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Screening of endophytic bacteria from the pharmacologically important medicinal plant *Gloriosa superba* for their multiple plant growth promoting properties

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Abstract
In our study, a total of 13 different bacterial endophytes (KV-1 to KV-13) isolated from *Gloriosa superba* during a previous study, were tested for different plant growth promoting properties like IAA (Indole-3-acetic acid) production by the method of Gordon and Weber; phosphate solubilisation on Pikovskaya's medium; siderophore production on Chrome azurol S (CAS) medium; Nitrogen fixation on Jensen's Nitrogen-free medium; ammonia production using Nessler's reagent; and Hydrocyanic acid (HCN) production on Nutrient Agar supplemented with 4.4 g/L glycine, having Whatmann No.1 filter paper strip soaked in 2% sodium carbonate in 0.5% picric acid held above it. Two promising isolates (KV-5 and KV-11) among the 13, were positive for all plant growth promoting traits tested except HCN production, whereas KV-13 was positive for phosphate solubilization, siderophore production and nitrogen fixation indicating their importance as potential candidates for the development of bioinoculants.

Keywords: *Gloriosa superba*, endophytes, IAA, phosphate solubilisation, siderophore, nitrogen fixation, PGPR

1. Introduction
The medicinal plant, *Gloriosa superba*, owes its most favored status in traditional medicine to its several biological activities such as antioxidant, antibacterial, antifungal and anthelmintic. [1] It has been used in the treatment of inflammatory disease, gout, ulcers, fever, piles, blood disorders and hemorrhage. It causes uterine contractions and is a good abortifacient causing expulsion of fetus from the womb. [2] Various parts of this plant like rhizome, leaf, stem, etc. have been used for the extraction of valuable phytochemicals of pharmacological importance such as alkaloids (colchicine, gloriosine, superbine and colchicosides), flavonoids, glycosides, saponins, steroids, phenols and tannins.[1, 2, 3] Due to its pharmacological importance there is a real danger of *Gloriosa superba* being over-exploited resulting in it becoming an endangered species.[1, 3, 5] India, an economically developing country with a growing population, relies heavily on traditional plant-based medicines for ensuring the health of its population.[4] A rapid decrease in the density of medicinal plants results on the one hand from their over-exploitation while on the other hand, infertility of soil and fungal or insect pest attacks result in low productivity. Further, environmental damage results from practices such as spray of pesticides, use of chemical fertilizers etc. Thus, there is a need to improve medicinal crop productivity while safeguarding the health of the environment. Such problems can be addressed by improving the health of the soil by adding suitable bioinoculants to it. Such bioinoculants, if developed from amongst endophytes of these medicinal plants would have a higher probability of being successful in increasing the population of the medicinal plants. Plant growth promoting rhizobacteria have been reported to facilitate plant growth by: a) production of auxins such as IAA (Indole-3-acetic acid) which regulate and promote plant growth b) increasing the bioavailability of nutrients for plant uptake (by fixation of nitrogen, solubilisation of phosphorus, facilitated absorption of iron via production of siderophores), and c) production of lytic enzymes (like chitinases, cellulases, 1,3-glucanases and lipases) or antimicrobial compounds such as HCN to protect them from diseases (Vejan P, 2016). The use of such plant growth promoting endophytes as bioinoculants could reduce the use of chemical fertilizers, environmental pollution and at the same time increase the yield and productivity of medicinal and other agricultural plants [6]

We screened a total of 13 different bacterial endophytes (KV-1 to KV-13) isolated from rhizomes of *Gloriosa superba* during a previous study, for different plant growth promoting properties such as IAA (Indole-3-acetic acid) production; phosphate solubilisation; organic acid production (since organic acid production is a mechanism for solubilizing phosphate) siderophore production; Nitrogen fixation; ammonia production and Hydrocyanic acid (HCN) production.

2. Materials and Methods

2.1 Maintenance of cultures

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

2.2 Morphological characterization of Endophytic bacteria

Colony characteristics of the purified endophytes were noted, including Gram character and motility by hanging drop method.

2.3 Inoculum preparation

The cultures were grown on Standard Plate Count Agar at 30^0 C for a period of 20-24 h. Saline suspensions of these cultures were prepared and the culture density was adjusted to an optical density (O.D.) of 0.1 at wavelength of 540 nm using sterile saline as blank. These suspensions were then used as inoculum.

2.4 Characterization of Endophytic bacteria for their multiple plant growth promoting properties

The isolates were screened for the following plant growth promoting properties

2.4.1. Indole-3-acetic acid (IAA) production and quantitation [7]

Nutrient Broth (10 ml) (HIMEDIA Laboratories, India) supplemented with 0.5 g% tryptophan was inoculated with 0.1 ml [1% (v/v)] inoculum. It was then incubated for 48 h in a rotary shaker incubator, set at 30 ºC with a shaking speed of 120 rpm. After incubation, the broth culture was centrifuged at 10,000 rpm for 15 minutes at 4 ºC. To 1 ml of supernatant, 2ml Salkowski reagent (50 ml of 35% perchloric acid, 1 ml of 0.5 M $FeCl_3.6H_2O$ solution) was added. It was incubated in the dark for 30 minutes at room temperature. Uninoculated sterile medium served as the control. Development of cherry red colour indicated IAA production by the endophytic bacteria. The quantitative estimation of IAA was performed as per the method given by Gordon S. A. and Weber R. P. (1951).[7] Absorbance of the resultant cherry red colour was read at 540 nm using sterile uninoculated Nutrient Broth supplemented with 0.5 g% tryptophan as blank. 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.

2.4.2. Phosphate solubilisation [8]

A loopful of the culture inoculum was spot inoculated on sterile Pikovskaya's agar (HIMEDIA Laboratories, India) plate and incubated at 30 ºC. These plates were daily checked for zone of clearance around the colony upto 7 days. Uninoculated sterile medium served as the control. Development of a clear zone around the colony indicated phosphate solubilizing ability of the endophytic bacteria.

2.4.3. Organic acid production [9, 10]

A loopful of the culture inoculum was spot inoculated on sterile Pikovskaya's agar plate containing 0.01% Bromothymol blue as pH indicator and incubated at 30 ºC for 24 h. Uninoculated sterile medium served as control. Change in colour from light green (neutral) to yellow (acidic) around the colony indicated organic acid production by the bacteria which is required to solubilize the inorganic phosphate present in the medium.

2.4.4. Siderophore production [11]

All glassware used for detection of siderophore production were treated with/ immersed in 6N HCl overnight to remove residual iron and washed with double distilled water. All media and solutions were prepared in double distilled water.

Chrome Azurol S solution [60.5 mg Chrome Azurol S in 50 mL distilled water (D.W.)] and Hexadecyltrimethyl ammonium bromide solution (HDTMA 72.9 mg in 40 mL D.W.) were mixed and then the mixture was added to 10 mL of 1mM $FeCl_3.6H_2O$ solution prepared in 10 mM HCl. The solution thus obtained was autoclaved, cooled and then added to 900 mL of molten sterile Nutrient Agar base. This medium was then aseptically poured into sterile Petri plates. A loopful of the culture inoculum was spot inoculated on sterile Chrome azurol S (CAS) agar plates containing nutrient agar as basal medium and incubated at 30 ºC for 4 days. Uninoculated sterile medium served as the control. Development of orange halo around the growing bacterial colony indicated siderophore production.

2.4.5. Nitrogen fixation

The isolates were checked for their ability to fix atmospheric nitrogen by following methods:

2.4.5.1 Growth on Jensen's nitrogen free medium [12]

A loopful of the culture inoculum was streaked on sterile Jensen's nitrogen free medium (HIMEDIA Laboratories, India) and incubated at 30 ºC for 7 days. Uninoculated sterile medium served as the control. Growth on Jensen's nitrogen free medium indicated ability of the endophytic bacteria to fix atmospheric nitrogen.

2.4.5.2 Ammonia production [13]

Ten mL of 1% peptone water broth was inoculated with 0.1 mL [1% (v/v)] inoculum and incubated for 48 h in a rotary shaker incubator set at 30 ºC and shaking speed at 120 rpm. After incubation, 0.5 mL of Nessler's reagent was added in each tube. Uninoculated sterile medium served as the control. Development of brownish yellow colour indicated ammonia production and thereby the ability of the endophytic bacteria to fix atmospheric nitrogen.

2.4.6. Hydrogen cyanide (HCN) production [14]

Nutrient Broth (10 mL) supplemented with 4.4 g/L glycine was inoculated with 0.1 mL [1% (v/v)] inoculum, and incubated for 24 h in a rotary shaker incubator set at 30^0 C and shaking speed at 120 rpm.

A loopful from the broth was then streaked on a slant of Nutrient agar supplemented with 4.4 g/L glycine. The tube had a Whatman No.1 filter paper strip soaked in 2% sodium

carbonate in 0.5% picric acid placed above the medium. It was incubated for 4 days at 30 ºC. Uninoculated sterile medium with the soaked filter paper placed above the medium, served as the control. Change of colour of filter paper from yellow to orange brown would indicate hydrogen cyanide production by the bacteria.

3. Results

3.1 Morphological characterization of Endophytic bacteria

Colony characteristics of the 13 bacterial endophytes (KV-1 to KV-13) from *Gloriosa superba* were noted. They were circular, white, cream or yellow, transluscent to opaque, butyrous, 1-2 mm in size, flat, raised to convex; their Gram character and motility are shown table 1. Of the 13 endophytes, 7 were Gram negative rods, 4 were Gram positive rods and 2 were Gram positive cocci in chains. For motility by hanging drop method, 7 were motile and 6 were non-motile as shown in table 1.

Table 1: Gram character and motility of the isolates obtained from *Gloriosa superba*

Isolates	Gram character	Motility
KV-1	Gram negative short rods	Motile
KV-2	Gram positive short rods	Non-Motile
KV-3	Gram positive cocci in chain	Non-Motile
KV-4	Gram negative short rods	Motile
KV-5	Gram positive short rods	Non-motile
KV-6	Gram negative cocci in pairs	Motile
KV-7	Gram positive short rods	Non-motile
KV-8	Gram positive long rods	Non-motile
KV-9	Gram positive cocci in chain	Non-motile
KV-10	Gram negative short rods	Motile
KV-11	Gram negative Short rods	Motile
KV-12	Gram negative short rods	Motile
KV-13	Gram negative long rods	Motile

3.2 Plant growth promoting properties of the bacterial endophytes

3.2.1. Indole-3-acetic acid (IAA) production

Of the 13 isolates obtained from *Gloriosa superba*, 5 isolates KV-3, KV-5, KV-6, KV-9 and KV-11 tested positive for IAA production and showed development of cherry red colour on addition of Salkowski reagent as shown in Fig. 1.

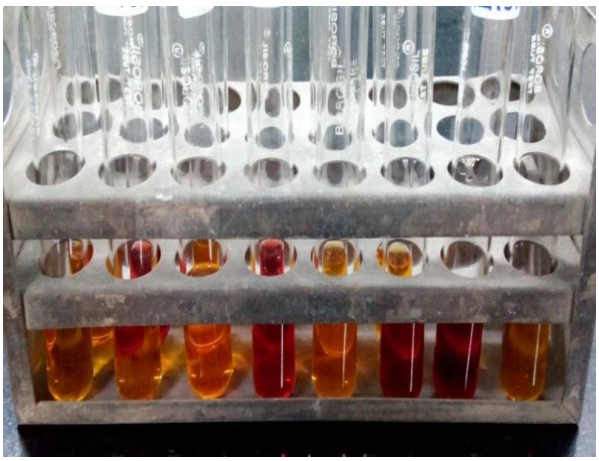

Fig 1: Detection of IAA production

The results of quantitative estimation of IAA are given in table 2.

Table 2: Quantitative estimation of IAA production

Isolate	Development of Cherry Red Colour	Absorbance At 540 nm	Concentration Of IAA (µg/mL)
KV-3	+	0.74	52.85
KV-5	+	1.25	89.28
KV-6	+	1.09	77.85
KV-9	+	1.17	83.57
KV-11	+	1.20	85.71
Control	-	0.00	-

Of these isolates, KV-5 showed maximum production of indole-3-acetic acid (89.28 µg/ml) followed by KV-11 (85.71µg/ml).

3.2.2 Phosphate solubilisation

Among 13 isolates, 3 of the isolates KV-5, KV-11 and KV-13 developed clear zones around their colonies on Pikovskaya's agar plate incubated at 30 ^{0}C upto 7 days indicating solubilization of phosphate.

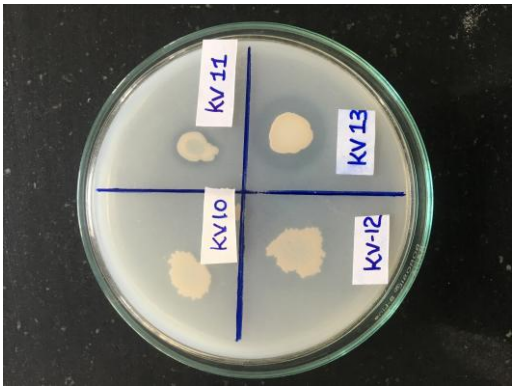

Fig 2: Detection of Phosphate solubilisation

3.2.3 Organic acid production

Of the 13 isolated bacterial endophytes, 5 isolates KV-5, KV-7, KV-11, KV-12 and KV-13 showed change in colour from light green (neutral) to yellow (acidic) around their colonies when grown at 30 ºC for 24 h on Pikovskaya's agar plate containing 0.01% Bromothymol blue as pH indicator dye, indicating organic acid production (required to solubilize the inorganic phosphate present in the medium).

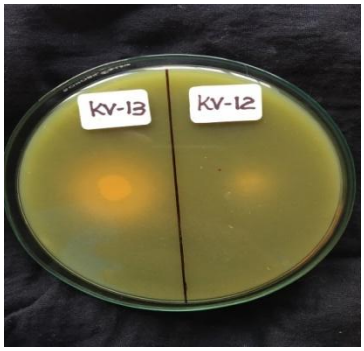

Fig 3: Detection of organic acid production

3.2.4 Siderophore production

A total of 7 isolates KV-2, KV-5, KV- 6, KV-10, KV-11, KV-12 and KV-13 showed an orange halo around their colonies, indicating siderophore production on CAS agar plates incubated at 30 ºC for 4 days.

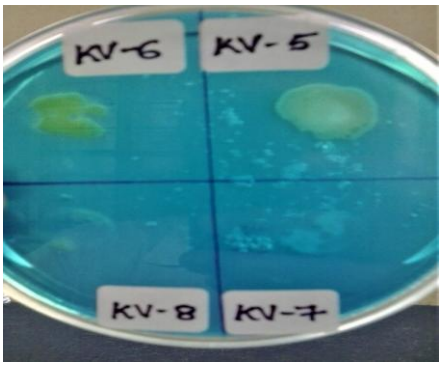

Fig 4: Detection of siderophore production

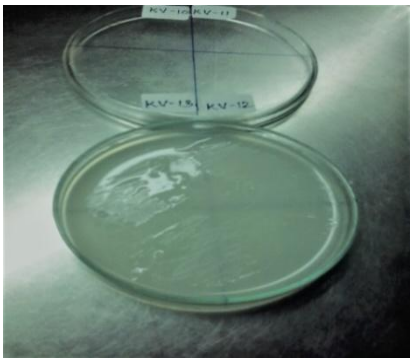

Fig 5: Detection of nitrogen fixation

3.2.5 Nitrogen fixation

3.2.5.1 Growth on Jensen's nitrogen free medium

A total of 11 isolates KV-1, KV-2, KV-3, KV-5, KV-6, KV-7, KV-9, KV-10, KV-11, KV-12 and KV-13 showed growth on Jensen's nitrogen free medium, when incubated at 30 ºC for 7 days, indicating the ability of the bacterial endophytes to fix atmospheric nitrogen.

3.2.6 Ammonia production

A total of 11 isolates KV-1, KV-2, KV-3, KV-5, KV-6, KV-7, KV-9, KV-10, KV-11, KV-12 and KV-13 tested positive for ammonia production by developing brownish yellow colouration on addition of Nessler's reagent, indicating ability of the bacterial endophytes to fix atmospheric nitrogen.

3.2.7 Hydrogen cyanide (HCN) production

Of the 13 bacterial endophytes tested, none tested positive for Hydrocyanic acid (HCN) production.

3.2.8 Summary of Results

Table 3: Plant Growth promoting properties of the isolates

ISOLATE	IAA production	Phosphate solubilization	Organic acid production	Siderophore production	Growth on Jensen's nitrogen free medium	Ammonia production	HCN production
KV-1	-	-	-	-	+	+	-
KV-2	-	-	-	+	+	+	-
KV-3	+	-	-	-	+	+	-
KV-4	-	-	-	-	-	-	-
KV-5	+	+	+	+	+	+	-
KV-6	+	-	-	+	+	+	-
KV-7	-	-	+	-	+	+	-
KV-8	-	-	-	-	-	-	-
KV-9	+	-	-	-	+	+	-
KV-10	-	-	-	+	+	+	-
KV-11	+	+	+	+	+	+	-
KV-12	-	-	+	+	+	+	-
KV-13	-	+	+	+	+	+	-

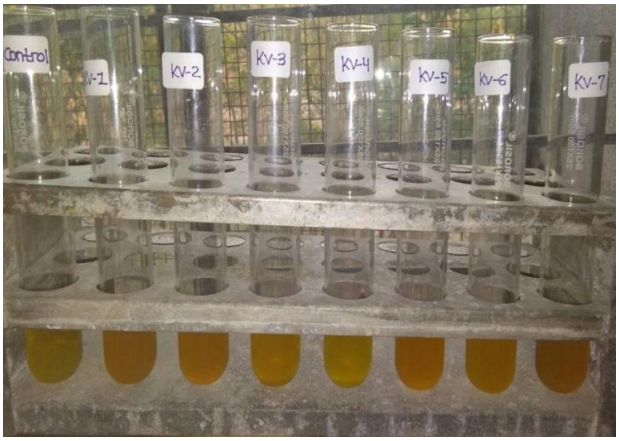

Fig 5: Detection of ammonia production

4. Discussion

Our report is one of the first few papers to report bacterial endophytes from the pharmaceutically important medicinal plant *Gloriosa superba* for their multiple plant growth promoting properties.

In the present study, a total of 13 bacterial endophytes from medicinal plant *Gloriosa superba* were screened for their multiple plant growth promoting properties like IAA (Indole-3- acetic acid) production, phosphate solubilisation, organic acid production, siderophore production nitrogen fixation, ammonia production and Hydrocyanic acid (HCN) production as shown in table 3. These endophytes as plant growth promoting rhizobacteria (PGPR) can promote the growth of plants directly, indirectly or synergistically [15, 16, 17]. Direct mechanism of plant growth promotion includes production of plant hormones like indole acetic acid (IAA) [18, 19]. phosphate solubilisation [20, 21] siderophore production [20] and atmospheric nitrogen fixation [19, 20]. Indirect mechanism of plant growth promotion includes production of antimicrobial compounds such as HCN or enzymes like chitinases to help the plant ward off infection [14].

4.1 Indole-3-acetic acid is a phytohormone that acts as an important signalling molecule which participates in the regulation of plant development including organogenesis,

tropic responses like phototropism, geotropism, cellular responses such as cell division, differentiation, enlargement, gene regulation, apical dominance, increases the rate of xylem and root development, controls the processes of vegetative growth, initiates formation of lateral and adventitious roots, affects photosynthesis, pigment formation, biosynthesis of various metabolites, and provides resistance to stressful conditions [18, 22]. IAA production also increases root growth and root length resulting in greater root surface area giving the plant greater access to soil nutrients and water uptake from the soil [24]. In our study, five of the 13 studied isolates could produce IAA and KV-5 showed maximum production of indole-3-acetic acid (89.28 µg/ ml). Malik *et al* (2011) [25]. have reported a maximum of 40.6 µg/ml of IAA production by their *Pseudomonas* isolate MPS77, upon 4 days of incubation.

4.2 Phosphate solubilisation

The bioavailability of phosphorus to the plants is very limited because the majority of phosphorus present in the soil is found in insoluble, immobilized, and precipitated form. Most of the insoluble forms of phosphorus exist as aluminum and iron phosphates in acidic soils [20] and calcium phosphates in alkaline soils [21]. The insoluble phosphorus exist either as an inorganic mineral such as apatite or as an organic mineral like inositol phosphate (soil phytate), phosphomonesters, and phosphotriesters [26 27]. The solubilization of inorganic phosphorus is due to the lowering of pH by the action of low molecular weight organic acids such as gluconate, citrate, lactate, succinate etc. release of protons during the assimilation of ammonia [20] and chelation of cations like calcium ions that release organic phosphorus and make it available for plant use. [28] Mineralization of organic phosphorus takes place through the synthesis of different phosphatases that catalyzes the hydrolysis of phosphoric esters. Therefore, phosphate solubilization and mineralization can coexist in the same strain of bacteria [29]. Phosphate solubilising bacteria are a group of beneficial bacteria that can solubilize insoluble phosphate into soluble form that can be absorbed as a nutrient by the plants for their overall growth and development. In our study, 5 isolates (KV-5, KV-7, KV-11, KV-12 and KV-13) showed organic acid production and three (KV-5, KV-11 and KV-13) of the 13 studied isolates could solubilize phosphate.

4.3 Siderophore production

Iron is involved in cellular growth and metabolism, ATP synthesis, as a cofactor of various enzymes etc. Siderophores are low molecular weight iron chelating compounds by which bacteria take up iron under iron limiting conditions [30]. They also form stable complexes with different heavy metals like Al, Cd, Cu etc. and radioisotopes such as neptunium and uranium that pose serious environmental threat thereby increasing soluble metal concentration. Therefore bacterial siderophores help to lower the stress imposed on plants that grow on soil contaminated with heavy metals [31]. These iron chelators improve plant growth by bringing ferric ion (Fe^{3+}) to the root surface where it is reduced to ferrous ion (Fe^{2+}) and immediately absorbed by the plant roots that prefer to absorb iron in the more reduced ferrous state thus promoting plant growth [24]. Siderophore producers have been reported to inhibit growth of various phytopathogenic fungi and bacteria [32]. In our study, of the 13 bacterial endophytes, 7 isolates viz. KV-2, KV-5, KV- 6, KV-10, KV-11, KV-12 and KV-13 showed siderophore production.

4.4 Nitrogen fixation

Most of the plants cannot take up atmospheric nitrogen. This atmospheric nitrogen is converted into plant-usable forms by Biological Nitrogen Fixation (BNF) which changes nitrogen into ammonia making it available to the plants by various nitrogen fixing endophytic bacteria.[31] In our study, a total of 11 isolates KV-1, KV-2, KV-3, KV-5, KV-6, KV-7, KV-9, KV-10, KV-11, KV-12 and KV-13 showed growth on Jensen's nitrogen free medium and ammonia production.

4.5 HCN production

Indirect mechanism of plant growth promotion includes production of compounds which help host plants combat infections such as hydrocyanic acid production which is a secondary metabolite formed by the decarboxylation of glycine [33]. It serves as an effective biological control agent against plant pathogens. HCN mainly inhibits electron transport chain and prevents energy supply to the cell, leading to death of the pathogen [30]. Of the 13 bacterial endophytes tested, none tested positive for Hydrocyanic acid (HCN) production.

4.6 Potential as bioinoculant

Kumar *et al.*, 2016 [34] isolated fourteen endophytic bacteria from the rhizomes of *Curcuma longa* L. (Turmeric) viz. *Bacillus cereus* (ECL1), *Bacillus thuringiensis* (ECL2), *Bacillus sp.* (ECL3), *Bacillus pumilus* (ECL4), *Pseudomonas putida* (ECL5) and *Clavibactermichiganensis* (ECL6). Except for *B. thuringiensis* ECL2, all the strains were capable of solubilizing tricalcium phosphate, all the strains produced IAA; siderophore production was seen only in *Bacillus* sp. (ECL3) and *P. putida* (ECL5). These isolates could be used as bioinoculants for increasing yield and productivity of medicinal crops. Shakeela *et al.*, 2017 [35] isolated forty phosphate solubilizing rhizobacteria and endorhizobacteria from the rhizosphere soil and rhizome/roots of the *Picrorhiza kurroa*. Among them, isolate PkR (7a) showed maximum phosphate solubilization whereas maximum IAA production was showed by PkR (34) and PkR (7b). Maximum siderophore production was observed in the isolate Pk12 (b) whereas high HCN production was seen in thee isolates viz., Pk14 (a), Pk14(c) and PC7 in which the colour of entire filter paper got changed from yellow to brown. They claimed that these PGPR could be used as bio-fertilizers or biocontrol agents to increase the survival and growth of medicinal plants. In our study, the bacterial endophytes KV-5 and KV-11 obtained from the medicinal plant *Gloriosa superba* tested positive for four (IAA production, phosphate solubilization, siderophore production and nitrogen fixation) of the five Plant Growth Promoting properties they were screened for. Whereas, the isolate KV-13 was positive for phosphate solubilization, siderophore production and nitrogen fixation. These promising isolates are strong contenders for the development of bioinoculants as individual strains or as consortia. They can be used to improve soil fertility and have potential to establish themselves as endophytes in medicinal plants such as *Gloriosa superba* resulting in greater yield and productivity of medicinal crops in an eco-friendly manner. This strategy would work towards solving the problem of over-exploitation of indigenous medicinal plants and thereby the threat of them rapidly becoming endangered species.

5. Conclusion

The current study indicated that pharmacologically important medicinal plant *Gloriosa superba* is an ecological niche for diverse bacterial endophytes. The endophytes KV-5, KV-11 and KV-13 displayed various plant growth promoting properties and are strong contenders for bioinoculant production either individually or as consortia thereby improving the health of the soil in an ecofriendly manner, resulting in an increase in the population of medicinal plants thus preventing them from becoming endangered species.

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