Advanced Journal of Microbiology Research Volume 2014
Available online at http://advancedresearchjournals.org/AJMR

Full Length Research Paper

Cytokinin production by some bacteria: Its impact on cell division in cucumber cotyledons

Anwar Hussain and Shahida Hasnain*

Department of Microbiology and Molecular Genetics University of the Punjab, Lahore-54590

Accepted 18 September, 2013

Bacterial strains isolated from rhizosphere of different plants were screened for cytokinin production by developing a simple and rapid bioassay technique. The assay was based on the ability of cytokinin to stimulate greening in etiolated cucumber cotyledons. Isolated bacterial strains were grown on half of 0.6% agar plate for 96 h at 30°C. Etiolated cucumber cotyledons were placed at 2 mm distance from bacterial culture under green light. After initial incubation for 20 h in darkness the plates were exposed to light for three hours. Greater chlorophyll contents were obvious in cotyledons exposed to *Bacillus licheniformis* Am2, *Bacillus subtilis* BC1 and *Pseudomonas aeruginosa* E2 strains. Sensitivity of the plate assay was 10^{-7} M of cytokinin. Cytokinin fractions in bacterial extract (BE) were separated by Thin Layer Chromatography (TLC) and quantified by High Performance Liquid Chromatography (HPLC). Major cytokinin species detected were zeatin and zeatin riboside. Bacterial extract enhanced cell division, fresh weight and cotyledon size in dark as well as light grown cucumber cotyledons against control.

Key words: *Bacillus*, *Pseudomonas,* cytokinin, cell division, cucumber, cotyledons, Trans-zeatin, HPLC, TLC.

INTRODUCTION

Phytohormones are signal molecules which regulate plant growth and development. Cytokinins are adenine derivative phytohormones that control cell division, cell cycle and stimulate developmental processes in plants (Srivastava, 2002). Stimulatory or inhibitory function of cytokinins in different developmental processes such as regulation of root and shoot growth as well as branching, control of apical dominance in the shoot, chloroplast development, and leaf senescence have been described (Werner et al., 2001; Oldroyd, 2007). Cytokinin influence cell division activity in embryonic as well as mature plants by altering the size and activity of meristems (Werner et al., 2001). Yang et al. (2002) showed that the rate of endosperm cell division is closely associated with cytokinin level in endosperm. They also reported that exogenous kinetin significantly increase the number of endosperm cells and grain weight.

Phytohormones including cytokinins were detected in the culture medium of several bacteria including *Halomonas desiderata*, *Proteus mirabilis*, *P. vulgaris*, *Klebsiella pneumoniae*, *Bacillus megaterium*, *B. cereus*, *B. subtilis* and *Escherichia coli* (Arkhipova et al., 2005; Karadeniz et al., 2006; Ali et al., 2009). Different Cytokinins are detected not only in the biomass of microorganisms (in free state or bound to certain tRNAs) but also in the culture medium in the form of either adenine derivatives, isoprenylated at N^6 position or their ribosides, such as 6-benzyladenine, N^6-isopentenyladenosine, zeatinriboside (Serdyuk et al., 2003). Transzeatine has also been found in the culture of *Agrobacterium tumefaciens* (Krall et al., 2002). Ryu et al. (2003) reported that cytokinin from bacterial origin improve growth in *Arabidopsis*. Inoculation of plant with bacteria producing cytokinin has been shown to stimulate shoot growth and reduce root/shoot ratio in droughted plants (Arkipova et al., 2007). Rhizobium was reported to enhance cytokinin production in plants by

*Corresponding author. E-mail: genetic@brain.net.pk.

Abbreviations: BE, bacterial extract; **BS,** bacterial suspension; **tZ,** trans zeatin; **ZR,** zeatin riboside.

regulating the expression of signaling pathway and trigger cortical sells to divide in plants (Oldroyd, 2007). We hypothesized that inoculation of plants with cytokinin producing bacteria may stimulate cell division rate in plants. To answer this question the present work was planned.

MATERIAL AND METHODS

Screening of rhizosphere bacteria for cytokinin production

At least thirty bacterial strains from rhizosphere of different plants including some important crop (maize, wheat, tomato, sugarcane, mustard, carrot and sunflower) and medicinal plants (*Ajuga* and *Nerium*) were isolated. The plants were uprooted with some non rhizosphere soil and brought in polythene bags to the lab. In the lab, roots were gently shaken to remove non rhizosphere soil, dipped in 500 ml flask containing sterile distilled water and vigorously jiggled. The Soil suspension obtained was serially diluted under sterile conditions. Fifty microliters suspension from dilutions (10^{-3} , 10^{-4} and 10^{-6}) was spreaded on Luria-Bertani agar plates (Tzfira et al., 1997) and incubated at 37°C. Growth was checked after 24 h. Colonies appeared on the plate were observed carefully under tube light and similar colonies were identified. Only 2 - 3 colonies which looked distinctly different were selected to avoid repeated selection of same strain. Selected colonies were streaked repeatedly on L-agar and monitored by gram staining for purity. Pure colonies obtained after 4 - 5 subcultures were maintained on L- agar plates supplemented with appropriate antibiotics at 4°C. Sub culturing was done on fortnight bases. Pure cultures were screened for cytokinin by developing a rapid and easy bioassay modified from Fletcher and McCuliagh 1971. Isolated bacterial strains were streaked on half of petri plate containing M9 medium supplemented with 0.2% Casamino Acids, 0.01% thiamine, and 2 pg of biotin per liter, solidified by 6 gL^{-1} agar (Akiyoshi et al., 1987). The plates were incubated at 28°C and 100 rpm for 96 h. Ten etiolated cucumber cotyledons were placed on each of the bacterial culture plate under green light. The plates were kept in light (intensity of 55 µ mol m^{-2} s^{-2}) for 3 h after initial incubation in darkness for 20 h. Minimal medium (9 M) was used as control. Chlorophyll was extracted from cotyledon grown on different bacterial culture plates or control with cold acetone and quantified by taking absorbance at 665 nm. The procedure was repeated three times for strains screened positive for cytokinin. To investigate the sensitivity of the plate assay varying amount of bacterial supernatant (equivalent to 10^{-9} to 10^{-5} M of kinetin) was added to the agar plates.

Bacterial strain identification

Genomic DNA was extracted by using DNA extraction kit (Fermentas) from overnight bacterial culture (LB) incubated at 37°C and 150 rpm. 1.5 Kb 16s rDNA fragment was amplified by method described by Hasnain and Thomas 1996. Forward primer 27f (5´-AGAGTTTGATCCTGGCTCAG- 3´) and reverse primer 1522r (5´-AAGGAGGTGATCCA (AG) CCGCA-3´) were used (Johnson 1994). Amplified fragment was extracted from gel by using aqua pure DNA extraction kit (Bio-rad) and sequenced with 27 f and 1522 r primers by ABI PRISM-3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences obtained with reverse primer were converted to reverse complementary sequence with Chromas Lite 2.01 (Technelysium Pty Ltd, Australia) and aligned with sequences obtained with forward primers using Basic local alignment search tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The aligned sequences were then submitted as a query to BLAST for comparison with the collection of 16S rRNA gene sequences present in the GenBank databases. Maximum homology of the query sequences to the database sequences was determined.

Extraction and detection of cytokinin

Bacterial strains were grown in M9 broth supplemented as described at 30°C and 150 rpm for five days. Extraction, fractionation and quantification of cytokinin in B E was done according to the flow chart (Figure 1). Cells free culture filtrate was obtained by centrifuging 50 ml culture at 16000 rpm for 10 min at 4°C. The supernatant obtained was neutralized with 7 N NaOH (pH 7.0), filtered through cellulose acetate filter (Millipore; 22 µm pore size; 47 mm Diameter; Australia Pty Limited, Australia), lyophilized to dryness and extracted three times with ethyl acetate/n-butanol. Organic phase was evaporated to dry and reconstituted in HPLC grade methanol (Sigma). Bound cytokinins were extracted after aqueous phase was adjusted to pH 11 and hydrolyzed. After drying the organic phase was re-dissolved in methanol. Pooled methanol fractions (Bacterial extract) were co-chromatogramed on Merck silica gel 60 PF_{254} with authentic cytokinins (Trans-zeatin, Zeatin riboside, Kinetin and Adenine) using n-butanol: acetic acid: water (12:3:5) (v/v) as mobile phase. TLC chromatograms were observed under 245 nm UV light to detect cytokinin as described by El-Tarabily et al., 2003. The *Rf* values were calculated for both standards and samples. Bands were then eluted separately with absolute methanol and run on C18 reverse-phase column using Syknm HPLC system with TZ and ZR standards. Solvent used was 70% methanol with flow rate 1ml/min and 8.6 MPa pressure. Different fractions were also checked for cytokinin activity through cucumber cotyledon bioassay. For comparison etiolated cotyledons were grown on kinetin standard (0.1, 1.0, 5, 10, 15 and 20 µM).

BE obtained at different time intervals (12, 24, 48, 72, 96, 120, 132 and 150 h) was subjected to TLC and HPLC analysis as described earlier. Quantification was done by comparing peak area of the tested compound to the standard curve made by plotting peak area against TZ and ZR (Sigma) concentration. Concentration (x) of TZ and ZR in the sample was determined by solving Straight line equation derived from regression analysis of the peak area of standards and respective concentration. Growth curve was made by taking OD of the culture at 600 µm for different time intervals. The procedure was repeated three times for *Bacillus licheniformis* Am2 only.

Plant materials and culture conditions

Cucumber seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min and rinsed five times with sterile distilled water. After germinating seeds for five days on distilled water in the dark at 25°C and 80% relatively humidity, the seedlings were divided into two groups. From one group of seedlings cotyledons were excised under green light and transferred (10 cotyledons per plate) to sterile Petri plates on double layers of filter papers soaked in distilled water. Plates were divided into three groups i.e. Positive control supplemented with 50µM standard cytokinin solutions, negative control without any supplement and experimental supplemented with 0.5, 1 and 1.5 ml of BE (equivalent to 24.64, 49.91 and 74.94 µM of tZ respectively). Each treatment was replicated four times. The plates were incubated in darkness for five more days at 25°C and 80% relative humidity. Seedlings of the second group were incubated on nutrient solution either inoculated with bacterial suspension or without inoculation for five days in 16 h light and 8 h dark

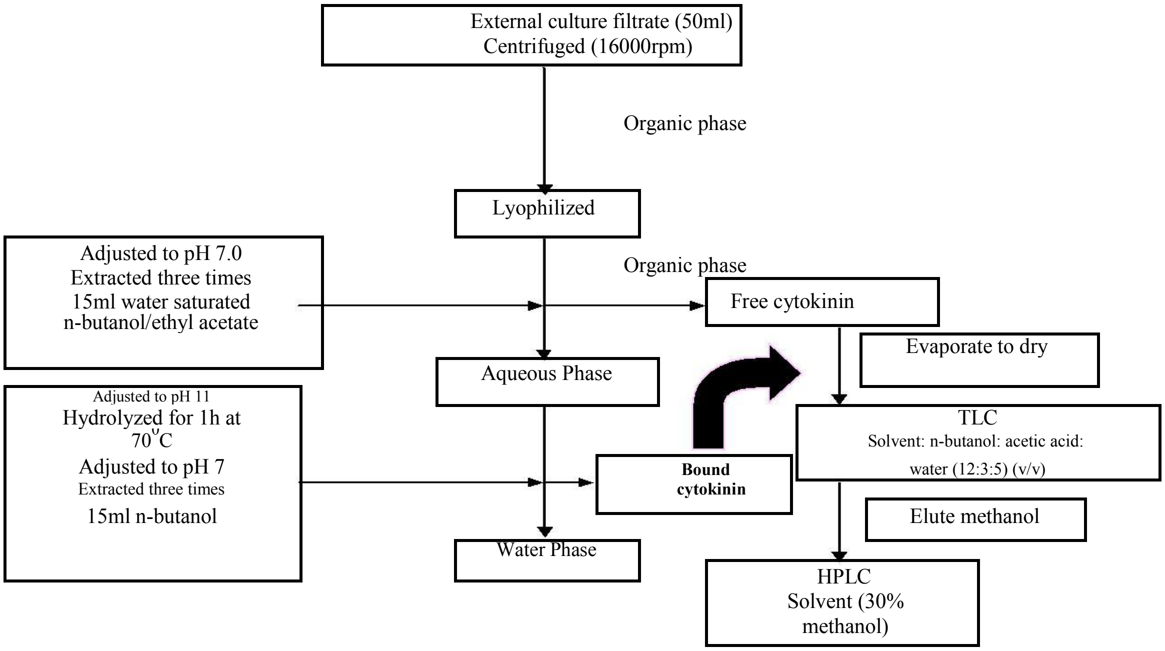

Figure 1. Flow chart for cytokinin extraction (modified from Tien et al., 1979).

photoperiod at 25°C. Each treatment was replicated four times. Cotyledons were processed for cell count after every 12 h interval. Fresh weight and cotyledon length were recorded after five days.

Cell division

Each group of 10 cotyledons was macerated in 40 ml of 5% (w: v) Cr_2O_3 at 37°C and 55 rpm for 24 h. Cells were then separated by stirring the mixture with magnetic bar for 60 minutes. A drop of the cell suspension after stirring was placed on hemocytometer and the no of vacuolated as well as non vacuolated cells were counted. Cell division per meristematic cell per 12 h was calculated as described (Brown and Rickless, 1949). Vacuolated (non meristematic) and non vacuolated (meristematic) cells in one milliliter of the cell suspension were determined by equation 1 and division rate was calculated by equation 2.

$$\text{vacuolated/non vacuolated cells} = \frac{\text{cells on slide X 1000}}{\text{volume used}} \qquad (1)$$

$$\text{Rate of division} = \frac{T2 - T1}{m1 + m2} X2 \, . \qquad (2)$$

RESULTS

Bacterial strains isolation and screening for cytokinin production

The strains isolated and their source plants are listed in Table 1. Important crop plants from agriculture land including maize, wheat, tomato, sugarcane, mustard, carrot and sunflower along with some medicinal plants from forest (*Ajuga* and *Nerium*) were selected for bacterial isolation. The isolated strains were named according to the botanical name of the source plants. Purified isolates were screened for cytokinin like activity by cucumber cotyledon bioassay. Cucumber cotyledons were incubated on stationary phase bacterial culture in the dark for 14 h. Greening occurred in cotyledons when shifted to light for 3 h (Figure 2). Enhanced chlorophyll formation (73.08, 61.54 and 51.28%) was obvious in etiolated cucumber cotyledons exposed to *Bacillus licheniformis* Am2, *Bacillus subtilis* BC1 and *Pseudomonas aeruginosa* E2 respectively against control (Figure 3a) . cytokinin like activity was not detected in the rest of the strains by the method adopted. Detectable concentration of cytokinin in bacterial supernatant was 10^{-7} M.

Strains homology

Sequences homology study was done by comparing partial sequence of 16 S rDNA from three bacterial strains Am2, BC1 and E2 with nucleotide sequence database (GeneBank) through BLAST (www.ncbi.nih.nlm.gov/BLAST). The strains showed maximum homology with *Bacillus licheniformis*, *Bacillus subtilis* and *Pseudomonas aeruginosa* respectively. The nucleotide sequence from the three strains has been submitted to GeneBank under accession No. FJ190075, EF600045 and EU418740 respectively.

Extraction and quantification of cytokinin

Recovery of TZ was 76 and 65.2% with ethyl acetate

Table 1. List of bacterial isolated from rhizosphere of different plants

S. No.	Strain	Plant	S. No.	Strain	Plant
1	Zm1	*Zea mays*	16	Am2	*Amaranthus* sp
2	Zm2	*Zea mays*	17	Te1	*Triticum aestivum*
3	Sl1	*Solanum lycopersicum*	18	Te2	*Triticum aestivum*
4	Sl2	*Solanum lycopersicum*	19	St1	*Solanum tuberosum*
5	Ap1	*Ajuga parviflora*	20	St2	*Solanum tuberosum*
6	Ap2	*Ajuga parviflora*	21	E1	Euphorbia sp.
7	So1	*Saccharum officinarum*	22	E2	Euphorbia sp.
8	BC1	*Brassica campestris*	23	Fr1	*Fragaria* sp
9	BC2	*Brassica campestris*	24	Fr2	*Fragaria* sp
10	BC3	*Brassica campestris*	25	Ha1	*Helianthus annuus*
11	Dc1	*Daucus carota*	26	Ha2	*Helianthus annuus*
12	Dc2	*Daucus carota*	27	Ha3	*Helianthus annuus*
13	Dc3	*Daucus carota*	28	No1	*Nerium oleander*
14	Dc4	*Daucus carota*	29	No2	*Nerium oleander*
15	Am1	*Amaranthus* sp	30	Tf1	*Trifolium* sp

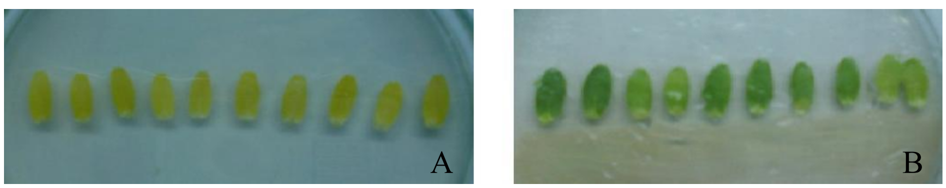

Figure 2. Bioassay for cytokinin with Cucumber cotyledons A: Control plate, B: stationary phase culture plate of *B. licheniformis* Am2.

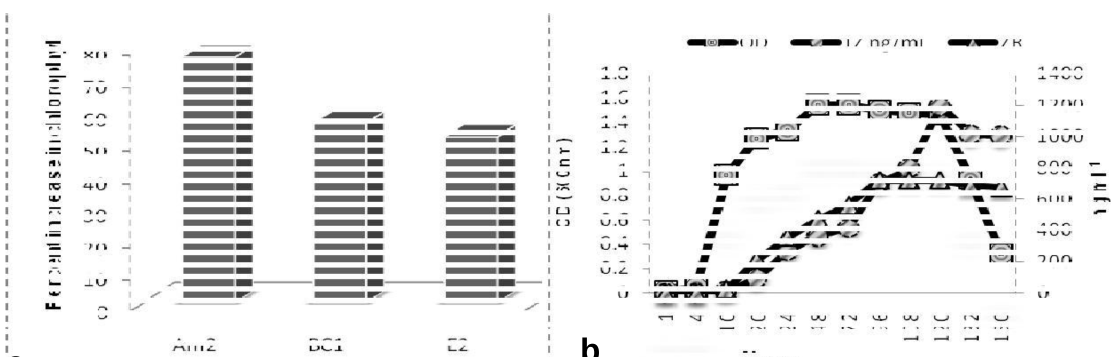

Figure 3. (a) Percentage increase chlorophyll synthesis enhanced by bacterial strains in excised Cucumber cotyledons relative to control. (b) *Bacillus licheniformis* Am2 strain growth curve and cytokinin production.

and n-butanol respectively. Ethyl acetate was selected for extraction on behalf of its efficient extraction and quick evaporation. Portions of methanol fraction (BE) chromatogramed on Merck silica gel 60 PF_{254}. Three compounds in BE (*Rf* values 0.5, 0.54 and 0.58) showed cytokinin activity in bioassay (Table 2). A total of 20 µl of BE eluted from TLC plates was injected on a BDS hypersil C18 reverse phase column (Thermo-hypersil; dimensions; 200 x 4.6 mm; particle size; 5 µm) and eluted with 70% methanol. Trans-Zeatin and ZR were eluted after retention time 2.55 min and 3 min respectively (Figure 4), recorded with UV detector at 270 nm. Maximum concentration recorded was 1091.9 ng ml^{-1} (TZ) and 521 ng ml^{-1} (ZR) in the late stationary phase culture of Bacillus licheniformis (Am2 strain) after 120 and 96 h incubation respectively (Figure 3b). Maximum

Table 2. Activity of different *Rf* fraction (equivalent to µM of kinetin) in cucumber cotyledon bioassay. Results are means ± S.E $\bar{x}$.

	Rf					
Strain	0.29	0.43	0.50	0.54	0.58	0.8
Bacillus licheniformis Am2	0	0	-	5.7 ± 0.88	20.1 ± 1.1	0
Bacillus subtilis BC1	0	0	-	-	19.5 ± 1.23	0
Pseudomonas aeruginosa E2	-	-	4.6 ± 0.81	-	19.2 ± 0.96	0

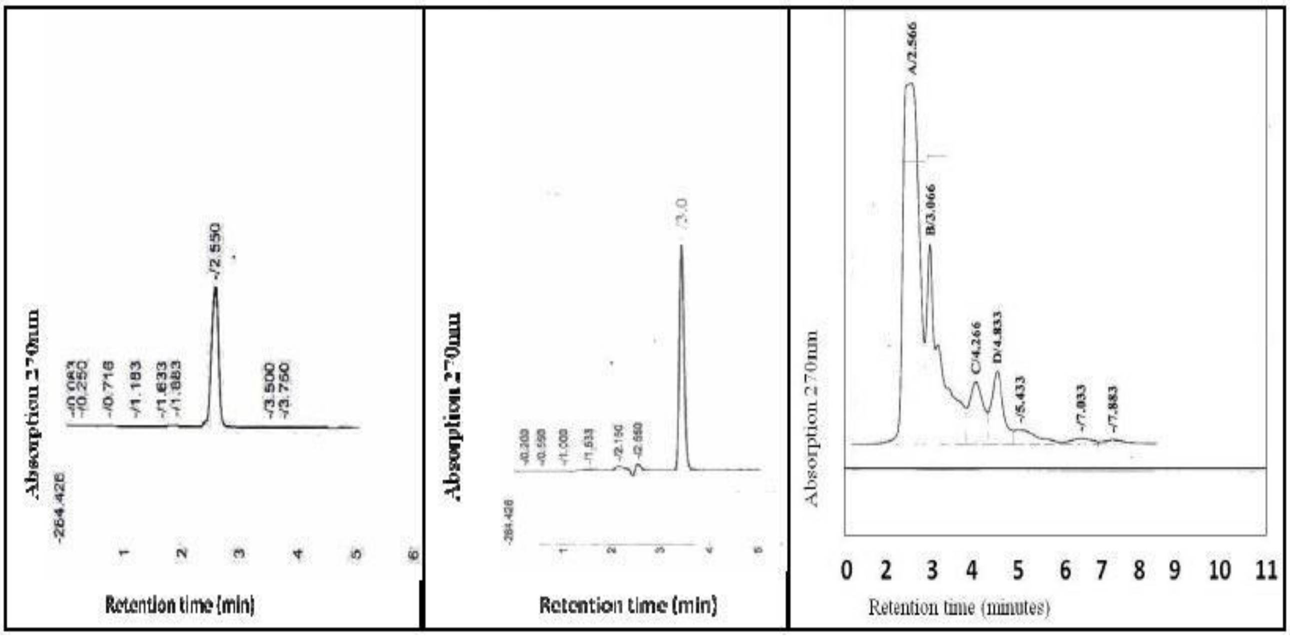

Figure 4. HPLC chromatographs (a) tZ, (b) ZR and (c) *Bacillus licheniformis* Am2.

cytokinin detected in 120 h old culture filtrate of BC1 and E2strains was 984 ng ml^{-1} and 640 ng ml^{-1} respectively.

Growth and cell division in etiolated cucumber cotyledons

When incubated with bacterial extract supplemented to distilled water for five days Cucumber cotyledons showed enhanced growth as compared to control cotyledons grown on water alone (Figure 5) . 50 µM TZ was used as authentic cytokinin supplement. Increase in cotyledon area and fresh weight was observed along with increase in total cell no and cell division rate (Table 3 and 4). In all cases studied, supplement of 1.0 ml BE (extracted from 50 ml culture supernatant) to 5 ml distilled water (final concentration equivalent to 49.91 µM of TZ in case of Am2) showed the finest results as compared to the other two treatments that is 0.5 and 1.5 ml (final concentration equivalent to 24.64 µM and 74.94 µM of trans-zeatin respectively in case of Am2) BE. The most efficient strain was *Bacillus licheniformis* Am2 which generated growth response in excised cotyledons as TZ standard supplemented in final concentration equal to 50 µM (Least significant difference $_{0.05}$). Mean cells per ml of cotyledon suspension were 36.6 x 10^4 and 33.2 x 10^4 in cotyledon grown on exogenous TZ and 1.0ml Am2 BE respectively. The strains BC1 and E2 increased total cell no. up to 28 x 10^4 and 20.6 x 10^4 respectively when 1.0 ml BE was used as a supplement. Mean No. of cells ml^{-1} suspension obtained from cotyledons grown on distilled water alone (9.55 x 10^4) was not significantly greater than cells present initially (7.75 x 10^4) . Number of divisions per meristematic cell per 12 h was 0.144188, 0.070025, 0.137863, 0.124191 and 0.100442 in dark incubated cotyledons on TZ standard, water, Am2, BC1 and E2 strains respectively (Table 4).

B. licheniformis Am2, *B. subtilis* BC1 and *P. aeruginosa* E2 enhanced gain in fresh weight of light grown cotyledons by 39.23, 36.96 and 28.07% respectively as compared to control. Dark grown cotyledons showed significant stimulation in size under additional supply of TZ or BE. No significant expansion in cotyledon size was witnessed in light grown cotyledons under similar condi-

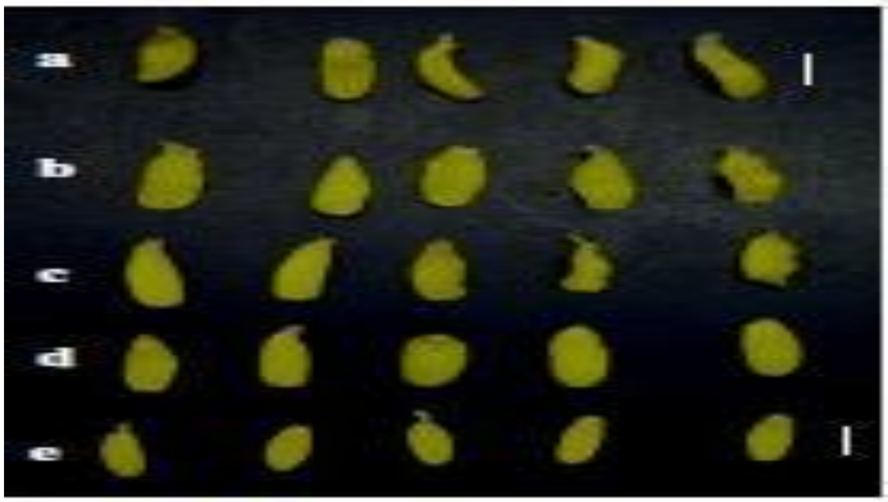

Figure 5. Excised cucumber cotyledons grown in the dark for five days on water supplemented with (a) TZ, 1.0 ml BE (49.91µM) from (b) *Bacillus licheniformis* Am2 strain (c) *Bacillus subtilis* BC1 strain (d) *Pseudomonas aeruginosa* E2 strain and (e) water alone (bar = 10mm)

Table 3. Expansion in excised and intact Cucumber cotyledons grown for five days on BE and BS from different bacterial strains or control.

Treatment	**Dark grown cotyledons (BE)**		**Light grown cotyledons* (BS)**	
	Area (mm^2±SE)	**Gain in fresh weight (%)**	**Area (mm^2±SE)**	**Gain in fresh weight (%)**
Water	40.09 ± 4.05a	100	64.39 ± 3.35a	100
TZ	72.75 ± 3.82cd	134.31	-	-
B. licheniformis Am2	70.49 ± 5.00cd	131.55	77.46 ± 4.71b	139.23
B. Subtilis BC1	60.73 ± 3.07bc	129.61	68.36 ± 4.63a	136.96
P. aeruginosa E2	55.7 ± 4.72b	124.11	65.55 ± 3.86a	128.07
5%LSD	12.70		5%LSD	6.49
r (p=0.01)	0.88**		r (p = 0.01)	0.551

tions. Significant correlation was obvious between TZ concentration and cell division rate in dark as well as light grown Cucumber cotyledons (Table 3). However cotyledon expansion was significantly correlated to TZ concentration in darkness only (Table 3).

Effect of BS on cell division in light grown cotyledons

To demonstrate the impact of bacterial inoculation on intact cotyledons cucumber cotyledons were grown in photoperiod of 16 h light and 8 h darkness in the presence of bacterial suspension adjusted to 10^6 cfu. Seedlings grown in the presence of BS showed enhanced cell division rate by contrast to seedlings grown on nutrient solution alone. Both groups of seedlings (treated and control) showed similar pattern of cell increase after 12 and 24 h intervals. Beyond 24 h significant increase in total cell number was observed for BS treated cotyledons (Figure 6). After 36 h cell division rate was 0.1435 divisions per meristematic cell per 12 h in control cotyledons which was significantly less than cotyledons treated with BS (0.1628, 0.1594 and 0.1553 divisions per meristematic cell per 12 h in Am2, BC1 and E2 respectively). The rate of division was maintained for the next 120 h with non significant fluctuations.

DISCUSSION

Plant rhizosphere is a rich environment that hosts a wide

Table 4. Impact of different treatment on cell division rate in cucumber cotyledons.

Treatment	Cell division rate (Division MC^{-1} 12h^{-1}±SE) in Cucumber cotyledon	
	Dark grown (BE)	Light grown* (BS)
Water	0.0538 ± 0.00432a	0.1515 ± 0.000814a
TZ	0.144 ± 0.00343c	-
B. licheniformis Am2	0.137 ± 0.0078c	0.1687 ± 0.001332bc
B. Subtilis BC1	0.124 ± 0.00725bc	0.1632 ± 0.000945b
P. aeruginosa E2	0.1 ± 0.0108b	0.1594 ± 0.000869b
5%LSD	0.0345	0.0097
r (p = 0.01)	0.853**	0.914**

Alphabets represent significant difference between means using Duncan multiple range test (p=0.05)
**Significant correlation between TZ concentration and (1a) cotyledon area (1b) cell div rate (1c) cell size.
*16h light and 8 h dark photoperiod.

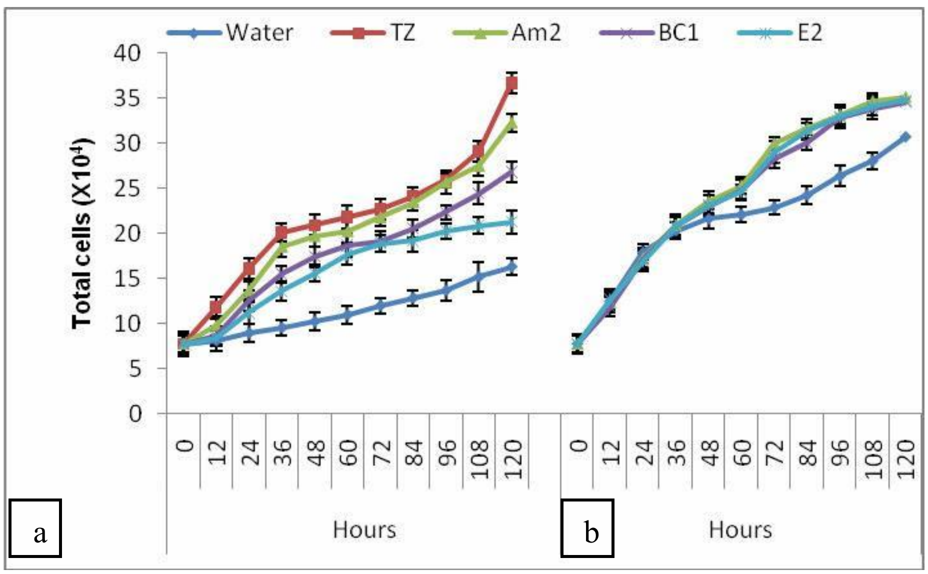

Figure 6. Total cell No. in Cucumber cotyledons (a) Excised cotyledons grown on KCl alone, treated with TZ (56µM) or 1ml BE (49.91µM); (b) From seedlings grown on nutrient solution alone (control) or supplemented with BS (*Bacillus licheniformis* Am2, *Bacillus subtilis* BC1and *Pseudomonas aeruginosa* E2). Cell count was based on samples taken every 12 hours interval.

Bars represent means± S.E (mean of five replicates).

array of bacteria including PGPR. Phytostimulatory effect of PGPR may be initiated by several ways but in cytokinin production by such bacteria is the direct mechanism to improve plant growth (Ortíz-Castro et al., 2008; Remans et al., 2008). In majority of studies PGPR were isolated form crop plants (Hynes et al., 2008; Ashrafuzzaman et al., 2009). In this study not only crop plants but also herbaceous wild type plants including medicinal plants were selected. Screening of PGPR bacteria for cytokinin production is a critical step in studying such bacteria because of the laborious extraction and bioassay procedures. We established an easy and quick screening technique for cytokinin producing bacteria bypassing extraction procedure modified from Fletcher and McCuliagh 1971. The technique can be used to detect cytokinin in the bacterial cultural plates as less as 10^{-7} M. The mentioned test helped us to select three strains out of total thirty strains very swiftly. It was evident that *Bacillus licheniformis* Am2 strain isolated from crop plant *B. campestris* was the most efficient cytokinin secreting bacteria among the strains studied. Two species of cytokinins were detected in the culture media of *Bacillus licheniformis* Am2 strains. The main species was TZ (equivalent to 1091.9 ng ml^{-1}; of authentic TZ) also shared by the other two strains that is *Bacillus subtilis* BC1 and *Pseudomonas aeruginosa* E2. Stationary phase culture (120 hours old) contained maximum amount of TZ. Zeatin riboside on the other hand hit the highest point (equivalent to 521 ng ml^{-1} of authentic ZR) after 96 h of incubation at 30°C and upheld till 120 h before decline in concentration started. Arkhipova et al. (2005) reported cytokinin as equivalent to 1.2 mg of Zeatin per litre in *Bacillus subtilis* culture medium. In another study, Taller and Wong (1989) determined cytokinins as equivalent to 0.75 µg of kinetin per litre in *Azotobacter vinelandii* culture medium while Barea and Brown (1974) reported 20 µg of cytokinin equivalent per liter for *Azotobacter paspali* and 50 µg L^{-1} for *A. vinelandii*.

Exogenous cytokinin enhances cell division rate in plants (Riou-Khamlichi et al., 1999; Cecchetti et al., 2007). However impact of cytokinin producing bacteria on plant cell division is investigated mainly in root nodules formation (Phillips and Torrey, 1972; Markmann and Parniske, 2009). *Azospirillum brasilense* has been reported to enhance cell division in root tips of inoculated wheat (Molina-Favero et al., 2007). Our results showed that bacterial extract significantly enhances cell division rate in cucumber cotyledons. Extract from Am2 strain enhanced sell division rate up to 0.1378 divisions meristematic $cell^{-1}$ in 12 h which is analogous to that of 56µM of standard trans-zeatin. In contrast to 0.5 ml (equivalent to 24.64 µM of tZ) and 1.5 ml BE (equivalent to 74.94 µM of tZ), 1.0 ml BE (equivalent to 49.91 µM of tZ) triggered the cells to divide with maximum rate and induced significant cotyledon expansion. 1.5 ml BE had supraoptimal concentration of TZ and the resulted decline in cell division rate may be due to the same reason. But this hypothesis was not supported by the results obtained in the experiment performed on Cucumber seedling inoculated with BS in photoperiod of 16 hour light and 8 h dark. The cell division was followed for five days (period of maximum TZ secretion by bacteria) at 12 h intervals. Cucumber seedlings inoculated with BS showed significantly enhanced cell division as compared to non inoculated seedlings after 24 h lag time. The lag may be due to least amount of exogenous cytokinin secreted by bacteria during 24 h incubation. The cell division rate was maintained after the lag period for 120 h and no decline was recorded. The consistent cell division in seedlings supplied with BS may be attributed to the fact that light and cytokinin synergistically effect cotyledons growth as reported by several authors (Brenner et al., 2005; Zubo et al., 2008). It is also reported that exogenous cytokinin is inhibitory to cell expansion in light grown cotyledon but stimulatory to cell division resulting in small cells (Stoynova-Bakalova et al., 2004). We found that bacterial cytokinin in the form of BE was significantly correlated to cell division as well as cotyledon expansion in the dark. However in light grown cotyledons bacterial cytokinin was only significantly correlated to cell division but not to the cotyledon expansion. It may be concluded that PGPR isolated from crop plants and other herbaceous plants were capable of cytokinin production which essentially affected plant cell division in the same way as exogenous cytokinin. The most efficient cytokinin producing strain was *B. licheniformis* Am2 isolated from the rhizosphere of *Amaranthus* sp.

REFERENCES

Akiyoshi DE, Regier DA, Gordon MP (1987). Cytokinin Production by *Agrobacterium* and *Pseudomonas* Spp. J. Bacteriol. 169: 4242-4248.

Ali B, Sabri AN, Ljung K, Hasnain S (2009). Auxin production by plant associated bacteria: impact on endogenous IAA content and growth of *Triticum aestivum* L. Lett. Appl. Microbiol. 48: 542-547.

Arkhipova TN, Prinsen EA, Veselov SU, Martinenko EV, Melentiev AI, Kudoyarova GR (2007). Cytokinin producing bacteria enhance plant growth in drying soil. Plant. Soil. 292: 305–315.

Arkhipova TN, Veselov SU, Melentiev AI, Martynenko EV, Kudoyarova GR (2005). Ability of bacterium Bacillus subtilis to produce cytokinins and to influence the growth and endogenous hormone content of lettuce plants. Plant. Soil. 272: 201–209.

Ashrafuzzaman M, Hossen FA, Ismail MR, Hoque MA, Islam MZ, Shahidullah SM, Meon S (2009). Efficiency of plant growth-promoting rhizobacteria (PGPR) for the enhancement of rice growth. Afr. J. Biotechnol. 8: 1247-1252.

Barea JM, Brown ME (1974). E ects on plant growth produced by *Azotobacter paspali* related to synthesis of plant growth regulating substances. J. Appl. Bacteriol. 37: 583–593.

Brenner WG, Romanov GA, Kollmer I, Burkle L, Schmulling T (2005). Immediate-early and delayed cytokinin response genes of Arabidopsis thaliana identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J. 44: 314-333.

Brown R, Rickless P (1949). A new method for the study of cell division and cell expansion with some preliminary on the effect of temperature and nutrients. Proc. Royal Soc. Lond. Series B. Biol. Sci. 136: 110-125.

Cecchett V, MM Altamura, Serino GM, Pomponi FG, Costantino P, Cardarelli M (2007). *ROX1*, a gene induced by *rolB*, is involved in procambial cell proliferation and xylem differentiation in tobacco stamen. Plant. J. 49(1): 27–37.

El-Tarabily KA, Nassar AH, Hardy GES, Sivasithamparam K (2003). Fish emulsion as a food base for rhizobacteria promoting growth of radish (*Raphanus sativus* L. var. *sativus*) in a sandy soil. Plant. Soil. 252: 397–411.

Fletcher RA, Mccullagh D (1971). Cytokinin-induced chlorophyll formation in cucumber cotyledons. Planta. 101: 88-90.

Hasnain S, Thomas CM (1996). Two related rolling circle replicating plasmids from salt-tolerant bacteria. Plasmid. 36: 191-199.

Hynes RK, Leung GC, Hirkala Y, DLM, Nelson LM (2008). Isolation, selection, and characterization of beneficial rhizobacteria from pea, lentil, and chickpea grown in western Canada. Can. J. Microbiol. 54:248-258.

Johnson JL (1994). In Methods for general and molecular bacteriology. Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds.). American Society for Microbiology –Washington –DC. pp. 625–700,

Karadeniz A, Topcuoglu SF, Inan S (2006). Auxin, gibberellin, cytokinin and abscisic acid production in some bacteria. World. J. Microbiol. Biotech. 22(10): 1061-1064.

Krall L, Raschke M, Zenk MH, Baron C (2002). The Tzs protein from *Agrobacterium tumefaciens* C58 produces zeatin riboside 50-phosphate from 4-hydroxy-3-methyl- 2-(E) -butenyl diphosphate and AMP. FEBS. Lett. 527: 315–318.

Markmann K, Parniske M (2009). Evolution of root endosymbiosis with bacteria: how novel are nodules? Trends Plant Sci. 14: 77-86.

Molina-Favero C, Creus CM, Lanteri ML, Correa-Aragunde N, Lombardo MC, Barassi CA, Lamattina L (2007). Nitric oxide and

plant growth promoting rhizobacteria: Common features influencing root growth and development. Adv. Bot. Res. 46: 1-33.
Oldroyd GED (2007). Nodules and hormones. Science 315(5808): 52-53.
Ortíz-Castro R, Valencia-Cantero E, López-Bucio J (2008). Plant growth promotion by *Bacillus megaterium* involves cytokinin signaling. Plant Signal. Behav. 3: 263-265.
Phillips DA, Torrey JG (1972). Studies on Cytokinin Production by *Rhizobium* 1. Plant Physiol. 49:11-15.
Remans R, Beebe S, Blair M, Manrique G, Tovar E, Rao I, Croonenborghs A, Torres-Gutierrez R, El-Howeity M, Michiels J (2008). Physiological and genetic analysis of root responsiveness to auxin-producing plant growth-promoting bacteria in common bean (*Phaseolus vulgaris* L.). Plant Soil. 302: 149-161.
Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JAH (1999). Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Sci. 283: 1541-1544.
Ryu C, Farag MA, Hu C, Reddy MS, Wei H, Pare PW, Kloepper JW (2003). Bacterial volatiles promote growth in *Arabidopsis*. Proc. Natl. Acad. Sci. 100(8): 4927-4932
Serdyuk OP,Smolygina LD, Ivanova EP, Adanin VM (2003). Phototrophic Purple Bacterium *Chromatium minutissimum* does not synthesize cytokinins under optimal growth conditions. Doklady. Biochem. Biophys. 392(5): 700-702.
Srivastava LM (2002). Plant Growth and Development: Hormones and Environment. San Diego Academic Press.
Stoynova-Bakalova E, Karanov E, Petrov P, Hall MA (2004). Cell division and cell expansion in cotyledons of Arabidopsis seedlings. New Phytologist. 162: 471-479.
Taller BJ, Wong TY (1989). Cytokinins in *Azotobacter vinelandii* culture medium. Appl. Environ. Microbiol. 55: 266–267.
Tien TM, Gaskins MH, Hubbell DH (1979). Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl. Environ. Microbiol. 37(10): 16-1024.
Tzfira T, Jensen CS, Wang WX, Zuker A, Vinocur B, Altman A, Vainstein A (1997). Transgenic *Populus tremula*: a step-by-step protocol for its Agrobacterium-mediated transformation. Plant Mol. Biol. Rep. 15: 219-235.
Werner T, Motyka V, Strnad M, Schmulling T (2001). Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA. 98: 10487–1049.
Yang J, Zhang J, Huang Z, Wang Z, Zhu Q, Liu L (2002). Correlation of cytokinin levels in the endosperm and roots with cell number and cell division activity during endosperm development in Rice. Ann. Bot. 90: 369-377.
Zubo YO, Yamburenko MV, Selivankina SY, Shakirova FM, Avalbaev AM, Kudryakova NV, Zubkova NK, Liere K, Kulaeva ON. Kusnetsov VV (2008). Cytokinin stimulates chloroplast transcription in detached barley leaves. Plant Physiol. 148: 1082-1893.