Extraction of Indole-3-acetic Acid from Plant Growth Promoting Rhizobacteria of Bamboo Rhizosphere and Its Effect on Biosynthesis of Chlorophyll in Bamboo Seedlings

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KEYWORDS: Bacillus, Bamboo, Biofertilizer, Indole-3-acetic acid, Plant growth promoting rhizobacteria.

ABSTRACT
Background: Indole-3-acetic acid (IAA), a principal phytohormone, controls several crucial physiological processes of plants. Itameliorates plant growth by stimulating cell elongation, root initiation, seed germination and seedling growth. Alteration of IAA level byplant growth promoting rhizobacteria leads to varied impacts on plant growth and development.
Methods: Soil samples were collected from bamboo (Bambusa tulda, B. nutans subsp. cupulata, B. balcooa and Dendrocalamus strictus) rhizosphere. Altogether five bacterial isolates were screened by serial dilution method and subjected to biochemical analysis. The isolate BUX1 with high IAA production capacity was optimized for IAA production. IAA was partially purified and quantified from the bacterial extract by thin layer chromatography (TLC). The influence of extracted bacterial IAA on chlorophyll biosynthesis in bamboo seedlings of B. tulda was compared with uninoculated control plants.
Results: Biochemical analysis revealed that all the isolates belonged to genus Bacillus which were found capable of producing IAA. During optimization, BUX1 isolate produced 99.13 µg ml⁻¹ of IAA at 37°C, pH 7, 3 mg l⁻¹ concentration of L-tryptophan and 150 rpm agitation rate after 192 hour of incubation. The RI value of the bacterial IAA during TLC was identical to that of standard IAA (0.425) indicating that IAA was present in crude extract of Bacillus (BUX1). The influence of bacterial IAA on chlorophyll biosynthesis in bamboo seedlings was significant in comparison to uninoculated plants. Therefore, this isolate could be a prospective candidate to be employed as biofertilizer.

INTRODUCTION
Indole-3-acetic acid (IAA) is a vital plant hormone that governs several facets of plant growth and development (Tan et al. 2007). It is the most common naturally available and physiologically crucial phytohormone of the auxin class, although there are various compounds like 4-chloroindole-3-acetic acid, phenylacetic acid, indole-3-butyric acid and indole-3-propionic acid with similar activity. The role of bacterial IAA is diverse in relation to the interaction between plants and microorganisms. Previously, bacterial auxin production was believed to be mainly associated with pathogenesis, specifically with bacterial gall formation. However, it is obvious that several phytopathogenic bacteria including gall inducing as well as plant growth promoting bacteria are capable of synthesizing IAA. Besides being produced by plants, it is also synthesized by root associated bacteria like Rhizobium sp., Pseudomonas sp. and Azospirillum sp. (Sreaopen et al. 2007).

Synthesis of IAA by bacteria has been directly associated to the development of the plant host root system and increased root growth and branching (Biswaes et al. 2018). Therefore, auxins have been discovered in the culture media of Bacillus pumilus 8N-4 from rhizosphere of Triticum aestivum, Zea mays and Oryza sativa (Hafeez et al. 2006), Pantoea agglomerans from Legumes (Sergeeva et al. 2007), Rhizobium sp. from root nodules of Sesbania sesban (Sridevi and Mallaiah, 2007), Bacillus sp. from Solanum tuberosum (Ahmed and Hasnain, 2010), Pseudomonas sp. from apple rhizosphere (Sharma et al. 2014), Pseudomonas fluorescens from Gossypium hirsutum (Nehra et al. 2014), Fluorescens pseudomonads from Oryza sativa (Suressh et al. 2014) and P. fluorescens and P. putida from Hordeum vulgaris (Meliani et al. 2017).

There is no research documented on auxin producing bacteria from bamboo rhizosphere. However, Ruananksa (2014) isolated and identified the phosphate solubilizing...
plant growth promoting rhizobacteria (PGPR) from bamboo rhizosphere. In context of Nepal, very scanty works have been done regarding PGPR. To mention some of them, the diversity of PGPR was documented from rhizosphere of Glycine max (Adhikari et al. 2012) and Oryza sativa (Shrivastava, 2013). Pathak et al. (2017) isolated phosphate solubilizing PGPR from rhizosphere of Lycopersicon esculentum and documented their biocontrol activities. Kunwar et al. (2018) isolated phosphate solubilizing PGPR from rhizosphere of Coffea arabica and observed its growth promoting activities in coffee seedlings and Puri et al. (2018) studied growth promoting activities of endophytic PGPR from Ipomea batatas tuber in Nepal. Therefore, our objective was to isolate IAA producing bacteria inhabiting bamboo rhizosphere (Bambusa tulda, B. balcooa, B. nutans sub sp. cupulata and Dendrocalamus strictus) and to evaluate its efficiency for chlorophyll formation in bamboo seedlings. As per the objectives, in this part of the research we have isolated the bacteria from the bamboo rhizosphere, characterized and analyzed the secondary metabolite produced by them as auxin.

**MATERIALS AND METHODS**

**Soil sample collection**

The sampling site was Sasapur of Hariwon Municipality, Sralahi district, province number 2, Nepal located at an altitude of 138m (N 27°05'03.9” and E 085°36'59.9”) towards Northern side of Lakhandehi River. Three soil samples were taken from the rhizosphere of bamboos (B. tulda, B. balcooa, B. nutans sub sp. cupulata and D. strictus) from different depth (5, 10, 15cm) during June, 2017 according to Barillot et al. (2013). The soil samples were kept in sterilized falcon tubes in ice bag, transported to laboratory of Organic Farming and Natural Product Research Centre (ONRC) of Kathmandu University, Dhulikhel, Nepal and stored at 4°C until the time of isolation. All the laboratory experiments were conducted at ONRC.

**Isolation and purification of bacterial isolates**

One gram rhizosphere soil sample was suspended in 9ml of sterilized distilled water followed by serial dilution under aseptic condition. 0.1ml of diluted samples (10^{-2} to 10^{-6}) were spread on the sterile Luria- Bertani (LB) agar medium (HiMedia) and incubated for 48 hours at 30°C. Morphologically distinct colonies were selected from agar plates to avoid repetition of same strain. Selected colonies were streaked repeatedly on LB agar and evaluated by Gram staining for purity. Pure colonies were stored in LB broth containing 80% glycerol at -20ºC for further studies. These isolates were maintained by transferring them to LB broth after every 30-60 days.

**Screening of bacterial isolates for IAA by spectro photometric quantification**

Isolated colonies were inoculated into LB broth containing 3mg I^{-1} L-tryptophan (L-trp) and incubated at 30°C with continuous shaking at 150 rpm for 24 hours (Rahman et al. 2010) with slight modification. For each isolate, the experiment was repeated three times with three replicates. After 24 hours, 2 ml of cultured solution with triplicates were centrifuged at 10000 rpm for ten minutes at 4°C and amount of IAA per ml of culture was estimated by adding 2 ml of culture supernatant to 4 ml of Salkowski’s reagent (2 ml of 0.5 M FeCl_{3} in 98 ml of 35% HClO_{4} (Asghar et al. 2002) and incubated for one hour in darkness at room temperature for color development. Intensity of color was measured by spectrophotometer at 535nm (Hussain and Husnain, 2011). IAA concentration was estimated using a standard curve prepared with known amount (10-100µg ml^{-1}) of filtered IAA (HiMedia). A propitious isolate exhibiting more intensity of color was selected for further study and nominated as BUX1 and identified up to the genus in accordance with Bergey’s Manual of Systematic Bacteriology (Garrity et al. 2004).

**Optimization of growth condition for IAA production**

Optimization of pH (5 to 9), incubation time (24 to 240 hours), media [LB broth, Pikovskaya’s (PVK) broth and Nutrient broth (NB)], temperature (25 to 40°C) and L-trp concentration (0 to 4 mg I^{-1}) were carried out in triplicate and experiments were repeated three times for improved yield of IAA. IAA production was determined according to Rahman et al. (2010).

**Extraction and quantification of IAA by thin layer chromatography (TLC)**

A single colony of BUX1 isolate was inoculated in 250 ml of LB broth containing 3 mg I^{-1} L-trp with pH 7 and incubated at 37°C for 196 hours on a shaking incubator at 150 rpm. Bacterial cells were separated by centrifugation at 10000 rpm for 20 minutes at 4°C. The supernatant was acidified to pH 2.5 with 1M HCL and extracted twice with ethyl acetate. Extracted ethyl acetate fraction was filtered by using syringe filter and evaporated in rotatory evaporator at 35°C. The extract was dissolved in 200 ml methanol and stored at -20°C. Ethyl acetate fraction of 25 ml were spotted on TLC plate and developed in the mixture of chloroform, ethyl acetate and formic acid (77:22:1 v/v) and continuously by preparative TLC. Spots with retention factor (R_f) value identical to standard IAA (HiMedia) were identified under UV light (254nm) by spraying the plates with Salkowski’s reagent (Rahman et al. 2010).

**Effects of crude bacterial IAA on chlorophyll formation of bamboo seedlings**

B. tulda was selected for this experiment because it was mostly preferred by the local communities for construction and rehabilitation of fallow land as observed during the field visit and the bacterial isolate of its rhizosphere demonstrated the best production of IAA during spectrophotometric quantification. For in vitro estimation of chlorophyll of bamboo seedlings, seeds of B. tulda were surface sterilized with 1% sodium hypochlorite for 1 minute and washed three times with autoclaved distilled water and soaked in bacterial crude IAA (2µl, 4µl, 6µl, 8µl and 10µl). In case of control, surface sterilized seeds were treated with sterilized LB broth.
and water as negative controls and standard IAA (HiMedia) as positive control. Each treatment was repeated three times with three replications and they were arranged based on completely randomized design and placed in growth chamber at 28±10°C adjusted to 16 hours light and eight hour dark period for 24 days. Total chlorophyll was extracted from the leaves of bamboo seedlings and estimated according to Ammon (1949).

Statistical analysis

Statistical analyses were performed using IBM Statistical Package for the Social Science (SPSS) version 25. Comparative assessment of IAA production capacity of isolates as well as the biosynthesis of chlorophyll by the bamboo seedlings at various treatments were analyzed by ANOVA using Duncan’s Multiple Range Test (DMRT) at 5% (P ≤ 0.05) probability level.

RESULTS AND DISCUSSION

Isolation and biochemical characterization of bacterial strain

Altogether ten rhizobacterial isolates were isolated from bamboo rhizosphere. All isolates were processed for biochemical tests and detection of plant growth promoting substances (IAA). Five isolates were selected based on their biochemical tests and ability to produce IAA in a preliminary screening. These isolates were rod shaped, circular colony, translucent/opaque, smooth/irregular margin, gram positive, catalase positive, oxidase negative and citrate positive. For the competitive root colonization and bacterial maintenance in roots, both the flagellar motility and citrus usage are considered to provide a significant contribution (Weisskopf et al. 2011). Based on morphological and biochemical characteristics, five isolates belonged to the genus Bacillus (Table 1). Most of the researches have documented the occurrence of Pseudomonas and Bacillus as common genera of PGPR (Hallmann and Berg, 2006; Zahid et al. 2015).

The biochemical application of Salkowski’s reagent is a better option for the qualitative detection of IAA in the culture (Mohite, 2013). The same reagent was used for confirmation of IAA synthesized by our five isolates and the isolate BUX1 was found to be the best producer of IAA. Maximum IAA production was detected in BUX1 isolate followed by BUX14 (Table 1). The selected bacterial isolates demonstrated IAA producing activity in the range of 9.94 to 89.65 µg ml⁻¹. Hence, isolate BUX1 was selected as promising candidate for further experiments.

Optimization of IAA production by (BUX1)

Optimization of IAA production was carried out among different media, pH, temperature, concentration of L-trp and incubation period. The effect of pH showed a gradual increase in its production from pH 5-7, maximum at pH 7 (101.28 µg ml⁻¹) and gradually decreased thereafter. Isolate BUX1 was able to produce auxin over a temperature range from 25°C to 40°C and the optimum temperature for the experimental microbial synthesis of IAA was at 37°C (99.17 µg ml⁻¹). Considering L-trp as an important modulator in IAA production, experiments at various concentration of L-trp were designed. IAA produced was found to be at negligible amount in L-trp free medium. It revealed that the maximum production reached at concentration of 3 mg l⁻¹ of L-trp in LB broth medium (99.13 µg ml⁻¹) at 37°C in pH 7 after 192 hours of incubation at the agitation rate of 150 rpm. When the IAA production was measured at different incubation period (24 to 240 hrs), the least production was recorded at 24 hrs (72.89 µg ml⁻¹) while the maximum production was at 192 hrs incubation (98.36 µg ml⁻¹) followed by gradual decrease thereafter (Fig 1 A-E). Extracellular supply of L-trp facilitates capacity of the cell to accumulate an increased tryptophan pool which is used for biosynthesis of IAA (Malhotra and Srivastava, 2008).

Detection of bacterial IAA by TLC

TLC analysis of the crude extract affirmed the presence of IAA with similar Rf value (0.425) as that of the standard IAA. It unveils the fact that IAA was present in the crude extract of Bacillus (BUX1). The result was supported by previous studies (Mohite 2013; Sharma et al. 2014). It has been reported that IAA production by PGPR can fluctuate among different species and also affected by culture condition, growth stage and substrate ability (Mirza et al. 2001). The level of microbial IAA biosynthesis of isolated strains remained variable, however, storage of bacteria under mineral oil for long time affected their IAA production capacity differently (Tsavkelova et al. 2006).

Measurement of total chlorophyll

Total chlorophyll content measured in bamboo seedlings were found to be influenced by inoculation of extracted IAA

### Table 1: Biochemical analysis of bacterial isolates.

| Sources of isolates | Isolates | Geographical location | Gram reaction | Citrate | KOH | Catalase | Oxidase | Biochemical analysis |
|---------------------|---------|-----------------------|---------------|--------|-----|---------|---------|---------------------|
| B. tulda            | BUX1    | N 26°57’07.5”E 085°45’03.9” | + ve          | + ve   | + ve | + ve    | - ve    | 89.65±0.57         |
| B. balcooa          | BUX2    | N 26°57’07.5”E 085°45’03.9” | + ve          | + ve   | + ve | + ve    | - ve    | 9.94±0.04          |
| D. strictus         | BUX3    | N 26°57’48.2”E 085°44’32.1” | + ve          | + ve   | + ve | - ve    | - ve    | 7.81±0.07          |
| B. nutans sub sp. cupulata | BUX4 | N 26°57’41.2”E 085°44’01.6” | + ve          | + ve   | + ve | + ve    | - ve    | 45.41±0.28         |
| B. tulda            | BUX14   | N 26°57’48.2”E 085°44’32.1” | + ve          | + ve   | - ve | + ve    | - ve    | 87.34±0.11         |

+ ve: positive response, – ve: negative response. Values are means ± SE. Levels not connected by same letter are significantly different (P < 0.05) according to DMRT. Experiment was repeated three times with three replicates for each isolate.
at various treatments. All the treatments except at 6µl of extracted IAA, showed significantly low level of production of total chlorophyll (1.12% in 2µl, 14.81% in 6µl, 14.51% in 8µl and 13.49% in 10µl) in comparison to positive control (Fig 2). But when compared with the negative controls (LB broth and water), all the treatments revealed statistically significant increase in the yield of total chlorophyll at various treatments (25% in 2 µl, 10% in 4µl, 42% in 6µl, 60% in 8µl and 127% in 10µl). The highest yield of total chlorophyll was recorded from inoculants treated at 10µl of extracted IAA. PGPR increased chlorophyll biosynthesis in plant leaves enhancing the rate of photosynthesis (Nadeem et al. 2009). Our extracted microbial IAA significantly increased the content of total chlorophyll (1.26 µg mg⁻¹) in comparison
to negative controls. Similar kind of experiment was conducted by Ahmed and Hasnain (2010) in which they documented that *Solanum tuberosum* treated with *B. flexus* (Amb7) exhibited 26% accretion in chlorophyll ‘a’ and 82% in chlorophyll ‘b’. Sandeep *et al.* (2011) reported higher amount of chlorophyll content (1.70 mg g⁻¹) from *Lactuca sativa* treated with *B. megaterium*. Increase of chlorophyll content may be an indicative of interaction that triggers the chlorophyll related enzymes for enhanced production of chlorophyll (Kang *et al.* 2014). They also reported the higher amount of chlorophyll in mustard plants treated with *B. megaterium* (mj1212) as compared to untreated plants. Similarly, Gul *et al.* (2019) reported significant increase of chlorophyll content along with other growth parameters in a combined treatment of urea and *Rhizobium* sp. in *Cyanopsis tetragonoloba*.

**CONCLUSION**

The production of IAA by PGPR isolated from bamboo rhizosphere is confirmed by TLC. This is the very first kind of research carried out regarding the production of IAA by PGPR isolated from bamboo rhizosphere. This study extends the knowledge on auxin producing PGPR inhabiting the bamboo rhizosphere. Genus *Bacillus* (BUX1) produces maximum IAA in LB medium with 3mg l⁻¹ concentration of L-trp in pH 7 at 37°C after 192 hour of incubation. It demands similar kind of research in rhizosphere of other bamboo species distributed across the country. Bacteria associated with bamboo rhizosphere may be a good source for development of biofertilizer for organic production. Therefore, we conclude that genus *Bacillus* (BUX1) can be considered as a potential source for the commercial production of IAA which is environment friendly to mitigate chemical pollution of soil. However, an intensive and extensive study in the field condition is expected particularly for the economically important crops with different types of soil in the field at varying climatic condition.

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**Conflict of interest**

All authors declare that they have no potential competing interests.

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