Mysore J. Agric. Sci., 57 (1) : 360-366 (2023)

Isolation and Screening of Bacterial Endophytes Against *Alternaria solani* - Causing Early Blight Disease in Potato (*Solanum tuberosum* L.)

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Received : August 2022
Accepted : February 2023

Abstract

The use of endophytes as biocontrol agents has been gaining importance towards the sustainable agriculture and eco friendly farming. The present study is designed to isolate and screen the bacterial endophytes that inhibit the fungal pathogen *Alternaria solani* that causes early blight disease in the potato and in most of the solanaceous crops. The bacterial endophytes (62) were isolated from different parts of potato plant *viz.,* leaf, stem, roots and tubers. Among them, ten isolates were taken as efficient isolates which significantly inhibited the growth of fungal pathogen in dual culture method. The highest inhibition percentage against the pathogen was recorded in the isolate PEL-4 (71.46%) followed by PES-5 (69.38%). All the ten efficient isolates were found to be Gram positive. Six isolates tested positive for starch hydrolysis. Eight isolates tested catalase positive. Only three isolates were tested positive for HCN production.

Keywords : Endophytes, Pathogen, Biocontrol, *Alternaria solani,* Solanaecous crops

The extensive use of chemicals for the cultivation of crops has been causing many adverse effects on the human health and also on the environment in the recent times. In this regard, bio-control strategies are gaining importance as a remedy or as an alternate towards sustainable agriculture and organic farming. Off-late, endophytes have been gaining importance to combat the various biotic and abiotic stresses that cause damage to the crops by various means. The term 'Endophyte' is derived from the Greek words 'Endon' (within) and 'Phyte' (plant). Endophytes are the microorganisms (bacteria or fungi), present in the plants for the whole or a part of their lifecycle, residing inter and intra-cellular healthy tissues of the host plant, without causing any noticeable disease symptoms.

Biological control by microbial endophytes is the best way to control pathogens as they are inherently safe, cost-effective and environmental friendly (Rabiey *et al.,* 2019). These endophytes colonize the plant tissues and fight against other microbial pathogens on the same ecological habitats. Therefore, the plant-endophyte association promotes plant health *via* several mechanisms and potentially contributes to the host plant resistance against pathogens (Malhadas *et al.,* 2017). These endophytes not only act as biocontrol agents but also possess various plant growth promotion traits, or helping the host plant to tolerate stress (Ullah *et al.,* 2019) and help in the overall growth and development of the crop plants. Endophytic bacteria have been isolated from roots, leaves, stems, a few from flowers, fruits and seeds (Imran *et al.,* 2019).

Potato (*Solanum tuberosum* L.) is the third most important food crop after rice and wheat interims of its usage as a stable food around the globe. India is the second largest producer of potato in the world behind China. The world wide consumption of potato was 241 million tonnes, third after rice and wheat (FAOSTAT, 2020). Potato occupies premier place in the list of vegetable crops in the world including India. It is a member of Solanaceae family (chromosome

number 2n = 48). Potatoes are an important source of carbohydrate, protein, vitamins and minerals. It is used as a staple food in many countries of the world.

Even though, there are many reports available against the early blight disease, only a few have been reported in potato, especially the reports with respect to the beneficial effect of endophytes isolated from various parts of potato plant are scarce. The present study highlights the potential of bacterial endophytes isolated from potato for the control of early blight disease causing *Alternaria solani*. Potato plants are susceptible to a wide range of diseases *viz.,* late blight, early blight, wilt, scab, *etc*. Among them, *Alternaria solani* is a kind of fungal pathogen that causes early blight disease of tomato, potato and many other vegetable crops and lead to huge losses in agricultural production. Early blight disease in potato can cause up to 80 per cent of annual yield losses in some regions of the world (Peters *et al.,* 2008). In the present study, the bacterial endophytes were isolated from the potato plants collected from various places and were screened *in-vitro* against the pathogen *Alternaria solani*.

Material and Methods

The present study was carried out in the Department of Agricultural Microbiology, University of Agricultural Sciences, Gandhi Krishi Vigana Kendra (GKVK), Bengaluru.

Sample Collection

The plant and tuber samples of potato were collected from different regions of Bengaluru Rural, Bengaluru Urban, Chickaballapura and Kolar districts (Table 1).

Isolation of Endophytic Bacteria from Leaf / Stem / Roots / Tubers of Potato

The isolation of endophytes was carried out as per the standard procedures given by Bacon *et al*. (2002). The randomly selected plants were uprooted manually and washed in running tap water to remove the attached portion of soil. Shoot / root / leaf / tuber sections of 2 cm length were excised using flame sterilized scalpel. The surface sterilization of the shoot / root/leaf / tuber pieces was carried out with the following immersion sequence: 70 per cent ethanol and three per cent sodium hypochlorite. They were then rinsed four times with sterile water and dried in laminar flow. The cut ends of surface sterilised segments were removed with flame sterilized scalpel and were placed in the plates with nutrient agar medium with the cut surface touching the agar. The plates were incubated for 24 h at 27 ˚C.

Table 1

Geographical information of the regions where plants were collected for endophytes isolation

Place (District)	Location	Lattitude (N)	Longitude (E)	Number of bacterial isolates
Bengaluru Urban	Samandur	12° 73' 792''	77° 74' 967''	6
	Halehalli	12° 74' 964''	77° 74' 964''	5
	Harohalli	12° 75' 335''	77° 75' 438''	8
	Arehali	12° 92' 023''	77° 49' 346''	7
Kolar	Malur	13° 06' 025''	76° 50' 045''	6
	Vatrakunte	13° 07' 753''	78° 12' 968''	4
Chickaballapura	Shidlaghatta	13° 25' 104''	77° 58' 062''	5
	Hunasenahalli	13° 34' 395''	77° 82' 368''	5
Bengaluru Rural	Vijayapura	13° 30' 377''	77° 80' 231''	6
	Bhatrenahalli	13° 31' 920''	77° 81' 813''	8
	Total			62

The Mysore Journal of Agricultural Sciences

For sterility check, 0.1 ml aliquot from the final wash was inoculated to the nutrient agar plate (Gyaneshwar *et al.,* 2001). Samples were discarded if any growth was detected in the sterility check.

Screening of the Endophytic Isolates *in-vitro* Against *Alternaria Solani*

The isolated bacterial endophytes were tested against the pathogen in dual culture method to check the inhibition of the pathogen by the endophytes. The pathogen, *Alternaria solani* was procured from the Department of Plant Pathology, University of Agricultural Sciences, GKVK, Bengaluru.

Dual Culture Method

Dual culture technique was followed for *in-vitro* screening of bacterial endophytes against *Alternaria solani*. The endophytes were streaked in the same plate parallel to the inoculated pathogen and kept for incubation. The zone of inhibition was measured and the percent inhibition of the growth of pathogen was calculated using the formula.

$$I = \frac{(C-T)}{C} \times 100$$

Where,

I = Per cent inhibition

C = Growth of fungal pathogen in control (mm)

T = Growth of fungal pathogen in dual culture plate (mm)

The best isolates showing high inhibition of the pathogen were selected for further study following the method given by Gravel (2005).

Characterization of the Endophytic Isolates

The efficient isolates that inhibited the higher per cent growth of pathogen in the dual culture method were selected for further characterization.

Morphological Tests for Bacterial Endophytes

The following morphological tests *viz*., cell shape, Gram reaction were carried out to characterize the tentatively identified endophytes.

Cell Shape

The purified cultures, at log phase were observed microscopically for the cell morphological characteristics.

Gram Staining

Gram staining was carried out using the procedure given by Harrigan and McCance (2014). The slides were viewed with the light microscope under oil-immersion. Gram-positive bacteria appear violet and gram-negative bacteria appear pinkish red.

Biochemical Characterization of Efficient Endophytic Isolates

The ability of the isolates to hydrolyze starch was examined in the petriplates containing starch agar which were inoculated with test cultures and incubated at 30 °C for three days. After incubation the plates were flooded with Lugol's iodine solution and allowed to stand for 15-20 minutes. The clear zone around the colony was considered as positive for the test.

The nutrient agar slants were inoculated with test organisms and were incubated at 30 °C for 24 hours. After incubation the slides were flooded with one mL of three per cent hydrogen peroxide and observed for production of effervescence. The occurrence of effervescence was scored positive for catalase activity (Yunting *et al*., 2013).

Hydrogen cyanide production was detected as described by Bakker and Schippers (1987). Petri plates containing 10 per cent Trypticase soya agar supplemented with 4.4 g of glycine per litre were inoculated with the bacterial endophytes and inverted with a lid containing filter paper, impregnated with 0.5 per cent picric acid and two per cent sodium carbonate, over each petri plate. The plates were incubated at 28 °C for three to five days. A change in colour of the filter paper from yellow to orange-brown on the filter paper indicated cyanide production.

Statistical Analysis

The data was statistically analysed using WASP: 2.0 (Web Agri Stat Package 2) statistical tool

(www.icargoa.res.in/wasp2/index.php) and the means were separated by Duncan's Multiple Range Test (DMRT).

Results and Discussion

Isolation of Endophytic Bacteria from Leaf / Stem / Roots / Tubers of Potato

A total of sixty two endophytic bacterial isolates were obtained from different parts of the potato plant collected from different regions. They varied among themselves in colony morphology, color and other characters and were further evaluated for screening and biochemical characterisation. In the same way, Shuang *et al.* (2022) isolated and characterised an endophyte *Bacillus* sp. K-9, from the tubers of potato and studied its biocontrol potential against potato scab disease.

Screening of the Endophytic Isolates *in vitro* against *Alternaria solani*

The sixty two isolates obtained were tested for their antagonistic activity against the pathogen *Altenaria solani* by dual culture method *in vitro.* The pathogen and the bacterial endophyte were inoculated in the same plate and the zone diameter was measured both in the control plate inoculated with pathogen only and in the plate with pathogen and endophyte. Later, the inhibition percentage was calculated and ten isolates *viz.,* PEL-4, PEL-5, PEL-6, PEL-8, PES-5, PER-6, PER-10, PEL-13, PEL-20 and PEL-22 were found to be significantly superior among the sixty two

Table 2

Screening of the endophytic isolates *in vitro* against *Alternaria solani* by dual culture

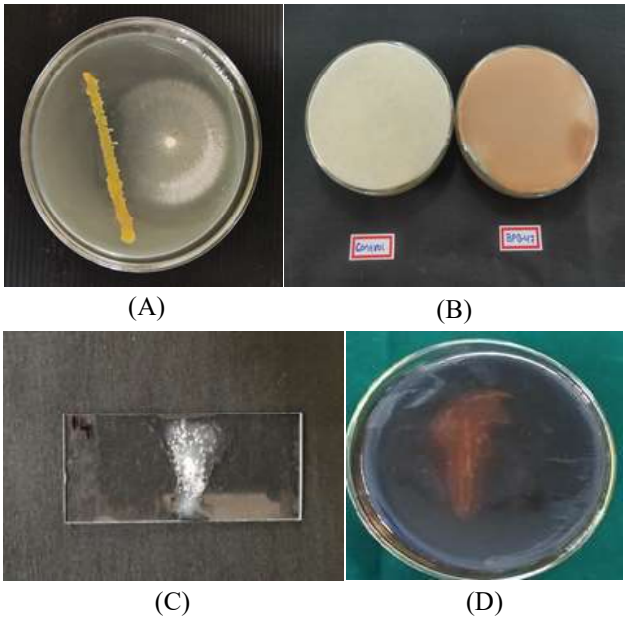
Sl. No.	Bacterial Isolate	Per cent Inhibition	Sl. No.	Bacterial Isolate	Per cent Inhibition	Sl. No.	Bacterial Isolate	Per cent Inhibition
1	PEL-1	32.26 ^{A}	22	PEL-11	40.14 ^{s}	43	PEL-14	35.41 ^{w}
2	PEL-2	42.3 ^{pq}	23	PES-8	33.32 ^{xy}	44	PEL-15	29.1 ^{D}
3	PEL-3	33.19 ^{xyz}	24	PES-9	35.2 ^{w}	45	PEL-16	52.48 ^{i}
4	PEL-4	71.46 ^{a}	25	PES-10	43.55 ^{mn}	46	PER-11	36.43 ^{v}
5	PES-1	44.27 ^{lm}	26	PET-1	31.93 ^{AB}	47	PER-12	42.46 ^{op}
6	PEL-5	62.07 ^{f}	27	PET-2	16.42 ^{J}	48	PER-13	32.47 ^{zA}
7	PEL-6	64.13 ^{e}	28	PET-3	33.52 ^{x}	49	PES-15	49.08 ^{j}
8	PEL-7	46.9 ^{k}	29	PES-11	32.42 ^{zA}	50	PES-16	43.28 ^{n}
9	PEL-8	65.67 ^{d}	30	PES-12	41.04 ^{r}	51	PEL-17	23.55 ^{F}
10	PES-2	43.17 ^{no}	31	PEL-12	52.25 ^{i}	52	PEL-18	25.22 ^{E}
11	PER-1	15.25 ^{K}	32	PER-5	31.35 ^{B}	53	PET-6	31.89 ^{AB}
12	PES-3	44.38 ^{l}	33	PER-6	68.26 ^{c}	54	PEL-19	52.66 ^{i}
13	PES-4	38.66 ^{t}	34	PER-7	37.12 ^{uv}	55	PEL-20	58.39 ^{g}
14	PES-5	70.08 ^{b}	35	PER-8	45.06 ^{l}	56	PET-7	22.52 ^{G}
15	PER-2	37.48 ^{u}	36	PER-9	28.65 ^{D}	57	PET-8	41.64 ^{pqr}
16	PEL-9	52.09 ^{i}	37	PER-10	62.29 ^{f}	58	PER-14	24.89 ^{E}
17	PEL-10	40.26 ^{s}	38	PET-4	30.21 ^{C}	59	PER-15	32.63 ^{yzA}
18	PES-6	20.21 ^{I}	39	PET-5	42.18 ^{pq}	60	PET-8	41.55 ^{qr}
19	PES-7	38.96 ^{t}	40	PES-13	56.43 ^{h}	61	PEL-21	21.45 ^{H}
20	PER-3	62.07 ^{f}	41	PES-14	52.41 ^{i}	62	PEL-22	69.36 ^{b}
21	PER-4	42.2 ^{pq}	42	PEL-13	69.38 ^{b}			

Note: P- Plant, E- Endophyte, L- Leaf, R- Root, S- Stem

Means with same letter, in a column do not differ significantly at P= <0.05 as per Duncan Multiple Range Test (DMRT)

The Mysore Journal of Agricultural Sciences

isolates with the values ranging from 15.25 per cent to 71.46 per cent. The highest inhibition percentage was found with the isolate PEL-4 (71.46%) and the lowest percentage was observed with the isolate PER-1 (15.25%) (Table 2) (Fig. 1A). This might be due to the production of secondary metabolites and volatile organic compounds by the bacterial endohytes that suppressed the fungal pathogen. These results obtained are in line with the study made by Sahu and Brahmaprakash (2018), where the inhibition percentage of the fungal pathogens by the various endophytic bacterial isolates varied from 51 per cent to 78.02 per cent against *Scelrotium rolfsii* and nine per cent to 70.81 per cent against *Rhizoctonia solani*. Also, similar results were reported by Maruti and Sriram (2021), while working with both bacterial and fungal endophytes against the wilt pathogen of pomegranate.



(A) (B)

(C) (D)

Fig. 1: (A) Dual culure method; (B) HCN Production; (C) Catalase Test; (D) Starch Hydrolysis

Note : P - Plant, E - Endophyte, L - Leaf, Pathogen - *Alternaria solani*

Characterization of Endophytic Isolates

The morphological characters of the endophytic isolates are mentioned in the Table 2. Among the 10 efficient isolates, eight were Gram negative and two were Gram positive (PER-10 and PEL-20) (Table 3).

Biochemical Characterization of Efficient Bacterial Endophytic Isolates

The primarily screened 10 isolates were subjected to various biochemical tests such as starch hydrolysis, catalase test and HCN production to tentatively characterise and identify the bacteria (Fig. 1). Among the 10 isolates, six tested positive for starch hydrolysis (PEL-4, PES-5, PER-10, PEL-13, PEL-20 and PEL-22) and four were found negative (PEL-5, PEL-6, PEL-8 and PER-6).

Among the 10 bacterial isolates, nine were found positive for catalase activity, where as only one isolate (PER-10) tested negative (Table 4) (Fig. 1C).

TABLE 3

Morphological characteristics of the endophytic bacteria isolated from various parts of potato plant

Isolates	Colony colour	Colony shape	Colony surface	Cell shape
PEL-4	White	Round	Smooth glistening	Rods
PEL-5	Creamish white	Irregular	Smooth glistening	Rods
PEL-6	Yellow	Round	Glistening	Rods
PEL-8	Creamish white	Round	Smooth glistening	Rods
PES-5	Creamish white	Round	Smooth glistening	Rods
PER-6	Yellow	Round	Smooth	Rods
PER-10	Bright white	Round	Smooth shiny	Coccii
PEL-13	White	Irregular	Dull	Rods
PEL-20	White	Round	Smooth glistening	Rods
PEL-22	Creamish white	Round	Smooth	Rods

Note: P- Plant, E- Endophyte, L- Leaf, R- Root, S- Stem

TABLE 4

Biochemical characteristics of endophytic bacteria *in vitro*

Isolate code	Gram reaction	Catalase Test	Starch Hydrolysis	HCN Production
PEL-4	-	+	+	+
PEL-5	-	+	-	-
PEL-6	-	+	-	-
PEL-8	-	+	-	-
PES-5	-	+	+	+
PER-6	-	+	-	-
PER-10	+	-	+	-
PEL-13	-	-	+	-
PEL-20	+	+	+	-
PEL-22	-	+	+	+

Note: P- Plant, E- Endophyte, L- Leaf, R- Root, S- Stem

Later, when the isolates were subjected to HCN production, the filter papers turned to brown in only three isolates (PEL-4, PES-5 and PEL-22) and were taken as positive and the other seven isolates were recorded as negative due to the absence of colour change which indicated that there was no HCN production (Table 4) (Fig. 1B). From these results we can conclude that the isolated bacterial endophytes showed positive results to various biochemical tests separately. These results are in confirmation with the results of Sahu and Brahmaprakash (2018). The isolates possessing various biochemical characteristics have a wider application and usage and are used for further studies. In the present study, the bacterial endophytes inhibit the growth of the pathogen. The efficient isolates which showed highest inhibition per cent of the pathogen in dual culture technique are used for future studies and molecular characterization of the isolates were carried out.

The endophytes are present in each and every plant. They possess various plant growth promoting traits and confer innate resistance to the plants against disease causing pathogens and also against various abiotic stresses. They aid in the growth and development of plants in a positive way and confer numerous beneficial characteristics that are needed to be harnessed commercially. These endophytes are widespread and inhabitate in almost every part of the plants and are acting as natural soldiers to the plants. The biocontrol and growth promotion properties of the endophytes prove to be very useful and improve the plants resistance to the diseases and confer ability to withstand varying environmental conditions.

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