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# Indole-3-acetic Acid Production and Effect on Sprouting of Yam (*Dioscorea rotundata* L.) Minisetts by *Bacillus subtilis* Isolated from Culturable Cowdung Microflora

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Abstract

*Bacillus subtilis* strains (CM1-CM5) isolated from culturable cowdung microflora were investigated for indole-3-acetic acid (IAA) production in nutrient broth (NB). All the strains tested produced IAA in NB; albeit in very low concentrations (0.09–0.37 mg/l). The addition of L-tryptophan (0.1–1.0 g/l) into NB substantially enhanced IAA production (6.1–31.5 folds) indicating that L-tryptophan was the precursor for IAA biosynthesis by these bacterial strains. Maximum IAA production was observed after 8 days of incubation (in late stationary phase of bacterial growth). The variation in IAA production was attributed to the genetic make up of these strains as evaluated by RAPD analysis of these isolates and *B. subtilis* type strain MTCC 441. Application of *B. subtilis* suspension ($8 \times 10^9$ CFU/ml) on the surface of yam (*Dioscorea rotundata* L.) minisetts increased the number of sprouts, roots and shoots length, root and shoot fresh weights and root: shoot ratio over those minisetts not treated with bacterial suspension. Fresh cowdung slurry treatment on yam minisetts also produced similar results as obtained with *B. subtilis* application.

Key words: *Bacillus subtilis*, Cowdung, yam (*Dioscorea rotundata* L.), indole-3-acetic acid, L-tryptophan

## Introduction

There is firm evidence that indole-3-acetic acid (IAA) and other growth regulators ($GA_3$) produced by plants and essential to their growth and development are produced by various bacteria, which live in soil as well as in association with plants (Glick, 1995). There is also evidence that growth regulators such as IAA produced by bacteria can in some instances increase and improve yields of the host plants. Bacterial production of IAA has been studied not only regarding its physiological effects on plants but also regarding its possible role as a phytohormone in plant – microbe interaction (Barbieri and Galli, 1993; Xie *et al.*, 1996; Patten and Glick, 2002).

Cowdung is a mixture of faeces and urine in a ratio of around 3:1. It contains crude fibre, crude protein, cellulose, hemicellulose and 24 types of minerals such as N, K, S, traces of P, Fe, Ca, Mg, Co, Mn *etc.* (Nene, 1999). It is normally used as an organic fertilizer for *e.g.* enhancing soil fertility, dressing seeds and plastering cut ends of vegetative propagated sugarcane (Kesavan, 2006). Generally cowdung treated seeds are spared from pathogenic fungal and bacterial attack, because bacteria, and particularly *Bacillus* spp. in cowdung microflora play a significant role in controlling the growth of pathogenic microorganisms by colonizing the surface area of the seeds (Basak and Lee, 2000 a, b). In a previous study, several strains of *Bacillus subtilis* isolated from cowdung inhibited the growth of *Fusarium oxysporum* and *Botryodiplodia therobromae*, two important post harvest pathogens of yam (*Dioscorea rotundata* L., Family: *Dioscoreaceae*) tubers (Swain and Ray, 2007)

In India, farmers apply cowdung traditionally on yam tubers before planting, which promotes sprouting and seedling growth, and prevents them from rotting (Naskar *et al.*, 2003; Swain and Ray, 2007). However, the mechanism how cowdung promotes sprouting is not yet understood. In this paper, the production of IAA by *B. subtilis* strains isolated from culturable cowdung microflora has been explored and the effect of exogenous application of *B. subtilis* culture and cowdung slurry on sprouting of yam tubers has been studied.

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## Experimental

### Materials and Methods

***Bacillus subtilis* strains.** Five *Bacillus subtilis* strains (CM1, CM2, CM3, CM4 and CM5), showing antagonistic action against *Fusarium oxysporum* and *Botryodiplodia therobromae*, were isolated earlier from the culturable microflora of cowdung (Swain and Ray, in press) and maintained on Nutrient Agar slants.

**Cowdung.** Fresh cowdung was collected from a nearby cow shade and brought to the Microbiology Laboratory in sterile polyethylene bags.

**Screening *B. subtilis* strains for IAA production.** All five *B. subtilis* strains (n = 2) were evaluated for IAA production (Bentley, 1962) by inoculating 1 ml of each of cells suspension ($1 \times 10^6$ CFU/ml) in 50 ml of nutrient broth (NB) [peptone, 5.0; yeast extract, 3.0; beef extract, 1.0 and NaCl, 5.0 (g/l)] kept in 250 ml Elenmeyer flasks. L-tryptophan was added at 100 mg/l NB to half (n = 3) of the flasks and the others were kept without L-tryptophan. The flasks were incubated for 8 days at 30°C while being shaken at 120 rpm in an incubator-cum-shaker (Remi India Pvt. Ltd. Bombay, India) and the culture filtrates were analyzed for IAA production as described in the section 'plant growth hormones extraction and estimation'.

**Experiment 1: Effect of L-tryptophan concentration on IAA production**. Strains CM4 and CM5 of *B. subtilis* were used in this experiment. One ml of each bacterial cell suspension ($1 \times 10^6$ CFU/ml) was inoculated in 250 ml Elenmeyer flasks containing 50 ml of the NB and supplemented with or without L-tryptophan (0–2 g/l). The flasks (n = 3) were kept shaken at 120 rpm at 30°C and the culture filtrates were analyzed for IAA concentration at the end of 8 days incubation period. The growth of *B. subtilis* strains was determined by measuring the optical density of the growth medium at 600 nm in a UV-Vis Spectrophotometer (Model No 302, Cecil Instrument, UK).

**Experiment 2: Effect of incubation period on IAA production.** Effect of incubation period (2–10 days) was studied by inoculating *B. subtilis* (CM4 or CM5) in NB containing 1 g/l L-tryptophan. The experimental conditions were otherwise the same as in the previous experiment.

**Plant growth hormones extraction and estimation.** After being centrifuged (8000 g for 20 min) bacterial cells were extracted thrice by adding the same amount of ethyl acetate after adjusting pH to 2.5 with 1N HCl (Hansan, 2002). The extracted fractions were mixed together and reduced to 2 ml by evaporation at 45°C using a Rotary Vacuum Evaporator (Model No 102, Strike, Italy). The concentrated extracts were re-dissolved in acetone before thin-layer-chromatography (TLC). TLC was carried out using 0.5 mm-thick preparative silica gel plates and solvent used was a mixture of isopopanol, ammonium hydoxide, and water (10:1:1; v/v/v) to separate plant growth hormones (IAA and $GA_3$). IAA was detected on TLC plates by spraying with Ehrlich reagent (10% p-dimethyl aminobenzyldehyde in 70% perchloric acid) which resulted in development of pink colour visualized under normal light (Bentley, 1962). To detect $GA_3$, extracts were spread on the plates using ethanolic sulphuric acid (90:10; v/v) and heated to induce fluorescence of the compounds in ultraviolet light (MacMilan and Suter, 1963). The identified spots for growth regulators on the TLC plates were eluted in methanol. The colour absorbency for IAA was measured at 565 nm using UV-Vis Spectrophotometer (Model No 302, Cecil Instrument, UK). The IAA content was measured from a standard curve prepared with a known concentration of IAA and expressed as mg/l. The flow-chart for extraction and bioassay of growth regulators from bacterial culture is given in Figure 1.

**Random Amplified Polymorphic DNA (RAPD) analysis of *B. subtilis* stains.** DNA of *B. subtilis* strains (CM1-CM5) were isolated from cultures grown overnight in 10 ml NB by the method described by Lampe (1998). A single RAPD primer OPA-15 (5'TTCCCGACC3') specific for *Bacillus* spp. was used for DNA amplification (Lampe, 1998). The reaction mixture (20 ml) contained 40 ng of genomic *B. subtilis* DNA and 40 pmol of RAPD primer. PCR cycles were performed in a DNA thermal cycle (Model No 200, MJ Research, UK) as follows: four cycles at 94°C for five min, 30°C for five min and 72°C for five min, 30 cycles at 94°C for 30 s, 35°C for 15 s and 72°C for one min and finally extension of 72°C for 10 min. PCR mixture was electrophoresed on 1.5% agarose gel in tris-borate-EDTA buffer to confirm the RAPD typing (Darling *et al*., 1998)

**Experiment 3: Effect of *B. subtilis* culture and cowdung on sprouting of yam minisetts.** Healthy and diseases free yam tubers harvested within 20–30 days were collected from the farm of Regional Centre of Central Tuber Crops Research Institute, Bhubaneswar during the month of March, 2006 (day temperature, 30±2°C and night temperature, 24±2°C). The tubers were washed under running tap water and its surfaces were sterilized for 10 min in 1% NaOCl followed by a washing step with sterile water. The tubers were dried under laboratory conditions (room temperature, 30±2°C; relative humidity, 70–80%). The tubers were cut into cubes (minisetts) of approximate sizes of $5 \times 6 \times 8$ $cm^3$ weighing between 130–150 g each. The yam minisetts (n = 6) were dipped in *B. subtilis* (CM4 and CM5) culture suspensions separately ($8 \times 10^9$ CFU/ml) for 2 h, planted 5 cm below the sand, and kept for 15 days under laboratory conditions.

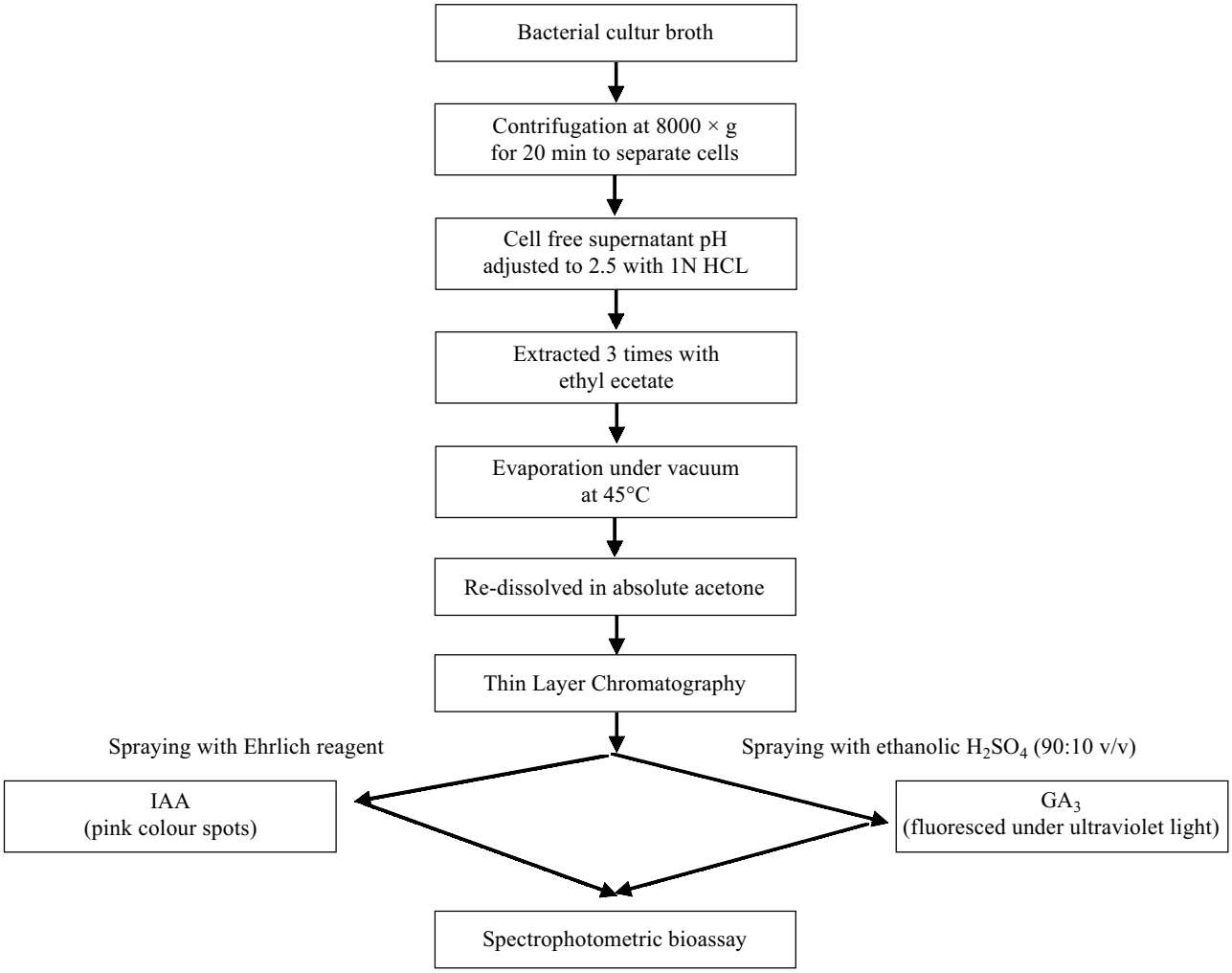


Fig. 1. Procedure used for extraction and bioassay of growth regulators (IAA and $GA_3$) from bacterial cultures.

Cowdung slurry was infused with water (1:1; w/v), and homogenized by shaking for 2 h at 120 rpm. The yam minisetts prepared as above were dipped in cowdung slurry for 2 h and then planted on sand bed for 15 days. Minisetts without any (bacterial or cowdung) treatment served as control in this experiment. Six replicates (n = 6) were maintained for control as well as for each treatment and mean data with standard deviations were calculated for number of sprouting, root and shoot length, root and shoot freshweight, root and shoot dry weight and root: shoot ratio per minisett.

**Solid-state bioprocessing of cowdung for IAA production by *B. subtilis*.** Twenty grams of oven-dried cowdung kept in 250 ml Erlenmeyer flasks was adjusted to 60% water holding capacity (WHC) by the addition of sterile distilled water and autoclaved at 15 lb for 15 min. The sterilized cowdung dust was cooled at room temperature and inoculated with strain of CM4 and CM5 mixture at 10% rate ($1 \times 10^6$ CFU/ml) and culture flasks (n = 3) were incubated at 30±2°C for 10 days. The contents in Erlenmeyer flasks were mixed periodically by gentle tapping and distilled water was supplemented to replenish the loss of water evaporated to maintain WHC of 60%. After 2 days of incubation triplicate flasks were taken out. To determine IAA content, 50 ml distilled water was added to each flask and the mixtures (cowdung and water) were homogenised thoroughly at 200 rpm for 30 min in a shaker. The homogenate was filtered through a cheese cotton cloth and centrifuged at 5000 g for 20 min. The supernatant was then filtered through a Whatmann No 1 filter paper and taken for IAA extraction. The IAA content in cowdung was expressed as mg IAA/gram dry substrate (gds).

## Result and Discussion

In our earlier studies, the microbial composition of cowdung which includes several bacteria (*i.e. Bacillus* spp., *Corynebacterium* spp., *Lactobacillus* spp., *etc*), fungi (*i.e. Aspergillus*, *Trichoderma*, *etc*) and yeasts (*i.e. Saccharomyces*, *Candida*, *etc*) has been

discussed (Swain *et al*., 2006; Swain and Ray, 2007). Of these, *B. subtilis* strains are the predominant microorganisms of agricultural importance (Swain and Ray, 2007). Preliminary studies using TLC and spectrophotometric bioassay showed that *B. subtilis* strains (CM1-CM5) produce only IAA while $GA_3$ could not be detected (MacMilan and Suter, 1963). In the absence of L-typtophan, *B. subtilis* strains CM 1, CM2 and CM3 produced negligible amounts (0.12–0.22 mg/l) while strains CM4 and CM5 produced 0.38 mg/l (62.3% more than CM1-CM3) of IAA. Furthermore, the addition of L-tryptophan (0.1 g/l) enhanced IAA production by CM4 and CM5 strains to 2.1–2.5 mg/l (5.6–6.8 fold). Consequently, these two strains were chosen for further studies.

**Effect of L-tryptophan concentrations on IAA production.** L-tryptophan is generally considered as an IAA precursor, because its addition to IAA producing bacterial culture promotes an increase in IAA biosynthesis (Costacurta and Vanderleyden, 1995; Tien *et al.*, 1979). Figure 2A shows the effect of L-tryptophan concentrations on IAA production by *B. subtilis* CM4 and CM5. With the increase in L-tryptophan concentration from 0 to 2 g/l, there was a linear increase in IAA production in the case of strain CM4 (Fig. 2A). However, in the case of CM5, an increase in IAA production was observed up to concentration of tryptophan 1 g/l and there was a slight decrease at higher concentrations. This might be explained by the fact that a single bacterial strain often uses more than one biosynthesis pathway for IAA production (Patten and Glick, 1996). Concomitant with increased IAA biosynthesis due to increasing L-tryptophan concentration, the growth of *B. subtilis* was also stimulated (Fig. 2B). A similar result was reported in for *Azospirillum brasilense* (Tien *et al*.,

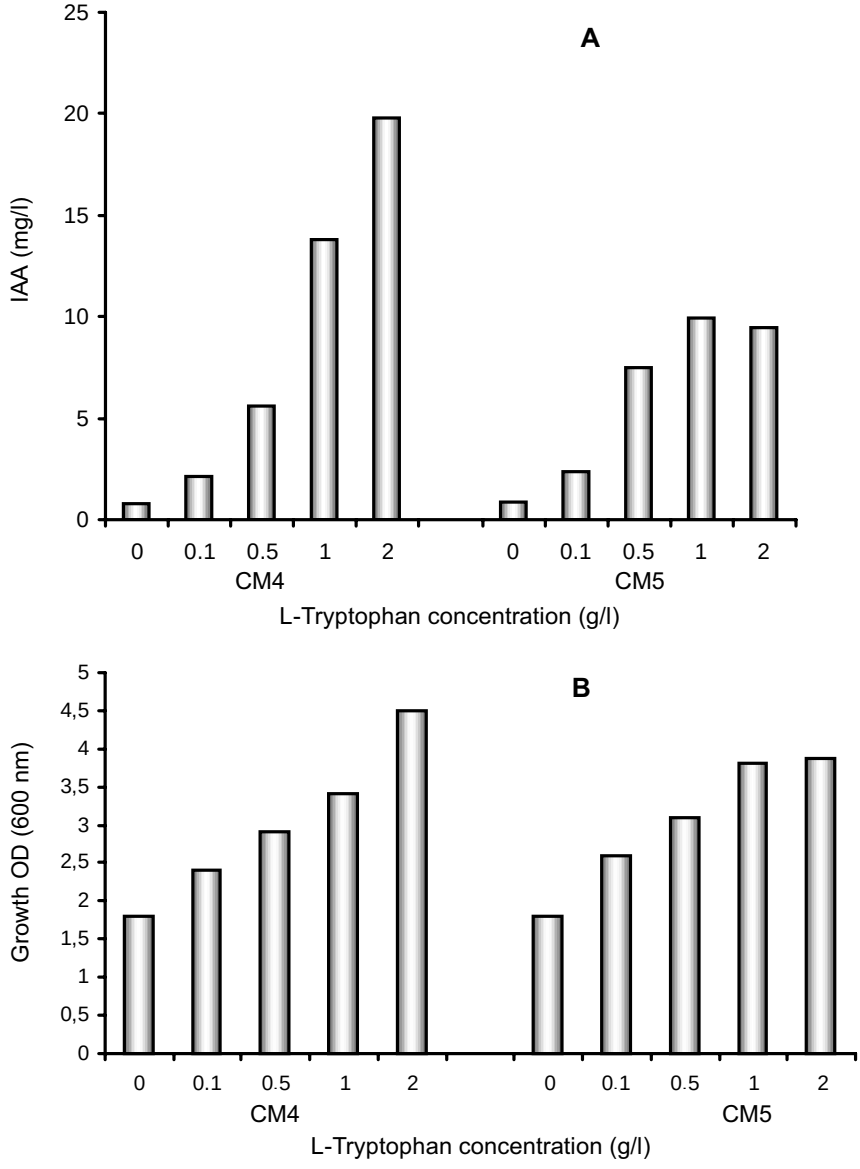


Fig. 2. Effect of L-tryptophan concentration on (A) IAA production and (B) growth by *B. subtilis* strains (CM4 and CM5).

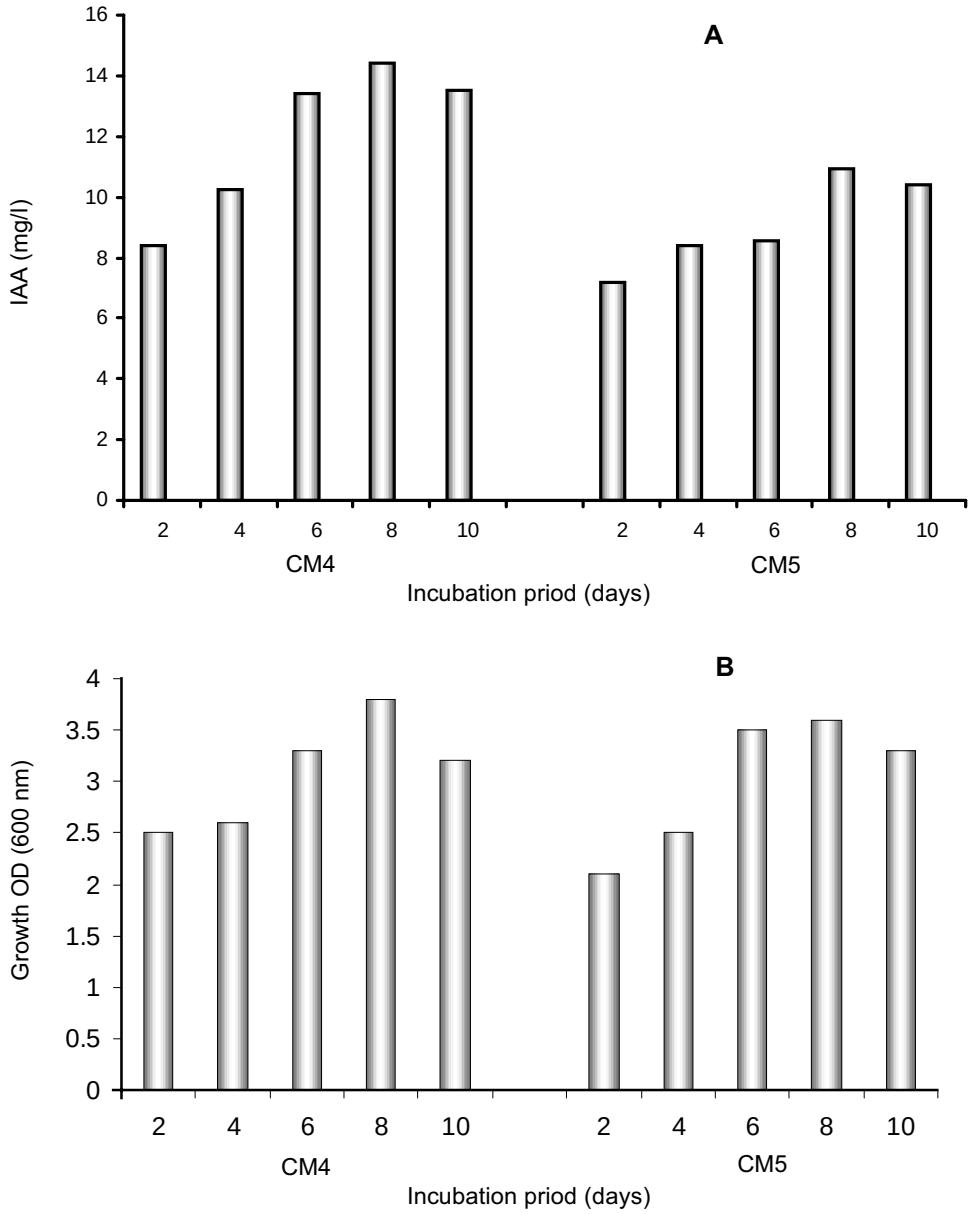


Fig. 3. Effect of incubation period on (A) IAA production and (B) growth by *B. subtilis* strains (CM4 and CM5).

1979). Likewise, several strains of *B. subtilis* produced IAA in culture media (Tang, 1994; Ghosh *et al*., 2003). Tryptophan-dependant IAA synthesis has been also determined in several other bacteria (Patten and Glick, 1996). For example, in *Enterobacter cloacae*, IAA was synthesized *via* indole-3-pyruvic acid (Koga *et al*., 1991); in *Pseudomonas syringae,* IAA biosynthesis occurs mostly from tryptophan *via* indole-3-acetamide (Hutcheson and Kosuge, 1985; Kosuge and Sanger, 1987) and in *Pseudomonas fluorescens*, tryptophan bypassing the indole-3-pyruvic acid step, is directly converted to indole-3-acetaldehyde, which is further converted to IAA (Oberhansli *et al.*, 1991). IAA synthesis has also been found to occur *via* tryptamine in *Agrobacterium tumefaciens* and *via* indole-3-acetonitrile in *Alcaligenes faecalis* and *A. tumefaciens* (Costacurta and Vanderleyden, 1995; Kobayashi *et al.*, 1995).

**Effect of incubation period on IAA production.** Figure 3A shows IAA production by *B. subtilis* strains CM4 and CM5 in tryptophan (1 g/l) – supplemented culture medium. The production of IAA was almost linear from 2 to 8 days; after that the IAA biosynthesis marginally decreased. This was concomitant with the growth of *B. subtilis* in tryptophan-supplemented medium (Fig. 3B). Unyayar *et al*., (2000) and Hansan (2002) reported for *Pseudomonas* spp. that the maximum amount of IAA was synthesized during stationary phase of growth. The reason hypothesized was that during stationary phase the bacterium might be able to get maximum tryptophan from the dead bacterial mass, which could result in more IAA production.

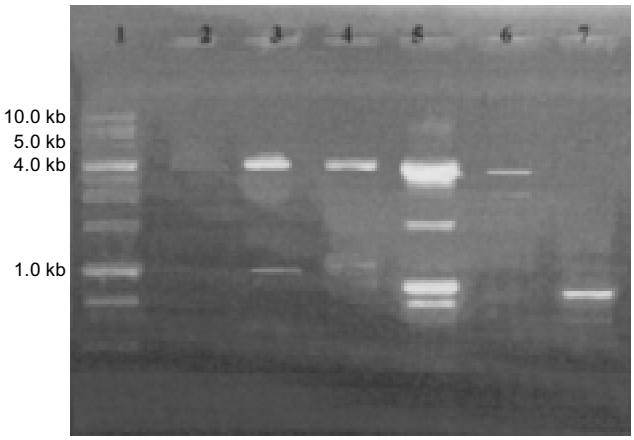


Fig. 4. RAPD finger printing of DNAs from *B. subtilis* strains amplified with primer OPA 15.

From left: Lane1-molecular size markers (1-kb ladder), Lane 2 – CM4, Lane 3 – CM1, Lane 4 – CM5, Lane 5 – CM2, Lane 6 – CM3 and Lane 7 – Type strain MTCC 441. A photograph of gel was scanned with a HP 800 Scanner.

**RAPD amplification.** *B. subtilis* strains (CM1 to CM5) were compared with standard *B. subtilis* (MTCC 441) by RAPD fingerprinting using a single primer (Fig. 4). Lane 1 represented standard marker, lanes 2 to 6 represented *B. subtilis* strains (CM1 to CM5) and lane 7 represented type strain *B. subtilis* (MTCC 441). The band patterns obtained with RAPD were all different except for a single common band detected at 4.0 kb. The result indicated that all the isolates belong to *B. subtilis* strains but at gene level they were different from each other, as evident from the production of IAA in the present study.

**Effect of *B. subtilis* strains and cowdung on sprouting of *Dioscorea* minisetts.** When yam minisetts were dipped in *B. subtilis* suspension, an increase in root and shoot length as well as fresh and dry weights in comparison with control (minisetts not treated with *B. subtilis*) could be observed (Table I). For example, yam minisetts treated with *B. subtilis* strain CM 4 showed 63.5 and 83.3% more root and shoot elongation, respectively, in comparison with those not treated with the bacterial suspension (Fig. 5A). Similarly, 76.2 and 75.7% more root and shoot fresh weights, respectively, were observed in *B. subtilis* CM4 treated yam minisetts in comparison with the control minisetts. Similar results were observed also for CM 5 strain treated minisetts. In general, the average root: shoot ratio was higher in *B. subtilis*-treated yam minisetts in comparison to those not treated with the bacterial culture. In earlier reports, root elongation was found to occur in *Sesbania aculeata* by inoculation with *Azotobacter* sp. and *Pseudomona*s sp. (Ahmad *et al.*, 2005), in *Brassica campestris* by *Bacillus* spp. (Ghosh *et al.*, 2003), in *Vigna radiata* by *Pseudomonas putida* and in (Patten and Glick, 2002), *Pennisetum americanum* by *Azospirillum brasilense* (Tien *et al.*, 1979). Like *B. subtilis* treatment, increase in shoot and root number and length was observed in the case of cowdung-treated minisetts (Table I). However, the effect was smaller than obtained with the *B. subtilis* treatment (Fig. 5B). Since *B. subtilis* strains in the present study did not produce $GA_3$, which could stimulate root and shoot elongation (Hopkins, 1999), this indirectly confirmed the involvement of IAA synthesized by the bacterial strains in enhancing the sprouting of yam minisetts.

**IAA production by *B. subtilis* in solid-state fermentation.** To examine whether cowdung could serve as a solid substrate for the production of bioinoculants such as *B. subtilis* that produces IAA, moistened cowdung was inoculated with CM4 and CM5 strains and IAA production was followed for 10 days. IAA production was marginally higher on days 2–4 (42–45 mg/gds) by CM 4 strain and remained more or less stable during the incubation days 6 to 10 (30–36 mg/gds). The synthesis of IAA by CM5 strain was almost the same during the course of study (2–10 days) (38.0–43.0 mg/gds).

**Conclusion.** Cowdung is traditionally used in Asian agriculture as an organic fertilizer. The goal of the 'evergreen revolution' necessarily involves components, which don't adversely affect soil health, water quality, biodiversity, atmosphere and renewable energy sources (Kesavan, 2006). In this context, organic farm-

Table I
Effect of application of *B. subtilis* strains (CM 4 and CM5) and cow dung slurry on sprouting of *Dioscorea* minisetts

| Parameters | Control | *B. subtilis* (CM4) | *B. subtilis* (CM5) | Cowdung |
|---|---|---|---|---|
| Root: shoot ratio | 5.02 | 6.16 | 6.2 | 5.13 |
| Root length (cm) | 15.7 ± 1.23 | 43.08 ± 3.12 | 44.09 ± 3.01 | 34.00 ± 2.02 |
| Shoot length (cm) | 2.58 ± 0.49 | 15.0 ± 0.90 | 13.8 ± 1.32 | 7.60 ± 1.02 |
| Root fresh wt. (g) | 1.24 ± 0.23 | 5.21 ± 2.3 | 5.22 ± 1.80 | 3.54 ± 1.32 |
| Shoot fresh wt. (g) | 1.02 ± 0.03 | 4.19 ± 1.12 | 4.20 ± 0.80 | 3.04 ± 0.42 |
| Shoot dry wt. (g) | 0.12 ± 0.01 | 0.47 ± 0.04 | 0.47 ± 0.01 | 0.30 ± 0.10 |
| Root dry wt. (g) | 0.11 ± 0.02 | 0.39 ± 0.02 | 0.41 ± 0.1 | 0.23 ± 0.02 |

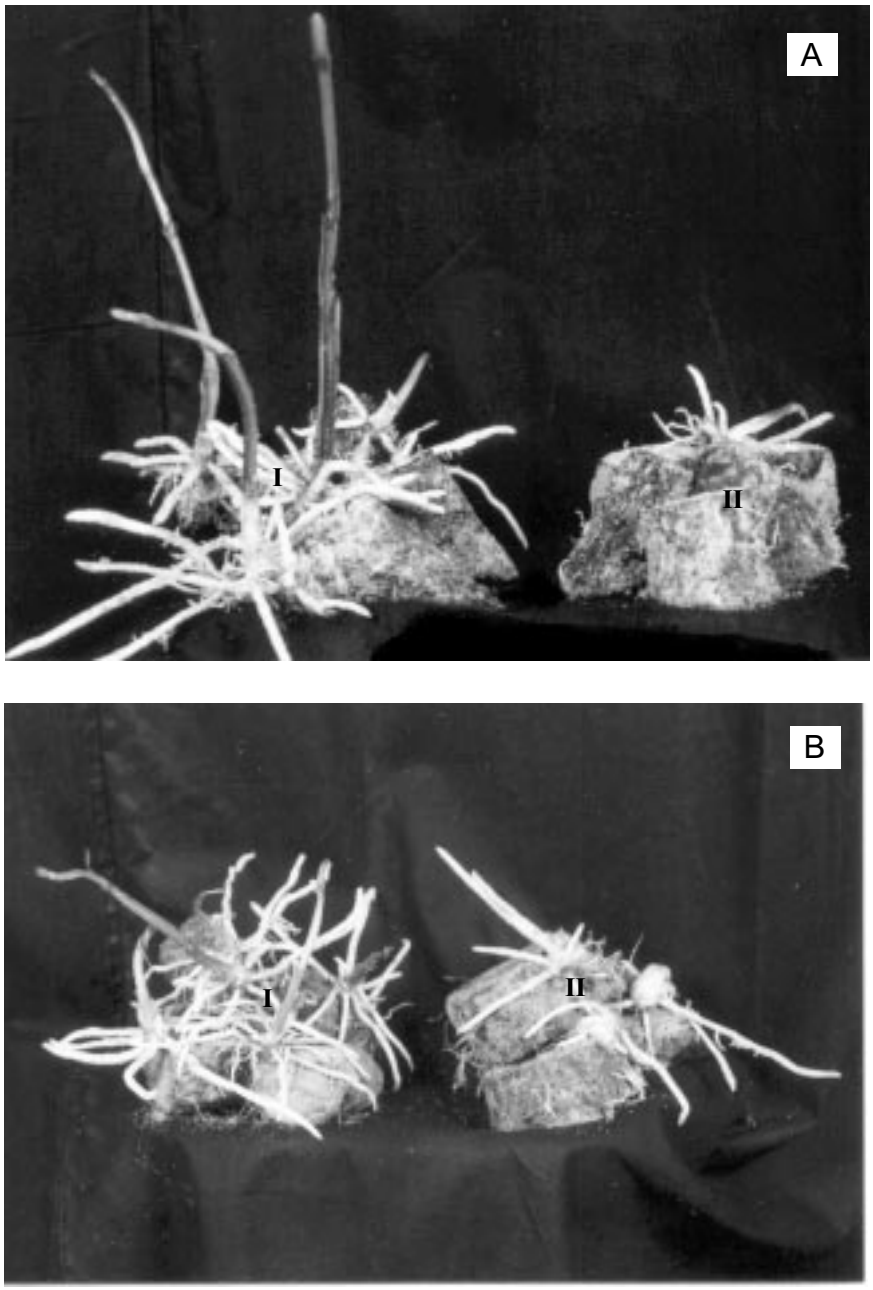


Fig. 5. Sprouting of *Dioscorea* minisetts after 15 days planting on sand bed.
A. (I) Minisetts with *B. subtilis* CM4 treatment (II) Minisetts without *B. subtilis* CM4 treatment.
B. (I) Minisetts with cowdung treatment (B) Minisetts without cowdung treatment.

ing using natural substances such as cowdung and effective microorganisms such as *B. subtilis* would sustain and even increase agricultural productivity without affecting soil health. This is implicit in the activity of cowdung microflora such as *B. subtilis* by producing growth regulator such as IAA which promoted sprouting (in the present study), biocontrol activity against plant pathogens (Basak and Lee, 2000a, b; Swain and Ray, 2007) and production of agriculturally important enzymes such as α-amylase (Swain *et al.*, 2006) which have manifold beneficial impacts on crop growth and production.

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