

Original Research Article

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Pseudomonas aeruginosa KPSE 3 – An Endophytic PGPR for Bio Control of Potato Phytopathogens

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ABSTRACT

Endophytic bacteria residing within the living plant tissue without causing harm to it have promise to be practically used in agriculture and included as active components of Integrated Disease Management (IDM). In present study 4 strains of pseudomonad were isolated from surface sterilized plant parts viz. leaf, root, tuber and stem of potato (*Solanum tuberosum* L. cv. Kufri Badshah) by using King's B agar and preliminary characterized as *Pseudomonas* on the basis of morphological and biochemical characteristics. Among all the isolates KPSE3 was emerged as best PGP strain showing production of siderophore and IAA (9.79 µg/ml). Moreover, KPSE3 has capacity of nitrogen fixation (23.57 mg N / g glucose consumed) and phosphate solubilization (21.20 P µg/ml at 3 DAI). The isolate KPSE3 also have broad spectrum antifungal activity with 15 % growth inhibition of *Pythium* sp. 34% of *Fusarium* sp. and 40 % of *Macrophomina* sp. In pot trial conditions potato tuber bacterization with KPSE3 in presence of phytopathogens viz. *Fusarium* sp., *Macrophomina* sp. and *pythium* sp., significantly influenced growth of Potato cv. Kufri Badshah. The endophytic isolate KPSE3 exhibiting the best PGP activity was further characterized at molecular level and identified as *Pseudomonas aeruginosa* (Gene Bank Accessions Number- KC787580) which can be used for developing a cost - effective eco-friendly multifunctional biofertilizer and biocontrol agent for sustainable agriculture.

Keywords

Endophytic,
Siderophore,
Phytopathogen,
Eco-friendly.

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Introduction

Phytopathogenic fungi are the most visible threats for sustainable food production, their control can be achieved by either chemicals or biological measures. Over use of chemicals may cause soil, water and air pollution as well as have harmful effects on human beings and living organisms. Due to increasing awareness among consumers about food

safety, scientists are in search of alternative biological control that lowers down the use of chemicals in agriculture (Gerhadson, 2002). Biological control of plant diseases involving the use of antagonistic microorganisms offers an excellent alternative to chemical control. Pseudomonads have special mechanisms for inhibition of pathogenic fungi by various

mode of action like production of antibiotics, iron chelating compound like siderophores (Meyer, 2000), hydrolytic enzyme and biosurfactants (Nielsen *et al.*, 2002). Competition for favorable nutritional sites (Suslow and Schroth, 1982) β -(1,3)-glucanase and chitinase (Renwick *et al.*, 1991) and cyanide (Flaishman *et al.*, 1996) induction of systemic resistance (Parmar and Dadarwala, 1999) and ability to effectively colonize roots is responsible for plant growth promotion (Defago and Duffy, 1999). Keeping this in mind, the main goal of the current study was to look for the best endophytic plant growth promoting bio-agents which could be applied to the potato to get a higher yield and also work as biocontrol against soil borne pathogen like *Fusarium* sp., *Macrophomina* sp. and *Pythium* sp.

In the present study, an attempt has been made to isolate endophytic bacteria mainly belonging to *Pseudomonas* genus from potato possessing ability to work as plant growth promoting bacteria (PGPB) as well as biocontrol agent.

Materials and Methods

Isolation of endophytic *Pseudomonas* from potato

In present study, isolation of endophytic pseudomonads was attempted from various plant parts (root, leaf, stem and tuber) of potato (*Solanum tuberosum* L.) cv. Kufri Badshah. For isolation potato plant samples were surface sterilized and inoculated in to King's B medium and plates were incubated at 37°C for 48 to 72 h.

Characterization of isolates

Isolates were characterized on the basis of morphological, cultural and biochemical characteristics (Halt *et al.*, 1994).

Antagonistic effect on pathogenic fungi (Biocontrol)

The antagonistic activity of isolates against *Fusarium* sp., *Macrophomina* sp. and *Pythium* sp. was tested by dual culture technique. The radial growth of mycelium was measured and per cent inhibition (PI) was calculated as follows.

$$\text{Per cent inhibition (PI)} = \frac{C - T}{C \times 100}$$

Where, C- mycelial growth of pathogen in control and T- mycelial growth of pathogen in dual plate.

In vivo inhibitory activity of isolates

Small sized potato tubers surface sterilized and injected with 100 μ l of bacterial isolate suspension (10^7 bacterial cells /ml) at one corner and *Fusarium* sp. was inoculated after 24 h at same corner and each treatment was repeated three times. Inoculated tubers were kept in growth chamber at 25-27°C for 21 days at 70% RH. After incubation period, tubers were cut longitudinally from the sites of inoculation and dry rot induced maximal width (W) and depth (d) was noted. The pathogen penetration in to tubers was calculated using formula Penetration (mm) = $(w/2 + (d-6))/2$ (Lapwood *et al.*, 1984).

PGP traits of endophytic isolates viz. N fixation, P Solubilization, IAA and Siderophore production

Nitrogen fixing capacity

For detection of nitrogen fixing capacity of isolates the bacterial cultures were inoculated in nitrogen free media containing glucose as carbon source. Inoculated media were incubated at $28 \pm 2^\circ\text{C}$ for one week and nitrogen fixation rates were measured by

Micro-Kjeldahl method (A.O.A.C., 1965) and sugar utilization was estimated by Fehling's method. The rate of nitrogen fixation was expressed as mg nitrogen fixed/gram of glucose consumed.

Phosphate solubilizing efficiency of endophytic bacteria

Isolates were tested for their phosphate solubilizing capacity in PKVK Medium. Here, PKVK broth was inoculated with 100 µl bacterial culture and soluble phosphate content was estimated as per the method given by APHA, 1995 at 3 and 5 days after inoculation.

Estimation of growth hormone IAA

Isolates were grown in respective medium with 0.1% tryptophan and incubated at a 28 ± 2°C temperature during 24 h on a rotary shaker (90 rpm), after that they were centrifuged at 5000 rpm for 25 min. The supernatant liquid mixed with Salkowski's reagent (1:2) and the colors were measured by spectroscopy at 530 nm after 30 min. The levels of IAA production were estimated by a standard IAA graph.

Detection of siderophore

Detection of siderophore producing organism was carried out by using the method as described and recorded by Schwyn and Neilands (1987).

Efficacy of proven endophytic isolates against Phytopathogenic fungi in potato

In pot experiment the best proven *Pseudomonas* isolate was tested for antifungal activity against soil borne fungi viz. *Fusarium* sp., *Macrophomina* sp. and *Pythium* sp. following completely randomized design with eight treatments viz. T₁: Uninoculated control,

T₂: isolate (seed tuber bacterization @ 5 ml/kg seed), T₃: isolate + *Fusarium* sp., T₄: isolate + *Macrophomina* sp., T₅: isolate + *Pythium* sp., T₆: *Fusarium* sp., T₇: *Macrophomina* sp. and T₈: *Pythium* sp. each with three replications.

Results and Discussion

Isolation of microorganisms, screening for desirable traits and selection of efficient strains are important steps to optimize high crop yields and improve the sustainability of the ecosystem controlling plant pathogen. Endophytic bacteria are one of the most potential biological control agents for plant disease management. The aim of this work is to evaluate the antimicrobial activities of endophytic bacteria isolated from potato (*Solanum tuberosum* L.) and to check their plant growth promoting ability as well as *in vitro* and *in vivo* efficacy for antagonistic characters.

Isolation of endophytic pseudomonads

Plant is the prime source of nutrition for microorganisms in soil providing them nutrients indirectly from root exudates or dead tissues or directly when microorganisms colonize the interior of plant roots or other organs. In this study, after 2-3 days of the inoculation due to limitation of nutrients inside plant part, microorganisms ooze outside the plant part and grow luxuriously in the selective medium.

KPSE1-KPSE4 gave shiny colony appearance and all isolates produced yellow-green diffusible pigment of variable intensities on King's B medium. These observations are supported by findings of Kuarabachew *et al.*, 2007 who isolated *Pseudomonas* spp. from soil samples collected from potato growing areas around Wolyata, Shahamane and Wonodogenet, Ethiopian. Moreover, all the

growth patterns were confirmed keeping standard strains *Pseudomonas fluorescens* as check.

Morphological characterization

The colonies of the four isolates (KPSE1-KPSE4) were irregular, undulate, convex, smooth and slimy, transparent and characteristic smell on KB plate. Isolates produced yellow-green diffusible pigment of variable intensities on King's B medium. The cells of all the four isolates were G⁻ve, rods, which is characteristic feature of genus *Pseudomonas*.

Biochemical characterization

Results of carbohydrate utilization pattern of all isolates are presented in Table 1. Isolates KPSE1-KPSE4 can utilize almost all the tested sugars *viz.* D-glucose, ribose, mannitol, lactose, sucrose, inositol, maltose, arabinose, raffinose, galactose, cellobiose, fructose and xylose. Kuarabachew *et al.*, 2007 reported *P. fluorescens* utilized arabinose, galactose, glucose, fructose, mannose, trehalose. The data revealed that all the tested isolates were positive for catalase, indole production and negative for H₂S production, while all the isolates are positive for gelatinase and urease (Table 1).

Sakthivel and Karthikeyan (2012) studied 30 bacterial isolates obtained from *Coleus forskohlii* rhizospheric soil of Perambalur and Salem districts in Tamil Nadu and discriminated isolates in different genus as *Azospirillum* spp., *Bacillus* spp., *Pseudomonas* spp., and *Azotobacter* spp. on the basis of biochemical characterization. All the biochemical tests for characterization of endophytic bacterial isolates have well established similarities of KPSE1-KPSE4 with genus *Pseudomonas*.

PGP traits of endophytic isolates viz. N fixation, P solubilization, IAA and siderophore production

Nitrogen fixing capacity

Comparison of endophytic diazotrophic bacteria with free living nitrogen fixers showed that internalized bacteria are much more likely to contribute significantly to nitrogen economy of the plant (Cocking, 2003) (Table 2).

In vitro nitrogen fixation efficiency of isolates showed that all the isolates were confirmed to have ability of fix atmospheric nitrogen. It was revealed from the result that nitrogen fixing potentiality of these isolates ranged from 11.23-23.57mg N / g of glucose consumed and isolate KPSE-3 was showing maximum nitrogen fixation capacity among all the isolates (23.57mg N/g of glucose consumed). Akond *et al.*, 2007 studied *Azotobacter* isolates recovered from wheat fields among them highest amount of N was found to be fixed by the isolate M1 (9.26 mg N/g substrate) and the lowest by the isolate M4 (5.45 mg N/g substrate).

Phosphate solubilization efficiency of endophytic isolates

Estimation of P in the PKVK broth revealed that all the strains released P from tri calcium phosphate (TCP). Isolates KPSE3 recorded maximum available phosphate 21.20 P µg/ml at 3 DAI, whereas, all isolates that reduce the phosphates solubilization after 5 DAI compare to 3 DAI because of utilization of solubilized phosphate by microorganisms. The present findings established the phosphate solubilization as an additional benefit of endophytic bacterial isolates, apart from fixing atmospheric nitrogen all the isolates can also improve the availability of phosphorous in crop's rhizosphere.

Estimation of growth hormone IAA

Data regarding IAA production of isolates are presented in Table 2. All tested isolates grown in a culture medium contain tryptophan as carbon source. IAA production was detected by the Salkowski reagent under spectrophotometer in the range of 1.07 µg/ml to 9.79 µg/ml IAA. The higher concentration of IAA was obtained by KPSE3 strain 9.79 µg/ml.

Similarly Pedraza *et al.*, (2004) found that *Azospirillum* strains produced the highest concentrations of IAA (16.5–38 µg IAA/mg protein) whereas, *Gluconacetobacter* and *P. stutzeri* strains produced lower concentrations of IAA ranging from 1-2.9 µg IAA/mg protein in a culture medium supplemented with tryptophan.

Detection of siderophore

Isolates KPSE1-KPSE4 were found positive for siderophore production. Similarly, Dave and Dube (2000) isolated six rhizobacterial *P. fluorescens* on Chrom Azurol S agar medium. Among six isolates, two gave maximum siderophore production. Siderophore chelates iron and other metals contribute to disease suppression by conferring competitive advantages to biocontrol agents.

Antagonistic effect on pathogenic fungi (Biocontrol)

***In vivo* inhibitory activity of isolates**

Out of 4 native endophytic *Pseudomonas* isolates, KPSE3 and KPSE4 showed inhibition of the phytopathogenic fungus *Fusarium* sp. (Figure. 1) compared with control. Results showed that isolates KPSE3 (2.22 mm) showing minimum Penetration of *Fusarium* sp. compare with the control (5.9 mm) (Table 3).

Similar type of results was also reported by Remadi *et al.*, (2006). They reported that bacillus application into sites of infection, 24 h before inoculation by pathogen, reduced dry rot and population of plant pathogenic fungi *viz.* *F. oxysporum*, *F. graminearum*, *F. Solani* in presence of an active host resistance by whole tuber assay.

***In vitro* interaction of isolate**

All native endophytic *Pseudomonas* isolates were tested for antifungal activity against plant pathogenic fungi *Pythium* sp., *Fusarium* sp. and *Macrophomina* sp. by dual culture assay. After seven days incubation, fungal hyphae of phytopathogenic fungi were unable to reach the bacterial culture and inhibition zone was established with the dimension of inhibition circle, ranging from 5 to 40 mm. Highest antifungal activity was recorded by isolate KPSE3 (40 %) followed KPSE4 (10%) against *Macrophomina* sp. Highest antifungal activity against *Fusarium* sp. was recorded for isolate KPSE3 (34%) and the highest antifungal activity was recorded by isolate KPSE3 (15%) against *Pythium* sp. Among all the tested isolates, KPSE3 found to have broad spectrum of antifungal activity against of *Macrophomina* sp. (40 %), *Fusarium* sp. (34%) and *Pythium* sp. (15%). So from antifungal activity results, the most promising culture KPSE3 was selected for bacterization in a replicated pot trial (Table 4). Similar type of results were recorded by Singh *et al.*, 2008 they reported isolate *B. subtilis* BU 1 strongly inhibited growth of *M. phaseolina* with maximum inhibition of 74 % at 5 DAI. They also reported inhibition of *F. oxysporum* and *Pythium* sp. by the same isolate. At the same time, Naik *et al.*, (2008) reported antifungal potential of *P. fluorescens* isolate towards number of plant pathogenic fungi including *F. oxysporum*, *M. phaseolina* and reported that the fungal growth was inhibited with zone diameter of 10 and 36 mm (Table 4).

Table.1 Characterization of native isolates

Sr NO.	Test	KPSE 1	KPSE 2	KPSE 3	KPSE 4
Morphological characteristics					
1	Shape	Irregular	Round	Round	Round
2	Margin	Undulate	Entire	Entire	Entire
3	Elevation	Convex	Slightly raised	Slightly raised	Slightly raised
4	Texture	Smooth & slimy	Smooth	Smooth	Smooth
5	Opacity	Transparent	Transparent	Transparent	Transparent
6	Microscopic characterization	Gram negative short rod	Gram negative short rod	Gram negative short rod	Gram negative short rod
Biochemical characteristics					
7	Glucose	+	+	+	+
8	Ribose	+	+	+	+
9	Mannitol	+	+	+	+
10	Lactose	+	+	+	+
11	Inositol	+	+	+	+
12	Maltose	+	+	+	+
13	Arabionose	+	+	+	+
14	Raffinose	+	+	+	+
15	Galactose	+	+	+	+
16	Cellobiose	+	+	+	+
17	Fructose	+	+	+	+
18	Xylose	+	+	+	+
19	Sucrose	+	+	+	+
20	Gelatinase	+	+	+	+
21	Methyl red	-	-	-	-
22	Urease	+	+	+	+
23	Catalase	+	+	+	+
24	Indole production	+	+	+	+
25	Starch hydrolysis	-	-	-	-
26	H ₂ S production	-	-	-	-

Table.2 PGPR traits of isolates

No.	Isolate No.	N fixation	P solubilization		IAA production
		mg N / g of glucose consumed	P µg /ml 3 DAI	P µg /ml 5 DAI	µg/ml
1	KPSE1	14.33	10.2	5.8	6.07
2	KPSE2	13.60	12.7	8.0	4.92
3	KPSE3	23.57	21.2	17	9.79
4	KPSE4	11.23	15	5.0	1.07

Table.3 *In vivo* effect of endophytic isolates on *Fusarium* sp. causing potato dry rot

Sr. No.	Isolate No.	mean radial growth (Penetration(mm))
1.	KPSE3	2.2
2.	KPSE4	4.8
3.	Control	5.9

Table.4 *In vitro* biological control potential of native endophytic isolates against phytopathogenic fungi

Sr. No.	Name of isolate	<i>Fusarium</i> sp. (%)	<i>Macrophomina</i> sp. (%)	<i>Pythium</i> sp. (%)
1.	KPSE3	34	40	15
2.	KPSE4	29	10	0

Table.5 Effect of bacterization with the best isolate KPSE3 in potato to control phytopathogenic fungi

Tr. No.	Treatment	Germination (%)	30 DAS height (cm)	60 DAS height (cm)	Fresh biomass (g)	Dry biomass (g)
T1	Uninoculated Control	87.50	15.37 ^{ab}	37.00 ^{ab}	39.25 ^{ab}	15.25 ^b
T2	KPSE3	93.75	17.62 ^a	39.25 ^a	41.75 ^a	17.22 ^a
T3	KPSE3+ <i>Fusarium</i> sp.	87.50	15.22 ^{ab}	33.75 ^{cd}	35.70 ^{bc}	10.75 ^c
T4	KPSE3+ <i>Macrophomina</i> sp.	81.25	14.50 ^{ab}	30.25 ^e	32.50 ^{cd}	12.00 ^c
T5	KPSE3+ <i>Pythium</i> sp.	62.50	14.25 ^{ab}	35.00 ^{bc}	30.62 ^d	11.00 ^c
T6	<i>Fusarium</i> sp.	75.00	15.25 ^{ab}	32.00 ^{de}	21.50 ^e	8.00 ^d
T7	<i>Macrophomina</i> sp.	62.50	14.12 ^{ab}	27.75 ^f	25.00 ^e	6.50 ^{de}
T8	<i>Pythium</i> sp.	50.00	12.87 ^b	21.75 ^g	17.50 ^f	5.50 ^e
	S.Em	-	1.28	0.74	1.17	0.52
	CD At 5 %	-	3.70	2.14	3.37	1.50
	C.V %	-	17.15	4.62	7.65	9.61

Note: Treatment means with the letter/letters in common are not significant by Duncan's New Multiple Range Test at 5% level of significance

Figure.1 Radial growth inhibition of *Fusarium* by isolate KPSE 3 and KPSE 4



Figure.2 Phylogenetic tree of partial 16S rRNA genes of pseudomonas spp. KPSE3 isolate from closely related of bacteria obtained after BLAST

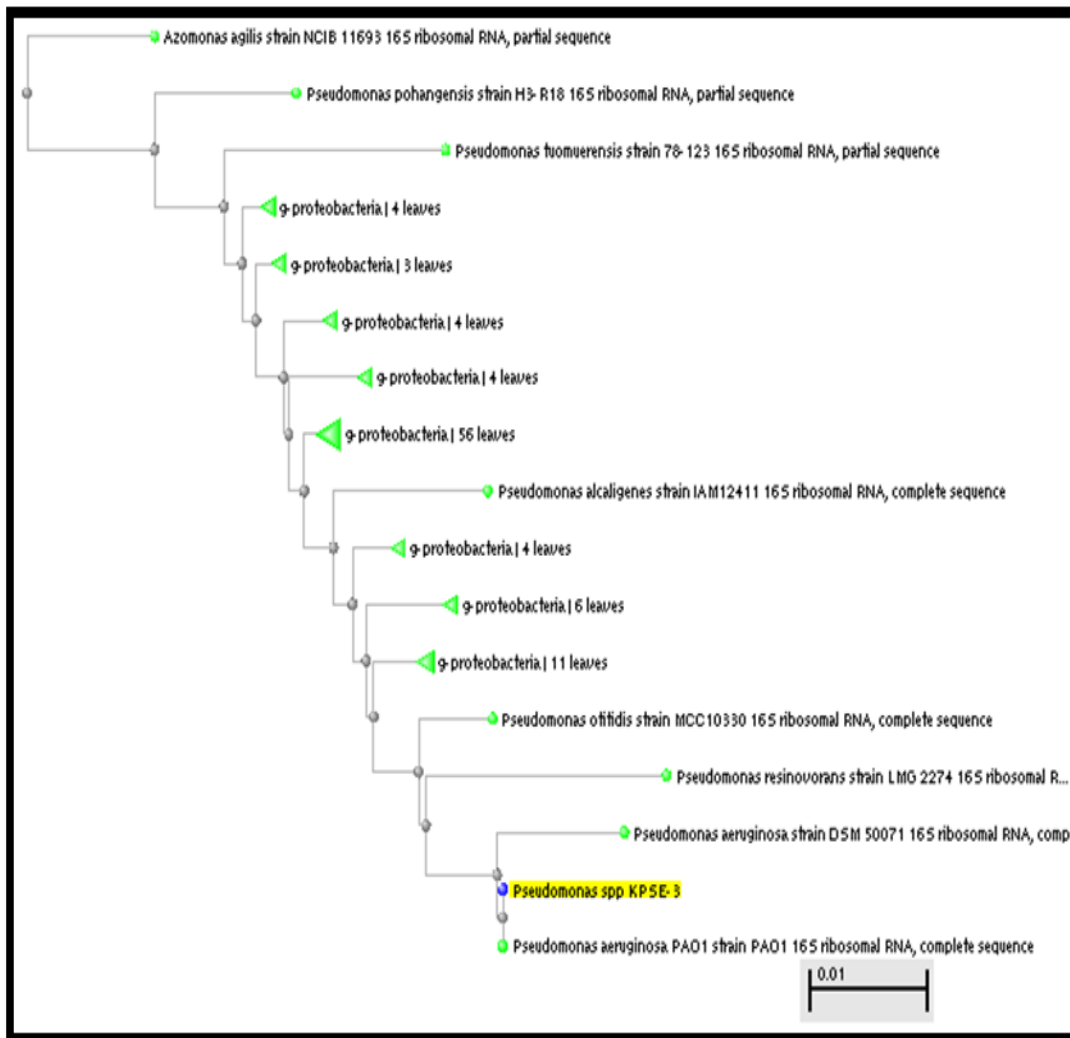
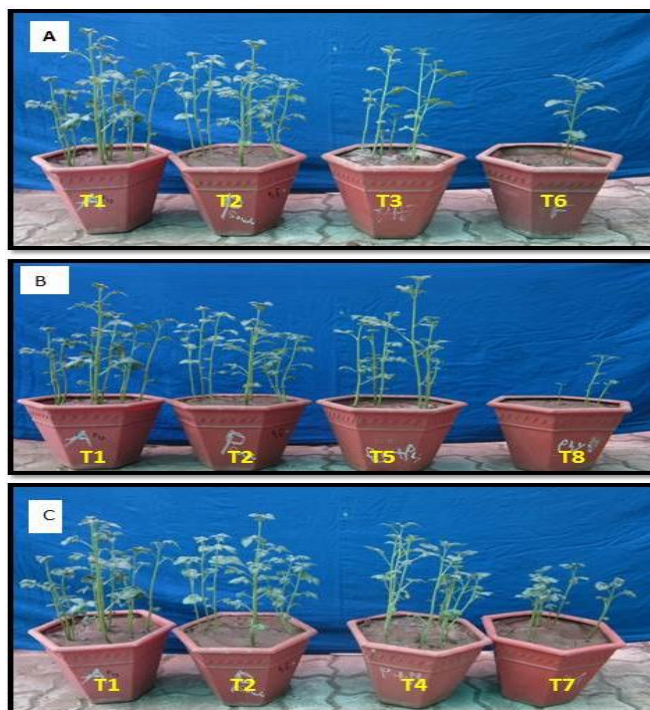


Figure.3 Pot efficacy of PGPB (KPSE3) in presence of pathogenic fungi on potato crop at 30 DAS



16S *rRNA* gene sequencing and identification of native potential KPSE3 isolate

16S *rRNA* partial gene sequence identified isolate KPSE3 as *Pseudomonas* sp. with 100% similarity and 100 % query coverage to *Pseudomonas aeruginosa* (GeneBank Accn. No: KC787580). Additionally, the phylogenetic position of the isolate was also worked out within the available database of NCBI (presented as phylogenetic tree in figure 4.4). The phylogenetic tree indicated that KPSE3 belong to *Pseudomonas aeruginosa* PAO1 γ - proteobacteria and grouped with other *Pseudomonas* sp. The tree formed two major group/clusters within available known γ - proteobacterial sp. Isolate KPSE3 was placed in cluster one with more than 99 % similarity with known *Pseudomonas* sp. indicating phylogenetic closeness (Figure 2).

Efficacy of proven endophytic isolate KPSE3 (*P. aeruginosa*) (GeneBank Accn. No: KC787580) against phytopathogenic fungi in potato

In pot experiment the best proven isolate KPSE3 was tested for antifungal activity against phytopathogenic fungi viz. *Fusarium* sp., *Macrophomina* sp. and *Pythium* sp.

A pot trial was carried out with recommended dose (220 kg /ha (RDN) +110 Kg/ha (RDP) +220(RDK)) to study the effect of endophytic bacterial isolate KPSE3 on Potato cv. Kufri Badshah and also to demonstrate plant protection aspect in potato due to bio-inoculants.

It was observed from the pot experiment that KPSE3 treated plants were noticeably taller and had more fresh and dry weight (Table 5).

Effect of endophytic isolates KPSE3 on potato plants germination per cent, plant height at 30 and 60 DAI, fresh and dry biomass

Results presented in table 5 indicted that treatment T3, T4, T5 receiving seed tuber inoculation of KPSE3 along with pathogenic fungi viz. *Pythium* sp., *Fusarium* sp. and *Macrophomina* sp. showed significant increase in germination per cent (Figure 3), plant height, fresh and dry biomass of potato as compared to treatment T6, T7, T8 pathogenic control.

Singh *et al.*, 2010 reported that fluorescent *P. aeruginosa* PN1 increased plant germination percentage, shoot and root length, shoot and root fresh as well as dry weight compared to non- bacterized control in pot trial containing *M. phaseolina*-infested soil. PN1 exhibited strong antagonistic property against *M. phaseolina*, suppressed the disease *in vitro*.

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