Screening of endophytic bacteria from potato tubers and their antagonistic activity against soil-borne potato pathogens

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Abstract

In order to appraise the bacterial endophyte communities that help resist disease in potato tuber, the separation, the population density, biodiversity and the antagonistic activity of endophytic bacteria, from the tuber peel of potato cultivars (Fontan90, Agria, Sante'a and Jeli89), were examined in the Fars province in Iran. In this study, the bacterial endophyte Colony Forming Units (CFU) were counted based on the most suitable dilution in petri dishes and expressed per g of wet weight of tuber tissue. The presence of bacteria was found mostly in the outer layer. A wide variety of endophyte species biodiversity was in Agria cultivar. To estimate the antagonistic effect of potato associated endophytic bacteria, 115 bacterial isolates were evaluated by dual culture method against main soil-borne potato pathogens *Fusarium oxysporum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Streptomyces scabies* and *Ralstonia solanacearum*. Endophyte strains were identified based on physiological, morphological and chemical characteristics and the 16S rRNA gene sequence analysis. The highest degree of the inhibitory activity in all layers of potato cultivars was related to *Bacillus subtilis*, *Bacillus mojavensis* and *Klebsiella variicola*. Antagonistic activity of endophytic bacteria against the pathogens was significantly higher ($p<0.01$) in the examined strains from the outermost layer of tuber peel and decreased progressively toward the center of the tuber. In this research, *Klebsiella variicola* was reported as endophyte bacteria in the four commercial potato cultivars mentioned above, for the first time.

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Introduction

Plants host a specific group of microorganisms called endophytic bacteria, able to both promote growth and prevent infection by pathogens.[1] They are found in plant tissues, such as fruits,[2] stems,[3] roots,[4] flowers[5] and tubers.[6] The interaction between endophytic bacteria and their host plants is not completely understood. However, many isolates seem to have beneficial effects on their hosts.[4] Endophyte communities ameliorate disease development, and in some instances, plant-endophyte relationships have been found to be tissue type and tissue site specific.[7] Some research has also shown a link between endophyte bacteria and phytopathogens in a variety of plants. For example, Lastochkina *et al*.[8] indicated that the presence of endophytic bacteria can control the infection caused by fungal pathogens in infected potatoes. More attention has been paid to endophytic bacteria regarding antagonistic bioactivities, including biological control of plant pathogens, plant growth promoting, nitrogen-fixing.[9]

The connection between endophytic bacteria and their hosts makes them an attractive option for biocontrol agents and organic agriculture.[7] In recent decades, manipulation of plant-microbial ecosystems by inoculating seeds with beneficial bacterial endophytes, or encouraging the early development of beneficial endophyte communities has been suggested in sustainable crop produc-

tion systems as a method to improve crop productivity, as well as helping to acclimatize plants to environmental stresses.[8] Sturz *et al*.[10] showed that endophyte bacteria affected the resistance of potato tubers to soil-borne diseases. In their study, soft rot disease development was negatively correlated with the density of tuber populations of endophytic bacteria able to inhibit growth of *Erwinia* sp. *in vitro*. Endophytic bacteria have already been implicated in the *de novo* synthesis of structural compounds and fungitoxic metabolites that occur at the sites of phytopathogen attack.[11]

Nowadays, it is known that the use of endophytic bacteria leads to severe reduction of soil borne pathogens on a large scale and their use is a new alternative to control the plant diseases. The ability to produce secondary metabolites, ecological niche and nutrient competition and the induction of systemic acquired resistance are the biocontrol mechanisms of endophytic bacteria.[12] However, the existence of an inhibition zone of bacteria in successive layers would be beneficial to counter invasions by soil-borne pathogens. Currently, little is known on endophytes associated to potato, their beneficial effects on the crop and the influence of the environment on the bacterial population. This research examined biodiversity, population density and antagonistic activity of endophytic bacteria to evaluate whether the location of bacterial endophyte communities contributes to disease resistance in potato tubers.

Materials and Methods

Preparation of potato tubers

The study was done at a greenhouse in Shiraz Islamic Azad University in 2020. The soil was sandy-loam prepared using a minimum tillage system. Cultural practice and fertilizer application practice were made. The potato tubers were grown in a randomized complete-block experimental design with three replicates. Twenty healthy excrescences of each type of potatoes were selected for planting.[13] All potato tubers (approximately 30-40 g) of each cultivar (Fontan90, Agria, Sante'a and Jeli89) were cultivated in rows 1 meter apart from each other, with a plant spacing of 20-25 centimeters. All of them were harvested by hand 120 days later.

Distribution of bacteria in potato peels

Endophytic bacterial isolation was done according to the method explained by Shi *et al*.[14] Tubers were randomly sampled and cleaned with 2% aliquots of commercial detergent (safeguard fruit & veggie wash) and running tap-water for the purpose of removing soil from the outside of them. They were then surface-sterilized with sodium hypochlorite solution (5% available Cl^{-}) and hydrogen peroxide solution (3%) for three minutes each, and then rinsed three times in sterile distilled water. The surface tension depressant polyoxyethylene sorbitan monolaurate (Tween 20, Fisher Scientific, Montreal, Quebec, Canada) was used in all of the hypochlorite and rinsing solutions (1mL Tween per 20 L).[15] To verify that the sterilization process was successful and that no biological pollution from the surface of the potato was transmitted into the tuber, the surface tissues were pressed onto Potato Dextrose Agar (PDA) and Tryptic Soy Agar (TSA). They were then incubated at 22°C for 5 days and the cultures were examined for the presence or absence of growth of bacteria colonies. Three sets of potato peel, 3 mm thick, were sampled sequentially from the outer skin (periderm) surface towards the tuber center (designated Peel 1, Peel 2 and Peel 3, respectively).

Isolation and enumeration of bacteria

Potato layers were weighed and macerated for 3 minutes in a commercial blender. They were then shaken for 1 hour. Serial dilutions were made, and 0.1 mL of each diluted solution was spread on the TSA plates (replicated three times). The samples were incubated at 22°C for 3 days. Following incubation, the most suitable dilution series were selected and the number of Colony Forming Units (CFU) per Petri plate was counted, then expressed per g of wet weight tuber tissue.[16] Bacterial isolates were grouped by their phenotypic characteristics (color, shape, rate of growth and colony morphology) and gram staining reaction.[17,18] Representatives were selected from each group for antagonistic tests and their inhibition was measured.

Antagonistic Test

Three strains of fungal and two strains of bacterial phytopathogens for antimicrobial activities assays were used in this study. The tested isolates, *Fusarium oxysporum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Streptomyces scabies* and *Ralstonia solanacearum* were collected in Persian Type Culture Collection (PTCC), Tehran, Iran. Potato Dextrose Agar (PDA) was used for maintaining fungal phytopathogens and Nutrient Agar (NA) was used for bacteria culture.

For the antifungal assay, the dual cultures method was used. A plug of mycelium of each fungus (5 mm diameter) was plated at the center of the petri dishes containing PDA. A circular line, made with a 6 cm diameter Petri dish dipped in a suspension of endophytic bacteria (5×10^{-9} cfu mL^{-1}), was placed surrounding the fungal inoculum. All the plates were incubated at 22°C for 5 days and the inhibition effects were assessed by measuring the diameters of the inhibition zones.[19]

For the antibacterial assay, the pathogen was cultured as a slime layer in the Petri dish containing NA and an actively growing culture of the antagonist was introduced as a spot 5 mm in diameter.[20] Petri dishes were incubated at 30°C for 24-48 hours after interactions were examined and the distance of the inhibition growth were measured and recorded (all the experiments were scored on a scale of 1–4, where 1= no growth, 2= low growth, 3= moderate growth and 4= overgrowth).[10]

Identification and characterization of antagonist bacteria

The physiological, biochemical and morphological characterization of antagonist bacteria with high activity levels were done by the protocols in Bergey's manual of systematic[17] and by using standardized methods of bacteriology.[18] The physiological identification was done on the basis of colony color, shape, size, pigment and gram staining. Biochemical characterization included catalase production, starch hydrolysis, oxidase, gelatinase, indole test, urease, citrate and nitrate utilization were investigated for each bacteria species that finally led to the identification of antagonist bacteria at the genus level.[18] The best antagonists were selected and identified at the species level by a 16S rRNA analysis.

16S rRNA gene amplification and sequencing

Genomic DNA was prepared by the pure individual isolates by means of purification using a Genomic DNA extraction kit (Bioneer, Seoul, Korea) according to the manufacturer's protocol. PCR was performed with the universal 16S rRNA gene primers: 27f: 5′ GAGAGTTTGATCCTGGCTCAG-3′ and 1495r: 5′-CTACGGCTACCTTGTTACGA-3′.[21] The PCR mixture (50 µL) contained 5 µL of 10× PCR buffer with 15 µmol L^{-1} $MgCl_2$, 200

μmol L^{-1} of each deoxynucleotide triphosphate (Takara), 10 pmol of each primer, 1.5 units of Taq DNA polymerase and 1 μL of DNA template. The PCR was performed in a thermocycler, with a thermal profile of 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 45 seconds with an extension at 72°C for 1.5 minutes, and a final elongation at 72°C for 6 minutes.[22] An approximate 1500 bp PCR fragment of the 16S rRNA gene was obtained for each isolate. DNA sequencing was performed through the Macrogen sequencing service (Seoul, Korea). In this research, the sequences obtained were deposited in the NCBI GenBank. Each sequence was compared to the reference sequences in GenBank, using a BLAST search. The phylogenetic tree was constructed for endophytic bacterial isolates along with the sequences from selected references strains. The analysis was conducted with MEGA (version 7.0) using neighbor-joining method (Bootstrap analysis with 1000 replicates).[23]

Statistical analyses

The plant infectivity studies were made under a Complete Randomized block Design (CRD), with each treatment replicated twice. Raw data was imported to Microsoft Excel 2010 program for calculations and graphical representation. The SPSS version 17.0 program was used for analysis of variance. Quantitative changes of different parameters were analyzed through Analysis Of Variance (ANOVA), with Duncan's multiple range test at $p<0.01$ being used to determine significant differences among treatments.

Results

Colony counts

In these experiments, peel layers were colonized by a total of 32 genera comprising 115 species. Among the obtained genera and according to their phenotypic properties, only 8 isolates were selected (Table 1). There were no statistically significant differences in the population densities of endophytic bacteria and peel layers (1, 2 and 3). As it was observed, the population density of endophyte bacteria after the end of the incubation period and the appearance of colonies were plenty more variable in all 3 layers. There was a general sequence from the outermost peel (peel 1) to the innermost peel (peel 3) and the highest population density was in peel 1. The most common endophytic bacteria were *Bacillus* sp., followed by *Pseudomonas* sp. and large variety of endophyte species biodiversity was in Agria peels.

Identification of screened antagonists

Among the studied endophytic bacteria, eight isolates that had the highest abundance and antagonist behaviors in all layers were selected. There was great diversity in colony morphology, including Gram reaction, size, shape and color. Nearly half of the bacterial isolates were Gram positive, all of them were rod shaped and produced pigments (Table 2). Biochemical tests and physical properties showed that strain SKK_1, strain SKK_2, strain SKK_3 and strain SKK_4 were mostly related to genus *Bacillus* sp.; strain SKK_5 belongs to the

Table 1. Identification and relative percent frequency of bacterial species recovered within each peel layer (top 3mm: peels 1-3, peel 1 outermost) in four potato cultivars. SKK: Strain codes of bacterial species isolated from potato peels. Based on theexamination of 115 antagonisticbacteriaisolates recoveredfromcvs Fontan90, Agria, Sante'a and Jeli 89 peels, respectively.

Species	Fontan 90			Agria			Sante'a			Jeli 89			Cumulative
	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	Total
Bacillus sp. SKK_1	-	30.1	5	-	16.67	10	20	25	23.1	-	28.58	24	182.45
Bacillus sp. SKK_2	33.33	23.1	20	-	33.33	30	-	25	30.77	-	21.42	20	236.95
Bacillus sp. SKK_3	-	-	30	-	8.33	10	-	-	-	-	14.28	8	70.61
Bacillus sp. SKK_4	-	-	5	16.67	-	-	-	8.33	7.7	28.58	7.14	-	73.42
Klebsiella sp. SKK_5	33.33	15.40	25	33.33	25	20	-	16.67	23.1	28.58	-	16	236.41
Pseudomonas sp. SKK_6	33.33	15.40		16.67	-	10	40	16.67	-	42.85	-	12	186.92
Pseudomonas sp. SKK_7	-	15.40	5	16.67	8.33	5	20	8.33	15.39	-	21.42	12	127.54
Xanthomonas sp. SKK_8	-	-	10	16.67	8.33	15	20	-	-	-	7.14	8	85.14
CFU per g fresh weight	3.9×10^3	3.2×10^3	3.4×10^3	6×10^4	5×10^3	2.3×10^4	3.3×10^3	3×10^3	5×10^2	2.3×10^3	2×10^3	2×10^1	1.07×10^5

Table 2. Morphological characteristics of the endophytic bacterial strain (SKK: Strain codes of bacterial species isolated from potato peels).

Strain	Gram reaction	Shape	Size	Margin	Elevation	Textures	Pigmentation
Bacillus sp. SKK_1	Positive	Rod	1.0-4.0 μm	Irregular	Flat	Mucoid	White
Bacillus sp. SKK_2	Positive	Rod	2.0-4.0 μm	Irregular	Flat	Smooth	Cream
Bacillus sp. SKK_3	Positive	Rod	1.0-5 μm	Entire	Convex	Rough	White
Bacillus sp. SKK_4	Positive	Rod	1.0-4.0 μm	Entire	Raised	Smooth	Yellow
Klebsiella sp. SKK_5	Negative	Rod	0.5-1 μm	Entire	Convex	Mucoid	Pink
Pseudomonas sp. SKK_6	Negative	Rod	1.5-3 μm	Irregular	Raised	Smooth	Yellow
Pseudomonas sp. SKK_7	Negative	Rod	1.5-5 μm	Entire	Convex	Smooth	Green
Xanthomonas sp. SKK_8	Negative	Rod	2-2.5 μm	Entire	Convex	Smooth	Yellow

genus *Klebsiella* sp., while strain SKK_6 and strain SKK_7 were placed in the genus *Pseudomonas* sp. and strain SKK_8 isolate was assigned to the genus *Xanthomonas* sp., based on these characteristics (Table 3). The biochemical characterization studies for the pathogen and antagonists were done twice with three replications.

To identify three of the eight above antagonistic isolates that appeared to be the most effective for suppression - *Bacillus* sp. SKK_1, *Bacillus* sp. SKK_2 and *Klebsiella* sp. SKK_5 - the 16S rRNA gene sequence was amplified and compared.

The partial 16S rRNA gene sequences of the isolates were deposited in the NCBI GenBank with the accession number KP109754, KP109755 and KP109756. Using partial 16S rRNA sequencing, strain *Bacillus* sp. SKK_1 was determined to be *Bacillus subtilis,* strain *Bacillus* sp. SKK_2 as *Bacillus mojavensis,* and strain *Klebsiella* sp. SKK_5 as *Klebsiella variicola*.

Sequence analysis indicates that isolates *Bacillus subtilis*, *Bacillus mojavensis* and *Klebsiella variicola* had 100.0%, 99.3% and 99.7% similarity to related bacteria in the NCBI database, respectively. A Phylogenetic tree was constructed using the partial 16S rRNA sequences of the putative endophytic bacterial isolates and representative bacterial type strains of related taxa generated by neighbor-joining method with 1000 bootstrap replications is presented in (Figure 1). With 8 identified species, *Klebsiella variicola* is reported as endophytes of potato cultivars for the first time. To explore the potential applications of these bacterial endophytes, we intend to make this isolate available to scientists who are interested in doing further research.

Antagonistic Test

All bacterial antagonists tested inhibited mycelial growth of fungal pathogens and bacterial pathogens, but they had different antagonistic behaviors against soil-borne pathogens so that, some of them were more active than the others. However, there were significant differences among the bacterial strains (Table 4 and Table 5).

Fusarium oxysporum

Endophytic bacteria with the highest degree of inhibition examined were obtained from Peel 1 of Fontan90. For *Fusarium oxysporum*, the greatest degree of antibiosis was obtained from Peel 1, followed by Peel 2. This was related to *Bacillus* sp. strain SKK_1 and *Bacillus* sp. strain SKK_2, with 25 mm diameter inhibition zones ($p<0.01$; Table 6).

Rhizoctonia solani

For *Rhizoctonia solani*, the most antibiosis bacteria were recovered from the outermost (peel 1) of Sante'a. This was related to *Klebsiella* sp. strain SKK_5 with 22 mm diameter inhibition zones. Although the difference in degree of antibioses between Peel 1 and 2 was not significant, their difference in reaction of the *Rhizoctonia solani* to the endophytes was statistically significant ($p<0.01$; Table 7).

Verticillium dahliae

The degree of endophytes inhibition to *Verticillium dahliae*, overall, declined from the outermost peel to the innermost peel in Fontan90 and Agria. This was related to *Klebsiella* sp. strain SKK_5, which had inhibition zone diameters of 22 mm. For Fontan90 and Jeli89, endophytes from Peel 2 were the most effective. This was related to *Bacillus* sp. strain SKK_1 and *Bacillus* sp. strain SKK_2, where the diameters of inhibition zones were 20 mm. Those from Peel 3 were the least effective ($p<0.01$; Table 8).

Ralstonia solanacearum

Three of these antagonists' bacteria indicated a statistically significant effect on the degree of antibiosis when they were tested

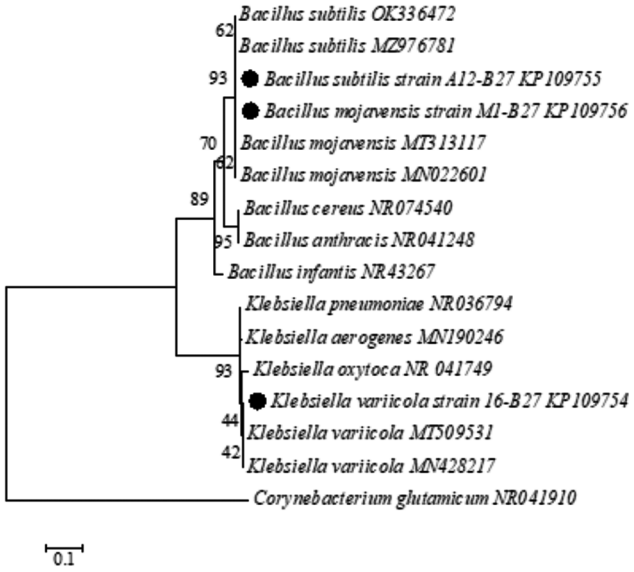

Figure 1. Phylogenetic tree of partial 16S rRNA sequences of the endophytic bacteria strains isolated from potato tubers along with the sequences from NCBI and based on neighbour-joining method using MEGA 7 with 1000 bootstrap replications. *Corynebacterium glutamicum* (NR041910) was used as an outgroup in the analysis.

Table 3. Biochemical characteristics of the endophytic bacterial strain (SKK: Strain codes of bacterial species isolated from potato peels).

Strain	Oxidase	Catalase	Gelatinase	Urease	Starch	Indole	Citrate	Nitrate
Bacillus sp. SKK_1	–	+	+	–	+	–	+	+
Bacillus sp. SKK_2	+	+	+	–	+	–	+	+
Bacillus sp. SKK_3	+	+	+	+	+	–	+	+
Bacillus sp. SKK_4	+	+	+	–	+	–	+	+
Klebsiella sp. SKK_5	–	+	+	+	+	+	–	+
Pseudomonas sp. SKK_6	+	+	+	–	+	–	+	+
Pseudomonas sp. SKK_7	+	+	–	–	–	–	+	–
Xanthomonas sp. SKK_8	+	+	+	–	+	–	–	–

+, Positive reaction; –, Negative reaction.

Table 4. Levels of *in vitro* antagonistic activity in endophyte bacteria recovered from different peel layers (top 3mm, peels 1–3, peel 1 outermost) of potato tubers of four cultivars (Fontan90, Agria, Sante'a and Jeli89) against a plug of fungal pathogens on a scale of 1–4, from 1= complete inhibition of fungal growth, to 4= no effect. SKK: Strain codes of bacterial species isolated from potato peels.

Species		*Fusarium oxysporum*					*Rhizoctonia solani*					*Verticillium dahliae*					
	Peel layer	Fontan 90	Agria	Sante'a	Jeli 89	Mean	Fontan 90	Agria	Sante'a	Jeli 89	Mean	Fontan 90	Agria	Sante'a	Jeli 89	Mean
Bacillus sp. SKK_1	1	1	1	3	2	1.7	2	3	1	2	2	1	1	2	3	1.7
Bacillus sp. SKK_1	2	1	3	1	2	1.7	2	3	3	4	3	1	2	1	3	1.7
Bacillus sp. SKK_1	3	1	2	1	3	1.7	4	3	2	1	2.5	1	2	1	1	1.2
Bacillus sp. SKK_2	1	1	1	2	2	1.5	2	3	1	2	2	1	1	3	3	2
Bacillus sp. SKK_2	2	1	4	1	3	2.2	2	3	1	2	2	2	1	1	2	1.5
Bacillus sp. SKK_2	3	2	1	1	3	1.7	3	3	2	2	2.5	1	3	1	1	1.5
Bacillus sp. SKK_3	1	2	2	4	1	2.2	3	3	1	4	2.7	4	4	4	2	3.5
Bacillus sp. SKK_3	2	3	3	4	2	3	4	4	4	3	3.7	4	4	3	3	3.5
Bacillus sp. SKK_3	3	3	4	3	4	3.5	4	4	4	2	3.5	4	4	2	3	3.2
Bacillus sp. SKK_4	1	1	3	4	2	2.5	3	4	3	3	3.2	4	1	4	2	2.7
Bacillus sp. SKK_4	2	3	3	4	4	3.5	4	4	4	3	3.7	4	3	3	4	3.5
Bacillus sp. SKK_4	3	4	3	4	4	3.7	4	4	4	2	3.5	4	1	3	3	2.7
Klebsiella sp. SKK_5	1	1	1	2	1	1.2	1	3	1	2	1.7	1	1	3	1	1.5
Klebsiella sp. SKK_5	2	1	2	2	4	2.2	2	2	1	2	1.7	1	2	3	1	1.7
Klebsiella sp. SKK_5	3	1	1	1	3	1.5	2	3	1	1	1.7	3	2	1	1	1.7
Pseudomonas sp. SKK_6	1	4	3	4	3	3.5	4	3	4	3	3.5	3	3	3	4	3.2
Pseudomonas sp. SKK_6	2	4	3	3	4	3.5	4	4	4	3	3.7	4	4	2	4	3.5
Pseudomonas sp. SKK_6	3	4	3	2	4	3.2	4	4	4	3	3.7	4	3	2	2	2.7
Pseudomonas sp. SKK_7	1	4	3	4	3	3.5	3	2	4	3	3	2	3	4	4	3.2
Pseudomonas sp. SKK_7	2	4	4	4	4	4	4	4	4	2	3.5	4	3	4	4	3.7
Pseudomonas sp. SKK_7	3	4	4	4	4	4	4	4	4	2	3.5	4	3	4	3	3.5
Xanthomonas sp. SKK_8	1	4	4	4	1	3.2	4	3	3	4	3.5	3	3	2	4	3
Xanthomonas sp. SKK_8	2	4	4	3	4	3.7	4	4	3	4	3.7	3	4	3	4	3.5
Xanthomonas sp. SKK_8	3	4	4	3	4	3.7	4	4	4	3	3.7	3	2	3	4	3

Table 5. Levels of *in vitro* antagonistic activity in endophyte bacteria recovered from different peel layers (top 3mm, peels 1–3, peel 1 outermost) of potato tubers of four cultivars (Fontan90, Agria, Sante'a and Jeli89) against a plug of bacterial pathogens on a scale of 1–4, from 1= complete inhibition of fungal growth, to 4= no effect. SKK: Strain codes of bacterial species isolated from potato peels.

Species		*Ralstonia solanacearum*					*Streptomyces scabies*				
	Peel layer	Fontan 90	Agria	Sante'a	Jeli 89	Mean	Fontan 90	Agria	Sante'a	Jeli 89	Mean
Bacillus sp. SKK_1	1	1	1	1	1	1	1	1	2	1	1.2
Bacillus sp. SKK_1	2	1	3	1	1	1.5	1	2	1	4	2
Bacillus sp. SKK_1	3	2	3	1	1	1.7	2	3	1	1	1.7
Bacillus sp. SKK_2	1	1	1	3	1	1.5	1	1	2	1	1.2
Bacillus sp. SKK_2	2	1	2	3	2	2	1	1	2	1	1.2
Bacillus sp. SKK_2	3	1	3	3	3	2.5	2	1	1	2	1.5
Bacillus sp. SKK_3	1	3	4	4	3	3.5	4	4	4	2	3.5
Bacillus sp. SKK_3	2	4	4	4	4	4	4	4	3	2	3.2
Bacillus sp. SKK_3	3	4	4	4	4	4	4	4	2	3	3.2
Bacillus sp. SKK_4	1	4	4	4	4	4	4	2	4	3	3.2
Bacillus sp. SKK_4	2	4	4	4	4	4	4	4	4	3	3.7
Bacillus sp. SKK_4	3	4	4	4	4	4	4	4	3	4	3.7
Klebsiella sp. SKK_5	1	1	1	2	1	1.2	1	1	2	2	1.5
Klebsiella sp. SKK_5	2	2	1	2	2	1.7	1	2	1	1	1.2
Klebsiella sp. SKK_5	3	2	1	3	2	2	3	2	1	3	2.2
Pseudomonas sp. SKK_6	1	2	2	3	4	2.7	2	3	4	4	3.2
Pseudomonas sp. SKK_6	2	3	3	4	4	3.5	3	3	4	3	3.2
Pseudomonas sp. SKK_6	3	4	4	4	4	4	4	3	4	3	3.5
Pseudomonas sp. SKK_7	1	2	3	4	3	3	3	2	4	4	3.2
Pseudomonas sp. SKK_7	2	2	4	4	4	3.5	4	3	4	2	3.2
Pseudomonas sp. SKK_7	3	3	4	4	4	3.7	4	4	4	3	3.7
Xanthomonas sp. SKK_8	1	4	4	2	2	3	2	2	4	4	3
Xanthomonas sp. SKK_8	2	4	4	4	3	3.7	3	2	4	4	3.2
Xanthomonas sp. SKK_8	3	4	4	4	4	4	3	3	4	4	3.5

against the *Ralstonia solanacearum* for inhibition. In Jeli89, *Bacillus* sp. strain SKK_1 from Peel 1 and 2 with inhibition zones over 25 mm (p=0.01) was generally more effective against all phytopathogens than the *Bacillus* sp. strain SKK_1 recovered from layer 3 (p<0.01; Table 9).

Streptomyces scabies

In Fontan 90, *Bacillus* sp. strain SKK_1 and *Bacillus* sp. strain SKK_2 were the antagonists that indicated the best inhibitory effect, with inhibition zones over 25 mm (p=0.01) from layer 1. In comparison, there was no significant difference in inhibitory effect between *Streptomyces scabies* isolated from layer 2 and 3. Antibiosis to pathogens was the greatest in bacterial isolates obtained from layer 1 (p<0.01; Table 10).

Discussion

Many features of a plant's surface function as physical barriers to pathogens penetration. The epiderm as a physical barrier, is the first line of defence in plants. Pathogens must break down this layer and physical barriers to enter the plant.[8] The presence of endophytes in host tissues can enhance their resistance against pathogens by eliciting the host response or by producing antagonistic metabolites themselves. Endophytes and the host plant thus work in tandem to protect the plant from pathogens.[24] In the present study, a total of 115 endophytic bacterial isolates of potato tubers' periderm (cultivars Fontan90, Agria, Sante'a and Jeli89) were identified from peels 1, 2 and 3 in respect to their potential as

Table 6. Variation in antibiosis (inhibition zone mm) by *Fusarium oxysporum* based on the examination of the best antagonistic bacteria recovered from different peel layers (top 3mm, peels 1–3, peel 1 outermost) of potato tubers.

	Bacillus subtilis			*Fusarium oxysporum* *Bacillus mojavensis*			*Klebsiella variicola*			Effect against pathogen
	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2 2	Peel 3	Peel 1	Peel 2	Peel 3	
Fontan 90	25^{a}	22^{a}	17^{d}	25^{a}	19^{c}	13^{e}	20^{b}	20^{b}	17^{d}	**
Agria	18^{c}	9^{g}	11^{f}	18^{c}	17^{d}	17^{d}	14^{de}	7^{i}	0^{k}	**
Sante'a	10^{g}	17^{d}	22.7^{a}	13^{ef}	19^{c}	19^{c}	13^{ef}	13^{f}	19^{c}	**
Jeli 89	15^{d}	11^{g}	7^{h}	15^{d}	9^{h}	9^{g}	17^{c}	17^{d}	9^{g}	**
Mean	17^{a}	14.7^{b}	14.2^{b}	17.7^{a}	16^{a}	14.5^{b}	16^{a}	14.2^{b}	8.7^{c}	**

Different lowercase letters in each column indicate significant difference between variables at p<0.01 probability level. Values followed by same letters have no significant differences in the same column of the table. **Indicates that comparison differences are significant between variables at each column (p<0.01).

Table 7. Variation in antibiosis (inhibition zone mm) by *Rhizoctonia solani* based on the examination of the best antagonistic bacteria recovered from different peel layers (top 3mm, peels 1–3, peel 1 outermost) of potato tubers.

	Bacillus subtilis			*Rhizoctonia solani* *Bacillus mojavensis*			*Klebsiella variicola*			Effect against pathogen
	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	
Fontan 90	15^{e}	11^{f}	5^{h}	15^{e}	15^{c}	10^{ef}	19^{c}	11^{f}	11^{e}	**
Agria	10^{gh}	7^{h}	7^{g}	10^{gh}	10^{f}	10^{ef}	10^{gh}	14^{d}	9^{f}	**
Sante'a	20^{b}	10^{f}	11^{e}	20^{b}	17^{b}	11^{e}	22^{a}	20^{a}	20^{b}	**
Jeli 89	14.2^{e}	17^{b}	17^{c}	11^{gh}	11^{f}	11^{e}	12^{f}	14^{d}	25^{a}	**
Mean	14.8^{b}	11.2^{b}	10^{c}	14^{b}	13.2^{b}	10.5^{c}	15.7^{b}	14.7^{b}	16.2^{a}	**

Different lowercase letters in each column indicate significant difference between variables at p<0.01 probability level. Values followed by same letters have no significant differences in the same column of the table. **Indicates that comparison differences are significant between variables at each column (p<0.01).

Table 8. Variation in antibiosis (inhibition zone mm) by *Verticillium dahliae* based on the examination of the best antagonistic bacteria recovered from different peel layers (top 3mm, peels 1–3, peel 1 outermost) of potato tubers.

	Bacillus subtilis			*Verticillium dahliae* *Bacillus mojavensis*			*Klebsiella variicola*			Effect against pathogen
	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	
Fontan 90	18^{c}	18^{b}	17^{d}	22^{a}	13^{d}	20^{b}	20^{b}	20^{a}	7.2 j	**
Agria	22^{a}	14^{d}	14^{e}	20^{b}	15.7^{c}	7^{k}	16^{e}	11^{e}	15^{e}	**
Sante'a	12^{f}	17^{b}	20^{b}	10^{g}	17^{b}	17^{d}	10^{g}	10^{e}	20^{b}	**
Jeli 89	8^{i}	8^{g}	23^{a}	10^{g}	14^{d}	19^{c}	20^{b}	20^{a}	20^{b}	**
Mean	15^{b}	14.2^{b}	18.5^{a}	15.5^{b}	14.9^{b}	15.7^{b}	16.5^{a}	15.2^{b}	15.5^{b}	**

Different lowercase letters in each column indicate significant difference between variables at p<0.01 probability level. Values followed by same letters have no significant differences in the same column of the table. **Indicates that comparison differences are significant between variables at each column (p<0.01).

biofertilizers. The presence of bacteria was found mostly in the outer layer. It is speculated that the greater abundance of CFU in peel 1 may be due to the higher sugar content in this layer.[25,26] This is the first report of endophytic bacteria isolated from potato cultivars of Fontan90, Sante'a and Jeli89. These bacteria were seen mostly from Agria cultivar. The selected isolates could be grouped into four genera, *i.e.*, *Bacillus* (4 strains), *Klebsiella* (1 strain), *Pseudomonas* (2 strains) and *Xanthomonas* (1 strain). Similarly, other researchers have done reports about the isolation of endophytic bacteria from maize,[27] carrots,[28] common bean,[29,30] and sugar beet.[31] There was significant diversity in the types of endophytes bacteria, both genotypic and phenotypic.[32] There could be many different factors, for example, plant age, geographical distribution, host specificity, plant age and tissue type.[33] The greater population density of bacteria in the periderm of potato tubers shows the colonization of the periderm by bacterial endophytes. The population densities of culturable endophytic bacteria in this study were similar to the population densities of isolates that Costa *et al*.,[29] obtained from soybean leaves in herbicide-free soil. Many of the endophytic bacterial found in this work were already reported by Lopez-Lopez *et al*.,[34] and many species of genus *Bacillus* were observed in bean seeds by Chimwamurombe *et al*.[35] The presence of certain genera in different potato cultivars indicates that they are better compatible to live as endophytic bacteria in *Klebsiella variicola* than as other genera.

There are many reports about the role of endophytic bacteria and isolation of them from different tissue of plants.[36] These roles included stimulating plant growth, combating phytopathogens and, inducing resistance.[37] In general, bacteria can have interactions with their host, such as symbiosis, cooperation, or antagonism. However, little is known about antagonist bacteria and their effects on plants. Research on the factors affecting antagonistic bacteria is important in order to be used for the biological management of plant diseases. Endophytic bacteria with the ability to stimulate growth and control of plant diseases can be successful in biological control and sustainable agriculture.[38]

This study evaluated the ability of antagonist bacteria *in vitro,* isolated from the periderm of different potato cultivars to control *Fusarium oxysporum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Streptomyces scabies* and *Ralstonia solanacearum*. Although highly variable, the lowest community of antagonists was always found in the third peel, while the highest percentage of antagonist bacteria resided in peel 1.[10] The effect that isolated bacteria have on the growth of pathogens were evaluated using standard methods. The presence and size of inhibition zones have been used as evidence for considering the antagonistic potential of the endophytic bacteria.[39] All of the tested pathogens' inhibition zone diameters were larger than 10 mm. The inhibition halo of *Fusarium oxysporum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Streptomyces scabies* and *Ralstonia solanacearum* were up to 25, 22, 22, 25 and 22 mm, respectively. Therefore, they may control various plant diseases and can also be considered as biological control agents for these three fungal pathogens and two bacterial pathogens. Based on a 16S rRNA gene sequence, three endophytes (*Bacillus subtilis*, *Bacillus mojavensis* and *Klebsiella variicola*) were identified and introduced. *Bacillus subtilis*,[40,41] *Bacillus mojavensis*[42] and *Klebsiella variicola*[43,44] are used as plant growth promoters, and they can play important roles in phytoprotection, phytoremediation, human health, environmental pollutions and applications of chemical fertilizer.

This research is the first report that identifies *Klebsiella variicola* as endophytic bacteria in potato cultivars (Fontan90, Agria, Sante'a and Jeli89). The use of these isolated antagonists can also be effective in reducing the chemical control of plant diseases and the negative environmental effects of pesticides on agricultural systems where edible plant products are grown. The use of soil-

Table 9. Variation in antibiosis (inhibition zone mm) by *Ralstonia solanacearum* based on the examination of the best antagonistic bacteria recovered from different peel layers (top 3mm, peels 1–3, peel 1 outermost) of potato tubers.

	Bacillus subtilis			*Ralstonia solanacearum* *Bacillus mojavensis*			*Klebsiella variicola*			Effect against pathogen
	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	
Fontan 90	20^{b}	17^{d}	15^{c}	20^{b}	20^{b}	20^{a}	18^{d}	14^{f}	12.7^{d}	**
Agria	18^{d}	10^{i}	10^{e}	20^{b}	12^{h}	7^{f}	19^{c}	19^{c}	16.5^{b}	**
Sante'a	19^{c}	22^{a}	18^{b}	10^{h}	7^{j}	7^{f}	14^{f}	13^{g}	7^{f}	**
Jeli 89	22^{a}	22^{a}	22^{a}	20^{b}	15^{e}	10^{e}	6.7^{e}	15^{e}	15^{c}	**
Mean	19.7^{a}	17.7^{a}	16.2^{a}	17.5^{a}	13.5^{b}	11^{b}	14.4^{b}	15.2^{b}	12.8^{b}	**

Different lowercase letters in each column indicate significant difference between variables at p<0.01 probability level. Values followed by same letters have no significant differences in the same column of the table. **Indicates that comparison differences are significant between variables at each column (p<0.01).

Table 10. Variation in antibiosis (inhibition zone mm) by *Streptomyces scabies* based on the examination of the best antagonistic bacteria recovered from different peel layers (top 3mm, peels 1–3, peel 1 outermost) of potato tubers.

	Bacillus subtilis			*Streptomyces scabies* *Bacillus mojavensis*			*Klebsiella variicola*			Effect against pathogen
	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	Peel 1	Peel 2	Peel 3	
Fontan 90	25^{a}	18^{c}	11^{f}	25^{a}	20^{b}	14.7^{d}	20^{d}	18^{c}	8^{h}	**
Agria	19^{e}	14^{d}	10^{f}	23^{b}	20^{b}	19^{c}	22^{c}	15^{d}	15^{d}	**
Sante'a	14^{g}	17^{c}	23^{a}	14^{g}	14^{d}	19^{c}	14^{g}	17^{c}	21^{b}	**
Jeli 89	20^{d}	22^{a}	19^{c}	17^{f}	20^{b}	14^{d}	12^{h}	18^{c}	9^{g}	**
Mean	19.5^{a}	17.7^{a}	15.7^{b}	19.7^{a}	18.5^{a}	16.6^{a}	17^{a}	17^{a}	13.2^{b}	**

Different lowercase letters in each column indicate significant difference between variables at p<0.01 probability level. Values followed by same letters have no significant differences in the same column of the table. **Indicates that comparison differences are significant between variables at each column (p<0.01).

derived antagonists to protect plants and increase yields is a promising approach in new sustainable farming systems. Future research should lead to details of how these strains work, and field studies should be conducted to confirm the effectiveness of these isolated antagonists under natural conditions.

Conclusions

In this research, we described the main soil borne diseases affecting potato production. For the control of potato diseases, previous research has focused predominantly on chemical methods, prophylactic strategies, and genetic selection. More recently, there has been a surge in the exploration of biological control methods albeit, with limited in-depth studies. Biological control is especially worthy of exploration in view of current trends to limit the use of environmental pollution due to pesticides use. The study of endophytic microorganisms is important to comprehend their interaction with their host plants. We found that the population densities of bacteria from peel layers were highly variable, and there was a fairly consistent relationship between depth of tuber peel layer and antifungal activity of the bacteria recovered. This can be utilized in future applications and considered an interesting and potentially useful selection criterion for plant protection programs. Additionally, the results of this study indicated that *Bacillus subtilis*, *Bacillus mojavensis* and *Klebsiella variicola* are broad-spectrum antagonist bacteria and provide us with new insights in the biological control of plant disease which are good for growth stimulation and diseases prevention.

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