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Evaluation of *Withania somnifera* Endophytic Bacterial Isolates under In-Vitro Conditions

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***Abstract*:** Endophytes are colonized with the internal tissues of their host plants and can form a range of different types of relationships including symbiotic, mutualistic and trophobiotic. Endophytic bacteria can promote plant growth, yield and can act as biocontrol agents. To explore biocontrol potential of various bacterial isolates an attempt was made to isolate endophytic bacteria and examined for hydrogen cyanide (HCN) production, siderophore production and cellulase activity. Thirty-two endophytic bacterial isolates (ARBE1-ARBE32) were retrieved from the roots of *Withania somnifera* grown at Medicinal and Aromatic Plant Section, Chaudhary Charan Singh Haryana Agricultural University , Hisar. Under *in vitro* conditions siderophore production was observed in isolates ARBE5, ARBE9, ARBE11, ARBE13, ARBE14, ARBE17, ARBE18, ARBE19, ARBE22, ARBE30, ARBE31 and ARBE32. Hydrogen cyanide production indicated by a change in color of alkaline picrate-soaked filter paper was observed in isolates ARBE5, ARBE9, ARBE11, ARBE13, ARBE14, ARBE16, ARBE18, ARBE19, ARBE23, ARBE27, ARBE28, ARBE29 and ARBE32. The isolates, ARBE13, ARBE20, ARBE26, ARBE29 and ARBE30 were positive for cellulase activity. The isolates possessing multi biocontrol traits can further be exploited for management of plant diseases in integrated manner.

***Keywords*:** Ashwagandha root bacterial endophyte, Biocontrol activities, Endophytic, Wilt, *Withania somnifera*

Pesticide residues possess harmful effects, visible since the onset of green revolution. Therefore, researchers of the present era are focusing on environment-friendly and sustainable options for managing pests (Tao et al 2022). In this sequence, endophytic microbiota catches the interest of researchers as well as environmentalists due to its sustainability and environmental friendliness. Endophytic colonization is a subsystem of the soil microbiome that plays an important role in the synthesis of biologically active compounds that protect plants from soil-borne pathogens (Cays 2021). The first report of the presence of endophytes was by Vogl (1898), who found a mycelium residing in grass seed *(Lolium temulentum* L.). Endophytic micro-biota plays an important role in plants' metabolic activities. They trigger various biochemical pathways in the host plant by producing physiologically important chemical compounds. They produce a diverse range of secondary metabolites with vital medicinal values and can be used in medicine, agriculture and industry (Siegel et al 1987). Endophytes are the microorganisms (fungi, bacteria, actinomycetes, slime-molds, archae a, viruses, phytoplasmas and other protozoan species) that colonize the host tissues and establish a relationship where both partners get benefit from their interactions which spend at least a part of their life cycle inside the plant tissues without causing any diseases (Prasad et al 2014, Esmael & Goodwin 2022, Mahnkopp-Dirks et al 2022). The specific benefits of many endophytes are, however unknown. Generally, the bacterial endophytes are linked with the plants and can perform three functionalities – biotic and abiotic stress alleviation and phytohormones production. The population of plant endophytes is generally less than the rhizosphere bacteria because roots favour the growth of several microbial communities (Samuel et al 2022). Plants mainly attract microorganisms from microbial hubs around them. Plants not only absorb water and nutrients from the soil but they also absorb a variety of microorganisms through their roots (Hardoim et al 2015).

Although, there was a misconception among the researchers to consider the presence of any bacteria within plant tissues as symptomatic of a particular pathological condition, however, this perception was changed by one famous scientist Perotti, 1926 who described the occurrence of a non-pathogenic flora in root tissues. Endophytic microorganisms may be present in all parts of a healthy plant at a specific life stage or throughout their life and they generally do not cause diseases in plants. They are mutually beneficial to their host (Shurigin et al 2022). Endophytes enhance host plants' resistance by producing valuable substances and inducing systemic resistance (Zaghloul et al

2016, Alsultan et al 2019). They also possess various powerful bioremediation effects (Miao et al 2022). In the last few decades, medicinal plants are becoming popular due to their excellent remediation characteristics and no side effects as compared to allopathic medicinal systems. Medicinal plants provide valuable therapeutic agents in traditional medicines used at the global level for human health. Ashwagandha (*Withania somnifera*) also known as Indian ginseng is one of the most important medicinal plants (Shabbir and Mohammad 2014). It is reported in literature that plant root endophytes can produce metabolites with antifungal properties to avoid pathogen infection by competing with pathogens or by direct antagonism through the production of antimicrobial compounds (Compant et al 2005). However, little information is available regarding antagonistic mechanism of bacterial root endophytes, which is needed to be fully explored (Herrera et al 2022). To accomplish this, the present study was designed to assess various biocontrol attributes *viz.,* siderophore, cellulase activity and HCN production of bacterial endophytes of ashwagandha for development of eco-friendly approach for disease management.

MATERIAL AND METHODS

Root samples: Root samples of ashwagandha were collected during crop season 2019-2020. An intact root system was dug out and the roots of ashwagandha plants were carefully taken in plastic bags for various studies.

Isolation of bacterial root endophytes from ashwagandha roots: The roots were washed with running tap water and surface sterilized sequentially in 75 % (v/v) ethanol for 2 min, 2.6 % (w/v) sodium hypochlorite solution for 5 min and 75 % (v/v) ethanol for 1 min. One gram of plant tissue was crushed in a pestle and mortar with 10 ml of sterile distilled water to get a homogenous paste and allowed to settle down for 20 minutes. The supernatant was diluted serially and approximately 10µl was placed on nutrient agar (NA) plates and incubated at 28±2°C for 3 days. The bacterial colonies appearing on the plates were considered to be endophytes. Colonies were characterized according to different visual observations, purified on plates using streak plate technique and maintained at 4 ±1°C for further studies.

Screening of Endophytic Bacterial isolates for Biocontrol Activities

Siderophore production: Siderophore production by bacterial endophytes of ashwagandha was observed by Chrome azurol S (CAS) assay (modified method of Schwyn and Neilands 1987). The presence of siderophore (iron chelator) was indicated by the decolourization of blue-coloured ferric dye complex, resulting in yellow halo zones around the bacterial colonies.

Hydrogen cyanide production: Hydrogen Cyanide production by bacterial endophytes was assessed using the method of Alstrom and Burns (1989). Active culture of different bacterial endophytes was prepared by inoculation of 48 h old culture from nutrient agar slants to freshly prepared Kings B broth. The production of cyanide was detected after 72 h of incubation at 28±2°C, using alkaline picrate-soaked filter paper fixed underside of the test tube. A change of colour from yellow to light brown, brown and reddish-brown was recorded as an indication of weak, moderate or strong cyanogenic potential.

Cellulase activity: Ashwagandha root bacterial endophytes were also screened for cellulase activity using the procedure described by Apun et al (2000). Freshly grown bacterial cultures were spot inoculated on carboxy-methyl-cellulose (CMC) agar plates, incubated at 28±2°C for 48 h and then flooded with 0.1% aqueous solution of Congo red for 15-20 minutes followed by washing with 1 M NaCl. Cellulase production was indicated by a clear zone around the colony.

RESULTS AND DISCUSSION

Screening of ashwagandha root bacterial endophytes for biocontrol activity: The biocontrol activity of ashwagandha root endophytes was assessed under laboratory conditions by examining their potential for siderophore production, HCN production and cellulase activity.

Siderophore production by endophytic bacterial isolates: The isolates ARBE5, ARBE9, ARBE11, ARBE13, ARBE14, ARBE17, ARBE18, ARBE19, ARBE22, ARBE30, ARBE31 and ARBE32 were found positive for siderophore production (Table 1, Fig. 1). Joshi et al (2018) isolated 10 bacteria from the roots, stems and leaves of *Aloe vera* and *Ocimum sanctum* and observed that only three of the isolates, TNR15, TKR 1 II and AVJR7 II were able to produce siderophore on CAS-medium. Etminani and Harighi (2018) observed that only five isolates, namely Pb1, Pb71, Pb78, Sp15 and Bp108 were able to produce siderophore out of ten isolates extracted from the leaves and stems of healthy wild pistachio trees. Arora and Singh (2016) observed ashwagandha endophytic bacteria (*Pseudomonas* sp.) as a growth-promoting agent as well as for their siderophore production activities and revealed *Pseudomonas* PSE-1 strain as potent siderophore producer.

Hydrogen cyanide production: Among 32 isolates a change in the colour of alkaline picrate-soaked filter paper was observed in isolates ARBE5, ARBE9, ARBE11, ARBE13, ARBE14, ARBE16, ARBE18, ARBE19, ARBE23, ARBE27, ARBE28, ARBE29 and ARBE32 (Table 2, Fig. 2).

Table 1. Siderophore production by ashwagandha root bacterial endophytes

Isolate	Siderophore production	Isolate	Siderophore production	Isolate	Siderophore production
ARBE1	-	ARBE12	-	ARBE23	-
ARBE2	-	ARBE13	+	ARBE24	-
ARBE3	-	ARBE14	+	ARBE25	-
ARBE4	-	ARBE15	-	ARBE26	-
ARBE5	++	ARBE16	-	ARBE27	-
ARBE6	-	ARBE17	+	ARBE28	-
ARBE7	-	ARBE18	+	ARBE29	-
ARBE8	-	ARBE19	++	ARBE30	+
ARBE9	+	ARBE20	-	ARBE31	+
ARBE10	-	ARBE21	-	ARBE32	++
ARBE11	++	ARBE22	+		

'+' Orange zone formation (light colour), '-' No zone formation'++' Orange zone formation (dark colour)

Inducing resistance and serving as a plant defence mechanism against pathogens, hydrocyanic acid works as an inducer of resistance. This volatile substance prevents electron transportation, interferes with cell energy delivery and ultimately kills pathogens. This substance indirectly makes phosphorus and iron more available to plants, which results in faster plant development. Etminani and Harighi (2018) showed that out of 10 isolates extracted from the leaves and stems of healthy wild pistachio trees only isolate Ba66, having a closer similarity to *B. anthracis*, was able to produce HCN. Etesami et al (2014) reported that amongst 200 bacterial isolates retrieved from the berseem clover plant's rhizosphere, roots and nodules only five were positive

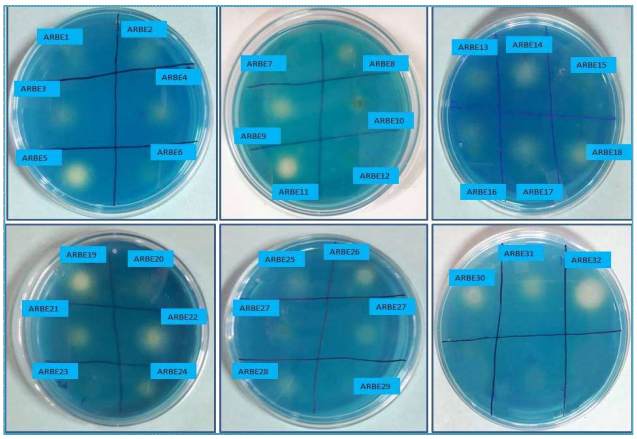

Fig. 1. Siderophore production by bacterial endophytes

Fig. 2. HCN production by bacterial root endophytic isolates

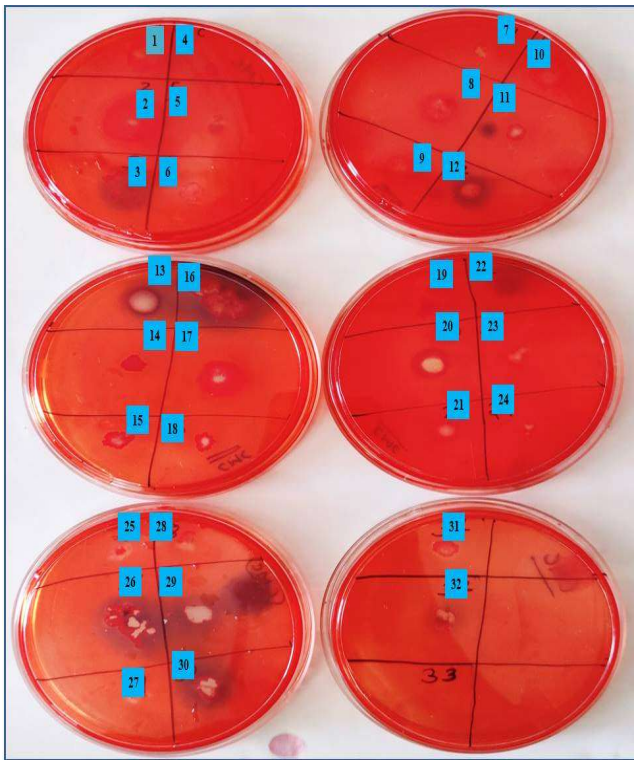

Fig. 3. Cellulase activity of ashwagandha root bacterial endophytic isolates

Table 2. Hydrogen cyanide production by endophytic bacterial isolates retrieved from ashwagandha roots

Isolate	HCN production	Isolate	HCN production	Isolate	HCN production
ARBE1	-	ARBE12	-	ARBE23	+
ARBE2	-	ARBE13	++	ARBE24	-
ARBE3	-	ARBE14	+	ARBE25	-
ARBE4	-	ARBE15	-	ARBE26	-
ARBE5	++	ARBE16	+	ARBE27	+
ARBE6	-	ARBE17	-	ARBE28	+
ARBE7	-	ARBE18	+	ARBE29	+
ARBE8	-	ARBE19	++	ARBE30	-
ARBE9	+	ARBE20	-	ARBE31	-
ARBE10	-	ARBE21	-	ARBE32	++
ARBE11	++	ARBE22	-		

'+' Orange zone formation (light colour), '-' No zone formation'++' Orange zone formation (dark colour)

for HCN production. Abdallah et al (2016) reported that the endophyte bacterium retrieved from ashwagandha was able to produce hydrogen cyanide.

Cellulase activity: Amongst 32 isolates, ARBE13, ARBE20, ARBE26, ARBE29 and ARBE30 were found positive for cellulase activity (Fig. 3). Microbes mediate nutrient cycling in soils in which extracellular enzymes *e.g.,* protease, lipase, amylase, cellulose, phosphatases, chitinase, urease, *etc.* play a significant role by mineralizing organic compounds (Das and Varma 2010). Soil enzyme activity measurements have been used as an indicator of soil quality and health (Badiane et al 2001). The results of the present study showed the diversity of culturable endophytic bacteria that reside in the interior root tissues of the ashwagandha plant. Ntabo et al (2018) isolated 42 bacteria from the leaves and roots of mangrove plants. They reported endophytes, including *Bacillus*, *Myroides, Pseudochrobactrum* and *Serratia* isolated primarily from leaves and suggested their potential role in colonising the leaf tissues and expressed cellulase activity. Kukla et al (2014) isolated twenty-nine endophytic bacteria from ryegrass and evaluated them for their cellulase enzyme, siderophore and hydrogen cyanide production.

CONCLUSION

A total of 32 endophytic bacterial isolates were retrieved from the ashwagandha roots. Present study focused on suitable screening for the selection of the promising endophytic isolates capable to produce cellulase, siderophore and hydrogen cyanide. The efficient isolates could be exploited as a biocontrol agent, for management of plant diseases and to overcome the fungicidal load in agricultural sector. The use of endophytic microorganisms as bioinoculants could also be a promising alternative to synthetic fertilizers, especially for the cultivation of medicinal plants.

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