Int.J.Curr.Microbiol.App.Sci (2018) *7*(4): 1048-1056

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 04 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.704.115

Establishment of Antifungal Phyllospheric Bacteria in Potato (*Solanum tuberosum* L.)

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Keywords

Potato, Leaf, Antifungal, Bacterial count

Article Info

Accepted:
10 March 2018
Available Online:
10 April 2018

A B S T R A C T

The phyllosphere supports a large and complex bacterial community that varies both across plant species and geographical locations. Phyllosphere bacteria can have important effects on plant health. Many fungal diseases leads to important crop loss and due to limited antifungal availability and effectiveness in agriculture practices, it appears necessary to develop alternative control strategies. A total of 46 bacteria isolates were obtained from phyllosphere of different crops (Wheat, Pearl millet, Cotton, Mungbean and Potato). All the bacterial isolates were characterized for antifungal activity against *Aspergillus niger* and *Rhizoctonia solani*. Only Eighteen bacterial isolates showed the growth inhibition of phyto-pathogenic fungi against *A. niger*. and *R. solani*. On the basis of plant growth promotion traits, a total of seven isolates (POK-3, WHN-2, WHN-1, PMK-3, WHK-2, COJ-4, and MUK-1) were applied as foliar spray. The plants inoculated with the phyllospheric bacterial isolates recorded as biocontrol against phyto pathogenic fungi *A. niger* and *R. solani*. Therefore, it can be concluded that application of PGPB has immense potential to be used as biocontrol as they promote plant growth as well as improve the health and yield of the plants.

Introduction

Potato (*Solanum tuberosum* L.) is a member of solanaceae family and considered as one of the most valuable and widely distributed crops that is used for human food in most part of the world. It yields exceptionally high, produces more energy that is edible and protein per unit area and time than many crops. This also fits well in multiple cropping systems prevalent under tropical and subtropical agro-climatic conditions. Bacterial-host interaction can be harmful for the host (pathogenic interaction) or useful (symbiotic interaction). To have an optimal interaction, bacteria have to fine-tune itself with the biotic and abiotic environmental conditions of the host plant. This can be achieved via expression or repression of essential traits, such as host detection, motility towards the host, colonization and resistance

to host defences, growth and reproduction. The ability to quickly adapt to host environments is therefore critical for bacteria to have successful association with host. The phyllosphere refers to leaf surfaces, or total above ground surfaces, of plants as a habitat for microorganisms. Phyllosphere term was given by Ruinen (1961). Phyllosphere is the leaf surfaces or total above-ground surfaces of a plant as a habitat for microorganisms. All plants are host to a numerous and diverse community of microorganisms including bacteria, fungi and yeasts. The study of the characteristics of microbial life in the phyllosphere is of great commercial importance to the agricultural industry because of two reasons. One is to understand the survival of plant disease-causing bacteria and fungi while other is to develop new ways to control their spread (Lindow and Brandl, 2003; Leveau, 2006). Phyllosphere bacteria can promote plant growth and both suppress or stimulate the colonization and infection of tissues by plant pathogens (Lindow and Brandl, 2003). Phyllosphere microbiology has much to offer to the field of microbial ecology and promises to contribute the more effective and less environmentally damaging means of plant protection. Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism by several microorganisms. Also, indole-3-acetic acid (IAA), the major naturally occurring auxin, is a signalling molecule in microorganisms because IAA affects gene expression in some microorganisms (Spaepen and Vanderleyden, 2016). They can also be involved in plant protection, which is due to direct interactions of microorganisms through the production of antibiotic compounds and competition for resources (Berg, 2009). Additionally, microorganisms may protect plants against the pathogens by inducing systemic resistance (Conrath *et al.,* 2006; Pieterse *et al.,* 2012). PGPB can act as biocontrol agents by the formation of secondary metabolites like siderophore, HCN and by certain enzyme activity like chitinase, cellulase, protease and peroxidase *etc.* that showed toxic effect against plant pathogens (antifungal activity) (Labuschagne *et al.,* 2010; Nabti *et al.,* 2014). So, the internal and external foliar microbiota and their likely key roles in plant performance, growth and health, currant studies to understand better plant functioning and its responses and effects in a changing world, the importance and role of bacteria in phyllosphere make it available to the plants.

Materials and Methods

Isolation of bacteria from the phyllosphere of different crops

Bacteria were isolated from the phyllosphere of different crops (Wheat, Potato, Cotton, Mung bean and Pearl millet) grown at CCS HAU research farm. For isolation, 10 g of fresh leaves were added into 90 ml sterilized distilled water and placed on rotary shaker for half an hour. Serial dilutions (up to 10^{-5}) of samples were made in 9.0 ml sterilized water blanks and 0.1 ml of appropriate dilution was spread on different (Nutrient agar, King's B medium and Jensen's N_2 free medium) media plates. The plates were incubated at 28 ± 2^0C in a BOD incubator for 2-3 days. Based on the morphotypes, different bacterial colonies were selected, maintained and purified on respective media slants.

Determination of antifungal activity

The antagonistic interaction of bacterial isolates with *Rhizoctonia solani* and *Aspergillus niger* was studied by the spot test method on potato dextrose agar (PDA) medium plates (Sindhu *et al.,* 1999). Fungi were grown on PDA slants for 4 days and spore suspension was harvested in 2 ml sterilized water. About 0.1 ml of fungal spore

suspension was spread over fresh PDA medium plates. Loopful growth of 48-hours old cultures of different bacterial isolates was spotted on each plate. The growth inhibition of phyto pathogenic fungi around the spotted bacterial growth was recorded after 4 days old incubation at 28±2^{0}C.

Establishment of antifungal bacterial isolates vis-à-vis PGPB in phyllosphere of potato

A pot house experiment was conducted to test the survival and growth of antifungal bacterial isolates in the phyllopshere of potato. Total viable count in different treatments reflects the establishment of particular isolates in phyllopshere of potato under screen house.

Total bacterial population in phyllosphere

Total viable counts in phyllosphere of potato plant were taken at an interval of 30, 60 and 90 days. 10 g of fresh leaves were added into 90 ml sterilized distilled water and further serially diluted and hundred µl of each sample from various dilutions (10^{-3}, 10^{-4} and 10^{-5}) were spread over nutrient agar plates. The plates were incubated for 3-5 days at 28±2ºC and colonies appeared were counted. The counts were calculated on per g leaf basis using formula:

No. of cfu (colony forming units) x dilution factor/ volume taken (ml)

Results and Discussion

Isolation of bacteria from the phyllosphere of different crops

A total of forty six bacterial isolates were retrieved from five phyllospheric leaf samples (wheat, potato, cotton, pearl millet and mung bean) using dilution plating on Nutrient agar media, King's B media and Jensen's N_2 free media (Table 1). Microorganisms in the phyllosphere can promote plant growth by different mechanisms, e.g. through production of hormones. They can also be involved in plant protection, which is due to direct interactions of microorganisms through the production of antibiotic compounds and competition for resources (Wu *et al.,* 2009). Additionally, microorganisms may protect plants against the pathogens by inducing systemic resistance (Pieterse *et al.,* 2012). Internal and external foliar micro biota have many other functions, including indirect protection against pathogens, through the interaction of commensal bacteria with the foliar plant pathogen (Arnold *et al.,* 2003) or communication through their contribution to different types and quantities of emissions of volatile organic compounds (Bulgerelli *et al.,* 2013).

Screening of different isolates for plant growth promoting traits

All the bacterial isolates were screened for antifungal activity. Figure 1 (a) and 1 (b) shows the growth inhibition of phyto pathogenic fungi around the spotted bacterial growth on PDA medium plates. Eighteen bacterial isolates showed the growth inhibition of phyto pathogenic fungi against *A. niger*. & *R. solani*. Maximum antifungal activity was observed in isolate PMK3 followed by COJ4 and WHN1.

Phyllosphere bacteria can affect plant growth indirectly by protecting plants against pathogens. These microbial communities may be involved in plant protection, due to direct interactions of resident microorganisms those produce antibiotic compounds and showed competition for resources. Phyllosphere bacteria can affect plant growth indirectly by protecting plants against pathogens. These microbial communities may be involved in plant protection, due to direct interactions of

resident microorganisms those produce antibiotic compounds and showed competition for resources (Berg, 2009). Such antagonistic effects have been demonstrated for several leaf-colonizing bacterial isolates. Large epiphytic strain collections were screened for inhibitory effects *in vitro*, e.g. by agar-diffusion assays or streak tests with laboratory cultures (May *et al.*, 1997; Adhikari *et al.*, 2001). Braun *et al.*, (2010) showed that the toxin-producing isolate *P. syringae* pv. *syringae* 22d inhibited growth of the near-isogenic foliar pathogen *P. syringae* pv. *glycinea in vitro*, but was not responsible for the antagonistic effects observed *in planta*. Abundant non-pathogenic soil microbes rapidly colonize plant surfaces and use most of the available nutrients, making it difficult for pathogens to grow. For example, in one series of experiments, researchers demonstrated that treatment of plants with the leaf bacterium *Sphingomonas* spp. prevented the bacterial pathogen *Pseudomonas syringae* pv. tomato from causing pathogenic symptoms (Innerebner *et al.*, 2011). However, for other strains it was shown that antibiosis is involved in plant protection. For instance, a Tn*5* mutant of *Pantoea agglomerans* Eh 252 deficient in antibiotic production was not as effective as the wild type strain to control fire blight in the field (Stockwell and Peterson, 2002). Antimicrobial compounds are produced by microorganisms to remain competitive in their environment by diminishing growth of other bacteria.

Establishment of antifungal bacterial isolates vis-à-vis PGPB in phyllosphere of potato

A pot house experiment was conducted to test the survival and growth of antifungal bacterial isolates in the phyllopshere of potato. After 20 days of sowing, selected bacterial isolates were sprayed on phyllosphere according to the treatments (Table 2).

Table.1 List of bacteria isolated from phyllosphere of different crops

Site of collection of leaf samples	Medium used for isolation			No. of isolates
	Nutrient agar	King's media	Jensen's N_2 free medium	
Wheat phyllosphere HAU research farm, Hisar	WHN1,WHN2, WHN3, WHN4, WHN5, WHN6	WHK1, WHK2, WHK3, WHK4, WHK5, WHK6	WHJ1, WHJ2, WHJ3	15
Potato phyllosphere HAU research farm, Hisar	PON1	POK1, POK3, POK4, POK5	POJ1, POJ2	7
Cotton phyllosphere HAU research farm, Hisar	CON1	COK2, COK3, COK4, COK5	COJ1, COJ4	7
Pearl millet phyllosphere HAU research farm, Hisar	PMN1	PMK1, PMK2, PMK3, PMK4, PMK5, PMK6	PMJ1	8
Mung bean phyllosphere HAU research farm, Hisar	MUN1, MUN2, MUN3, MUN4	MUK1, MUK2, MUK3, MUK4	MUJ1	9
Total no. of isolates	13	24	9	46

Table.2 Total viable count (bacteria) in the phyllosphere of potato crop under pot-house condition (log cfu/g leaves)

Treatment	DETAILS	30 DAS	60 DAS	90 DAS
T1	CONTROL	6.3	6.3	6.0
T2	75%RDF (Recommended dose of fertilizer)	6.6	6.5	6.6
T3	RDF (Recommended dose of fertilizer)	6.6	6.6	6.7
T4	75% RDF + Mac27 (Tuber treatment)	6.8	6.6	7.4
T5	RDF + Mac27 (Tuber treatment)	6.8	6.8	7.4
T6	75% RDF + Mac27 (Foliar treatment)	6.9	7.0	7.3
T7	RDF + Mac27 (Foliar treatment)	6.9	7.3	7.4
T8	75% RDF + Bacterial isolate POK-3 spray	7.8	7.5	7.5
T9	RDF + Bacterial isolate POK-3 spray	7.6	7.5	7.5
T10	75% RDF + Bacterial isolate WHN-2 spray	7.6	7.7	7.5
T11	RDF + Bacterial isolate WHN-2 spray	7.3	7.8	7.5
T12	75% RDF + Bacterial isolate WHN-1 spray	7.7	7.6	7.5
T13	RDF + Bacterial isolate WHN-1 spray	7.7	7.7	7.6
T14	75% RDF + Bacterial isolate PMK-3 spray	8.0	7.7	7.8
T15	RDF + Bacterial isolate PMK-3 spray	7.6	7.8	7.8
T16	75% RDF + Bacterial isolate WHK-2 spray	7.9	7.7	7.7
T17	RDF + Bacterial isolate WHK-2 spray	7.4	7.8	7.7
T18	75% RDF + Bacterial isolate COJ-4 spray	7.5	7.8	7.5
T19	RDF + Bacterial isolate COJ-4 spray	7.8	7.6	7.7
T20	75% RDF + Bacterial isolate MUK-1 spray	6.9	7.5	7.4
T21	RDF + Bacterial isolate MUK-1 spray	7.4	7.5	7.4
C.D. at 5%		0.28	0.26	0.30

Fig.1 (a) Antifungal activity exhibited by bacterial isolates against *Aspergillus niger*

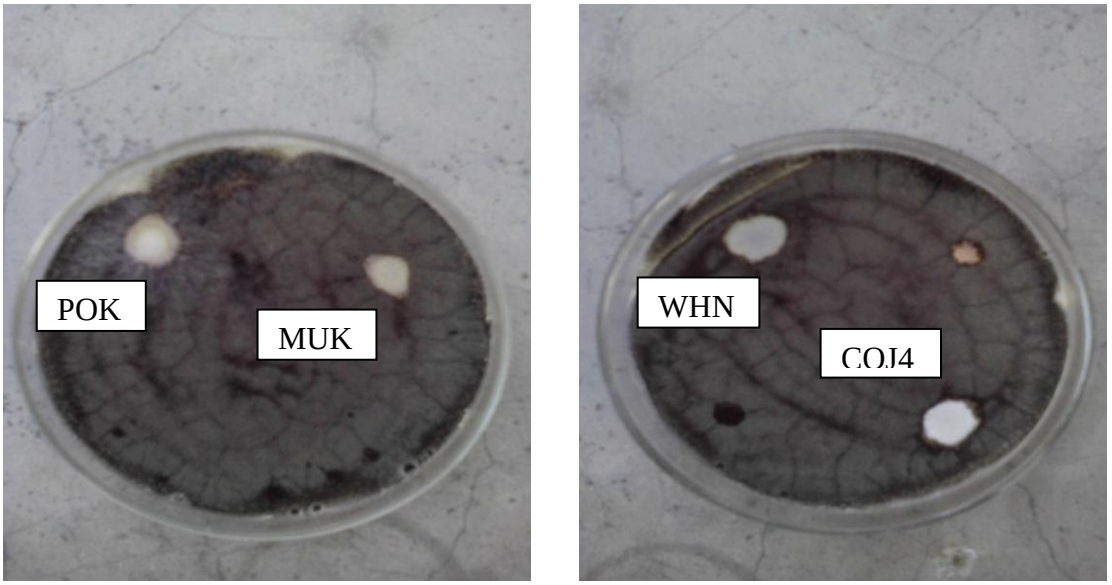

Fig.1 (b) Antifungal activity exhibited by bacterial isolates against *R. solani*

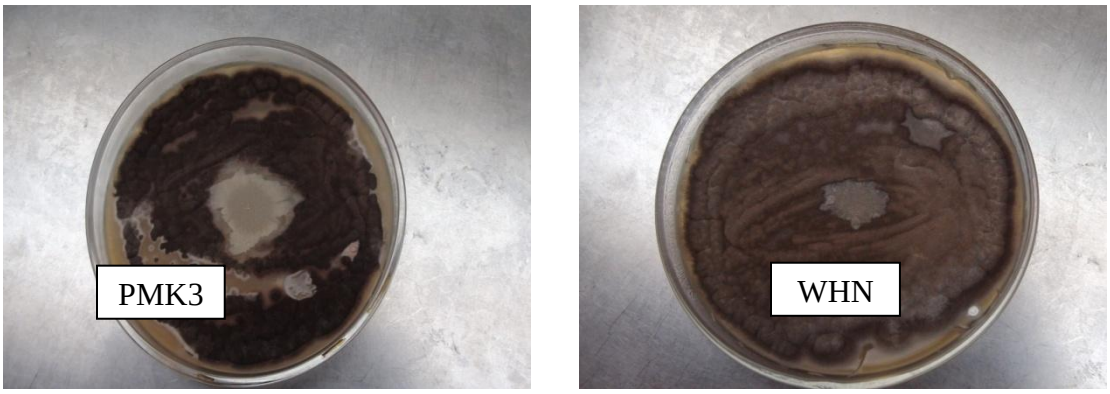

Fig.2 Comparative plant growth of potato crop in different treatments
(Before and after blight attack)

Bacterial count in phyllosphere of potato plant

The microbial communities of leaves are diverse and include many different genera of bacteria, filamentous fungi, yeasts and algae which are important for plant health and growth (Whipps *et al.,* 2008; Vorholt, 2012). The epiphyte population is dominated by bacteria which can be found in numbers ranging from 10^5 to 10^7 cells/g of plant material (Yadav *et al.,* 2005). Artur *et al.,* (2015) reported that a bacterial isolate called 49M, showing protective activity against fire blight caused by the bacterium *Erwinia amylovora,* was selected from a large

collection of isolates obtained from the apple phyllosphere and was identified as *Pseudomonas graminis,* based on its phenotype and sequence analysis of the 16S rRNA and rpoD genes.

Total viable count (phyllosphere) was determined in all the treatments at 30, 60 and 90 DAS. Total phyllospheric viable count was observed maximum in foliar application of isolate PMK3 along with RDF i.e. treatment T15 (7.8 log cfu/g leaves) followed by foliar application of isolate WHK2 and COJ4 along with RDF i.e. treatment T17 & T19 (7.7 log cfu/g leaves) at 90 DAS. The viable count in all the treatments increased till 90 DAS (Table 2). Total viable count ranged from 6.3-8.0 log no. cfu/leaves at 30 DAS, from 6.3 – 7.8 log no. cfu/leaves at 60 DAS and from 6.00 - 7.8 log no. cfu/leaves at 90 DAS which were non- significantly differed from one another.

The increased in phyllosphere viable count in treatment T15 indicates the establishment of isolate PMK3 in the phyllosphere of potato. The establishment of isolate PMK3 in potato phyllosphere results into better growth and yield attributing traits over the other treatments (Fig. 2).

Akter *et al.,* (2015) studied three bacterial isolates namely UMB20, KMB25 and BMB42 obtained from rice plants which showed the ability of biocontrol and plant growth promotion. Fungal growth inhibition by the isolates ranged from 86.85 to 93.15% in volatile and 100% in diffusible metabolites test. Among the isolates, BMB42 showed fungal growth inhibition significantly in the volatile metabolite test. Among the three isolates, KMB25 showed protease production and all of them were negative to pectinase and lipase and positive to the production of siderophore, and HCN, and were able to solubilize tricalcium phosphate.

Annalisa *et al.,* (2016) reported that sixty out of 162 bean rhizobacteria inhibited the growth *in vitro* of selected virulent strains of both varieties of *Xanthomonas axonopodis* pv. *phaseoli* and, when applied to seeds before sowing, six of them showed reduced disease symptoms on bean in *in vitro* and greenhouse pathogenicity assays. In order to deepen bacteria characterization, the six rhizobacteria were evaluated for lytic enzymes, hydrogen cyanide, ammonia, siderophores, indoles production, inorganic phosphates solubilisation and environmental adaptability in terms of salinity, pH and temperature gradients variation. Altogether the findings of this study indicate the above six rhizobacteria as potential biocontrol candidate.

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How to cite this article:

Satish Kumar, Deepika Chaudhary, Rashmi, Raman Jangra, Anju Kumari and Rakesh Kumar. 2018. Establishment of Antifungal Phyllospheric Bacteria in Potato (*Solanum tuberosum* L.). *Int.J.Curr.Microbiol.App.Sci.* 7(04): 1048-1056. doi: https://doi.org/10.20546/ijcmas.2018.704.115