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# Endophytic Fungi Isolated from *Citrullus Colocynthes*l. Leaves and Their Potential for Secretion of Indole Acetic Acid and Gibberellin

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## ABSTRACT

Fungal endophytes are the best known beneficial partners of all plants, having role in providing immunity against biotic and abiotic stresses as well as synthesizing biologically active secondary metabolites including plant growth promoting hormones. Twenty-nine (29) different fungal strains were isolated from xerophyte plant *Citrulluscolocynthes* L. (colocynth/bitter apple) leaves and screened on rice seedlings for their growth promoting or inhibiting activities. 20 strains were found to be growth promoter, 2 growth inhibitor and 7growth neutral. Growth promoting potential of endophytic fungi was further confirmed by detecting and quantifying gibberellic acid ($GA_3$) and indole acetic acid (IAA) in their culture filtrates via PerkinElmer Lambda 25 double beam spectrophotometer. Highest $GA_3$ concentration was found in the culture filtrate of CL 1-5-11 (146.06ng/ml) while, least in CL 1-2-1B (1.41ng/ml). Similarly, highest concentration of IAA was detected in the culture filtrate of CL 1-2-7 (50.66µg/ml) while, least in CL 1-2-6A (3.5µg/ml). Present work aims to replace artificial fertilizers by bio-fertilizersas they are cheap and non-hazardous to environment and humans as compared to artificial fertilizers.
**KEY WORDS:** Endophytic fungi, Gibberellin, Auxin, *Citrulluscolocynthes* L.

## INTRODUCTION

Man is in continuous struggle to fulfill the need of food for over-increasing population of the world. For the better quality and yield of crops, chemical fertilizers are used excessively. But these chemical fertilizers are hazardous to human and environment. Thus, use of bio-fertilizers in the agricultural world in the form of microbes like fungi, bacteria and cyanobacteria, may be one of the most suitable alternates and safe options [1]. Endophytes are mutualistic symbionts of all plants found completely inside them and cause no harm to host plant. High density of endophytic fungi has been recorded in the temperate and tropical rain forests[2]. Endophytic fungi are a diverse group and commonly belong to Ascomycota which reside in plant roots, stem, leaves, flowers, fruits and spines [3]. Endophytic fungi have positive role for host plants regarding phytohormones synthesis, $N_2$ fixation, P absorption and Phosphate solubilization [4]. They also release toxins to make plants unfit for herbivores feeding as well as enhance production of phytohormones (Cytokinin, Gibberellin and Auxin) that widens host potential for limited resources [5]. They have beneficial role for enhanced ecological adaptability of host plant and more biomass production [6]. It has been studied that *Phomaglomerata*and *Penicillium* sp., promote Waito-C and Dongjin-beyo rice (GAs deficient dwarf mutant) growth under salinity and other stresses as a result of biologically active secondary metabolites secretion (IAA and $GA_1$, $GA_3$, $GA_4$ and $GA_7$) as well as reduce ABA synthesis. Other stress related activities of plants like catalase, per-oxidase, polyphenol oxidase, ascorbic acid oxidase and glutathione also decreases under different stresses in fungal-endophyte-colonized-plants [7]. Phytohormones are secondary metabolites that function as signaling compounds and having role in important physiological activities of plants (growth and development). IAA and GAs are the two important plant hormones, but currently little is known about their secretion by endophytic fungi [8]. Current study aims to know the GAs and IAA secreting potential of endophytic fungi, a promo to replace costly and hazardous chemical fertilizers by safe, cheap and eco-friendly bio-fertilizers.

## MATERIALS AND METHODS

### Isolation of endophytic fungi

Leaves of *Citrulluscolocynthes* L. were collected from District Nowshera of Khyber Pakhtunkhwa, Pakistan, carried to plant-microbes-interaction (PMI) laboratoryand washed with water. Samples were then centrifuged inTween 80 solution to remove air born dust particles, at 120 rpm (revolutions per minute (rpm) for five (5) minutes at room temperature, in shaking incubator [9]. Then surface sterilization was done using ethanol (70%) for half minute, NaOCl (Sodium Hypo Chloride) (3%) for 5 minutes, and again (70%) ethanol for half minute. Leaves were cut into

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small discs (3mm) with- and without-midribs with the help of cork-borer [10]. Leaf pieces were then placed on Hagem minimal medium containing 80ppm streptomycin. Plates were kept at room temperature for 5 days [11]. After the emergence, endophytic fungi were inoculated on PDA (potato dextrose agar) medium for purification. For IAA and GAs collection, Czapek broth medium was used [12, 13].

**Growth medium and culture conditions**

Hagem, a minimal medium ($C_6H_{12}O_6$ 5gm/L, $KH_2PO_4$ 0.5gm/L, $MgSO_4.7H_2O$ 0.5gm/L, $NH_4Cl$ 0.5gm/L, $FeCl_3$ 0.1gm/L, Agar 15gm/L and Streptomycin 0.08gm/L (pH 5.6 ± 0.2), was used for the isolation of fungal strains from the leaves of *Citrulluscolocynthes* L. PDAmedia (Dextrose 20gm/L, agar powder 20gm/L, 300gm potato (sliced washed unpeeled) and 1L DW) was used for the pure culture of endophytic fungi in plates as well as slants. Slants were kept in refrigerator at 4ºC for storage. Then 50 ml Czapek media($C_6H_{12}O_6$ 10gm/L, peptone 10gm/L, KCl 0.5gm/L, $MgSO_4.7H_2O$ 0.5gm/L, $FeSO_4.7H_2O$ 0.5gm/L, pH 7.3 ± 0.2) was used in 250 ml flasks for the collection of active secondary metabolites ($GA_3$ and IAA). Flasks were kept at 30ºC in shaking incubator (120 revolution per minute) for 7 days [12, 13].

**Screening bioassay**

On the basis of colonial morphology and growth, different types of fungal strains were selected for further analysis. Culture filtrates of twenty-nine (29) strains out of 98, were screened on rice plants for their growth promoting potential. One ml supernatant obtained by growing endophytic fungi in Czapek broth media, was diluted in autoclaved DW and applied on the tip of rice plants at two leaves stage. Rice seedlings were grown in glass beakers having 30 ml media (0.8% water-agar medium) [12, 13]. With the help of micro-pipette, 100µl of fungal extracts were applied on the tips of experimental while, DW and Czapek medium on control rice seedlings. Rice seedlings were shifted to growth chamber (day/night cycle: 14 h—28 °C ± 0.3; 10 h—25 °C ± 0.3; relative humidity 70%;) six (6) plants per treatment. Root and shoots length, fresh and dry weight of rice plants were recorded after 7 days of the application of fungal extract [11].

**Determination of gibberellin and auxin**

A very simple and accurate bioassy of Ismail *et al*. [1] with some modifications, was used for the detection and quantification of $GA_3$ in the culture filtrate of endophytic fungi while, Salkowski reagent was used for the determination of IAA [14]. Salkowski reagent and fungal filtrate were mixed in 2:1 ratio and incubated for 30 minutes in the dark at $25^0$C. IAA was determined with PerkinElmer Lambda 25 spectrophotometer at 540 nm wavelength.

**Standard curve for $GA_3$**

Five different concentrations of pure $GA_3$ were made and their OD (optical density) were checked at 254nm with spectrophotometer (PerkinElmer Lambda 25).Standard curve of $GA_3$ was linear up to 5 order of magnitude with $R^2$ = 0.999998 (Figure 1a).

**Standard curve for IAA**

Ten different concentrations (starting from 10 µg/ml to 100 µg/ml) of IAA were made and OD was taken at 540nm with spectrophotometer ($R^2$ = 0.970196, Figure 1b).

**Data analysis**

Duncan Multiple Range (DMRT) was applied for data analysis. (IBM SPSS software version 21.0, SPSS Inc, Chicago, USA).

## RESULTS AND DISCUSSION

**Endophytic fungi isolated from *Citrulluscolocynthes* L.**

On the basis of morphological differences, twenty-nineendophytic fungi were isolated from *Citrulluscolocynthes* L. leaves (Figure S1). The fungal culture filtrates of these endophytes were bio-assayed on rice plants for growth promoting or growth inhibiting activities (Figure S2). 29 fungal filtrates were applied, of which 20 were found as growth promoter, 2 as growth inhibitors while, 7 as growth neutrals (Table 1).

**Table 1: Screening bioassay of fungal culture filtrates on rice seedlings isolated from*Citrulluscolocynthes* L.**

| Fungal isolates | Shoot length (cm) | Root length (cm) | Fresh weight shoot (g) | Fresh weight Root (g) | Dry weight shoot (g) | Dry weight Root (g) | Total chlorophyll (SPAD) | Growth status |
|---|---|---|---|---|---|---|---|---|
| **Control DW** | 7.7±0.3 | 4.9±1. | .03±0.0 | .088±0.03 | .005±0.01 | .015±0.01 | 14.3±3.4 | |
| **Control Czk** | 8.6±0.4 | 4.2±2. | .034±0.0 | .093±0.01 | .007±0.03 | .014±0.01 | 15.7±2.3 | |
| **CL 1-1-1A** | 13.9±0. | 5.4±1. | .067±0.0 | .099±0.09 | .01±0.01 | .026±0.01 | 19±2.1 | Promoted |
| **CL 1-6-4** | 13.1±1. | 6.1±1. | .071±0.0 | .099±0.02 | .009±0.02 | .026±0.01 | 18.9±1.7 | Promoted |
| **CL 1-3-5** | 11.7±1. | 4.5±2. | .046±0. | .061±0.08 | .005±0.03 | .015±0.02 | 15.9±1.2 | Promoted |
| **CL 1-5-11** | 13.4±0. | 7.6±0. | .084±0.0 | .088±0.03 | .01±0.04 | .031±0.01 | 18.4±1.8 | Promoted |
| **CL 1-5-10** | 14±0. | 5.2±1. | .096±0.0 | .097±0.07 | .013±0.04 | .029±0.01 | 19.7±3.2 | Promoted |
| **CL 1-6-3** | 11.2±1. | 6.5±2. | .046±0.0 | .061±0.04 | .005±0.01 | .015±0.01 | 15.9±2.6 | Promoted |
| **CL 1-6-6** | 5.5±1. | 2.7±1. | .013±0.0 | .04±0.06 | .0001±0.03 | .005±0.02 | 11±4.1 | Inhibited |
| **CL 1-2-2** | 7.5±0. | 3.5±2. | .036±0.0 | .056±0.05 | .003±0.04 | .009±0.03 | 13±2.9 | Neutral |
| **CL-1-1-3A** | 12.2±0. | 7.2±3. | .083±0.0 | .088±0.06 | .01±0.02 | .031±0.01 | 18.4±2.1 | Promoted |
| **CL 1-2-6B** | 12.7±1. | 6±1. | .082±0.0 | .087±0.05 | .009±0.02 | .033±0.01 | 18.5±5.3 | Promoted |
| **CL 1-6-2** | 9.2±0. | 3.5±0. | .034±0.0 | .026±0.07 | .008±0.01 | .004±0.02 | 13.7±3.2 | Neutral |
| **CL 1-2-4** | 13±0. | 4.4±0. | .08±0.0 | .089±0.03 | .009±0.04 | .031±0.01 | 18.4±2.7 | Promoted |
| **CL 1-4-2** | 14.1±1. | 6.6±1. | .096±0.0 | .097±0.08 | .013±0.03 | .029±0.02 | 19.7±3.4 | Promoted |
| **CL 1-1-3B** | 13.2±0. | 5.8±1. | .083±0.0 | .083±0.03 | .01±0.04 | .032±0.02 | 18.7±4.2 | Promoted |
| **CL 1-2-8** | 13.6±1. | 5.9±0. | .084±0.0 | .082±0.09 | .008±0.01 | .034±0.01 | 18±2.6 | Promoted |
| **CL 1-1-1B** | 11.4±0. | 4.5±1. | .047±0.0 | .061±0.02 | .005±0.01 | .015±0.01 | 15.9±3.2 | Promoted |
| **CL 1-5-2** | 11.5±0. | 4.7±0. | .045±0.0 | .06±0.01 | .006±0.03 | .013±0.01 | 15.2±1.5 | Promoted |
| **CL 1-1-4** | 7.5±0. | 6±1. | .013±0.0 | .04±0.09 | .0001±0.02 | .005±0.02 | 11±1.3 | Neutral |
| **CL 1-2-1B** | 13.5±1. | 6.7±0. | .081±0.0 | .088±0.02 | .009±0.02 | .031±0.02 | 18.4±1.8 | Promoted |
| **CL 1-2-3** | 10.5±1. | 5±0. | .046±0.0 | .061±0.08 | .004±0.04 | .015±0.01 | 15.9±4.3 | Neutral |
| **CL 1-2-7** | 13.5±0. | 7.3±1. | .084±0.0 | .083±0.03 | .009±0.05 | .031±0.01 | 18.4±2.7 | Promoted |
| **CL 1-2-6A** | 12.2±1. | 5.5±1. | .057±0.0 | .065±0.07 | .006±0.01 | .013±0.01 | 15.9±1.8 | Promoted |
| **CL 1-6-14** | 10.2±0. | 6±0. | .047±0.0 | .061±0.04 | .004±0.01 | .015±0.03 | 15.9±3.2 | Neutral |
| **CL 1-3-3** | 4.4±1. | 5±1. | .013±0.0 | .041±0.06 | .0001±0.02 | .004±0.01 | 11±1.6 | Inhibited |
| **CL 1-2-1A** | 11.5±0. | 4.5±0. | .045±0.0 | .065±0.05 | .004±0.01 | .013±0.01 | 15.9±2.5 | Promoted |
| **CL 1-2-9** | 14.5±1. | 7.5±1. | .095±0.0 | .094±0.04 | .014±0.05 | .029±0.02 | 19.7±3.4 | Promoted |
| **CL 1-3-1** | 9.8±1. | 4.5±0. | .047±0.0 | .06±0.06 | .004±0.01 | .013±0.02 | 15.9±1.3 | Neutral |
| **CL 1-1-2** | 11.4±0. | 5.5±1. | .045±0.0 | .063±0.03 | .004±0.03 | .015±0.01 | 15.9±2.6 | Promoted |
| **CL 1-6-11** | 10.7±1. | 4.9±1. | .041±0.0 | .06±0.07 | .004±0.01 | .013±0.01 | 15.9±1.7 | Neutral |

Note: Czk= Czapek Medium, D.W = Distilled Water. Values with different letters in the same column in that group are significantly different at the 5% level by DMRT (Duncan's Multiple Range Test). Values within the table refers to the mean ± SE (n = 3)

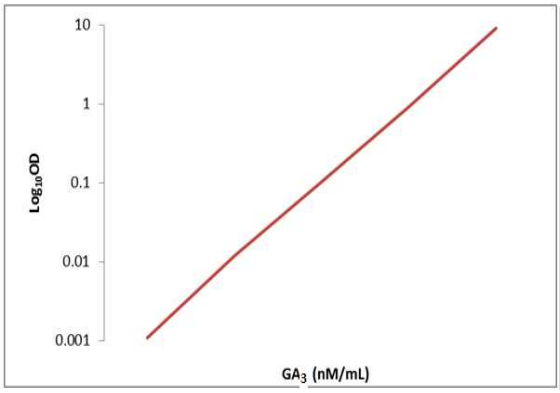


**Figure. 1(a).** Gibberellic acid ($GA_3$) standard curve

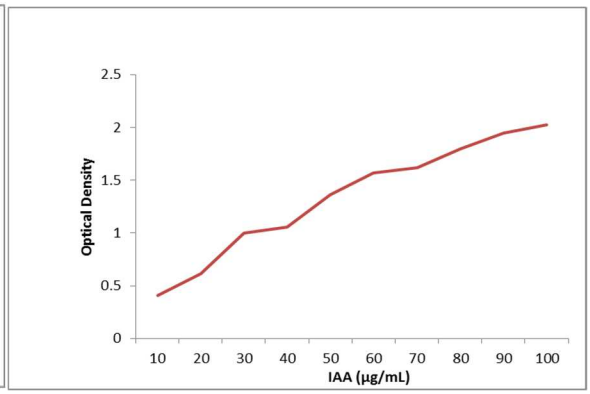


**Figure. 1(b).** Indole acetic acid (IAA) standard curve

**Gibberellic acid determination and quantification**

$GA_3$ detected and quantified using a very simple protocol, used by Ismail *et al*. This is a very simple but accurate bio-assay for $GA_3$. In this procedure embryo was totally discarded from the seeds of wheat. This bio-assay for $GA_3$ determination in fungal culture filtrate was first applied by Ismail *et al*. [1]. Highest $GA_3$ (146.06ng/ml) concentration was shown by strain CL 1-5-11 while, least (1.41ng/ml) by CL 1-2-1B (Figure 2).

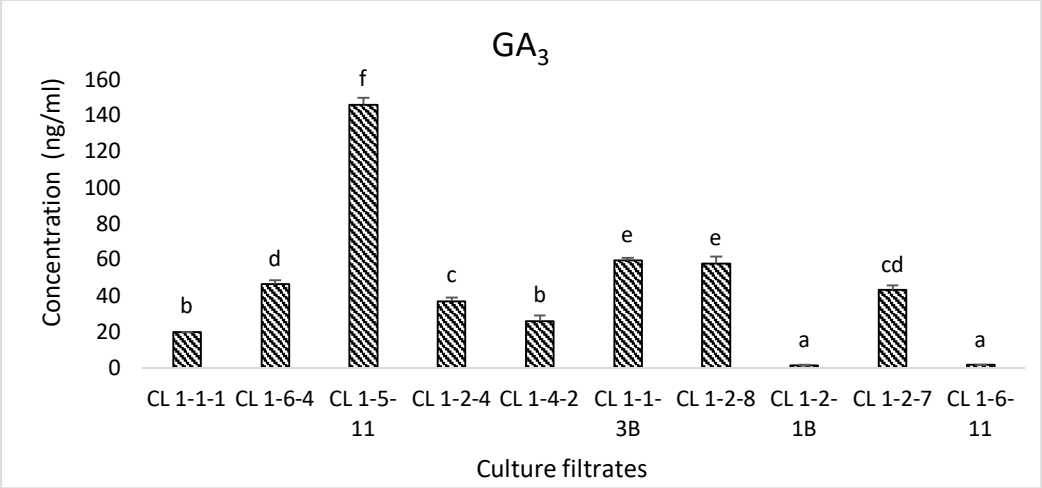


**Figure 2:** Quantification of $GA_3$ in fungal extracts. CL 1-5-11 have highest (146.06ng/ml) $GA_3$ concentration while, CL 1-2-1B have least (1.41ng/ml)

**Indole acetic acid determination and quantification**

IAA was determined in culture filtrates using Salkowski reagent. Salkowski solution gives a pink color in the presence of IAA which can be easily detected in the filtrates. Total of 29 fungal extracts were checked, 11 were found to have IAA. Two strains, CL 1-2-7 and CL 1-1-1 were maximum IAA producer 50.66µg/ml and 48.18µg/ml respectively while, CL 1-2-6A was the least producer strain (3.5µg/ml) (Figure 3).

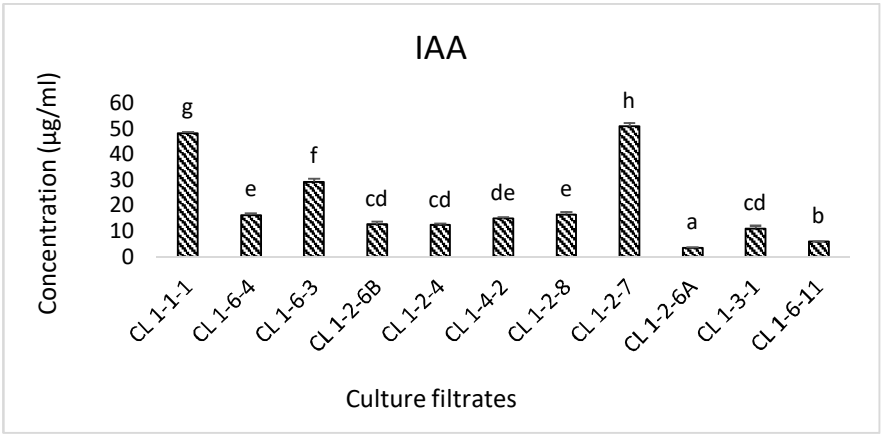


**Figure 3:** Quantification of IAAin fungal culture filtrates. Highest IAA (50.66µg/ml) concentration was found in strain CL 1-2-7 while, least (3.5µg/ml) in CL 1-2-6A strain

More than 0.3 million herbaceous and woody land plant species have been checked so for, for the presence of endophytic fungi. All of these plants are known to colonize one or more endophytic fungi [3]. All endophytes are known to have mutualistic relationship with their colonized host plant [15]. Endophytic fungi not only provide immunity to host plants against different stresses like pathogenicity, herbivory, drought, high and low temperatures and salinity but also having role in nitrogen fixation, phosphate solubilization, phosphorus absorption and phytohormones production [4]. In this work fungal filtrates were screened on rice seedlings for the presence of $GA_3$ and IAA as both hormones have potential role for plant growth promotion. Presence of $GA_3$ and IAA was also confirmed via PerkinElmer Lambda 25 spectrophotometer [16, 17]. Therefore, culture filtrate of endophytic fungi is considered to be the best source of biologically active secondary metabolites[14]. Screening bio-assay of fungal extracts on plants, is one of the easy and simple procedure for the determination and active secondary metabolites [18, 19]. Rim *et al.* [20] also used the same procedure for the screening of *Fusariumproliferatum* filtrate. Highest $GA_3$ (146.06ng/ml)concentration was shown by strain CL 1-5-11 while, least (1.41ng/ml) by CL 1-2-1B as compared to just 3.21ng/ml detected by [12]. Similarly, highest IAA concentration (50.66µg/ml), was detected in CL 1-2-7 strain while least (3.5µg/ml) in CL 1-2-6A strain.

## CONCLUSION

Present work indicates that fungal endophytes form mutualistic relationship with plant species. Presence of 29 endophytic fungi in one xerophytic plant suggests their diversity and adaptation to different habitats. Further, detection of biologically active secondary metabolites ($GA_3$ and IAA) in their culture filtrates, show their important ecological role for supporting host plants. Use of endophytic fungi in the agricultural field as bio-fertilizer, is suggested for the future because of hazardous effects of artificial fertilizers on the environment and human beings.

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