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Study of Microbial Cytokinin Production Under Various Enriched Conditions and its Effect on Plant Growth

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Abstract

BACKGROUD & OBJECTIVE: The present study highlights the effect of various nitrogen supplements responsible for the growth and production of microbial cytokinin. **METHODOLOGY:** During the experimental process it is compared with the production of cytokinin by different organisms, viz; *Rhizobium sp, Bacillus subtilis* BC1, *Pseudomonas aeruginosa* PAO1 and *E.coli* K12. The microbial cytokinin thus produced was tested for its effect on plant growth and shoot induction in a test plant, *Phylodendron xanadu*. **RESULTS:** According to the results observed *E.coli* K12 synthesized 0.186 mg/L amount of cytokinin in PM2. On the other hand, *Bacillus subtilis* BC1 showed the least production of cytokinin as compared to the other two. For this organism the amounts produced were 0.044 mg/L in PM1 and 0.0075 mg/L in PM2. **CONCLUSION:** The conclusion may be drawn as that PM1 is good for *Rhizobium* sp and *Bacillus subtilis* BC1. More shoot numbers was obtained in case of cytokinin sample from *E.coli* K12 with a height measuring to 3.5 cm in media with activated charcoal and 2.3 cm in media without activated charcoal. Similarly, PM3, PM4, PM5, and PM6 were also tried with various enrichment factors incorporated into the medium and the production was compared for cytokinin production.

Keywords: *Cytokinin, Rhizobium sp. Bacillus subtilis, Pseudomonas aeruginosa, shoot induction, nitrogen sources*

1. Introduction

Phytohormones are signal molecules, produced within the plant at very low concentration, which regulate the plant growth and development[1]. There are five major classes of PGHs (plant growth hormones): auxin, cytokinin, gibberellins, abscisic acid and ethylene. Cytokinins, an important class of these PGHs, are adenine derivatives, first identified as a by-product of DNA degradation, that control cell division, growth and differentiation, also dominance and leaf senescence. Large number of studies has indicated the involvement of PGHs produced by a microsymbiont in altering plant growth and development[2,3,4]. Cytokinin along with indole-3-acetic acid (IAA) and gibberellic acid (GA_3) has been reported to be produced by both plant and micro-organisms. Also, various plants like *Solanum dulcamara*, *Paulownia fortune,* pumpkin seeds etc. are also known to produce cytokinin[5,6,7]. The presence of these phytohormones in the culture media of bacteria, like *Bacillus megaterium, Proteus mirabilis, P. vulgaris, Rhizobium japonicum*, *Agrobacterium* and *Pseudomonas* spp, mosses, lichens and also in seeds of *Lupinus albus* L.[8] have been reported in the past[9,10,11,12,13].

The plant growth promoting bacteria (PGPB) also helps in accelerating phytoremediation[14,15,16]. For effective phytoremediation the concentration of available metals in the rhizosphere greatly influences the quantity of metal accumulation in plants, since large amount of heavy metals are bound to inorganic and organic constituents in the contaminated soil. PGPB can regulate the uptake of heavy metals in two ways directly through acidification, chelation, precipitation, immobilization and oxidation-reduction reactions in the rhizosphere and indirectly through their effects on plant growth dynamics[17] and directly. The phytohormones produced by plant -associated bacteria are IAA (Indole Acetic Acid), cytokinins and gibberellins. They can stimulate germination, growth, reproduction and protect plants against both biotic and abiotic stress[18]. Ethylene also a phytohormone modulates the growth and cellular metabolism of plants and is reported to be involved in disease-resistance, stress tolerance, plant –microbe partnership and plant nutrient cycle. Bacteria can reduce the stress impact on plants by ethylene by hydrolysis of 1-aminocyclopropane-1-carboxylic acid (ACC). The bacteria utilize the ammonia evolved from ACC as a sole source of nitrogen thus decreasing the ACC level within the plant due to which the ethylene synthesis is reduced. In absence of ACC-utilizing bacteria, ACC is oxidized by ACC oxidase to form ethylene, cyanide and CO_2[19,20]. Another important phytohormone studied majorly is IAA which is produced in the shoot of the plant and transported basipetally to the root tips associated with cell elongation and cell division and thus helps in the growth and defence system development. Plant-microbe interaction can be determined by IAA biosynthesis. The beneficial plant-associated bacteria synthesize IAA via indole-3 -pyruvate pathway and pathogenic bacteria follow using indole-3-acetamide pathway[21]. A 16% yield increase was observed in maize when inoculated with *Azotobacter chroococcum.* Although several studies have shown significant increase in crop yields resulting from the addition of microbial cultures to

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the rhizosphere, inconsistencies in plant growth are often noted and in most cases lacking in reproducibility [22].

In this study, 3 different micro-organisms, *Bacillus subtilis* BC1 and *E. coli* K12 and *Rhizobium* sp, that are most commonly found in the environment were selected and compared for their ability to produce cytokinin in the respective production media and also to check for their ability to assist in plant growth in ornamental plant *Philodendron xanadu*.

2. Experimental Methodology

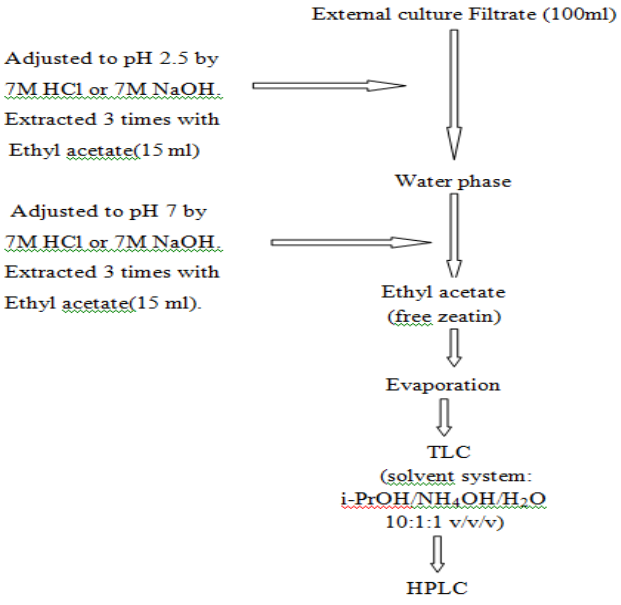
2.1 Bacterial strains and culture media

Rhizobium sp was isolated from *Mimosa pudica* on YEM (yeast extract mannitol, pH 7.0) agar plates and characterised using the various biochemical tests like IMViC tests, gelatin hydrolysis, glucose peptone agar (GPA), lactose assay and starch hydrolysis[23,24]. *Bacillus subtilis* BC1 and *E. coli* K12 cultures were obtained from MTCC, Hyderabad and were grown and maintained through subsequent sub culturing. The microorganisms were grown in two different production media with 1% inoculum from growth medium. Production Media 1 (PM1) (1% Starch, 0.3% peptone, 0.3% yeast extract, 0.3% maize extract 0.3% ammonium sulphate, 0.2% dipotassium hydrogen phosphate and 0.2% ammonium hydrogen phosphate) [23] and Production Media 2 (PM2) (6g/L disodium hydrogen phosphate, 3g/L potassium dihydrogen phosphate, 0.5g/L sodium chloride, 1g/L ammonium chloride, 2g/L sucrose, 1mM magnesium sulphate heptahydrate 0.1mM/0.1M calcium chloride, 0.003mM thiamine HCl and 0.003mM biotin) and incubated at 30°C and 37°C respectively at 150rpm for 72 hours. Secondly, the cultures were measured for microbial phytohormone synthesis and tested on *Philodendron xanadu.* Further to increase the production different nitrogen enriched medium by replacing maize extract with cornflour (PM3), raagi flour (PM4), soyabean granules (PM5) and wheat bran (PM6) were selected and evaluated for cytokinin synthesis.

2.2 Extraction and Quantification of cyltokinin

Bacterial cells were harvested from small cultures by centrifuging 100mL at 1000rpm for 10 mins and then extracted the samples. The extraction procedure is schematized in Figure I. The extracted cytokinin was detected by Thin layer chromatography (TLC) using a solvent system with isopropanol/ ammonia/distilled water (10:1:1 v/v/v)[25]. The bands developed were visualized under 254nm. The confirmation of the phytohormone from microbial origin was purified and quantified by HPLC (Waters510) and run on C18 reverse-phase column with 80% methanol as solvent and 1ml/ min as flow rate at 8.6 MPa pressure. The samples were detected at 278nm wavelength [25,26,27,28] (6-benzylamino purine) was used as standard.

Figure I: Modified procedure for extraction of cytokinin by solvent extraction method[25]



2.3 Plant material and culture conditions

Philodendron xanadu leaves were surface sterilized with 0.1% $HgCl_2$--- for 6 mins followed by rinsing five times with sterile distilled water. The explant along with the extracted cytokinin (0.05mg/mL) was inoculated in the MS medium. The explants were introduced in two different sets - one with charcoal and the other without charcoal along with a positive control of BAP (1mg/L) and a negative control (without hormones) . All the sets were incubated for 4 weeks and observed for the influence of the microbial synthesized-plant hormone.

3. Results

3.1 Isolation of *Rhizobium* sp from *Mimosa pudica*

Colonies of *Rhizobium* were obtained on YEM agar medium from the root nodules of *Mimosa pudica* after incubation at 30° C for 48 hours. The colonies were sticky in appearance, round and yellow in colour and gave positive results for both glucose peptone agar (GPA) and lactose assay[23].

3.2 Extraction, detection and quantification of cytokinin

Cytokinin was obtained by liquid-liquid extraction, from the ethyl acetate phase in the second step of the extraction procedure (Figure I).

Ethyl acetate was used for extraction because of it is an efficient solvent and evaporates faster. The ethyl acetate extracts were subjected to evaporation and re-dissolved in distilled water containing a few drops of 0.1N NaOH. This solution containing cytokinin was filtered through Whatman No.1 filter paper. TLC gave an R_f value for the samples close to that of the standard by using fluorescein dye. Table I represents the amount of cytokinin synthesized by different microbes after purification by HPLC.

3.3 Shooting in *Philodendron xanadu*

Growth of *Philodendron xanadu* under aseptic and controlled nutrient conditions were observed. Firstly, the effect of microbial synthesised-plant hormone on the shoot induction and plant growth, secondly, the effect of charcoal in association with the phytohormone was observed. There was a difference in the results obtained for media containing charcoal and the ones without charcoal with respect to the shoot height and the number of shoots. The plants incorporated with the microbial synthesized-plant hormone resulted in good number of shoots but less height where as the plant with synthetic hormone had comparatively less shoot number (Figure IIa; positive control) (Figure IIb; negative control). The medium with the charcoal in association with the hormone showed direct influence on the growth with maximum height of 3.5 cm and lustrous leaves in comparison to the plant in the medium without charcoal which had a maximum height of 2.5cm and less lustrous leaves. The shoot heights of the plants were recorded and have been presented in Table II (IIa for media without charcoal, IIb for media with charcoal) and the number of shoots represented in Figure IIIa, IIIb. The plant so grown was micro-propagated further and it was observed to have a healthy and lush growth.

Table I: Production of cytokinin from various microorganisms

Sample	*E.coli* K12 (PM2)	Rhizobium sp (PM1)	*Rhizobium* sp (PM2)	*Bacillus subtilis BC1* (PM1)	*Bacillus subtilis BC1* (PM2)
Concentration (mg/L)	0.186	0.093	Very low quantity	0.044	0.0075

Figure IIa: Positive control; with synthetic cytokinin (BAP), **b** Negative control; without hormone**.**

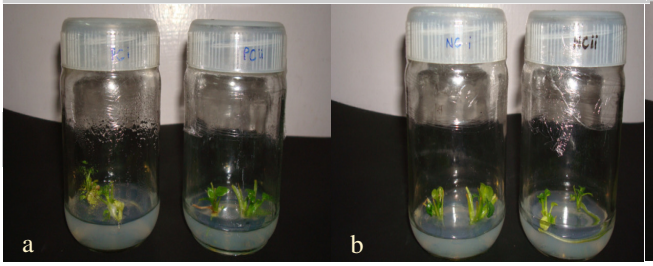
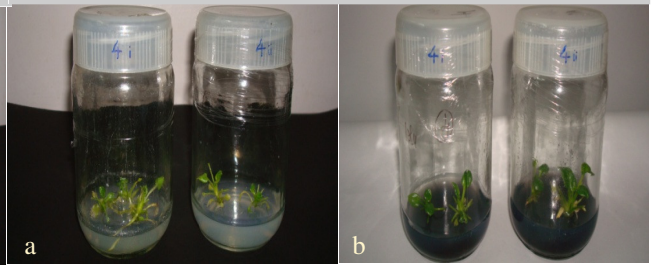


Figure III Shooting in Philodendron xanadu at the end of Week IV. **a** Cytokinin extracted from E.coli K12 in PM2 (without charcoal). **b** Cytokinin extracted from *E.coli* K12 in PM2 (with charcoal).



From the above experimental data it was observed that PM2 yielded higher plant height for *E.coli* K12 in comparison to PM1 but PM1 supported higher plant height for *Bacillus subtilis* BC1 and *Rhizobium* sp (Table I). The addition of calcium chloride controls the temperature fluctuation. By maintaining the viscosity and surface tension to low at a constant temperature increases the permeability of the cell membrane. Even at 0.1M solutions the production was found to me similar to 0.1mM concentration in PM2 medium, thus it can be interpreted that higher concentration of calcium chloride could result in accumulation of the ionic charges which could influence the change in the pH and temperature and also increase the viscosity of the medium.

Table IIa: Plant shooting in MS media without charcoal

Sample (without charcoal)	Average height (in cm)			
	Week 1	Week 2	Week 3	Week 4
Positive control	1.5	1.75	2.0	2.1
Negative control	1.0	1.5	1.5	1.5
E.coli K12 (PM2)	1.5	1.6	1.75	2.3
Rhizobium sp (PM1)	1.5	1.5	1.6	1.7
Rhizobium sp (PM2)	1.25	1.3	1.6	1.9
Bacillus subtilis BC1 (PM1)	1.5	1.5	1.9	1.9
Bacillus subtilis BC1 (PM2)	1.75	1.8	2.0	2.0

Table IIb: Plant shooting in MS media with charcoal

Sample (with charcoal)	Average height (in cm)			
	Week 1	Week 2	Week 3	Week 4
E.coli K12 (PM2)	1.0	1.9	2.4	3.5
Rhizobium sp (PM1)	1.0	1.6	2.3	3.0
Rhizobium sp (PM2)	1.0	1.7	2.3	2.0
Bacillus subtillis BCI (PM1)	1.0	1.4	1.8	2.3
Bacillus subtilis BC1 (PM2)	1.0	1.3	1.6	2.1

The enriched medium PM3. PM4, PM5 and PM6 were compared and it can be interpreted as *E. coli* K12 showed lesser adaptation to the crude form of the substrates. The maximum amount were observed for wheat bran (0.169mg/mL). *Bacillus subtilis* BC1 represented effective in cronflour (0.211mg/mL) and soyabean granules (0.316mg/mL) which is comparative significant to other organisms. *Pseudomonas aeruginosa* PA01 also showed higher production for cornflour, raagi flour and wheat bran (0.228, 0.266 and 0.231mg/L respectively).

4. Discussions

There has been research on cytokinin production by few microorganisms but very few have been reported. The presence of cytokinin has been reported in bacteria by different researchers who observed cytokinin production upto 14.88µg/ mL in *S. olivaceoviridis* among 24 bacterial strains of *Thallobacteria*[10]. In another study Donald[9] (1972) used soybean callus for determining cytokinin production by *Rhizobium japonicum* and reported a total of 0.3 µg KE/L production. Taller and Wong (1989)[13] determined cytokinin as equivalent to 0.75 µg of KE/L in *A. vinelandii* culture. Also, cytokinin producing microorganisms were inoculated in soil along with Wheat plant and it was observed that inoculation with such bacteria was beneficial for plant growth[28,29]. In our study we have found the macro and micro nutrients also have an effective role in the production and development of the phytohormone. As per our experimental data it is observed that in the absence of hormone the growth stops may be because the other ingredients support the initial growth (Figure IIb), later is found non-utilizable. Medium containing only microbial cytokinin gives more number of plantlets while medium with both microbial cytokinin and charcoal results in a better height and phenotypic appearance and also higher colour intensity and health. The addition of activated charcoal has helped the plant to grow vigorously and appear healthy with dark green coloured leaves because its acts as a purifying agent and provides the plant with the concentrated form of the growth hormone. *E.coli* K12 production of cytokinin in PM2 was found to be higher than that of *Rhizobium* sp and lowest in *Bacillus subtillis* BC1 in the same defined medium and vice versa results were obtained for the same microorganisms in the PM1. Height of the plant increased but number of plantlets for all the microorganisms were found to be minimum. Reports also reveal that micro and macro-nutrients provide necessary cofactor for many enzymatic reactions and virtually all biomass production is either elevated by or remains unaffected[15].

Most of the researchers have worked on production of IAA from microorganisms. Some researchers have also reported the production of other phytohormones by micro-organisms upto a level of 16.5-38 µg IAA/ mg protein. Karadeniz (2006)[25] reported production of phytohormones by various bacteria like *P. mirabilis, K .pneumonia, B. cereus, P. vulgaris* etc. upto a level of 50.68 µg IAA/100 ml of the culture filtrate by *K. pneumonia* and 11.21 µg Zeatin/ 100ml of culture filtrate by *P. mirabilis.*

Bacillus subtilis can maintain stable contact with higher plants and promote growth as a number of metabolites are released near the vicinity of the plant roots which increases nutrient availability for the plant[29]. Inoculation of PGPR promotes multiple effects on plant growth, enhancement on seedling germination, healthy growth in plant height, shoot

weight, nutrient content of shoot tissues, increased nodulation in legumes etc. The colonization of these beneficial microorganisms with the rhizosphere will enhance the growth and physiological features in plant. Inoculation of *Rhizobium* helps in increased growth and high yield in the number of nodules per root system. Many reports state the phytostimulatory effect of PGPR in various aspects in growth but use of cytokinin production by these bacteria is directly improving the plant growth[30,31]. Exogenous cytokinin enhances direct and fast cell division in root nodule formation.

All the test samples were used for induction and shoot development in *Philodendron xanadu* and the height of the shoot were concurrent with the amount of cytokinin obtained from the respective organism. *E. coli* K12, a conventional organism gave the highest amount of cytokinin in PM2 and also the maximum shoot height for both the type of medium (with and without charcoal). Higher shoot height were obtained in case of medium containing charcoal whereas without charcoal had more number of shoots. So, for this test plant, i.e., *Philodendron xanadu* medium with charcoal along with cytokinin produced by micro-organism can be considered better medium for growth and development. The variation in amount of production of cytokinin by these micro-organisms is a result of the difference in media composition and mechanism of the microorganism. It can be concluded that cytokinin produced from bacteria could be a source for primary and secondary metabolite production and also can be an efficient method for phytoremediation, for the removal of heavy metals from soil. The production method if modified for higher yield could also help in faster development and better quality of agricultural crop.

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