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Research Article

In vitro studies on the stress adaptation mechanisms of thermotolerant *Azospirillum*

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Abstract

Plant growth promoting rhizobacteria play a key role in nutrient cycling and maintenance of soil fertility, and establish positive interactions with plant roots in agricultural environments. In the present study, *in vitro* studies on the stress adaptation mechanisms of thermotolerant *Azospirillum* were carried out. The effect of thermotolerant *Azospirillum* strains on Poly β–hydroxyl butyrate (PHB) production, Proline content and Exopolysaccharides production (EPS) was studied at 30°C & 55°C during 5th & 10th day. It was observed that the PHB production, Proline content and EPS production was more at 55°C during the 10th day. The thermotolerant *Azospirillum* strains MZA – 36 exhibited maximum PHB production, Proline content and EPS production.

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Keywords: *Azospirillum*, Thermotolerant, Poly β–hydroxyl butyrate, Proline and Exopolysaccharides.

Introduction

The use of PGPR and symbiotic microorganisms has proved useful in developing strategies to facilitate plant growth in saline soils (Kohler *et al*., 2009). In general, inoculation with PGPR can enhance germination, seedling emergence and modify growth and yield of various cereal and non - cereal crops (Zarea, 2010). Regarding *Azospirillum*, the most researched associative bacterium (Bashan and Holguin, 1997), stress conditions appear to emphasize its growth-promoting effects on plants (Baltruschat *et al*., 2008). The damaging effects of NaCl on wheat seedlings were reduced by inoculation with *Azospirillum brasilense* (Casanovas *et al*., 2003), which partially reversed the negative effects on the relative elongation rate of shoots. Such reduction was accompanied by higher relative water contents (Tang *et al*., 2009). However, for several crops the tolerance to salt at one growth stage was not correlated to tolerance at another stage (Yang *et al*., 2009).

Azospirillum was first isolated from poor sandy soil in the Netherlands (Beijerinick, 1925). *Azospirillum* is a free living plant growth promoting bacterium (PGPB), capable of affecting growth and yield of numerous plant species, many of agronomic and ecological significance. The leading theory concerning its growth promotion lies in its ability to produce various phytohormones that improve root growth adsorption of water &minerals that eventually yield larger, and in many cases, more productive plants (Dobereiner and Day, 1976).

Bacteria of the genus *Azospirillum* are widely distributed in soil and associated with the roots of forage grasses, cereals and non gramineous plants

(Bashan and Holguin, 1997). *Azospirillum* sp. are widely distributed soil nitrogen fixing bacteria that play an important role in the promotion of plant growth (Steenhoudt and Vandeleyden, 2000). The genus *Azospirillum* comprises free - living nitrogen fixing rhizosphere bacteria to a group that exerts beneficial effects on plant growth, namely the plant growth promoting rhizobacteria (PGPR). Because of those properties, numerous studies on *Azospirillum* ecology, physiology and biochemistry have been carried out during the past 15 years (Vande Broek and Vandeleyden, 1995). *Azospirillum* are widespread in soils and comprise diverse diazotrophic rhizobacteria stimulating plant growth and development of polysaccharide components of the surface *Azospirillum* play an important role in the formation of associations with other rhizobacteria (Yogorenkova *et al*., 2001).

Members of the genus *Azospirillum* are capable of fixing nitrogen under microaerophilic conditions in association with the roots of several agriculturally important crops and cereals (Bashan and Levanony, 1990; Bashan and Holguin, 1997). They stimulate density and length of root hairs, rate of appearance of lateral roots and root surface area when present in required numbers (Okon and Labandera Gonzalez, 1994; Russo *et al.,* 2008). These changes cause roots to take up more water and minerals resulting in a faster plant growth and crop yield under appropriate agronomic conditions (Pereyra, 2006).

Materials and Methods

The thermo tolerant strains were studied for their efficiency in the production of poly-β-hydroxybutyrate (PHB), proline content and exopolysaccharide (EPS) production.

Determination of Poly-β-hydroxybutyrate (PHB) content

The thermo tolerant *Azospirillum* and the mesophilic reference strain Sp 7 were grown in 100 ml of yeast extract glucose broth prepared in 250 ml Erlenmeyer flasks under static condition at 30^{o}C and 50^{o}C separately the broth cultures were sampled on 5 days of incubation and centrifuge at 8000 x g for 20 min at 4^{0}C and the cell suspension was prepared. One ml of the cell suspension was added to one ml of 2 N hydrochloric acid and the mixture was digested at 100^{0}C for 2 hrs. After digestion, the contents were cooled and extracted twice with chloroform, volume of 5 ml the chloroform was then evaporated in a boiling water bath and to the sediment, 5.0 ml of sulphuric acid was added and then the sample was heated to 100^{0}C for 10 min over a water bath after cooling the absorbance was measured at 236 nm in a spectrophotometer 1001.

Determination of free Proline content

The *Azospirillum* strains and reference strain Sp7 were grown in 100 ml of yeast extract glucose broth in 250 ml Erlenmeyer flasks at two different temperatures at 30^{0}C and 55^{0}C. A quantity of 2 ml of the culture was mixed thoroughly with 5 ml of 3 percent aqueous salicylic acid the solution was filtered through Whatman No.2 filter paper a quantity of 2 ml of the filtrate was reacted with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid for 1 hrs at 100^{0}C and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml of toluene, mixed vigorously in a test tube and stirred for 15 to 20 sec the toluene phase was aspirated from the aqueous phase warmed to room temperature and the intensity of pink colour was determined at 420 nm in a spectrophotometer 1001 using toluene as blank. The proline concentration was determined using pure proline standards (Bates *et al*., 1973).

Determination of Exopolysaccharides (EPS) production

The thermotolerant *Azospirillum* strains and reference strain Sp 7 were grown in yeast extract glucose broth in 100 ml quantities in 250 ml Erlenmeyer flasks at two different temperatures 30°C and 55°C under static condition. After the incubation period, the cells were harvested by centrifugation at 8000 x g and used for estimation of alkali stable polysaccharides. The supernatant fraction was used for the analysis of water soluble polysaccharide. To 20 ml of supernatant fraction

equal quantity of 80 per cent ethyl alcohol was added and incubated overnight to get precipitate of water soluble polysaccharide. The precipitate was collected by filtering through preweighed Whattmann No. 1 filter paper and dried in an oven at 70°C for 24 hrs until a constant weight was recorded. The harvested cells were washed with distilled water and 1.0 ml of 80 per cent potassium hydroxide was added. The contents were heated for an hour at 100°C over a water bath and cooled to room temperature. Then, 20 ml of ethyl alcohol was added and shaken thoroughly. The precipitated polysaccharide was collected by filtering through preweighed Whattmann No. 1 filter paper and dried in an oven at 70°C for 24 hrs (Sutherland and Wilkinson, 1971).

Results and Discussion

Poly-β-hydroxy butyrate (PHB) is a common reserve material in prokaryotes, which is present in both Gram positive and Gram negative bacteria. PHB is a polymer of D(-)-β-hydroxy butyrate and had a molecular weight between 60,000 and 2,50,000. Polymer accumulation was initiated under nutrient imbalance and serve as an electron and carbon sink. PHB usually function as a carbon or energy source and is degraded under condition of stress and starvation.

The effect of thermotolerant *Azospirillum* strains on Poly β–hydroxyl butyrate (PHB) production was studied at 30°C & 55°C during 5^{th} & 10^{th} day, and the results were furnished in Table – 1. It was observed that the PHB production was more at 55°C during the 10^{th} day. The thermotolerant *Azospirillum* strains MZA – 36 (3.465 mg g^{-1} of dry weight of cells) exhibited maximum PHB production followed by the Reference strain Sp-7 (3.002 mg g^{-1} of dry weight of cells), MZA – 4 (2.830 mg g^{-1} of dry weight of cells), MZA – 13 (2.070 mg g^{-1} of dry weight of cells), MZA – 2 (1.160 mg g^{-1} of dry weight of cells) and least PHB production was observed in MZA – 3 (0.832 mg g^{-1} of dry weight of cells).

Later Vieille and Elmerich (1992) characterized PHB encoding NADPH linked acetoacetyl CoA reductase in *Azospirillum brasilense* Sp 7. Okon and Itzigsohn (1992) found PHB accumulation to the level of 70 per cent of cell dry weight with strains possessing high nitrogen fixation, establishment and survival. During nutritional stress particularly ammonium and phosphate limitation, the cells of *Methylobaclerium* sp. accumulated PHB in large amounts (Mothes *et al.*, 1997). The accumulation of PHB by stress tolerant strains of sunflower *Azospirillum* was upto 80 per cent by temperature tolerant strains of *Azospirillum* whereas the reference strain Sp 7 could accumulate only 27 per cent PHB (Elango and Sundaram, 2002).

Microbial inoculation enhanced proline accumulation in the roots and provides tolerance to plants under salinity stress. The higher proline contents in roots might be due to the fact that roots are the primary sites of water absorption and must maintain the osmotic balance between the water absorbing cells and external media as reported by Sharifi *et al*. (2007).

The effect of thermotolerant *Azospirillum* strains on accumulation of proline content was investigated at 30°C & 55°C during 5^{th} & 10^{th} day, and the results were presented in Table – 2. It was observed that the proline content was more at 55°C during the 10^{th} day. The thermotolerant *Azospirillum* strains MZA – 36 (272.53 mg g^{-1} of dry weight of cells) showed maximum proline content followed by the Reference strain Sp-7 (232.78 mg g^{-1} of dry weight of cells), MZA – 4 (161.58 mg g^{-1} of dry weight of cells), MZA – 3 (134.72 mg g^{-1} of dry weight of cells), MZA – 13 (133.17 mg g^{-1} of dry weight of cells) and least proline content was recorded in MZA – 2 (79.58 mg g^{-1} of dry weight of cells). Chen *et al*. (2007) correlated proline accumulation with drought and salt tolerance in plants. Introduction of proBA genes derived from *Bacillus subtilis* into *A. thaliana* resulted in production of higher levels of free proline resulting in increased tolerance to osmotic stress in the transgenic plants. Increased production of proline along with decreased electrolyte leakage, maintenance of relative water content of leaves and selective uptake of potassium ions resulted in salt tolerance in *Zea mays* co-inoculated with *Rhizobium* and *Pseudomonas* (Bano and Fatima, 2009).

Table - 1: Poly β–hydroxyl butyrate* production of thermotolerant *Azospirillum* strains

S. No	*Azospirillum* Strains	30^{0}C		55^{0}C		% increase over 30°C
		Period of incubation (days)		Period of incubation (days)		
		5	10	5	10	
1	MZA-2	0.614	0.862	0.924	1.160	41.19
2	MZA- 3	0.521	0.750	0.668	0.832	18.11
3	MZA-4	0.730	1.335	1.294	2.830	42.44
4	MZA-13	0.734	1.062	1.060	2.070	74.27
5	MZA-36	0.971	1.618	2.456	3.465	128.43
6	Sp-7	0.750	1.432	1.838	3.002	121.81
SE_D		0.06	0.13	0.27	0.43	-
CD (P = 0.05)		0.12	0.27	0.55	0.87	-

*****Expressed as mg g^{-1} of dry weight of cells

Table - 2: Accumulation of proline content* by thermotolerant *Azospirillum* strains

S. No	*Azospirillum* Strains	30^{0}C		55^{0}C		% increase over 30°C
		Period of incubation (days)		Period of incubation (days)		
		5	10	5	10	
1	MZA-2	44.53	75.58	61.08	79.58	11.52
2	MZA- 3	41.17	46.92	71.91	134.72	134.52
3	MZA-4	47.69	55.70	110.42	161.58	154.35
4	MZA-13	60.70	74.13	111.95	133.17	155.94
5	MZA-36	70.80	85.68	189.25	272.53	195.10
6	Sp-7	66.19	78.15	159.74	232.78	171.94
SE_D		5.03	6.04	20.22	29.03	-
CD (P = 0.05)		10.06	12.08	40.45	58.06	-

*****Expressed as mg g^{-1} of dry weight of cells

Table - 3: Exopolysaccharides (EPS) production of thermotolerant *Azospirillum* strains

S. No	*Azospirillum* Strains	EPS production (mg 100 ml^{-1})						% increase over 30°C
		30^{0}C		55^{0}C		Total EPS		
		Water soluble	Alkali stable	Water soluble	Alkali stable	30^{0}C	55^{0}C	
1	MZA-2	130.6	63.6	140.5	65.5	198.9	206.0	3.56
2	MZA- 3	108.5	60.2	125.6	63.5	168.7	189.1	12.09
3	MZA-4	135.3	70.5	155.6	75.6	201.1	231.2	14.96
4	MZA-13	138.2	73.6	168.9	86.3	211.8	249.2	17.65
5	MZA-36	165.5	98.2	265.6	156.4	263.7	422.0	60.45
6	Sp-7	153.9	85.6	235.0	121.5	239.5	356.5	48.85
SE_D		8.03	5.83	22.78	15.05	13.62	37.84	-
CD (P = 0.05)		16.06	11.66	45.56	30.10	27.24	75.68	-

The effect of thermotolerant *Azospirillum* strains on Exopolysaccharides (EPS) production was tested at 30°C & 55°C during 5th & 10th day, and the results were tabulated in Table – 3. It was observed that the EPS production was more at 55°C during the 10th day. The thermotolerant *Azospirillum* strains MZA – 36 (Water soluble – 265.6 mg 100 ml^{-1}, Alkali stable – 156.4 mg 100 ml^{-1}, Total EPS – 422.0 mg 100 ml^{-1}) showed maximum EPS production followed by the Reference strain Sp-7 (Water soluble – 235.0 mg 100 ml^{-1}, Alkali stable – 121.5 mg 100 ml^{-1}, Total EPS – 356.5 mg 100 ml^{-1}), MZA – 13 (Water soluble – 168.9 mg 100 ml^{-1}, Alkali stable – 86.3 mg 100 ml^{-1}, Total EPS – 249.2 mg 100 ml^{-1}), MZA – 4 (Water soluble – 155.6 mg 100 ml^{-1}, Alkali stable – 75.6 mg 100 ml^{-1}, Total EPS – 231.2 mg 100 ml^{-1}), MZA – 2 (Water soluble – 140.5 mg 100 ml^{-1}, Alkali stable – 65.5 mg 100 ml^{-1}, Total EPS – 206.0 mg 100 ml^{-1}) and least EPS production was recorded in MZA – 3 (Water soluble – 125.6 mg 100 ml^{-1}, Alkali stable – 63.5 mg 100 ml^{-1}, Total EPS – 189.1 mg 100 ml^{-1}).

The influence of EPS producing plant growth promoting rhizobacteria on the aggregation of root - adhering soils was recently reported by Alami *et al.* (2000). EPS producing plant growth promoting rhizobacteria can significantly enhance the volume of soil macropores and the rhizosphere soil aggregation, resulting in increased water and fertilizer availability to inoculated plants. EPS producing plant growth promoting rhizobacteria can also bind cations including Na^+. Therefore, an increase in the population density of EPS producing bacteria in the root zone is expected to decrease the content of Na^+ available for plant uptake, and thereby alleviate salt stress in plants growing in saline environments. However, relatively little is known about the influence of EPS producing rhizobacteria and their plant growth-promoting effect under soil salinity.

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