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Bioflocs: A novel bioformulation of PGPR cells with higher degree of stress tolerance

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Abstract
The positive effect of different bioformulations, namely, vegetative cell, natural and artificial bioflocs of PGPR cells, namely, *Azotobacter* and *Paenibacillus* to various environmental stresses, namely, temperature, desiccation and salt tolerance was studied under *in-vitro* condition.
It was observed that the natural and artificial bioflocs preparation of both *Azotobacter* and *Paenibacillus* cells exhibites higher tolerance to various environmental stresses, *viz*, temperature, desiccation and salt tolerance when compared to their respective vegetative cell formulations. Between the two bioflocs formulation, the natural biofloc formulation of both *Azotobacter* and *Paenibacillus* cells exhibited higher stress tolerance when compared to the artificial biofloc formulation. Between the two PGPR cells, the *Paenibacillus* cells in natural biofloc formulation exhibited higher stress tolerance than *Azotobacter* cells.
It was hypothesized that the "EPS mediated natural biofloc formulation" of PGPR cells exhibited higher tolerance to environmental stresses when compared to the respective "EPS deficient artificial biofloc and vegetative cell" formulations.

Keywords: Plant seed materials, Thermal tolerance, Desiccation tolerance, Generation time, Natural and Artificial biofloc

1. Introduction
Rhizosphere bacteria that favourably affect the plant growth and yield of commercially important crops are dominated as "plant growth promoting rhizobacteria" (PGPR) Van loon (2004) [32]; Dimkpa (2009) [6]. Several mechanism of plant-microbe interaction may participate in the association and affect the plant growth, including, 'N'-fixation, hormonal interaction, improvement in root growth, solubilization of nutrients, ACC-deaminase (acd) production and ethylene modulation at rhizosphere level, alleviation of soil salinity and biocontrol against phytopathogens. Thus, the PGPR affect the plant growth directly by producing and secreting plant growth promoting substances or eliciting root metabolic activities by supplying biological fixed nitrogen and indirectly by acting against phytopathogenic microorganisms Lugtenberg (2002) [17]; Morrissey (2004) [20]; Ehlers (2006) [7]. Bioinoculation of PGPR cells for the enhancement of growth and yield of agriculturally important crops has long been under practice (Neyra (1999) [23]. However, the lack of stress tolerance and poor survivability in soil are the most important critical factors which prevent the successful bioinoculation of field crops Esath Natheer (2013) [9].
An agricultural bioinoculant is a formulation containing one or more bacterial strains or species in an easy-to-use form. Higher degree of stress tolerance, long shelf life, enhanced survivability in soils and on seeds and consistent plant response to inoculation are the important characteristics of any agricultural bioinoculant Neyra (1997) [22]. Okon (1994) [24] suggested the importance of the physiological status of microorganisms in agricultural bioinoculant preparation rather than the cell numbers to ensure more survival in carriers, survival in soil and on seed, colonization in the rhizosphere and positive plant response to bioinoculation. Moreover, the bioinoculant formulation has a crucial effect on the inoculation processes as the chosen formulation determines the potential success of the inoculant Bashan (1998) [2]. In the last few years, several new agricultural bioinoculant formulations have been proposed including, 1) microencapsulation of bacteria in alginate beeds 2) application of cells with high poly-ß-hydoxybutyrate content and 3) use of flocculated cells forms as

Delivery system (Olubayi (1998) [26]. Neyra (1995) [21] proposed the use of flocculated cell forms of *Azospirillum*, as novel delivery system, for the enhancement of growth and yield of crop plants. They described that *Azospirillum* biofloc containing high cell titre, increased adhesiveness to plant roots, enriched in encysted cells with thick capsules surrounded by EPS rich network which provided higher stress tolerance and longer shelf life to bioinoculant. They also proposed that the use of "flocculated cultures of *Azospirillum"* ensured the better establishment and interaction of the inoculated microbial cells in plant rhizosphere. The mechanism of exopolysaccharide (EPS) mediated bioflocculation of *Azospirillum* cells has been reported by many authors (Sadasivan (1985) [30]; Bleakley (1988) [3]; Madi (1989) [18]; Burdman (2000) [4]. However, the performance of PGPR biofloc *viz*., *Azotobacter* and *Paenibacillus* to various environmental stresses has not been exploited, so far.

Azotobacter and *Paenibacillus* are the two most important PGPR genera which are most frequently encoundered from the rhizosphere of many crop plants (Vonder wied (2000) [33]; Reddy (2007) [28]. The PGPR characteristics of the genus *Azotobacter* has already been well documented Saveetha (2009) [31]; Martinez-Viveros (2010) [19]; Ramezanpour (2011) [27]. *Paenibacillus* sp. a phytogenetic variant of the genus *bacillus* Ash (1994) [1] has been described as an effective PGPR Idris (2007) [14]; Richardson (2009) [29] and the ubiquitous occurrence of the same from the rhizosphere crop plans has already been reported Guemouri- Athmani (2000) [13]; Faria da mota (2002) [10].

However, there were no earlier reports on the comparative performance of different bioformulations of *Azotobacter* and *Paenibacillus* cells, to various environmental stresses, available. Hence, the present study has been undertaken with an aim to exploit the comparative performance of different bioformulations of *Azotobacter* and *Paenibacillus* cells, *viz.,* vegetative cells of *Azotobacter*, vegetative cells of *Paenibacillus,* natural bioflocs and artificial bioflocs of *Azotobacter* and *Paenibacillus* cells to various environmental streses.*viz*., thermal, desiccation and salt tolerance.

2. Materials and method

Culture condition of Azotobacter and Paenibacillus

The efficient strains of *Azotobacter* (AB-3) and *Paenibacillus* (PB-3), isolated from the rhaizophere of maize, grown at Keerapalaiyam, Cuddalore district, Tamil Nadu state, India, were used in the present study. The *Azotobacter* and *Paenibacillus* cells were maintained in base-77 and nutrient glucose agar Englesberg (1957) [8] slants, respectively, and incubated at 28 ± 2◦C, with monthly transfer.

Preparation of inoculum

The PGPR strains of *Azotobacter* and *Paenibacillus* isolates were individually grown in base-77 and nutrient glucose broth, respectively, in a shaking bath at 28 ± 2◦C for 24 hr. Then, the media were centrifuged separately at 5000× g for 10 min to harvest the log phase cells of the above stains. The pellets were washed three times with 0.1M phosphate buffer (pH 6.8), individually. Finally, the cells of *Azotobacter* and *Paenibacillus* were resuspented, separately, in the same buffer at a cell concentration of 1×10^7 CFU/mL by measuring the OD at 420 nm for *Azotobacter* and 540 nm for *Paenibacillus* and used as inoculum source.

Preparation of plant seed extract

The following plant seed extracts, namely, *Moringa oleifera, Strychnos potatorum, Allium cepa, Sappindus emaginatus and Aestracantha longifolia* were prepared, as stated below. Matured seeds of the above said plants were collected, crushed and sieved (0.8 mm mesh). The seed powder is mixed with a small amount of sterile water to form a paste. Then the paste is diluted to the required strength *viz.,* 5 per cent concentration before using it. The insoluble materials were filtered out using a fine mesh screen or muslin cloth. The milky white suspension was used for bioflocculation studies.

Preparation of Co-Ag buffer (Grimaudo and Nesbitt, 1997)

The co-aggregation buffer was prepared as stated below according to Grimaudo (1997) [11].

Estimation of flocculation Percentage

The flocculation percentage of *Azotobacter* and *Paenibacillus* cells were done according to the procedure of Madi (1989) [18].

Preparation of natural bioflocculation of Azotobacter and Paenibacillus cells

One mL aliquot of each PGPR strains (1 x 10^7 cells mL^{-1}) was mixed together in 10 mL of Co-Ag buffer Grimaudo (1997) [11]. The mixture was vortexed for 10 sec, shaken on a rotary platform shaker for 3 min and left undisturbed at room temperature for, 1h.

Preparation of artificial bioflocculation of Azotobacter and Paenibacillus using plant seed materials

The efficient strains of *Azotobacter* and *Paenibacillus viz*., AB-3 and PB-3, were grown in respective broth and the cells were harvested at log phase. One mL aliquot of each PGPR isolate (1 x 10^7 cells mL^{-1}) was mixed together in 10 mL of Co-Ag buffer Grimaudo (1997) [11] together with the addition of one ml of individual plant seed extract *viz*., *Moringa oleifera, Strychnos potatorum, Allium cepa, Sappindus emaginatus* and *Asteracantha longifolia.* The mixture was vortexed for 10 sec, shaken on a rotary platform shaker for 3 min and left undisturbed at room temperature for, 1h whereas the salt tolerance of the same biofloc was estimated according to Esath Natheer (2013) [9].

Thermal and desiccation tolerance

The thermal and desiccation tolerance of *Azotobacter* and *Paenibacillus* bioflocs were done according to Sadasivam (1985) [30].

Statistical analysis

The experimental results were statistically analyzed in randomized block design (RBD) and in Duncan's multiple range test (DMRT) as per the procedure described by Gomez (1984) [12].

3. Results and discussion

The addition of plant seed flocculants to CO-AG buffer could augment the flocculation of *Azotobacter* and *Paenibacillus* cells within a short period of time, namely, 1hr. Among the different plant seed materials tested, the seed materials from *Strychnos potatorum* could augment the flocculation of *Azotobacter* and *Paenibacillus* cells to a

higher level followed by *Sappindus emaginatus*, *Moringa oleifera, Allium cepa,* and *Asteracantha longifolia* (Table-1) Okuda (2000) [25] characterized some of the traditionally used plant seed materials, as plant seed flocculant, and emphasized the role of Indian nirmali seed (*Strychnos potatorum*) for primary water treatments. The water soluble protein released from the seed kernel of *Strychnos potatorum* might be the reason for the flocculation of *Azotobacter* and *Paenibacillus* cell in a shorter period and the mechanism is unknown.

The comparative performance of different bioformulations, namely, vegetative cell, natural and artificial bioflocs of PGPR cells, namely, *Azotobacter* and *Paenibacillus* was studied for their temperature and desiccation tolerance and the results of presented in Table 2 and 3. The temperature and desiccation tolerance of both natural and artificial bioflocs of *Azotobacter* and *Paenibacillus* was found to be more when compared to their respective vegetative cell formulation. Between the two bioflocs preparation, the natural biofloc of *Azotobacter* and *Paenibacillus* cells exhibited higher temperature and desiccation tolerance when compared to the artificial biofloc preparation. Between the two PGPR biofloc preparation namely, *Azotobacter* and *Paenibacillus*, the biofloc formulation of *paenibacillus* cells exhibited higher temperature and desiccation tolerance than *Azotobacter* biofloc formulation. In this laboratory, Kannan (2000) [16] studied the thermal and desiccation tolerance of natural and artificial coaggregates of *Azospirillum* and *Paenibacillus* and reported the higher performance of natural coaggregates of *Azospirillum* and *Paenibacillus* on thermal and desiccation tolerance when compared to artificial coaggregates. However, there were no earlier reports available on the thermal and desiccation tolerance of natural and artificial coaggregation of *Azotobacter* and *Paenibacillus* and this is the first comprehensive report on the subject.

The effect of different salinity levels on different bioformulations, namely, vegetative cell, natural and artificial bioflocs of PGPR cells namely, *Azotobacter* and *Paenibacillus* on generation time (GT) of the same was studied under *in-vitro* condition and the results presented Table-4. It was observed that the generation time (GT) of both PGPR genera vary with salinity levels in the growth medium. The PGPR cells in all the bioformulations exhibited a higher generation time in the growth medium at 0.8M NaCl level. It was also observed that the increasing of salinity level increased the generation time of the PGPR cells in all the bioformulations. Interestingly, there was a slight variation in generation time upto 0.3M concentration of NaCl whereas on the generation time varied widely beyond this level. Between the two bioformulations of PGPR cells, the natural biofloc formulation of *Paenibacillus* cells recorded a very low generation time 2.3 at 0.3M salinity level when compared to other formulations. The results of the present study clearly revealed the positive salt tolerance of natural biofloc preparation when compared to artificial biofloc and vegetative cell preparation. Ding (2005) [5] reported the interstrain differences of five isolates of *Paenibacillus polymyxa,* namely, C-4, C-5, G-1, T-1, and W-5 on salt tolerance and reported that NaCl tolerance limit of *Paenibacillus polymyxa* was upto 1-7%. However, there were no earlier report regarding the salt tolerance of different bioformulation of PGPR cells namely, *Azotobacter* and *Paenibacillus*, available for discussion.

Table 1: Effect of Different Plant Seed Materials on Co-Flocculation[d] of *Azotobacter* and *Paenibacillus* Cells

Addition of plant seed material[a]	*Azotobacter*		*Paenibacillus*	
	Percentage of bioflocculation[b]	Statistics[c]	Percentage of bioflocculation[b]	Statistics[c]
Moringa oleifera	93.3 ± 0.3	c	94.2 ± 0.2	c
Strychnas potatorum	96.2 ± 0.2	a	97.4 ± 0.1	a
Allium cepa	92.8 ± 0.3	d	93.6 ± 0.2	d
Asleracantha longifolia	91.6 ± 0.1	e	92.2 ± 0.3	e
Sappindus emaginatus	94.6 ± 0.2	b	95.1 ± 0.2	b
LSD(P=0.05)	0.6		0.5	

a. PGPR cells *viz., Azotobacter* (AB-3) and *Paenibacillus* (PB-3), collected from Base-77 and NGA, respectively at log phase of growth used for bioflocculation assay at inoculum level of 1×10^{7} CFU mL^{-1}.
b. Addition of plant seed material at a concentration of 5% level.
c. Assayed according to Madi and Henis (1989) after 24 hr of incubation time.
d. Values followed by different letters are significantly differed at 5% level according to student't' test.

Table 2: Effect of Different Bioformulations of *Azotobacter* and *Paenibacillus* Cells on Thermal Tolerance

Treatment*[a]	No. of viable cells/mL after 50◦C treatment for 20 min[b,c]
Azotobacter vegetative cell	$3.85 \pm 0.22 \times 10^{5}$
Paenibacillus vegetative cell	$4.98 \pm 0.23 \times 10^{5}$
Azotobacter biofloc (Natural)	$9.27 \pm 0.24 \times 10^{6}$
Paenibacillus biofloc (Natural)	$8.20 \pm 0.15 \times 10^{6}$
Azotobacter biofloc **(**Artificial)	$7.92 \pm 0.14 \times 10^{6}$
Paenibacillus biofloc **(**Artificial)	$6.05 \pm 0.10 \times 10^{6}$

*- natural and artificial bioflocs, obtained from the respective PGPR cells, by subjecting the same to natural flocculation in CO-AG buffer and artificial flocculation by plant seed materials.
a. At 1×10^{7} CFU / mL inoculum level.
b. Values are mean of three replication ± SD.
c. Values followed by different letters are significantly differed at 5% level according to student't' test.

Table 3: Effect of Different Bioformulations of *Azotobacter* and *Paenibacillus* Cells On Desiccation Tolerance

Treatment*[a]	No. of viable cells/mL after 1 week incubation on desiccation tolerance[b,c]
Azotobacter vegetative cell	$4.35 \pm 0.32 \times 10^{5}$
Paenibacillus vegetative cell	$5.86 \pm 0.11 \times 10^{5}$
Azotobacter biofloc (Natural)	$9.15 \pm 0.30 \times 10^{6}$
Paenibacillus biofloc (Natural)	$8.56 \pm 0.12 \times 10^{6}$
Azotobacter biofloc (Artificial)	$7.93 \pm 0.32 \times 10^{6}$
Paenibacillus biofloc (Artificial)	$6.20 \pm 0.22 \times 10^{6}$

*- natural and artificial bioflocs, obtained from the respective PGPR cells, by subjecting the same to natural flocculation in CO-AG buffer and artificial flocculation by plant seed materials.
a. At 1×10^{7} CFU / mL inoculum level.
b. Values are mean of three replication ± SD.
c. Values followed by different letters are significantly differed at 5% level according to student't' test.

Table 4: Effect of Different Bioformulations of *Azotobacter* and *Paenibacillus* Cells On Generation Time (Gt) At Different Salinity Lelels

Treatment*[a]	Generation time (h)				
	Salinity level				
	- NaCl	0.1M NaCl	0.3M NaCl	0.5M NaCl	0.8M NaCl
Azotobacter vegetative cell	2.0	2.3	3.0	14.2	34.3
Paenibacillus vegetative cell	1.8	2.1	2.9	13.7	33.8
Azotobacter biofloc (Natural)	1.4	1.7	2.5	10.9	30.7
Paenibacillus biofloc (Natural)	1.3	1.5	2.3	10.2	29.8
Azotobacter biofloc (Artificial)	1.6	1.9	2.6	11.8	31.5
Paenibacillus biofloc (Artificial)	1.5	2.0	2.7	12.5	32.6

*- natural and artificial bioflocs, obtained from the respective PGPR cells, by subjecting the same to natural flocculation in CO-AG buffer and artificial flocculation by plant seed materials.

a. At 1×10^7 CFU / mL inoculum level.
b. Values are mean of three replication ± SD.

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