Role of certain cationic compounds on the enhancement of flocculation in *Azospirillum brasilense* MTCC-125: Bioinoculation effect on growth of sunflower

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Zur Bedeutung bestimmter Kationenverbindungen im Ausflockungsverhalten von *Azospirillum brasilense* MTCC-125: Bioinokulationsversuche mit Sonnenblumen

1 Introduction

Azospirillum spp. are free-living plant growth-promoting rhizobacterium (PGPR) capable of promoting growth and yield of numerous plant species through the production of plant growth hormones (DOBBELAERE et al., 2003; BASHAN et al., 2004). This growth promotion activity has been positively exploited in the enhancement of growth and yield of many agriculturally important crops through bioinoculation (OKON and LABERANDERA-GONZALEZ, 1994). However, the major limitation in the field level application of *Azospirillum* or any other bioinoculant is the prevalence of harsh conditions such as frequent droughts, lack of sufficient irrigation, high salinity and soil erosion (VANVEEN et al., 1997). This harsh condition may quickly diminish the population of *Azospirillum* introduced into the soil (HEIJNEN et al., 1992).

However, *Azospirillum* spp. does display a range of very efficient physiological adaptations that may enable them to survive under unfavorable conditions. These include cyst formation (SADASIVAN and NEYRA, 1985), polysaccharide synthesis (DEL GALLO and HAEGI 1990), poly-β-hydroxybutyrate synthesis (OKON and ITZIGSOHN, 1992) and floc formation (NEYRA et al., 1995).

One among these physiological adaptations is the ability of *Azospirillum* cells to aggregate or flocculate (BURDMAN et al., 2001). This aggregated or flocculated cells formed by the modification of entire vegetative cells, often accumulates additional cell wall as well as intracellular polymers such as poly-β-hydroxybutyrate (PHB) (SADASIVAN and NEYRA, 1985; BAHAT-SAMET et al., 2004).

These flocculated cells due to the high level accumulation of these components have increased resistance to envi-

Zusammenfassung

Das Phänomen der Ausflockung bzw. der Aggregation von Azospirillum brasilense MTCC-125 wurde durch Zugabe bestimmter Kationenverbindungen erhöht. Unter allen untersuchten Kationenverbindungen erreichte die Zugabe von Aluminiumsulfat den höchsten Grad an Ausflockung (80.00 %), und führte zu einer Ausbeute von 1,26g/l (Trockengewicht). Diese ausgeflockten Zellen zeigten im Vergleich zu den Kontrollzellen einen höheren Anteil an EPS und PHB, während der Proteingehalt niedriger war. Ausgeflockte Zellen zeigten in Stress-Toleranz-Versuchen hohe Beständigkeit sowohl gegenüber osmotischer oder thermischer Belastung als auch gegenüber Austrocknung. Die Überlebensfähigkeit dieser ausgeflockten Zellen in verschiedenen beimpften Trägeratermaterialien war ebenfalls höher als in den Kontrollzellen. Der Behandlungseffekt dieser ausgeflockten Zellen wurde für folgende lebensnotwendige Setzlingseigenschaften untersucht: Vitalität, Keimfähigkeit in Sonnenblumenpflanzen gefolgt von einer Studie mit Topfpflanzen, um die Effektivität dieser ausgeflockten Zellen mit jener normaler Zellen bezüglich des phytostimulierenden Effekts zu vergleichen. Es konnte festgestellt werden, dass eine Behandlung des Saatgutes mit diesen ausgeflockten Zellen einen höheren Prozentsatz an Keimfähigkeit und Vitalität zeigte. Ebenso konnte ein positiver Trend in der Steigerung bestimmter Wachstumsparameter von Sonnenblumenpflanzen gezeigt werden.

Schlagworte: Azospirillum brasilense Sp-7, Aggregation, Flokkulation, Poly-β-hydroxybutyrat (PHB), exozelluläre Polysaccharide (EPS), Toleranz, Trägermaterial, Sonnenblumen.

Summary

The phenomenon of flocculation or aggregation in *Azospirillum brasilense* MTCC-125 was enhanced by the addition of certain cationic compounds. Among different cationic compounds studied, addition of aluminum sulphate favoured the highest aggregation percentage (80.00), and a floc yield of 1.26 g L^{-1} dry wt. These flocculated cells, when compared with control exhibited high levels of EPS and PHB content, while the protein content was found to be low. Flocculated cells when subjected to stress tolerance experiments exhibited a high degree of osmotic, thermal, and desiccation resistance. The survivability of these flocculated cells in different inoculant carrier material was also found to be higher, when compared with control. The treatment effect of these flocculated cells was studied for vital seedling parameters such as vigour index and germination percentage in sunflower crop followed by a pot culture study to compare the efficiency of these flocculated cells with normal cells for its phyto-stimulatory effect. It has been found that seed treatment with these flocculated cells exhibited a higher germination percentage and vigour index and a positive trend on the enhancement of certain growth parameters in sunflower crop.

Key words: *Azospirillum brasilense* Sp-7, aggregation, flocculation, poly-β-hydroxybutyrate (PHB), exocellular polysaccharides (EPS), tolerance, carrier material, sunflower.

ronmental factors, such as starvation, desiccation and they remain metabolically dormant as long as the stress conditions persist (BAHAT-SAMET et al., 2004). Hence, this phenomenon of flocculation in *Azospirillum* may play a vital role in production, storage and survival of bacterial inoculants for agricultural application (SADOFF, 1975).

This flocculation in *Azospirillum* may be induced either by altering the nutrient culture conditions under which the cells are grown or by the addition of various chemical agents (BARRY et al., 1969).

Though flocculation through the alteration of nutrient conditions through the modification of C/N ratio in the bacterial medium has been studied extensively under batch culture condition, in which the bacterial cells are induced to aggregate towards the end of the exponential growth phase in a medium containing high C/N ratio (SADASIVAN and NEYRA, 1985; BURDMAN et al., 1988, 1999, 2000). However, the yield of the flocculated cells using this protocol was very low. Furthermore, the generation time was long and this mechanism of physiological induction played no role during the large-scale production of flocculated cells.

This led us to search for other alternatives for the artificial induction of flocculation in *Azospirillum* and we came with this idea of using cationic compounds for this phenomenon. Moreover, the use of chemicals or cationic compounds for this purpose has not been studied extensively.

This motivated us to undertake the present study with the following objectives:

1. To study the influence of different cationic compounds on the aggregation/ flocculation in *Azospirillum.*
2. To investigate the role of these compounds on certain cellular reserve material such as EPS, PHB and protein of flocculated cells.
3. To evaluate the ability of these flocculated cells to with stand osmotic, thermal and desiccation tolerance and to screen for its survivability in different carriers.
4. Bioinoculation effect of these flocculated cells on germination percentage, vigour index and growth on sunflower crop was also determined.

2 Material and methods

2.1 Culture and growth conditions

Azospirillum brasilense (MTCC-125) obtained from IMTECH, Chandigargh, India was used throughout this study. The strains were maintained on nutrient agar slants at 30 °C. The stock cultures were routinely sub cultured in semisolid N-free malate medium.

For flocculation experiments one mL log phase (initial inoculation load 1 × 10^9 CFU mL^{-1}) culture of *Azospirillum brasilense* Sp-7 was grown in a 250 ml Erlenmeyer flasks, with 50 mL liquid medium containing a high C: N ratio (37.3 mM fructose and 4 mM ammonium chloride) as previously described by (BURDMAN et al., 1998) the medium was further enriched by the addition of a trace amount of yeast extract (0.20 g L^{-1}), Vitamin Solution (1 mL L^{-1})(containing Biotin-0.01 g and Pyridoxin-0.02 g), Trace element solution (2 mL L^{-1}) (containing $NaMoO_4$-0.02, $MnSO_4$-0.02, Boricacid-0.02, $CuSO_4$-0.008 and $ZnSO_4$-0.025 g L^{-1}) the pH was adjusted to 6.8 by using

KOH or with dilute HCl. The contents of the flask were incubated for 24 h on a rotary shaker (150 × *g*) at 30 ± 1 °C.

2.2 Artificial induction of aggregation in Azospirillum as influenced by cationic compounds studied under in vitro condition

Aggregation assay was carried out according to GRIMAUDO and NESBITT (1997). Briefly, one ml (initial inoculation load 1×10^9 CFU mL^{-1}) exponential phase cultures of *Azospirillum* MTCC-125 was inoculated into 10 ml buffer consisting of 20 mM Tris -HCl buffer (pH 7.8), 0.01 mM $CaCl_2$, 0.01 mM $MgCl_2$, 0.15 M NaCl, 0.02 % NaN_3. The addition of the cationic compounds to the buffer was made according to TOEDA and KURANE (1991), initially with Calcium chloride solution (0.05 ml; 1.6 mM) and it was replaced by various bivalant and polyvant cationic solutions, such as Aluminium sulphate, Magnesium sulphate, Ferric chloride and Sodium sulphate (0.05 mL; 1.6 mM). Uninoculated buffer served as control.

The cells containing suspension was vortexed for 10 s, shaken on a rotary platform shaker for 3 min, and left undistributed at room temperature for 24 h. The aggregation assay was performed in triplicate.

2.2.1 Estimation of aggregation

The degree of aggregation was monitored by a visual assay (CISAR et al., 1979). The degree of resultant aggregation was assigned a score ranging from 0 to 3+ by the following criteria: 0, no visible aggregates in the cell suspension; 1+ small uniform aggregates in suspension; definite aggregates seen but the suspension remained turbid; 3+ large aggregates that settled rapidly, leaving a clear supernatant

However the exact percentage of aggregation percentage was estimated by a spectrophometric assay. After degree of aggregation was scored visually, the reaction mixture was diluted with 0.5 ml of buffer, mixed gently with a Vortex mixer, allowed to stand for 30 min at room temperature and centrifuged at 7000 × *g* for 2 min. The supernatants were analyzed spectrophotometrically at 420 nm to estimate the aggregation percentage. The percentage of aggregation was calculated according to mathematical formulae described by MADI and HENIS (1989)

$$Percentage\ Aggregation = \frac{OD_t - OD_s \times 100}{OD_t}$$

Where OD_t = Total optical density after mechanical dispersion and OD_s = OD of aggregate after aggregate had settled.

2.2.2 Floc weight determination

The floc weight was determined according to SADASIVAN and NEYRA (1985). The net wt of the floc was obtained by subtracting the weight of dried filter paper from the weight of the filter paper with the floc obtained by filtration. The floc yield was expressed as mg L^{-1}. For dry wt determination the filter paper with the flocs were placed in a desiccator oven at 60 °C for 2 h.

2.3 Exocelluar polysaccharide, total protein and poly-β-hydroxybutyrate content of flocculated cells

Exocellular polysaccharide (EPS) was quantified according to DEL GALLO and HAEGI (1990). The bacteria were grown for 24 h. The cell fractions of both flocculated and normal cells were removed by centrifugation (4000 × *g*, 20 min.). The supernatant was then filtered through a 0.22 µm membrane filter in ethanol. Ethanol insoluble EPS was precipitated overnight at 4 °C with 3 volumes of ice-cold ethanol. After centrifugation (3000 × *g*, 20 min.) the EPS was re-suspended and dialyzed against distilled water for 3 days at 4 °C, using a dialysis membrane with a 2KDa molecular mass. The amount of sugar in the polysaccharide was determined by anthrone method, using glucose as a standard (DISHE, 1962). The amount of protein in the growth medium was determined according to BRADFORD (1976) using BSA as the standard.

For PHB determination 10.0 ml of cells was pelleted by centrifugation at 7000 × *g* for 20 min at 4 °C. PHB analysis was performed as per methods of LAW and SLEPECKY (1961).

2.4 Desiccation resistance

The *Azospirillum* flocculated and normal cells were transferred into sterile 1.5 mL Eppendorff micro capillary tubes and the tubes were kept open in a sterile petridish. The petridishes with the tubes were then placed in an incubator at 37 °C. After one-week incubation period, the dried cells from the capillary tubes were washed with one mL of sterile distilled water with vigorous agitation for the complete removal of the bacterial cells.

Cell numbers, before and after drying treatments were determined by MPN technique (COCHRAN, 1950).

2.5 Thermal tolerance

The *Azospirillum* flocculated and normal cells were taken in a test containing five mL of 10 mM phosphate buffer. The suspension was kept in a water bath adjusted to 50 °C temperature. After 20 min exposure the tubes were removed and cooled rapidly. Then one mL of sample from each plate was serially diluted and the bacterial viability was determined by MPN technique.

2.6 Osmotic tolerance and osmotic shock

The survivability of flocculated cells to osmotic tolerance and osmotic shock were done according to KADOURI et al. (2003). For osmotic tolerance experiments, twenty-five mL portion of 4 M glucose solution was added to the bacterial suspensions. The final glucose concentration was adjusted to 2 M. The bacteria were incubated at 30 °C for 24 h. One ml of sample was drawn and serially diluted and the bacterial viability was determined by MPN technique

Sensitivity to osmotic shock was determined by adding 25 mL of tris-glycerol solution (0.05 M tris, 4 M glycerol, pH 7.6) to 25 ml of cell suspension and incubated for 30 min at 30 °C. The cells were then centrifuged (4000 × *g*, 10 min) and re-suspended in 50 ml of distilled H_2O. Then one mL sample from each tube was drawn and serially diluted. Bacterial viability was determined by MPN technique.

2.7 Survivability in different inoculant carriers

The survivability in different carrier materials was tested according to FALLIK and OKON (1996). One ml of *Azospirillum* flocculated and normal cells were mixed with one of the following autoclaved carriers, such as vermiculite, lignite and peat. Inoculants were stored in sterile flasks at 30 °C. After seven days of incubation, the carriers have undergone desiccation to different extents. One gram of each sample was added to potassium phosphate buffer (0.06 M, pH 6.8) and stirred at 200 × *g* for 2 h at 30 °C. Bacterial viability was determined by MPN technique.

2.8 Bioinoculation effect of Azospirillum flocculated cells on sunflower

2.8.1 Germination percentage and vigour index

The treatment effect of *Azospirillum* flocculated cells were compared with the normal cells on the germination percentage and vigour index of sunflower. One hundred seeds were taken in a sterile petriplate and treated with ten ml of normal broth culture of either *Azospirillum* flocculated cells or normal cells (with an initial population 1×10^9 CFU mL^{-1}). The seeds were treated for 30 min and then shade dried. Then, these inoculated seeds were tested for the germination rate using paper towel method (ISTA, 1976). The germination percentage was calculated from eight days after sowing (DAS) to 12 DAS. The morphological characters like shoot length and root length was measured on 20 DAS. The vigour index (*VI*) of the seedlings was estimated as suggested by ABDUL-BAKI and ANDERSON (1973): $VI = RL + SL \times GP$, where *RL* is root length (cm), *SL* is shoot length (cm) and *GP* is germination percentage.

2.8.2 Seed bacterization for pot culture experiment

Seeds were surface-disinfected for three min in one per cent (w/v) NaoCl, rinsed in 70 % (v/v) ethanol for 3 min before finally rinsing three times in sterile distilled water. The efficacy of disinfection was tested by placing samples of the treated seeds on Potato dextrose agar (PDA) and Nutrient agar (NA) plates for any microbial growth. One ml (inoculation load 1×10^9 CFU mL^{-1}) of *Azospirillum* flocculated or normal cells of *Azospirillum* (control) were mixed separately with one gram of surface sterilized seed mixed with lignite and five ml of rice gruel to enhance the adhesiveness.

2.8.3 Pot culture experiment

Seeds of sunflower coated with either flocculated or normal cells of *Azospirillum* (control) were sown. The plants were thinned to three per pot, seven days after sowing. The plants were grown in green house and watered every three days with distilled water. The plants were maintained at 25 °C with 15 h light period. Three replications were maintained for each treatment.

2.8.4 Plant height

The height of the plants from each treatment was measured on 60^{th} day after sowing (DAS). The mean value of the plants from 5 replications was recorded.

2.8.5 Plant dry wt

The dry wt. of the entire plant was taken on the 60th day after sowing (DAS), five plant samples were drawn, washed and later dried to a constant wt. in an oven at 50 °C. The over dry wt of the plant sample was recorded.

2.8.6 'N' content of plant

The plant samples were collected on the 60th day after sowing (DAS), washed in water, air dried and later dried to a constant wt in an oven at 50 °C. Then they were powdered, sieved and 100 mg of sample was taken for analysis. The

Table 1: Effect of different cationic compounds on aggregation score, aggregation percentage and floc yield in *Azospirillum*
Tabelle 1*:* Einfluss verschiedener Kationenverbindungen auf das Aggregationsverhalten (Score, Prozente) und Niederschlagsausbeute bei *Azospirillum*

Polyvalant cation*	**Aggregation score****	**Aggregation (%)*****	**Floc yield g L^{-1}********	
			Fresh wt	**Dry wt**
Aluminum sulphate	3+	80.00^{a}	8.62^{a}	1.26^{a}
Calcium chloride	3+	72.84^{b}	7.82^{b}	1.10^{b}
Magnesium sulphate	2+	68.00^{c}	7.12^{c}	1.04^{c}
Ferric chloride	1+	54.00^{d}	5.92^{d}	0.94^{d}
Sodium sulphate	1+	51.00^{e}	4.82^{e}	1.36^{e}
Control	1+	40.00^{f}	4.20^{f}	0.88^{f}

* Addition of cationic compounds at a concentration of 1.6 mM.
** Visual assay according to CISAR et al. (1979).
*** Aggregation assay according to MADI and HENIS (1989).
**** Floc yield determined according to SADASIVAN and NEYRA (1985).
Values are a mean of three determinants ± S.D. Within a column different letters after values indicate that there is a significant difference at a P value of 0.05 as determined by a post hoc test.

Table 2: Resistance of *Azospirillum* cells* to osmotic pressure, osmotic shock, temperature and desiccation tolerance
Tabelle 2: Widerstandsfähigkeit von *Azospirillum*-Zellen* gegenüber osmotischem Druck, osmotischem Schock, Temperatur sowie Trockenheitsstoleranz

Morphological form	**Osmotic Pressure (Glucose 2M)[A]**	**Osmotic shock[B]**	**Thermal tolerance[C]**	**Desiccation Tolerance[D]**
Flocculated cells	$(2.62 \pm 0.2) \times 10^{8a**}$	$(7.20 \pm 0.6) \times 10^{6a}$	$(3.20 \pm 0.2) \times 10^{7a}$	$(2.62 \pm 0.4) \times 10^{8a}$
Normal cells	$(1.82 \pm 0.2) \times 10^{8b}$	$(3.82 \pm 0.6) \times 10^{6b}$	$(1.24 \pm 0.6) \times 10^{3b}$	$(1.22 \pm 0.2) \times 10^{6b}$

* Initial inoculation load 1×10^{9} CFU mL^{-1}
** No of survival cells mL^{-1}
A Osmotic tolerance at 2 M glucose
bB Osmotic shock determined in Tris glycerol solution (0.05 M Tris, 4 M glycerol) at pH 7.6
c For thermal tolerance experiment the cells were kept in a water bath at 50 °C for 60 min.
dD For desiccation tolerance experiment the cells were placed in a desiccator oven at 30 °C for one week incubation period.
Values are a mean of three determinants ± S.D. Within a column different letters after values indicate that there is a significant difference at a P value of 0.05 as determined by a post hoc test.

Table 3: Effect of *Azospirillum* flocculated cells on germination percentage, vigour index, plant growth and N uptake of sunflower
Tabelle 3: Einfluss von flokkulierten *Azospirillum*-Zellen auf die relative Auskeimungsdichte, den Vitalitätsindex, das Wachstumsverhalten und die Stickstoffaufnahme von Sonnenblumen

Treatment	**Germination (%)**	**Vigour index**	**Plant height***	**Plant dry wt g $plant^{-1}$***	**'N'** Uptake kg ha^{-1}***
Uninoculated Control	70.5^{c}	764.12^{c}	45.14^{c}	1400.72^{c}	170.42^{c}
Flocculated cells	92.5^{a}	1312.24^{a}	64.12^{a}	1322.70^{a}	198.72^{a}
Normal cells	86.5^{b}	1462.50^{b}	53.12^{b}	1362.42^{b}	184.70^{b}
LSD(P = 0.05)	2.54	12.46	4.12	16.74	5.74

* Observations at 60 DAS
** "N" uptake assayed according to Microkjeldahl assay
Values are a mean of six replications. Mean values followed by different letters are differed significantly according to least significant difference test ($P < 0.05$)

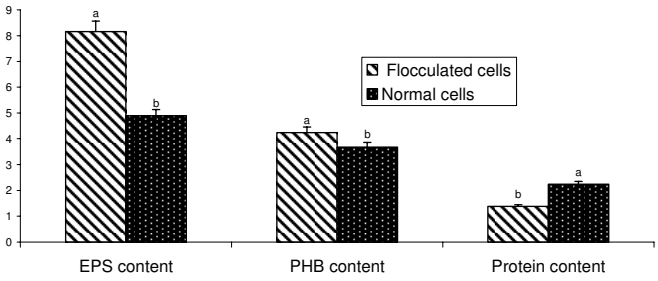
total nitrogen content was estimated by Microkjeldahl method (BREMNER, 1960).

2.8.7 Statistical analysis

The experimental results were statistically analyzed by analysis of variance (ANOVA) and the treatment means were compared relative to control following a post hoc test or least significant difference (LSD) test. Differences were only considered when significant at $P < 0.05$ as described by GOMEZ and GOMEZ (1984).

3 Results and discussion

The results of our present study showed that different cationic compounds are able to induce aggregation in *Azospirillum* (Table 1). Among the different cationic compounds studied, aluminum sulphate at a concentration of 1.6mM augmented the highest aggregation ability (80 %) followed by calcium ions (72 %), while the control treatment recorded (40 %). This increase in aggregation as influenced by cationic compounds is in conformity with the previous findings of TOEDA and KURANE (1991) that aggregation in *Alcaligenes cupidus* KT201 was synergistically stimulated by the addition of bivalent/trivalent cations such as Ca^{2+} and Al^{3+}. They further reported that trivalent cations were more effective in inducing flocculation, when compared with bivalent cations, with Al^{3+} being the most effective cation.



* EPS content as mg sugar (g dried bacteria)$^{-1}$

** PHB content as µL mL^{-1} of $CHCl_3$ extract

Values are a mean of for three replication ± SD. Within a column different letters after values indicate that there is a significant difference at p value of 0.05, as determined by one way analysis of variance followed by a post hoc test.

*** Protein content as mg protein (g dried bacteria)$^{-1}$

Figure 1: Total EPS, PHB and protein content of flocculated and normal cells of *Azosporillum*

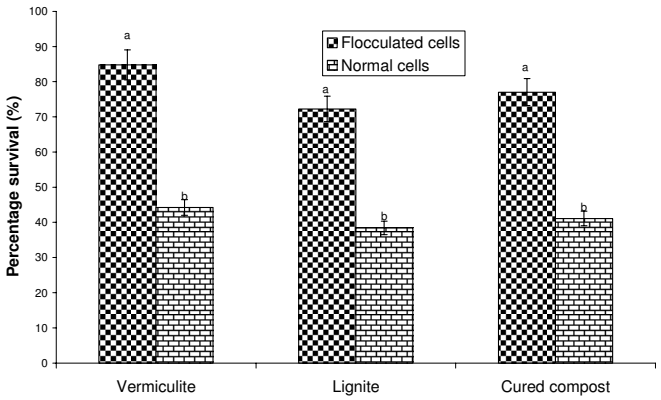
Abbildung 1: Gehalt an EPS, PHB und Protein in flokkulierten und normalen Zellen von *Azosporillum*

Figure 1 compares the EPS, protein and PHB content of the cationic induced flocculated cells with that of normal cells. It has been clearly evident from the figure that *Azospirillum* flocculated cells exhibited a higher level of EPS (9.42 mg sugar (g dried bacteria)$^{-1}$) and PHB content (0.13 g L^{-1} of cell dry wt). However, the protein content was low, when compared with normal cells.

The results of our present study is in conformity with the earlier studies that during flocculation *Azospirillum* cells; often accumulates additional cell wall or capsular material (BAHAT-SAMET et al., 2004) as well as intracellular polymers such as poly-β-hydroxy butyrate (PHB) (BURDMAN et al., 1999). The decrease in the protein content observed in our present study is in line with the earlier observations of OLUBAYI et al. (1998); reported a decrease in the cellular protein and a proportional increase in the cellular PHA and carbohydrates during flocculation.

The desiccation and thermal tolerance of *Azospirillum* flocculated cells studied under in vitro condition are presented in table 2. It was observed that *Azospirillum* flocculated cells exhibited a high level of thermal and desiccation tolerance, when compared with normal cells. The increase in the content of poly-β-hydroxybutyrate (PHB) during flocculation and the positive role of PHB in the enhancement of thermal and desiccation tolerance in *Azospirillum* has been reported earlier (SADASIVAN and NEYRA, 1985; BLEAKLEY et al., 1988; TAL and OKON , 1985; FALLIK and OKON, 1996; JOE et al., 2009). The result of our present study is in conformity with the above findings.

The survivability of the *Azospirillum* flocculated cells to glycerol-induced osmotic shock and osmotic tolerance (Glucose 2M) were studied under in vitro conditions and the results are summarized in Table 2. It was observed that *Azospirillum* flocculated cells exhibited a high degree of resistance to osmotic tolerance tests, when compared with normal cells. RUIZ et al. (2001) reported that guyanosine tetraphosphate (ppGpp) level occur concomitantly with PHA degradation. There is a strong evidence that ppGpp induces the expression of the rpoS gene (GENTRY et al., 1993). This gene encodes a transcription factor which activates the expression of genes involved in protecting against damaging agents, such as ethanol, H_2O_2, high temperature or high salt concentration (RAMOS-GONZALEZ and MOLIN, 1998). The increase in the resistance of *Azospirillum* flocculated cells to osmotic shock and osmotic tolerance observed in our present study may be due to the high level accumulation of ppGpp that induces the expression of rpoS gene, responsible for resistance against adverse conditions.



* Survival percentage after seven days of incubation at 30 °C.
Values are a mean of three replications ± SD, values followed by different letters are differed significantly at a p value of 0.05, as determined by one way analysis of variance followed by a post hoc test.

Figure 2: Survival rate of flocculated and normal cells of *Azosporillum* in different inoculant carriers

Abbildung 2: Überlebensrate flokkulierter und normaler Zellen von *Azosporillum* in unterschiedlichen Trägersubstraten

The *Azospirillum* flocculated cells exhibited a high level of survivability, when compared with normal cells in different carrier materials studied (Figure 2). Among the different carrier materials studied vermiculite sustained the highest survival population, followed by peat and lignite (Figure 2). FALLIK and OKON (1996) reported the survivability of inoculated bacteria has been significantly reduced after a six months storage period. This reduction that was observed in our studies too may probably be due to the stress that developed during storage under suboptimal conditions, such as lack of moisture, stress and available nutrients. Previous studies (BLEAKLEY et al., 1988; PEREG-GERK et al., 2000; KADOURI et al., 2003) have showed that flocculated cells of *Azospirillum* rich in PHB granules exhibited a high degree of resistance to desiccation. Furthermore, this phenomenon has increased resistance to environmental stress, as these cells remain metabolically dormant as long as the stress conditions persist (MADI et al., 1985).

Seed treatment with *Azospirillum* flocculated cells has significantly increased the germination percentage and vigour index of sunflower, when compared with normal cell treatment (Table 3). In the present study, the increase in vigour index and germination percentage of sunflower might be due to the increased survivability exhibited by the flocculated cells on the spermosphere and spermoplane of maize seed. This survivability may in turn enhance the production of growth hormones (auxins, gibberellins and cytokinins. Recent report by SIVAKUMAAR and JOE (2008) has showed that seed treatment with co-aggregated cells of *Azorhizobium* and *Azospirillum* has increased the vigour index in rice grown under in vitro conditions.

The results pertaining to the phyto-stimulatory effect of the aggregated cells of *Azospirillum* on sunflower crop are presented in the table 3. It has been found that these flocculated cells of *A. brasilense* positively augmented plant height; plant dry wt, N uptake and grain yield to a significant extent. Numerous studies have showed the indirect role of surface components associated with the flocculation process play a significant role in plant root colonization (BARRY et al., 1969; CROES et al., 1993; SADASIVAN and NEYRA, 1985; MICHIELS et al., 1990; KATUPITIYA et al., 1995; PEREG-GERK et al., 2000). This increase in root colonization as influenced by these cells increase the density and length of root hairs, as well as the appearance and elongation rates of lateral roots, thus increasing the root surface area (FALLIK et al., 1994). Further studies have shown that these morphological and physiological changes of the inoculated plant roots, lead to an enhancement of water and mineral uptake (OKON and KAPULNIK 1986).

4 Conclusion

The results of our present study are encouraging and have demonstrated that different cationic compounds are able to enhance the aggregation and also the floc yield in *Azospirillum*. These flocculated cells were also found to have increased resistance when subjected to different stress tolerance experiments and further, these cells also have an increased survivability in different inoculant carriers. The field results pertaining to the application of these flocculated cells are also quite encouraging. From the results it has been found that the application of these flocculated cells would be a promising approach for sunflower and other crops grown under rain fed conditions in temperate regions, where frequent droughts and lack of sufficient irrigation may prevail.

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