Pak. J. Bot., 54(5): 1813-1820, 2022. DOI: http://dx.doi.org/10.30848/PJB2022-5(22)

ISOLATION OF ENDOPHYTES THAT SUPPRESS PATHOGEN GROWTH OF CHICKPEA (*CICER ARIETINUM* L.)

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Abstract

Plant microbial pathogens destroy a significant percentage of the world's crops annually. The most common method of combating these pathogens is with chemical microbicides which can have toxic effects upon human health. Our objective was to isolate microbial biocontrol agents that had the ability to suppress plant pathogens. In this study, we isolated endophytic bacteria from leaves of tomato, potato, and pepper plants. Three of the strains were found to have antimicrobial activities and were identified as *Bacillus megaterium*, *Bacillus subtilis* and *Staphylococcus caprae* based on 16S rRNA sequencing. All three strains were tested for agar-based antibacterial activity against *Escherichia coli* and antifungal activity against pure isolates of *Fusarium oxysporum* and *Fusarium verticillioides.* *B. subtilis* had the most robust antimicrobial potential, followed by *S. caprae* and *B. megaterium*. Both *B. subtilis* and *S. caprae* conferred antifungal resistance to chickpea seeds. HPLC analysis of cell-free supernatants (CFS) from the endophytic bacteria cultured in different media demonstrated production of acetic, lactic, and propionic acid at various levels. Biochemical profiling by Biolog phenotypic microarray demonstrated that *B. subtilis* could utilize a wider range of carbohydrates, carboxylic acids, and amino acids as carbon sources compared to *S. caprae.* These endophytes can potentially be used as a pretreatment of chickpea seeds to reduce common fungal infections.

Key words: Antimicrobial, Biocontrol, Endophyte, *Bacillus subtilis*, *Staphylococcus caprae,* Chickpea.

Introduction

Endophytes are microbes that spend either their entire or a portion of their life cycle inside plant tissues without manifesting any apparent disease symptoms or damage (Saikkonen, 2004). Most plants are hosts to bacterial or fungal endophytes (Tan & Zou, 2001). These microbes are thought to originate from the microbial communities in the rhizosphere where they enter host plants through the sites of wounds or natural openings. Many tissues have been investigated for the isolation of endophytes, including roots, stems and leaves (Hallmann *et al.*, 1997), roots being the most populated source of microbes compared to the structures above ground (Rosenblueth & Martínez-Romero, 2006). Endophytes can interact more closely with their host plants compared to rhizobacteria because they reside within the plant tissue with readily available nutrients (Weyens *et al.*, 2009).

Endophytes have been investigated for their ability to protect plant health and increase crop yield. It has been demonstrated that these microorganisms promote plant growth through a variety of mechanisms, such as nutrient acquisition and hormone production (Santoyo *et al.*, 2016; Afzal *et al.*, 2019; Ghosh *et al.*, 2021). Endophytes can also allow plants to grow under stressful environmental conditions, such as drought, high salinity, and high metal concentration (Li *et al.*, 2020; Naveed *et al.*, 2020; Tseng *et al.*, 2020). Plant pathogens can be inhibited by various mechanisms, such as production of antibiotics, host defense induction, organic acids, and competitive exclusion (Ryan *et al.*, 2008; Eljounaidi *et al.*, 2016; Kovanda *et al.*, 2019; Coban, 2020). There are a variety of methods for inoculating the plants with endophytes. They can be applied to the aerial portions of plants using an aqueous spray (Rajab *et al.*, 2020). Endophytes have also been directly injected into the trunks of trees (Brooks *et al.*, 1994; Rabiey *et al.*, 2019). However, the most common strategy is to incorporate the endophytes directly into soil or coated onto the plant seeds (Lastochkina *et al.*, 2020; Rajab *et al.*, 2020; Rivas-Franco *et al.*, 2020). Multiple commercial endophyte formulations are available: AGTIV (Premier Tech Agriculture), ASCEND (BioScientific), BIO-N (NIMBB, Philippines), BioPower (NIBGE, Pakistan), BIOPROMOTER (Manidharma Biotech), Endomycorrhizal Inoculant (Bio-organic), FOSFORINA (Soils Institute, Cuba), MaxQ (Pennington Seed), Nitrofix (Ruchi Biochemicals), Optimize (Monsanto), Serenade (Bayer), and Symbion-N (T. Stanes Company) (Abbasi & Weselowski, 2014; Kauppinen *et al.*, 2016; Johnston-Monje *et al.*, 2019).

Like most other living organisms, plants are the target of several pathogenic species. *Fusarium* is one of the best known pathogenic fungi. Members of this genus are responsible for causing a number of diseases in a wide variety of plants, such as wilt in chickpea and banana and head blight in wheat and barley (Gopalakrishnan *et al.*, 2011; McMullen *et al.*, 2012; Ploetz, 2015). The primary approach to combat *Fusarium* is use of fungicides, but the toxicity of fungicides, such as tebuconazole and carbendazim, towards humans has been documented previously (Singh *et al.*, 2016; Zhou *et al.*, 2016). In this context, use of endophytes as biocontrol agent will be an environmental friendly approach against plant diseases (Gray & Smith, 2005). The biocontrol potential of endophytic bacterial such as bacilli, has been already demonstrated in previous studies (Janga *et al.*, 2017; Khan *et al.*, 2018).

This study focuses on isolation, identification and evaluating antimicrobial potential of endophytes residing within leaf tissue of various plants. They were further characterized based on their biochemical characteristics, 16S rRNA analysis, and ability to synthesize organic acids.

Materials and Methods

Isolation and identification of endophytes: Endophytes were isolated from leaf tissue of tomato, potato, and pepper. Potato samples were acquired from a greenhouse facility at the USDA-ARS in Albany, CA, USA. Tomato and pepper leaves were collected from an organic garden. Leaves from each plant were aseptically cut using a sterile blade inside a biosafety cabinet. The outer surfaces were sterilized by submerging the leaves in 1% solution of sodium hypochlorite (NaOCl) for 1 minute, followed by air drying for 10 minutes inside the cabinet. Afterwards, sterilized and air-dried leaves were chopped into smaller pieces, and cuttings from the midrib portion were added to deMan, Rogosa, and Sharpe (MRS) broth or Luria-Bertani (LB) broth and placed in an orbital shaker at 30°C and 37°C for overnight incubation. After signs of visible growth, aliquots were spread over MRS agar plates and incubated for 24-48 hours at 30°C and 37°C.

Colonies were screened for antibacterial and antifungal activities on agar plates as described below. Those colonies that had antimicrobial activities were further characterized by sequencing regions of the 16S rRNA gene which were amplified using the 27f and 1525r primer pair (Gond *et al.*, 2015) by colony PCR. 27f: 5'-agagtttgatcmtggctcag-3', 1525r: 5'-aaggaggtgwtccarcc-3'

A portion from a bacterial colony was dissolved in 5 µl of bacterial lysis buffer (CelLytic B Cell Lysis Reagent; Sigma-Aldrich, MO, USA) and incubated at room temperature for 40 min. Afterwards, 45 µl of deionized water was added, and 2 µl of the diluted cell lysate were used in a PCR reaction according to manufacturer's directions (Herculase; Agilent Technologies, TX, USA). PCR products representing the 16S rRNA genes were processed with the Clean & Concentrator kit (Zymo Research, CA, USA) and sequenced with the 27f and 1525r primers (Elim Biopharmaceuticals, CA, USA).

Antibacterial activity assays: The agar overlay method was used for the evaluation of antibacterial activity against *E. coli* (JM109; Promega, CA, USA) and overnight cultures of endophytes in MRS broth were grown. A loopful of inoculum from each was streaked on an MRS agar plate and incubated at 37°C for two days. Inoculum from an overnight culture of *E. coli* was added to 10 ml of soft LB agar (0.7 % agar) to an OD_{600}=0.2 and was then poured over the endophytic bacterial streaks. Plates were allowed to solidify and then placed at 30°C for overnight incubation. Antibacterial activity in the form of inhibition zones was observed the next day.

Antifungal activity assay - Co-culturing on agar plates: For the assessment of antifungal properties, a spot-on lawn method was used, and the target fungi were *Fusarium oxysporum* and *Fusarium verticillioides*. Briefly, fungal spores were dispensed in 20 ml of liquified warm potato dextrose agar (PDA) to a density of 10^5/ml. This PDA with fungal spores was then poured into Petri dishes and allowed to solidify. Afterwards, 5 µl spots of each overnight endophyte culture were applied to the surface of the agar. Plates were incubated at 30°C and were observed at day 3 for antifungal activity.

Antifungal activity assay - Culturing on chickpea seeds: Overnight cultures of endophytes grown in MRS broth were centrifuged, and the pellets were resuspended in sterile 0.1% carboxymethyl cellulose (CMC) solution to an OD_{600} of 0.7. Chickpea seeds were sterilized by soaking in 1% NaClO solution for 1 minute followed by air-drying. Afterwards, seeds were incubated in the CMC bacterial suspension for 40 minutes followed by air-drying inside the biosafety cabinet. The seeds were then placed in the incubator at 37°C for overnight incubation. Fungal spore suspensions for *F. oxysporum* and *F. verticillioides* were prepared in deionized distilled water at a concentration of 1×10^4 spores/ml. One ml of this fungal spore suspension was then applied onto the overnight incubating chickpea seeds using a spray bottle. Treated seeds were placed in Petri dishes that were sealed with Parafilm to avoid moisture loss. Plates were incubated at 25°C, and fungal growth was monitored for several days. Each plate contained approximately 50 seeds. All experiments were replicated.

Estimation of organic acids: Endophytic bacteria were inoculated in MRS and LB broth supplemented with 2% glucose and incubated in an orbital shaker (225 rpm) at 37°C for 48 hours. Cultures were centrifuged, and the supernatants were collected, filtered, and analyzed for the presence of organic acids. The acid analysis was performed on an Agilent HPLC system (Agilent, CA, USA) which was equipped with a quaternary pump, an autosampler with a 100 µl loop, and a refractive index detector. Flow rate was 0.5 ml/min at 40°C, and the analysis was performed isocratically with a 300 × 7.8 mm i.d. cation exchange column equipped with a cation H+ microguard cartridge (Bio-Rad Laboratories, CA, USA). The mobile phase was 0.01 M H_2SO_4. Solutions of analytical grade organic acids (lactic, acetic, and propionic acid) were used as standards. Unused media (MRS broth and LB broth supplemented with 2% glucose) were also analyzed to determine background organic acid levels. Acid concentrations were calculated on Microsoft Excel by the regression equation method.

Biochemical profiling by phenotypic microarray: Novel endophytes were analyzed for their biochemical attributes in Biolog Phenotypic Microarray (Biolog Inc., CA, USA) system as described by Mackie *et al.*, with slight modifications (Mackie *et al.*, 2014). Briefly, bacteria were subcultured for two consecutive cycles over BUG+B agar plates at 37°C. Cells were picked from the plates using sterile cotton swabs and suspended in growth media (IF-0a, PM1, 2, and Dye Mix F). One hundred µl of the cell suspension were added to each well of a PM1 microarray plate. The plates were placed in the Biolog

Omnilog incubator at 37°C for real time analysis up to 48 hrs. Microbial growth was measured in arbitrary Omnilog units (AOU), and the minimum threshold of microbial growth was set at 50.

Results

Endophyte isolation and identification: Endophytic strains isolated from various plant types (tomato, potato, and pepper) were screened for their antibacterial (Fig. 1) and antifungal (Fig. 2) activities as described below. Those strains that displayed antimicrobial activities were identified by isolating the 16S rRNA genes and analyzing the sequences by BLAST analysis to identify the microbes (Table 1) (Altschul *et al.*, 1990). The 16S rRNA sequence of each microbe had 100% identity to multiple strains of the same species.

Antimicrobial activity: Two of the endophytic isolates, *Bacillus subtilis* and *Staphylococcus caprae*, conferred observable antagonistic activity against *E. coli* on agar plates (Fig. 1). Co-inoculated plates showed clear halos around endophytic bacterial streaks which indicated the production of antibacterial compounds into the surrounding agar.

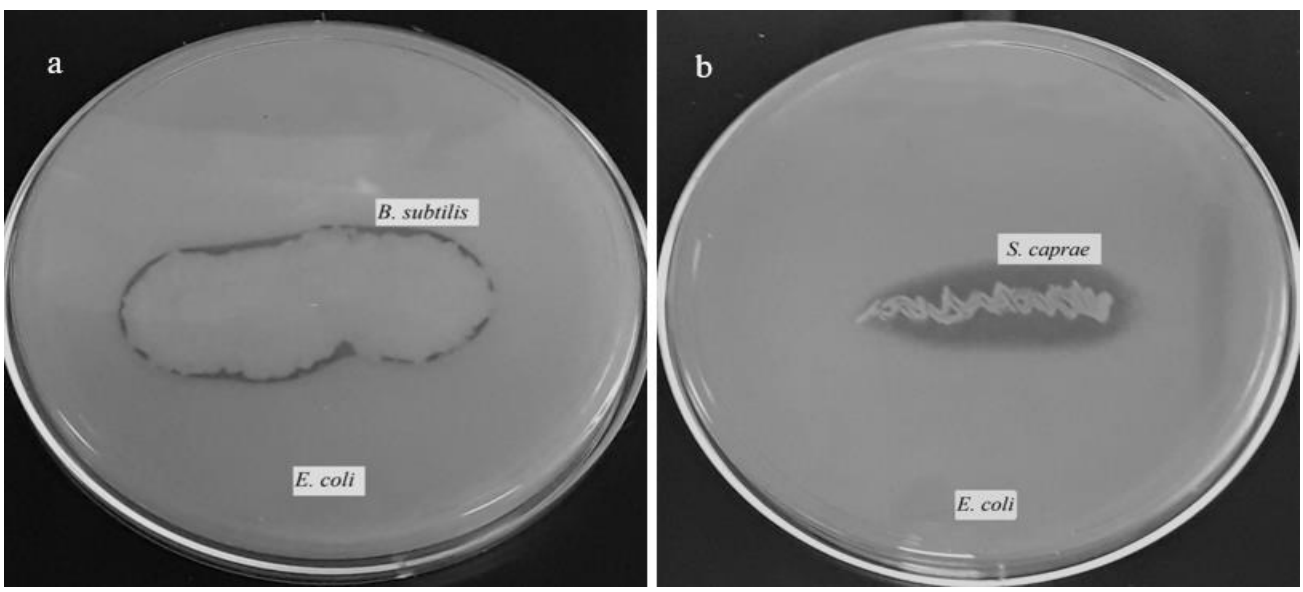

Fig. 1. Antibacterial activity of (a) *B. subtilis* and (b) *S. caprae* against *E. coli.* The agar overlay method was used for the evaluation of antibacterial activity against *E. coli*. Pure cultures of each endophyte were streaked and incubated on MRS agar plates. *E. coli* was added to soft LB agar and poured over the endophytic bacterial streaks. Plates were allowed to solidify and incubated at 30°C. Antibacterial activity in the form of inhibition zones was observed the next day.

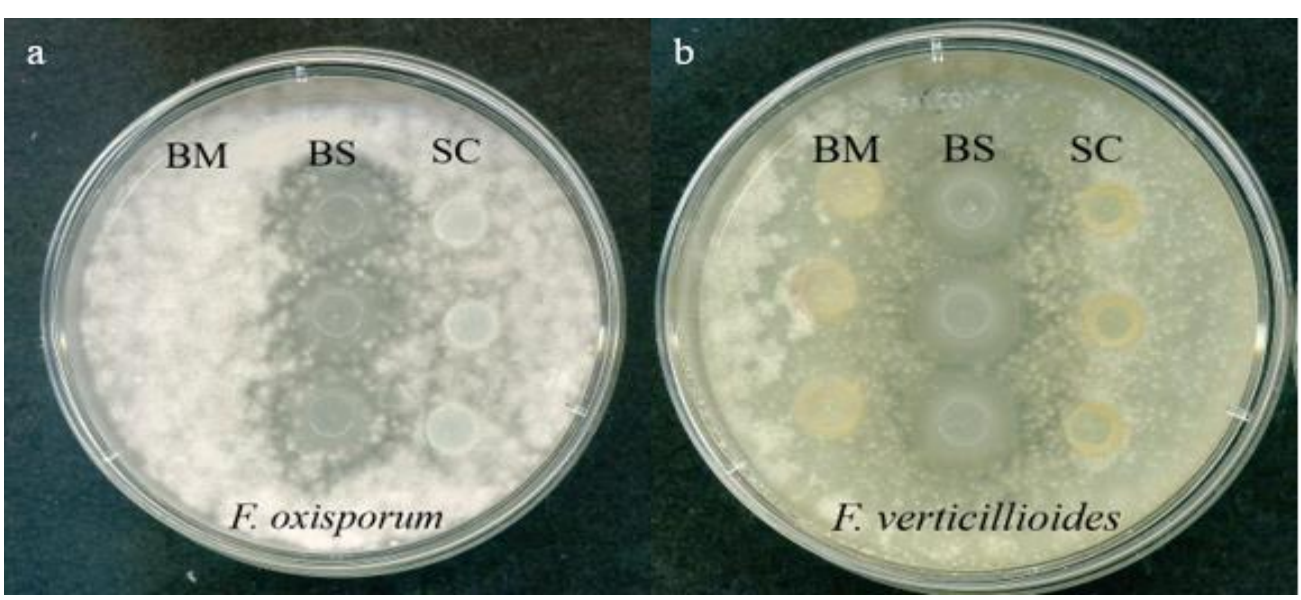

Fig. 2. Antifungal activity of endophytes against (a) *F. oxisporum* and (b) *F. verticillioides*. A spot-on lawn method was used to determine antifungal activity. *F. oxysporum* and *F. verticillioides* fungal spores were dispensed in liquified potato dextrose agar, poured into Petri dishes, and allowed to solidify. Afterwards, 5 µl spots of each overnight endophyte culture were applied to the surface of the agar. After 3 days incubation at 30°C, plates were observed for antifungal activity. (BM) *Bacillus megaterium*; (BS) *Bacillus subtilis*; (SC) *Staphylococcus caprae*.

The endophytes also demonstrated antimicrobial activity in an agar-based experiment against two fungal species: *Fusarium oxysporum* and *Fusarium verticillioides*. Antifungal activity was observed as halos around the bacterial colonies (Fig. 2). *B. subtilis* had the most robust antifungal activity against the two fusaria. *S. caprae* was also effective against both fusaria although to a lesser extent than that of *B. subtilis*. *Bacillus megaterium* had antifungal activity although it was much lower compared to the other two endophytes. *B. megaterium* had no inhibitory effect on *F. oxysporum* growth and a smaller inhibition against *F. verticillioides*.

Table 1. Microbial identification based on 16S rRNA analysis.

Isolate	NCBI acc. #	Source
Bacillus megaterium strain AN2	MT032309	Tomato leaves
Bacillus subtilis strain AN3	MT032310	Potato leaves
Staphylococcus caprae strain AN4	MT032311	Pepper leaves

The antifungal activity of *B. subtilis* and *S. caprae* was tested on chickpea seeds, a more natural, relevant substrate. Seeds were primed with *B. subtilis* or *S. caprae* cells and then inoculated with *F. oxysporum* and *F. verticillioides* fungal spores. After 7 days of growth at 30°C, the plates were analyzed for fungal growth (Figs. 3 and 4). Fungal growth appeared as a white powdery layer over the chickpea seeds. No fungal growth was observed on the control CMC-only seeds which were not treated with fungal spores (Figs. 3a and 4a), whereas, mycelial growth was clearly observed on plates inoculated with only fungal spores (Figs. 3b and 4b). Pretreating the chickpea seeds with *B. subtilis* greatly inhibited growth of both *F. oxysporum* and *F. verticillioides* on the chickpea seeds (Figs. 3c and 4c). Although *S. caprae* also inhibited growth of both fusaria, the degree of inhibition was less than that of *B. subtilis* (Figs. 3d and 4d). Overall, *B. subtilis* manifested better antifungal activity compared to *S. caprae* (Table 2). These results parallel the antifungal experiments conducted on the agar plates.

Table 2. Fungal growth on chickpeas seeds in the presence and absence of endophytic bacteria.

Plate	Fungal growth*
CMC only	-
F. oxysporum (Fo)	++++
F. verticillioides (Fv)	++++
B. subtilis + Fo	+
B. subtilis + Fv	+
S. caprae + Fo	++
S. caprae + Fv	++

*Arbitrary expressions of fungal growth: (-), no growth; (++++), dense growth with even spread; (++), less dense growth with uneven spread; (+), minimal growth with uneven spread

Organic acid production: The production of organic acids by these bacterial strains was investigated since these compounds are known to have antimicrobial properties (Kovanda *et al.*, 2019; Coban, 2020). MRS medium is a nutrient broth traditionally used to culture lactic acid bacteria (LAB) which are Gram-positive bacteria isolated from nutrient rich environments (milk, meat, plant, gastrointestinal tract, etc.). LAB are known for the fermentative conversion of sugars into lactic acid as a major product (Teusink & Smid, 2006). Because the *B. subtilis* and *S. caprae* endophytes were originally selected on MRS media, their ability to produce lactic and other organic acids was evaluated by high performance liquid chromatography (HPLC). The bacteria were cultured in either MRS or LB supplemented with 2% glucose (LB+glucose), and the cell free supernatant was collected and assayed.

The control sterile MRS broth had no propionic acid, a higher level of acetic acid, and, unexpectedly, a detectable amount of lactic acid (Table 3). When cultured in MRS, a low level of lactic acid production was observed in *S. caprae* culture broth, whereas, no lactic acid was detected in *B. subtilis* culture broth. *B. subtilis* did produce a low level of acetic acid; however, no acetic acid above that of the control MRS broth was produced by *S. caprae*. Finally, a significantly higher concentration of propionic acid (0.62%) was detected in *B. subtilis* culture broth compared to that of *S. caprae* (0.13%).

A different organic acid profile was observed from cultures in LB+glucose. There was 2.94% lactic acid in the *B. subtilis* culture broth which was in marked contrast to the lack of this acid's production when the bacteria were cultured in MRS broth. The major organic acid produced by *S. caprae* in LB+glucose was also lactic acid at 3.55%. Acetic acid was produced in a trace amount by *B. subtilis* and at a higher level by *S. caprae*. Propionic acid was also detected in the *B. subtilis* culture broth but the concentration was lower in LB+glucose (0.39%) as compared to the *B. subtilis* sample in MRS broth (0.62%). Only a trace amount of propionic acid was detected in the *S. caprae* sample grown in LB+glucose (0.03%).

Growth on variable carbon sources: Bacteria can rely on a variety of carbon sources for growth and survival. The Biolog Phenotypic microarray (PM) system was used to investigate the growth of endophytes on multiple carbon sources.

As shown in Table 4, carbohydrates, carboxylic acids, and amino acids are preferred by *B. subtilis* as carbon sources. Significant activity was observed in more than half of the carbohydrates, followed by amino acids (43.8%) and carboxylic acids (31.3%). Fewer carbon sources were utilized by *S. caprae* as only 5 carbohydrates, 1 carboxylic acid, and 1 amino acid supported growth above the threshold level (50 AOU). Amides, amines, esters, and fatty acids were not utilized by either bacterial strain.

Table 3. Detection of organic acids in endophytic cell-free supernatants in liquid media by HPLC method.

Sample (source)	Lactic acid (%)	Acetic acid (%)	Propionic acid (%)
B. subtilis CFS in MRS	0	0.51	0.62
S. caprae CFS in MRS	0.23	0.43	0.13
Control MRS broth	0.07	0.46	0.00
B. subtilis CFS in LB^{+*}	2.94	0.08	0.39
S. caprae CFS in LB^{+*}	3.55	0.24	0.03
Control LB broth^{+*}	0	0	0

*LB supplement ed with 2% glucose
CFS, Cell-free supernatants; MRS, Mann Rogosa Sharpe medium; LB, Luria Bertani medium

Table 4. Classes of carbon sources tested and used by endophytes in Biolog PM1 system (threshold 50 AOU).

C-source	Tested chemicals	*Bacillus subtilis*		*Staphylococcus caprae*	
		Used		Used	
		Number	%	Number	%
Carbohydrates	38	20	52.6	5	13.2
Carboxylic acids	32	10	31.3	1	3.1
Amino acids	16	7	43.8	1	6.3

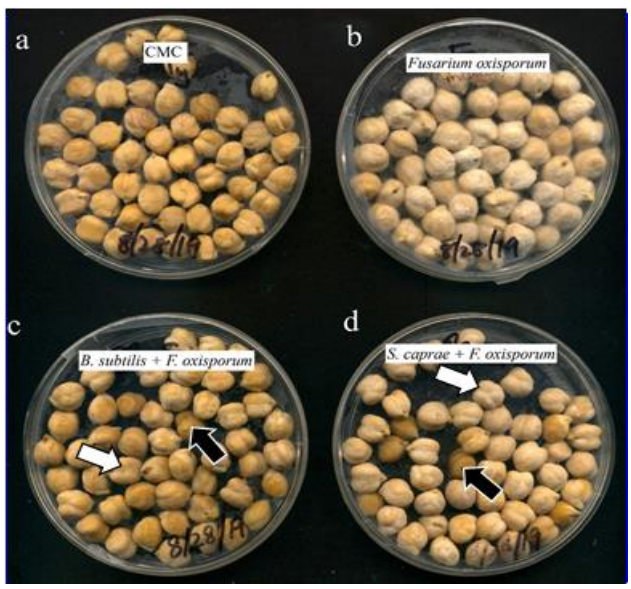

Fig. 3. Antifungal activity of endophytes against *F. oxisporum* on chickpea seeds. Seed treatments: (a) carboxymethyl cellulose (CMC)-only control plate, (b) *F. oxisporum* spore suspension, (c) *B. subtilis* pretreatment and *F. oxisporum* spore suspension, and (d) *S. caprae* pretreatment and *F. oxisporum* spore suspension. White arrows indicate seeds with white fungal patches while black arrows denote uninfected seeds. After 7 days of incubation, the fungal growth intensity can be described as b>d>c>a.

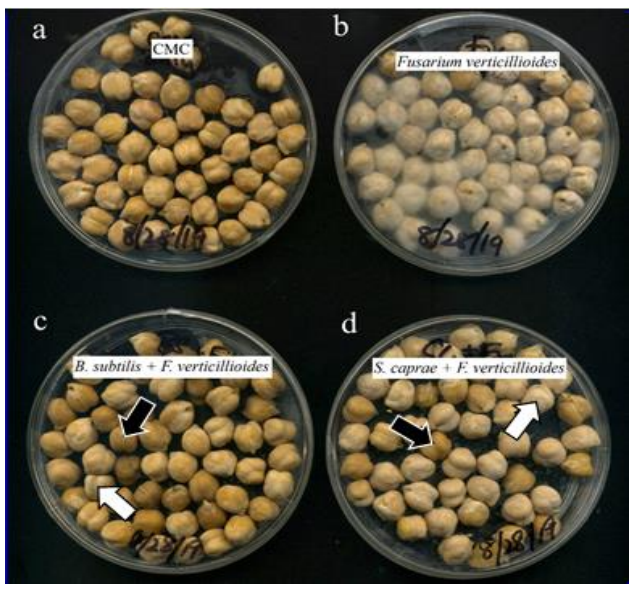

Fig. 4. Antifungal activity of endophytes against *F. verticillioides* on chickpea seeds. Seed treatments: (a) carboxymethyl cellulose (CMC)-only control plate, (b) *F. verticillioides* spore suspension, (c) *B. subtilis* pretreatment and *F. verticillioides* spore suspension, and (d) *S. caprae* pretreatment and *F. verticillioides* spore suspension. White arrows indicate seeds with white fungal patches while black arrows denote uninfected seeds. After 7 days of incubation, the fungal growth intensity can be described as b>d>c>a.

Discussion

Many diverse microbial groups have been reported to be associated with tomato, potato, and pepper plants (Manter *et al.*, 2010; Amaresan *et al.*, 2012; Paul *et al.*, 2013). In this study, endophytes were isolated from plant leaves, and three bacterial species (*Bacillus megaterium*, *Bacillus subtilis,* and *Staphylococcus caprae*) with antimicrobial activities were isolated. Although *B. megaterium* and *B. subtilis* have been found to be associated with tomato and potato plants (Porcel *et al.*, 2014; Hanif *et al.*, 2015), the isolation of *S. caprae* from pepper plant has not been reported to the best of our knowledge. *B. megaterium* and *B. subtilis* strains have both been previously reported to display antimicrobial activities (Aslim *et al.*, 2002; Lertcanawanichakul & Sawangnop, 2008), but the *B. megaterium* strain in this study did not have any antibacterial activity against *E. coli*. *B. subtilis* was found to have antibacterial activity against *E. coli* as well as having antifungal activity against both fusaria used in this study on both agar-based (PDA) and natural (chickpea seeds) growth substrates. With regards to antibacterial and antifungal activity, *S. caprae* has been understudied, although our study demonstrates its antibacterial and antifungal potential.

Many species from genus *Fusarium* are known to be associated with food crops. Dean *et al*. rank two *Fusarium* species among the top ten most important fungal pests (Dean *et al.*, 2012). *Fusarium oxysporum* has long been recognized as a threat to cereal and legume crops (Venuto *et al.*, 1995; Banerjee & Mittra, 2018). Chickpea (*Cicer arietinum* L.) is one of the primary targets of *F. oxysporum* where it causes wilt (Dubey *et al.*, 2010). *F. verticillioides* is also known as a fungal pest of legumes (Okoth & Siameto, 2010). With this history in mind, chickpea seeds were selected as a natural medium for our antifungal co-culturing study. Carboxymethyl cellulose (CMC) was used as a coating agent for bacterial cells on the seed surface. Although, other concentrations of CMC have been reported for this purpose (Kishore *et al.*, 2005; Raeisi *et al.*, 2015), we obtained promising results by using 0.1% aqueous solution of CMC.

Bacterial cell-free extracts in MRS medium were used for the detection of organic acids as previously discussed (Magnusson *et al.*, 2003). An interesting observation was that a detectable amount of lactic acid (0.07%) was measured by HPLC in sterile MRS medium which has not been previously reported to the best of our knowledge. Enzymatic conversion of sugars to lactic acid is a defining feature of lactic acid bacteria (Vries, 2006) and many members of these are in groups known to be probiotic for human consumption. Although, the phrase "lactic acid bacteria" is usually used to describe lactobacilli, bifidobacteria and similar genera, based on the studies (Du *et al.*, 2019), perhaps members of other microbial genera, like bacilli, could also be considered part of this group.

Biolog phenotypic microarray (PM) is a high throughput technique used to complement molecular identification of a wide variety of microbes (Shea *et al.*, 2012). In the current study, the PM1 system was used to observe the microbial responses to a variety of carbon sources. The consumption of a larger number of carbon sources by *B. subtilis* compared to *S. caprae* corresponds to its ubiquitous nature. It can be concluded by the biochemical profile that *B. subtilis* is more equipped to flourish on many carbon sources and habitats, as many members of this species have been isolated from diverse environments, ranging from oceans to plants to human gut (Hong *et al.*, 2009; Kim *et al.*, 2009; Liu *et al.*, 2010). Conversely, the use of a smaller number of carbon sources by *S. caprae* suggests the stricter nature of the microbe with regards to habitat diversity. Since its discovery in 1983, *S. caprae* has been reported to be associated with goat (Devriese *et al.*, 1983) and human microbiota (Vandenesch *et al.*, 1995). As this microbe is quite understudied, further research is needed.

Acknowledgements

This work was supported by the United States Department of Agriculture CRIS 2030-41000-067-000-D. and A.N. by a grant from the Higher Education Commission, Pakistan. We thank Dirk Sikkema for plant tissue and Antares Pham for technical support. Mention of trade names or commercial products in this report is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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(Received for publication 20 January 2021)