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SCREENING OF ENDOPHYTES OF *GLORIOSA SUPERBA* FOR THEIR PLANT GROWTH PROMOTING POTENTIAL AND OPTIMIZATION OF INDOLE-3-ACETIC ACID PRODUCTION

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ABSTRACT

Depletion of soils due to their repeated use in farming without replenishment leads to low productivity. This problem can be addressed with the application of fertilizers. Whereas chemical fertilizers are costly and can lead to deterioration of soil health, biofertilizers can prove to be an attractive alternative. Endophytes are endosymboints which enhance the growth of host plants by carrying out plant growth promoting (PGP) activities such as nitrogen fixation, phosphate solubilization and synthesis of auxins like indole-3-acetic acid (IAA). Hence they have a good potential in the development of biofertilizers or in the synthesis of auxins for use in horticulture/ agriculture. With this in mind, nine bacterial endophytes isolated from the rhizomes of *Gloriosa superba* (Kalalaavi) during a previous study, were used. They were screened for a) their ability to produce IAA in Nutrient broth supplemented with 5mg/ mL tryptophan (Salkowski's reagent was used to detect IAA); b) for nitrogen fixation by their ability to grow in Jensen's nitrogen-free medium and c) phosphate solubilisation as those cultures forming clear zones around their colonies on Pikovskaya's agar medium. All nine isolates demonstrated the ability to fix nitrogen, four to produce IAA, and two to solubilize phosphate. Optimization of parameters for the production of IAA was carried out for the selected isolates in Nutrient broth supplemented with tryptophan, by varying one factor at a time viz. pH, temperature, period of incubation and concentration of tryptophan added to the medium. The highest producer of IAA, isolate KV-P, produced 193.33 µg/ml of IAA under optimized conditions of pH 7.0, 30°C and 96 hours of incubation in Nutrient broth supplemented with 5mg/mL tryptophan.

KEYWORDS: *Gloriosa superba* endophytes, IAA, optimization, phosphate solubilization, Nitrogen fixation.

INTRODUCTION

When agricultural soil is used repeatedly for growing crops, it gets depleted of its nutrients and thus productivity suffers. Farmers resort to the use of chemical fertilizers to replenish the lost nutrients. These chemical fertilizers tend to be costly, may not be effective in the availability of necessary nutrients to the crops and add to the level of environmental pollution.[1] Application of bioinoculants to such soil/ seeds can greatly resolve this issue.

Bioinoculants can be developed from cultures of rhizobacteria/ endophytes which have proven capability to improve soil health by various mechanisms such as nitrogen fixation, phosphate solubilization, siderophore production etc. and the growth of the plant can be enhanced by bacteria which produce auxins such as indole-3-acetic acid (IAA).[2] Endophytes (bacteria which live in a symbiotic relationship with the host plant) have been reported with such capabilities as IAA production, phosphate solubilisation, nitrogen fixation, siderophore production etc.[3]

Nitrogen fixing bacteria play a very important role in the nitrogen cycle. They fix nitrogen using the enzyme nitrogenase. They produce reduced forms of nitrogen such as ammonia which is available to the plant.[4] They may be aerobic, facultatively anaerobic, microaerophilic or anaerobic in nature. Genera such as *Rhizobium* and *Frankia* are symbiotic.[5,6] nitrogen fixers whereas *Azotobacter and Clostridium* are non-symbiotic free-living nitrogen fixers.[6] Cyanobacteria such as *Anabaena* and *Nostoc* also fix nitrogen via specialized structures called heterocysts.[7]

Application of *Azospirillum*, a nitrogen fixing organism which also produces IAA, has been reported to promote branching of the roots, root-hair development, increased uptake of N, P, K and micronutrients and grain yield. IAA is produced by microorganisms by the tryptophan dependent or tryptophan independent pathways.[4] Its production can be detected by Salkowski's reagent.[8] Since this reagent also detects related compounds such as indolepyruvic acid and indoleacetamide, confirmation of IAA production can be done by Thin Layer Chromatography using standard IAA in comparison with the cell free supernatant.[9]

Inorganic phosphates in the form of rock phosphate are added to soil to replenish the lost phosphates. But they are unavailable to the plant since they are insoluble. Microorganisms have been reported to produce organic acids which result in the solubilization of such phosphates making them available to the plant.[10]

We explored the potential of nine endophytes of *Gloriosa superba* (Kalalaavi), a medicinal plant, in nitrogen fixation, phosphate solubilization and IAA production. Jensen's nitrogen-free medium is a suitable medium for the detection of non-symbiotic nitrogen fixers.[11] Pikovskaya's medium contains an insoluble precipitate of tricalcium phosphate. Solubilization of this results in the production of clear zones around the colonies of the organism.[10] We used Nutrient broth supplemented with tryptophan for the detection of IAA producing organisms.[12] Salkowski's reagent was used to detect the IAA produced in this medium as per method of Gordon and Weber.[13] Production of IAA was confirmed by Thin Layer Chromatography as described by Mohite.[14] We optimized the production of IAA by the selected isolates with respect to incubation period, incubation temperature, pH of the medium and concentration of tryptophan in the production medium by varying one factor at a time.

MATERIALS AND METHODS

Maintenance of Cultures

Nine bacterial endophytes, isolated from rhizomes of *Gloriosa superba* during a previous study, were maintained on Luria agar (HIMEDIA, India) and Standard Plate Count Agar (HIMEDIA, India).

Characterization and screening of endophytes

The endophytes were characterized with respect to their Gram character and screened for their plant growth promoting activities such as the ability to fix Nitrogen, solubilize phosphate and produce indole-3-acetic acid (IAA).

Nitrogen fixing ability was determined as its ability to grow on Jensen's Nitrogen-free agar medium (HIMEDIA, India), when incubated at 30°C; it was checked every 24 h for up to 96 hours.

Phosphate solubilization was detected as zones of clearance developed around the colonies on Pikovskaya agar (HIMEDIA, India) which contained insoluble tricalcium phosphate.

Production of IAA was determined by colorimetric method of Gordon & Weber.[13] The cultures were inoculated in Nutrient broth (HIMEDIA, India) supplemented with 5 mg/ mL tryptophan, incubated at 30°C for 24 h in a shaker incubator (120 rpm). The broth was then centrifuged at 10,000 rpm for 10 min at 4°C to get cell free supernatant. Two mL of Salkowski's reagent (2 ml 0.5M $FeCl_3$ added to 98 ml 35% perchloric acid) was added to one mL of cell free supernatant thus obtained. It was incubated in the dark for 30 min at room temperature. Development of pink color indicated production of IAA.

Extraction of IAA and its identification by Thin Layer Chromatography (TLC)[14]

The culture was grown in Nutrient broth supplemented with 5 mg/ mL tryptophan, at 30°C for 24 h under shaking conditions (120 rpm). The supernatant obtained after centrifuging the broth at 10,000 rpm for 10 min at 4°C was extracted with ethyl acetate (1:2) by vigorously shaking it and then allowing it to stand for 10 min. IAA was extracted in the solvent layer. It was detected by TLC. Standard IAA (100 µg/ mL) and the extracted sample were spotted on a prefabricated TLC plate (silica gel coated on aluminum foil; MERCK) which was then placed in a chromatography chamber saturated with solvent system (Propanol: Water:: 8:2). Chromatogram was developed with Salkowski's reagent after the completion of the run. Values of Retardation factor ($R_{f)}$ of the standard and IAA produced by the isolate were calculated as the ratio of the distance travelled by the centre of the spot to the distance travelled by the solvent front.

Optimization of parameters for the production of IAA

Parameters for the production of indole-3-acetic acid were optimized by 'one factor at a time' method. The parameters of incubation period, pH of the medium, temperature of incubation and tryptophan concentration were varied one at a time while the rest were kept constant.[15]

The cultures used were 18-20 h old. Their suspensions were made in sterile saline to a density corresponding to an OD_{600} (Optical Density taken at a wavelength of 600nm) value of 0.1. They were inoculated at a concentration of 5% v/v into sterile Nutrient broth supplemented with 5 mg/ mL tryptophan. They were incubated in a rotary shaker incubator set at 30°C and 120 rpm. Concentration of IAA produced was determined every 24 h up to a period of 96 h by colorimetric method of Gordon & Weber.[13] Two mL of Salkowski's reagent (2 ml 0.5M $FeCl_3$ added to 98 ml 35% perchloric acid) was added to one mL of cell free supernatant obtained after centrifuging the broth at 10,000 rpm for 10 min at 4°C. It was incubated in the dark for 30 min at room temperature. The intensity of pink color that developed (indicating

IAA production) was measured at a wavelength of 540 nm and the concentration of IAA produced was estimated by comparison with the standard dose response curve for IAA (10 µg/ mL to 100 µg/ mL) estimation by the method of Gordon & Weber.

1) For optimization of incubation period, the concentration of IAA produced was determined every 24 h up to a period of 96 h.
2) For optimization of pH, the medium was adjusted to pH values of 3, 5 and 7.
3) Incubation temperature was optimized in the range of 30°C, 37°C and 45°C.
4) Optimization of tryptophan concentration was done by using Nutrient broth supplemented with tryptophan in varying concentrations viz. 4 mg/ mL. 5 mg/mL and 6 mg/ mL.
5) The experiments were carried out in triplicates, the mean, standard deviation (SD) and Standard Error Mean (SEM) were determined. The results were plotted and thus the parameters were optimized for IAA production.

RESULTS

All the nine bacterial endophytes of *Gloriosa superba* were Gram negative rods. All nine isolates demonstrated the ability to fix N_2 as seen by their ability to grow on Jensen's nitrogen free agar medium (Fig. 1); two could solubilize phosphate as seen by the development of clear zones around the colonies on Pikovskaya medium (Fig.2) and four could produce IAA, seen by the development of rose-pink color upon the addition of Salkowski reagent according to the method described by Gordon and Weber.[13] (Fig. 3).

Table 1: Functional characterization of the endophytes of *Gloriosa superba.*

Sr. No.	Isolate	N_2 fixation (growth on Jensen's medium)	Phosphate solubilization (clear zones on Pikovskaya agar)	IAA production
1	KV-A1	+	--	+
2	KV-A4	+	--	+
3	KV-E	+	--	+
4	KV-P	+	--	+
5	KV-K	+	--	--
6	KV-A26	+	+	--
7	KV-G2	+	+	--
8	KV-B2	+	--	--
9	KV-S	+	--	--

Fig. 1: Growth of isolate on Jensen's medium

Fig.2: Growth of isolates on Pikovskaya agar

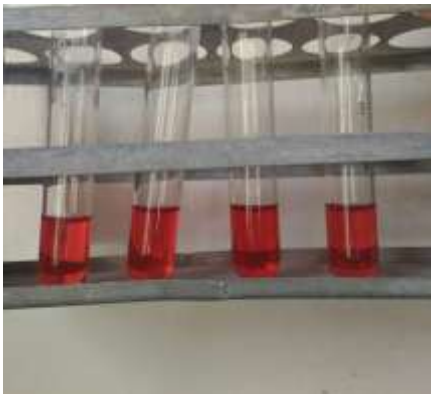


Fig. 3: Detection of IAA production by KV-A1, KV-A4, KV-E, KV-P.

Extraction of IAA and its identification by Thin Layer Chromatography (TLC)

1 2

Key:

Lane 1: Test sample

Lane 2: Standard IAA

Calculation of Retardation factor R_f:

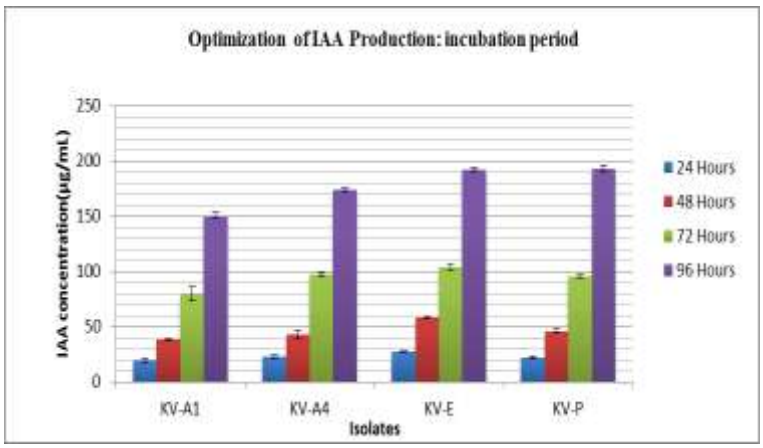
R_f of standard (100 µg/ mL IAA) = 0.76

R_f of test (rose pink colored spot) = 0.76

Fig. 4: Results of TLC: Test sample shows 2 spots one of which corresponds with standard IAA.

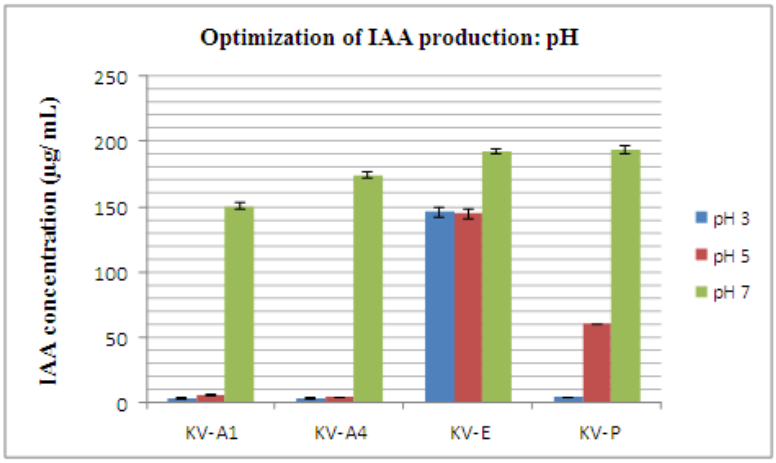
Optimization studies

a) Incubation period



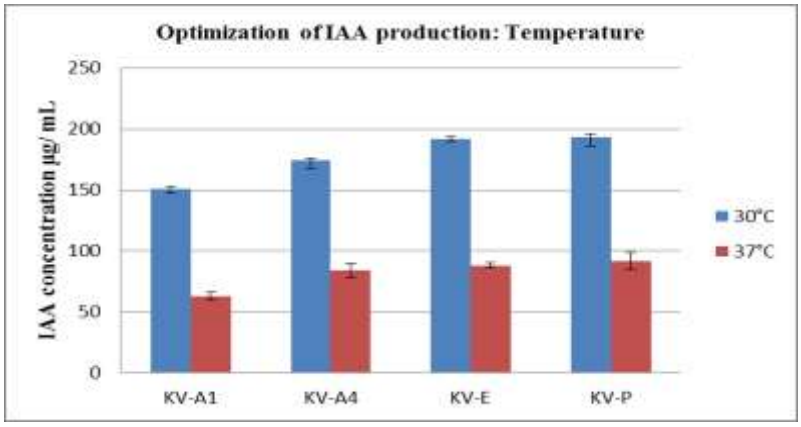
All isolates produced the maximum amount of IAA at 96 hours of incubation.

b) pH



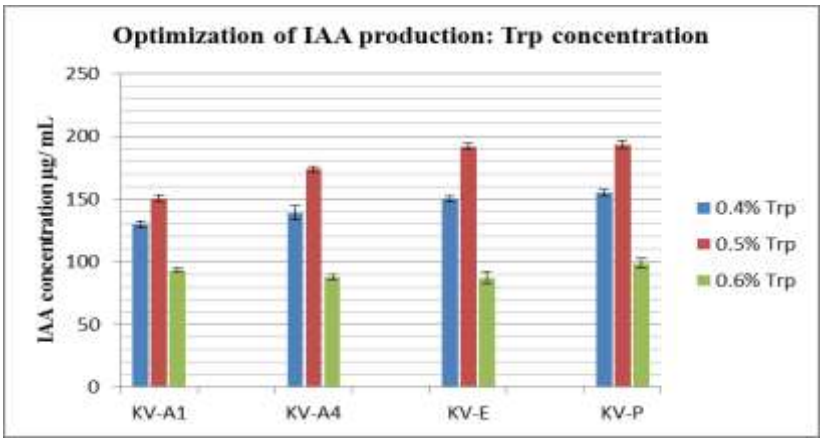
All isolates produced the maximum amount of IAA at pH 7.

c) Temperature



All isolates produced IAA optimally at 30°C.

d) Tryptophan concentration



All isolates produced IAA optimally at 0.5 g% concentration of tryptophan.

For the tested endophytes, optimum conditions for IAA production were determined to be pH 7.0, 30°C and 96 hours of incubation in Nutrient broth supplemented with 5mg/mL tryptophan under which conditions the isolates produced IAA in the range of 150 to 193.33µg/ml. The highest producer KV-P, produced 193.33 µg/ml of IAA under optimized conditions.

DISCUSSION

Nutrient composition of agricultural soil needs to be augmented for improving the productivity per hectare of land. Incorporation of environmentally friendly methods such as use of bioinoculants are indicated.[1] Hence various workers have reported the isolation and screening of Plant Growth Promoting Rhizobacteria (PGPR).[2,3,14,15,17] They have isolated Gram negative bacteria such as *Azotobacter, Pseudomonas* and *Mesorhizobium,* some of which solubilize phosphate (16% to 74% of isolates) and produce IAA (80% of isolates).[17] Nitrogen fixation and plant growth augmentation have been reported of *Azospirillum sp.*[4] As per our knowledge ours is the first report of the functional characterization of 9 bacterial endophytes of the medicinal plant *Gloriosa superba* with respect to their Plant Growth Promoting (PGP) activities and optimization of IAA production. We report here, nitrogen fixation by 9 (100%) isolates, phosphate solubilization by 2 (22.22%) isolates and auxin (IAA) production by 4 (44.44%) isolates. Budhiraja et al[18] have reported the production of anti-microbial and anti-cancer compounds by endophytic fungus of *Gloriosa superba*. Qin et al[19] have reported the isolation and identification of a novel species of the actinomycete *Saccharopolyspora*, from the stem of *Gloriosa superba.*

The endophytes of *Gloriosa superba* that we have characterized can be used in the development of bioinoculants/ biofertilizers. They may be used individually or as co-cultures as their capacity to fix nitrogen and solubilize phosphate would improve the nutrient composition of the soil leading to an increase in productivity. Some of these cultures also can produce indole-3-acetic acid, an auxin which would enhance the growth of the cultivated crops.

Various workers[15,17] have optimized the production of IAA by their isolates. Purified auxins are in high demand in the horticulture industry. Bharucha et al[15] reported that for their soil isolate, *Pseudomonas putida*, IAA production increased when the L-tryptophan containing medium for IAA production was supplemented with sucrose 0.5%, $(NH4)_2SO4$ 10 mg/ml, and tryptophan 0.2 mg/ml at pH 7.5. Maximum IAA production was achieved at 96 h.

According to Ahmad et al[17] a total of 11 selected isolates of *Azotobacter* (seven), fluorescent *Pseudomonas* (three) and *Bacillus* (one) were tested for the quantitative estimation of IAA in the presence of different concentrations of tryptophan. With no addition of tryptophan, production of IAA was not observed. With the addition of tryptophan from 50 to 500 mg/ml the production of IAA was increased. The production of IAA was highest in isolates of fluorescent *Pseudomonas,* followed by *Azotobacter* and *Bacillus*, respectively. Under optimal conditions the highest amount of IAA produced was 22.02±0.20 µg/ml by fluorescent *Pseudomonas* isolate.

We have optimized the production of IAA by our isolates and best results are obtained at pH 7.0, 30°C and 96 hours of incubation in Nutrient broth supplemented with 5 mg/ mL tryptophan under which conditions the isolates produced IAA in the range of 150 to 193.33 µg/ml of IAA. The highest producer KV-P, produced 193.33 µg/ml of IAA under optimized conditions.

CONCLUSIONS

1) Nine endophytes of *Gloriosa superba* were characterized for their PGP properties.
2) Nine (100%) endophytes of *Gloriosa superba* could fix Nitrogen, 2 (22.22%) could solubilize phosphate and 4 (44.44%) could produce IAA.
3) The highest producer KV-P, produced 193.33 µg/ml of IAA under optimized conditions.
4) These endophytes of *Gloriosa superba* are potential candidates for the development of bioinoculants/ biofertilizers which could improve health of soil as well as regulate/ enhance the growth of desired crops. They can also be used for the production of IAA which is in high demand in horticulture/ agriculture.[20]

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