBMB20; B-CBMB120; C-CW903) and in mixed cultures (A + C–CBMB20 and CW903; B + C-CBMB120 and CW903) (a) Growth expressed in CFU ml^{-1} of *Methylobacterium* and *A. brasilense* populations (b) Hydrolytic [Pectin lyase (PL), Pectin methyl esterase (PME) and Endoglucanase (EG)] enzyme production by pure and mixed bacterial cultures. (c) Autoaggregation/coaggregation under single and mixed culture conditions. Values are a mean of six replications ± SD. Different letters after values indicate that there is a significant difference at a *P* value of 005, according to Tukey's test. (a) (■) MSM + Pectin and (□) MSM + CMC. (b) (■) PL; (□) PME and (■) EG. (■) pection and (□) CMC.

tein^{-1} was recorded with mixed cultures of CBMB20 and CW903 strains. Low PME activity of 4·6 and 6·4 units mg protein^{-1} for CBMB120 and CW903 strains and no activity for the strain CBMB20 and no significant increase in PME activity under mixed culture conditions of either strain were observed. Higher Endoglucanase (EG) activity of 159·6 units mg protein^{-1} was recorded by the strain CBMB120 under pure culture condition, and no increase in activity under mixed culture conditions was observed. Highest aggregation of 46·4% was observed under mixed culture conditions of CBMB20 and CW903 strains in pectin (Fig. 3c).

Soil moisture content and water stress index in tomato plants

Soil moisture content was determined by watering to field capacity (FC) and then withholding irrigation. The initial water content of 38·4% at 24 h was decreased to a moisture content of 16·3% at 4·5 days (Fig. 4a). At the end of 4·5 days, water stress index scoring was carried out and the results are depicted graphically in Fig. 4 (b,c), showing their effects on plant growth. Among the different treatments, the highest stress index of 4·0 was recorded in control plants devoid of any bacterial inoculations that were subjected to stress. The lowest stress of 1·8 was recorded in plants inoculated with co-aggregated cells comprising of *Methylobacterium* CBMB20 and *A. brasilense* CW903 strains.

Peroxidase activity and lipid peroxidation, stress ethylene emission and dry weight of tomato plants

Inoculation effect of co-aggregated *Methylobacterium* strains on peroxidase activity, lipid peroxidation, stress ethylene emission and plant dry weight in tomato plants was studied under the background of water stress and the results are presented in Fig. 5 (a–d).

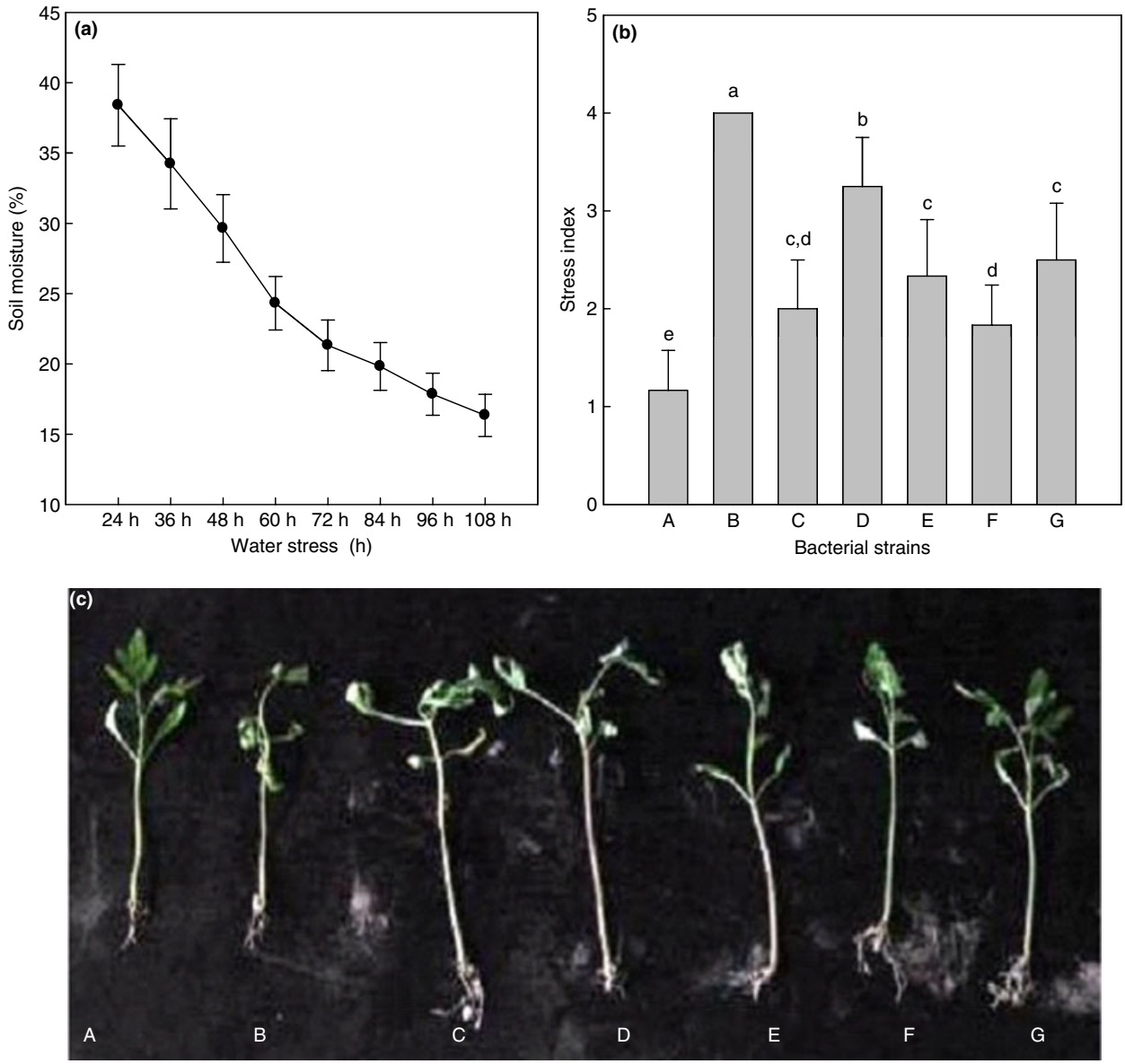


Figure 4 Soil Moisture content at different time intervals after withholding irrigation and water stress index in tomato plants mediated by single strain alone and coaggregated conditions (a) Soil moisture percent at different time periods after withholding irrigation. (b) Stress index in tomato plants as influenced by water stress and bacterial inoculation; the treatments include (A) Control without stress and bacterial inoculation (B) Negative control with stress and devoid of bacterial inoculation (C,D,E) plants inoculated with *Methylobacterium* (CBMB20,CBMB120) and *Azospirillum brasilense* (CW903) strains under normal or ample water present conditions (F) CBMB20 and CW903 strains under coaggregated conditions (G) CBMB120 and CW903 strains under coaggregated conditions and the figure (c) showing the respective treatments effects on the plants growth. Values are a mean of six replications ± SD. Different letters after bar values on the top of error bar indicate that there is a significant difference at a *P* value of 0·05, according to Tukey's test. (a) (–•–) Moisture content (%). (b) (□) stress index.

Peroxidase activity in water-stressed tomato plants as influenced by bacterial inoculation was studied and the results are presented in Fig. 5(a). Highest peroxidase activity of 3·82 U mg^{-1} of protein was recorded in control treatment subjected to water stress without any bacterial treatment. The reduction in the peroxidase activity (1·9 U) was recorded in plants treated with co-aggregated cells comprising of *Methylobacterium* CBMB20 and *A. brasilense* CW903 strains.

Lipid peroxidation in terms of MDA levels was determined in tomato plants grown under water-stressed conditions under the influence of bacterial inoculation was studied and the results are presented in Fig. 5(b). Although the bacterial treatment significantly reduced

MDA levels compared to the control treatments devoid of any bacterial inoculation, no significant differences among the bacterial treatments could be observed.

Ability to ameliorate water stress in tomato plants by bacteria was analysed based on the level of stress ethylene produced (Fig. 5c). In general, the bacterial treatments were found to significantly decrease the stress ethylene levels under water-stressed conditions. Better efficiency in stress reduction was observed in co-aggregated cell combination of CBMB20 and CW903 strains, which recorded a ethylene level of 1·8 [n mol (g Fw^{-1}) h^{-1}].

All bacterial treatments were found to significantly increase plant dry weight compared to control treatment without bacterial inoculation (Fig. 5d). Highest plant dry weight of 27·7 mg $plant^{-1}$ was observed in the plants treated with co-aggregated cells comprising of *Methylobacterium* CBMB20 and *A. brasilense* CW903 strains.

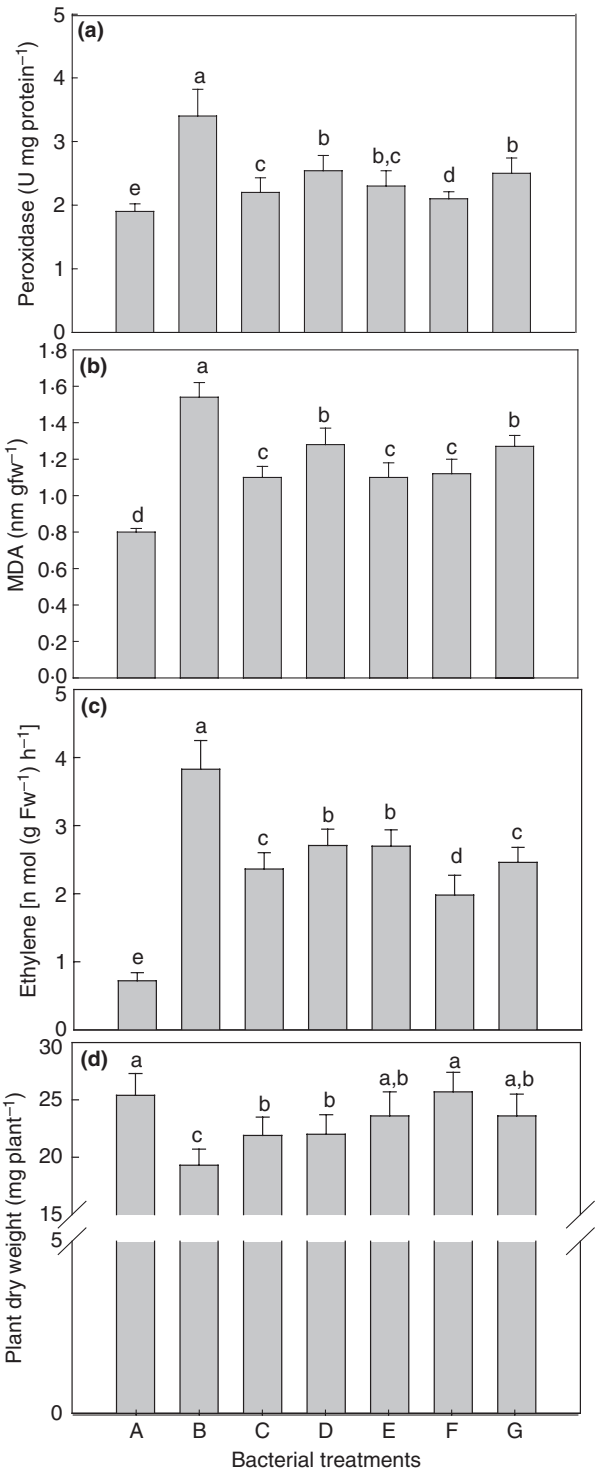


Figure 5 Peroxidase activity, malondialdehyde (MDA) content, stress ethylene levels, and plant height of tomato plants mediated by single strain alone and coaggregated bioformulations under water stressed conditions. The treatments include: (A) Control without stress and bacterial inoculation (B) Negative control with stress and devoid of bacterial inoculation (C,D and E) Single strains alone inoculated conditions (F) CBMB20 and CW903 strains under coaggregated conditions (G) CBMB120 and CW903 strains under coaggregated conditions. (a) Peroxidase activity (b) MDA content (c) Ethylene levels (d) Plant dry weight. Values are a mean of six replications ± SD. Different letters after bar values on the top of error bar indicate that there is a significant difference at a *P* value of 0·05, according to Tukey's test. (a) (▭) Peroxidase. (b) (▭) MDA. (c) (▭) Ethylene. (d) (▭) Plant dry weight.

Discussion

In the present study, the *Methylobacterium* strains CBMB20 and CBMB120 were able to form dual species biofilm with *A. brasilense* strain CW903 under static conditions in high C/N fructose minimal media. Both the *Methylobacterium* strains showed significant increase in aggregation percentage when co-aggregated with *A. brasilense* CW903. Although no co-aggregation studies have been carried out with the combination of *Methylobacterium* and *A. brasilense* strains, the ability of *A. brasilense* to co-aggregate with other PGPR strains was well documented by Neyra *et al.* (1995).

Scanning electron microscope observation shows that *A. brasilense* forms the major population in the co-aggregated bacterial inoculant comprising of *A. brasilense* and *Methylobacterium* strains. This observation was further supported by the plate count assay (see Fig. 1. F), using selective media. *Azospirillum* is known to form an aggregate type of colonization supported by massive fibrillar material (Bashan and Levanony 1990) and can colonize anything that comes into contact they may including sand, root surfaces, polystyrene beads and eukaryotic cell models such as *Chlorella vulgaris* (Bashan *et al.* 1991; Bashan and Holguin 1993, 1997; de-Bashan *et al.* 2011). These fibrillar materials play a vital role in anchoring the

bacterial cells to various surfaces and established connections between cells within bacterial aggregates (Bashan *et al.* 1991). In the presented study, a similar type of fibrillar attachment was observed in *A. brasilense* CW903 cells when they co-aggregated with *Methylobacterium* cells.

The better compatibility between CBMB20 and CW903 strains observed in our study is similar to the findings reported by Madhaiyan *et al.* (2010); they observed a synergistic association between *M. oryzae* CBMB20 with *A. brasilense* CW903 strains under co-inoculated conditions, and under such conditions, the N concentrations of the inoculated plants were also improved. Increase in co-aggregation reactions between *Methylobacterium* and *A. brasilense* strains as influenced by addition of calcium and magnesium ions is in accordance with the earlier findings of Toeda and Kurane (1991), and they reported that flocculation in *Alcaligenes cupidus* KT201 strain was synergistically stimulated by bivalent/trivalent cationic addition. The survivability and cell release from CBMB20 strain-based inoculant under co-aggregated conditions with *A. brasilense* was better compared to CBMB20 strain alone. Neyra *et al.* (1997) in his patent titled 'Flocculated microbial inoculants for delivery of agriculturally beneficial microorganisms' examined the survival of *Enterobacter* spp. and *Pseudomonas* sp. with *A. brasilense* flocs and reported that both the strains when cultivated as co-flocs with *A. brasilense* had a better survival rate compared to either strains-alone treatment. Possible explanation for reduction in population of CBMB120 strain under co-aggregated conditions with *A. brasilense* CW903 could be explained based the fact that certain mixtures of the bacterial strains do not show synergistic effects compared with the separate application of the bacteria, especially this property was noticed among the bacteria possessing biocontrol traits and also in *A. brasilense* (Schmidt *et al.* 2004; Felici *et al.* 2008).

Bashan and Gonzalez (1999) reported that 10% of *A. brasilense* original population entrapped in alginate beads survived a storage period of 14 years, as measured by three independent methods. Recent report by Trejo *et al.* (2012) demonstrated that *A. brasilense* viable cells accounting to a population of over 10^4 cells beads^{-1} from an initial bacterial population of $5{\cdot}89 \pm 0{\cdot}46 \times 10^5$ cells alginate beads^{-1}, survived after one year of storage period under dry conditions. In the present study in addition to aggregation, alginate entrapment has also contributed much to the better survivability and performance of these co-aggregated inoculants.

Cellulose is the major component of plant cell wall, whereas the middle lamella, which connects the cells, constitutes mainly of pectin (Verma *et al.* 2001). Hydrolytic enzymes such as cellulase and pectinase play a major role in the entry of bacteria into plant roots through degrading, thinning and solubilization of the plant cell wall materials (Arunika *et al.* 2007). Taking this into account, the presence of the enzymes including PL, PME and EG were analysed in *Methylobacterium* and *A. brasilense* strains using pectin and CMC as a substrate. An increase in population was noticed under mixed culture condition of *Methyobacterium* and *A. brasilense* strains compared to pure culture conditions and the activity of PL was also found to be increase under these conditions. Although no such increase in population or endoglucanase activity under mixed culture conditions was observed, the *A. brasilense* strain CW903 exhibited acetylene reduction activity in CMC when co-inoculated with either CBMB20 or CBMB120 *Methylobacterium* strain (Joe M.M and Sa T, unpublished data). In an earlier study, Khammas and Kaiser (1992) reported that co-cultures of *A. brasilense* species with *Bacillus polymyxa* or *Bacillus subtilis* allow the efficient utilization of pectin as carbon and energy sources for nitrogen-fixation process. Another study by Halsall and Goodchild (1986) showed that mixed cultures of *Cellulomonas* sp. and *A. brasilense* were capable of rapid growth and nitrogen fixation with either straw or cellulose as the carbon source. These authors reported that these co-cultures can be considered as metabolic associations, where in either *Bacillus* or *Cellulomonas* produces degradative by-products and fermentation products from pectin and cellulose, which can be used by *A. brasilense* species.

The optimal level for the inoculation of *A. brasilense* was suggested to be 10^5–l0^6 CFU ml^{-1} in wheat (Bashan 1986a); however, for seed treatment with alginate-based inoculants, a higher titre value of 10^9 CFU ml^{-1} has been recommended due to the slow release nature of alginate-based inoculants (Bashan 1986b). Similarly in the present study, slow release nature was also observed with the aggregated bacterial inoculant.

Inoculation with *Azospirillum* improved growth under water-stressed conditions (Bashan and Levanony 1990; Bashan and de-Bashan 2010). Creus *et al.* (1997) hitherto reported that *Azospirillum* inoculation in wheat plants under gnotobiotic conditions resulted in better water uptake as evident by faster shoot growth in seedlings grown under NaCl or osmotic stressed conditions. Recently, del Amor and Cuadra-Crespo (2011) showed that *A. brasilense*, in combination with *Pantoea dispersa*, partly ameliorated the effects of saline stress on growth of sweet pepper plants. These authors attributed this amelioration effect to higher stomatal conductance and increased photosynthesis in inoculated plants compared to un-inoculated plants. In the presented study, all bacterial inoculants that include both co-aggregated and single strain alone bacterial inoculants were found to reduce

water stress in plants compared to the un-inoculated control. Bacterial treatments reduced the peroxidase activity and MDA levels in plants. The bacterial treatments also helped the plant to sustain their growth in terms of a significant increase in plant dry weight with reduced ethylene levels under water-stressed conditions. Among the different treatments, better performance was recorded by the co-aggregated cell-based inoculant comprising of CBMB20 and CW903 strains.

Malondialdehyde, a major reactive aldehyde resulting from the peroxidation of biological membranes is used for the estimation of damage by reactive oxygen species (Vaca *et al.* 1988). In the present study, MDA content and peroxidase enzyme activity were found to be increased in tomato plants under stress conditions. However, in treatments applied with co-aggregated cells inoculant containing *A. brasilense* and *Methylobacterium* strains reduced peroxidase activity and MDA content was observed. An increase in the level of peroxidase enzyme as an antioxidative defence mechanism and lipid peroxidation in terms of MDA content was reported in many plants under diverse environmental stress conditions (Gaspar *et al.* 1982; Kohler *et al.* 2009). A recent study by Yim *et al.* (2013) reported that plants treated with ACC deaminase-positive *Methylobacterium* sp. showed significant reduction in disease incidence and ethylene levels when challenge inoculated with *Ralstonia solanacearum* (RS). Moreover, Joe *et al.* (2012) demonstrated through field studies conducted with flocculated cultures of *A. brasilense* promoted maize plant growth and yield under moisture stressed conditions through improved rhizoplane and rhizosphere colonization.

In this study, we attempted to formulate a consortium comprising of *A. brasilense* CW903 and ACC deaminase-positive *M. oryzae* strain CBMB20. The other inoculant that comprised of ACC deaminase-negative *Methylobacterium* strain CBMB120 was developed for comparative study. The concept of co-aggregation was adopted to develop the above-mentioned inoculants because the bacterial strains can co-aggregated only with certain genetically distinct bacteria and are able to attach to one another via specific molecules (Kolenbrander *et al.* 1993). Moreover, bacteria under aggregated condition have better stress tolerance and survivability in inoculants and soil compared to the normal cells due to the high level accumulation of exopolysaccharides and polyhydroxybutyrate granules under such conditions (Burdman *et al.* 1998, 2000). We report that plant growth promotion ability and stress reduction efficiency of the co-aggregated strains comprising of *Azospirillum brasilense* CW903 and ACC deaminase-positive *Methylobacterium* strain CBMB20 with the same inoculation load showed better performances compared to the normal cell inoculants of *Azospirillum brasilense* CW903, *Methylobacterium* CBMB20 strain and *Methylobacterium* CBMB120 strains. The reason for enhanced performance might be due to combined influence of aggregation potential of *A. brasilense* CW903 with *M. oryzae* CBMB20 that serves as protective microenvironment for CBMB20 strain under co-aggregated conditions and due to the influence of the enzyme ACC deaminase of CBMB20 strain that regulates the stress in inoculated plants by reducing the ethylene biosynthesis.

The results of the present study demonstrated that *Methylobacterium* strains CBMB20 and CBMB120 were able to co-aggregate with *A. brasilense* CW903. We observed biofilm formation and co-aggregation between *M. oryzae* CBMB20 and *A. brasilense* CW903 strains. Reduction in bacterial population after one year of storage at room temperature was least in co-aggregated combination of CBMB20 and CW903 strains compared to single strain inoculant. Growth and pectinolytic enzyme production in *Methylobacterium* CBMB20 and CBMB120 strains were enhanced when co-cultivated with *A. brasilense* CW903. In general, all bacterial inoculants were found to alleviate water stress in tomato plants with a better efficiency exerted in co-aggregated cells inoculants comprising *A. brasilense* CW903 and *Methylobacterium* CBMB20 strains. This finding opens up the avenue for the development of co-aggregated cell inoculant containing ACC deaminase-positive *Methylobacterium* and *A. brasilense* strains with a better shelf life and stress abatement in plants.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A2A1A01005294). Authors thank the unknown reviewers for the constructive comments in improving the manuscript. M. R. Islam is supported by the research grant of Inha University, Korea.

Conflict of interest

We declare no conflict of interests.

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