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Host specificity of plant endophytic bacterial interactions: Root and nodule colonization under sterilized sand conditions in disposable coffee cups

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ABSTRACT

Endophytes have a symbiotic relationship with the different parts of plants and could play a very important role in supporting the plant growth. In present study, 11 most efficient isolates were selected out of more than 200 endophytic bacteria isolated previously from roots of chickpea, field pea, Lucerne, wheat and oat and nodules of chickpea and field. To know their extent of establishment in different host and non-host tissues four host chickpea, field pea, wheat and oat were inoculated with these bacteria in sterilized sand in disposable coffee cups. To induce nodulation in chickpea and field pea were also inoculated with respective rhizobia. In chickpea at 15 d, isolate ORE 27, at 30 d isolates CNE 215 and ORE 35 and at 50 day in total 6 bacteria were able to enter the roots and exit as endophytic. In case of field pea all the 11 bacteria entered the roots after 30 d of growth, whereas in wheat and oat bacteria entrance was detected at 50d and not at 15 or 30d. In wheat 4 and in oat 6 bacterial isolates were detected as endophytic. Even at 50d, neither in chickpea nor in field pea nodules, no bacterial isolate was able to enter. In chickpea roots maximum endophytic colonization was observed by isolate PNE 92, in field pea by isolates CNE1 and PNE 17; in wheat and oat, isolate ORE 27 showed highest root endophytic colonization. No host specificity among endophytic bacteria and different hosts could be observed.

Keywords: Endophytes, Colonization, Plant endophyte interactions, Bacteria

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INTRODUCTION

Soil is a reservoir of microorganisms, which starts interacting with the emerging roots of plants. Depending upon the type of microorganism, beneficial or pathogenic relationship with plants is established. Root colonization is the critical step in establishment of plant-microbe association. Root exudates provides nutritional substances, specific metabolic products to promote specific microorganism, signals which cause colonization on roots by some bacteria while inhibits the other [1, 2]. Role of bacterial major outer membrane protein, cell surface proteins, chemical composition of lipopolysaccharides and Ca^{2+} signaling in host recognition, adhesion, binding leading to successful colonization [3]. Further bacteria may remain on the root surface or may enter into plant tissue, which may be root or nodules.

The mutualistic interaction of legumes with rhizobia involves finely tuned recognition steps which ultimately lead to the production of root nodules in which the plants accommodate the bacteria in a very coordinated manner [4, 5]. In case of legumes and legumes, some of bacteria, actinomycetes, fungi are capable of entering the plant roots as endophytes and establishes a mutualistic association. The processes of host-microbe signaling and colonization, and the mechanisms leading to endophytic association are less-well characterized [6]. Host endophyte relationship may be variable from host to host and endophyte to endophyte. Some research showed that host plant and endophyte relationship are able to balance pathogen host antagonism and are not truly symbiotic one [7].

Recent studies show that endophytes are not host specific [8]. Single endophytes can invade a wide host range. However others advocate for host specificity and involvement of a compatible host plant [9, 10]. Preliminary studies under liquid medium conditions showed that there is no host specificity between plant host and endophytic bacteria, but it is by chance and depends upon the availability of microbial population and plant host or tissue in the soil [11]. Therefore, present investigation was aimed to study the interaction of hosts with endophytic bacteria to know the level of host specificity existing among these interactions. In the present study 11 endophytic bacteria isolated from different tissues of five hosts were used to observe their interaction with four hosts under sterilized sand conditions in coffee cups.

MATERIALS AND METHODS

Endophytic bacterial cultures

About 200 endophytic bacteria isolated in the previous studies from nodules of chickpea (*Cicer arietinum*), field pea (*Pisum sativum*) and roots of chickpea, field pea, Lucerne (*Medicago sativa*), wheat (*Triticum aestivum*) and oat (*Avena sativa*) were used to select efficient isolates from all the sources. Out of these, 11 endophytic bacterial isolates, CRE1 (chickpea roots), CNE215 (chickpea nodules), PRE8 (field pea roots), PNE17 (field pea nodules), PNE92 (field pea nodules), LRE3 (lucerne roots), LRE7 (lucerne roots), WRE4 (wheat roots), WRE20 (wheat roots), ORE27 (oat roots), and ORE35 (oat roots) were selected for the present studies.

Screening of bacterial endophytes for presence of antibiotic markers

The endophytic isolates were screened for the presence antibiotic markers to find their colonization in roots and nodules of different hosts and non hosts in sterilized conditions. Each endophyte was grown in TSA medium [12] plates containing a particular antibiotic with particular concentration. A combination of different antibiotics with different concentrations (in which the endophyte was previously found to survive) was used in a single medium plate. In this way different multiple markers were prepared for different endophytes. These multiple antibiotic resistance markers were used for further studies.

Colonization studies in sterilized disposable coffee cups containing sand

Seeds of chickpea, field pea, wheat and oat were surface sterilized and kept for germination on 1% agar for 24 h at 28±2ºC in a BOD incubator [13]. River sand was thoroughly washed with acid followed by 6-7 washings with water and was sterilized in oven at 180ºC for one h, in trays. The sand was added in disposable coffee cups and nitrogen free nutrient solution was added and cups were covered with paper, held in position with help of a thread. These disposable coffee cup assemblies were again sterilized in autoclave at 15 lbs for one h. Germinated seedlings were transferred to sterilized disposable coffee cups containing sand, along with 1-2 mL of broth of different endophytic isolates along with *Mesorhizobium* CH1233 in case of chickpea and *R. leguminosarum* bv *trifoli* strain PS43 in case of field pea. These disposable coffee cup assemblies were kept in green house and watered daily with sterilized Sloger's nitrogen free watering solution [14]. After 15, 30 and 50 d of growth, plants were recovered and analyzed for roots as well as nodules colonization by endophytic bacterial. For colonization studies roots from each plant were removed from the disposable coffee cups after 15, 30 and 50 d of growth, were mildly sterilized using 95% ethyl alcohol and $HgCl_2$ and crushed aseptically. Contents were transferred to sterilized distilled water and after appropriate dilutions were plated on antibiotic containing plates. Log CFU g^{-1} of fresh root weight was determined. Similarly nodules were used to determine the endophytic count. To ensure the proper sterilization, the roots were placed on TSA medium plates and were incubated at 28±2ºC and observed for microbial growth. Fresh root and shoot biomass was also recorded.

RESULTS AND DISCUSSION

All the 11 endophytic bacteria were not resistant to higher concentration of single antibiotic, so all isolates were screened for multiple antibiotic markers. The different multiple antibiotic markers selected are shown in Table 1. During isolation of bacteria from root or nodule respective antibiotic markers were used the TSA plates. In case of chickpea, two controls were kept, one was absolute control without any inoculation and another was with *Mesorhizobium* sp. Strain CH1233. At 15d of inoculation, none of the isolate was able to enter the chickpea roots except isolate ORE27, while at 30d of growth, isolates CNE215 and ORE35 were also detected on their respective antibiotic marker containing plates (Table 2). At 50d of growth, isolates CNE215, PNE92, LRE7, WRE20, ORE27 and ORE35 were also detected in the chickpea roots and maximum number of 3.28 CFU plant $root^{-1}$ of isolate CNE 215 was observed. At 50d nodules were also screened for the presence of different endophytes but in none of the treatment endophyte could be recovered. After 50 d of growth highest fresh root and shoot growth (4.96 and 5.97 g $plant^{-1}$) was observed with isolate CNE 215.

In field pea also, two controls were kept, one was absolute control without any inoculation and another was with *Rhizobium leguminosarum* biovar *trifolii* strain PS-43. At 15d of inoculation, only isolates CNE215, LRE7, WRE4, ORE27 and ORE35 were detected in field pea roots, while at 30 and 50d of growth all the 11 endophytic bacterial isolates were detected on their respective antibiotic marker containing plates (Table 3). At 50d of growth maximum number of 3.97 CFU plant root^{-1} of isolate PNE92 was observed. At 50d nodules were also screened for the presence of different endophytes but in none of the treatment endophyte could be recovered. Highest fresh root growth of 3.96 g plant^{-1} was observed with isolates CNE1 as well as PNE17. Highest shoot growth of 5.28 g plant^{-1} was observed with isolate LRE3 after 50 d of growth.

In case of non-legume host, wheat and oat one absolute control was kept without any inoculation. In wheat at 15 and 30d of inoculation, none of the isolate was detected in wheat roots (Table 4). At 50d of growth four endophytic bacterial isolates i.e. LRE3, LRE7, WRE20 and ORE27 were detected on their respective antibiotic marker containing plates and maximum number of 3.97 CFU plant root^{-1} of isolate ORE27 was observed. Highest fresh root and shoot biomass of 1.88 and 1.99 g plant^{-1} was observed with isolate ORE27.

In case of oat also at 15 and 30d of inoculation, none of the isolate was detected in oat roots (Table 5). At 50d of growth 6 endophytic bacterial isolates i.e. CNE215, PRE8, LRE3, LRE7, WRE20 and ORE27 were detected on their respective antibiotic marker containing plates and maximum number of 3.98 CFU plant root^{-1} of isolate ORE27 was observed. Highest fresh root and shoot biomass of 1.73 and 2.01 g plant^{-1} was observed with isolate WRE20. The results showed that there was a statistically significant difference in root and shoot fresh weight over un inoculated controls as compared to when inoculated with endophytes in all the four crops i.e. chickpea, field pea, wheat as well oat.

The entrance of bacteria inside the roots was low at 15 d of incubation as compared to 30 and 50 d observation. Indicating that with an increase in age of the plant roots, increase in endophytic detection was observed in roots of chickpea, field pea, wheat and oat. This could be explained due to the reason that with increase in age of plant roots, probably cracks in roots occurs and this ultimately contributes to endophytic colonization. With all the crops, a statistically significant increase in colonization pattern as well as fresh root growth was observed as compared to control. Further to ensure the proper sterilization of roots, though plants were grown under sterilized conditions and were plated on respective antibiotic marker plates, even then roots were mildly sterilized and kept on the medium plates. In most of the cases, no growth on the plates was observed. Whenever some colonies appeared on these plates, the experiment was repeated again, so that only endophytic bacteria are observed. Even up to 50 d growth no bacteria could enter the nodules. Probably up to this stage no crack or injury of nodules was there and thereby bacteria was unable to enter the nodules. Zachow *et al*., 2010[15], also suggested that endophytes enter a plant tissue through natural cracks at the region where the lateral roots appear which further justify that with increase in age more cracks appeared in roots through which endophytes enter the roots. This mode of entry (often combined with active penetration) has also been suggested for *Azoarcus* sp. BH72.

Further increase in root or shoot biomass was not correlated with the existence of a strain as endophytic or in the rhizosphere but this was dependent on the ability of a strain for growth promotion. In literature no such studies has been reported [16].

Table I: Antibiotic Resistance pattern (ARP) of endophytes

Bacterial endophytes	Antibiotic resistance pattern
CRE1	$K_{25}+T_{30}+NA_{25}$
CNE215	$A_{50}+NA_{50}$
PRE8	$T_{30}+R_{25}+S_{200}$
PNE17	$K_{25}+NA_{25}$
PNE92	S_{200}
LRE3	$A_{50}+S_{200}$
LRE7	A_{50}
WRE4	$R_{25}+S_{400}$
WRE20	A_{30}
ORE27	S_{200}
ORE35	S_{400}

K= Kanamycin; T= Tetracyclin; NA= Nalidixic Acid; R= Rifampicin; A=Ampicillin; S= Streptomycin, Subscript= Denotes the antibiotic concentration ($\mu g\ mL^{-1}$)

Table II: Root and nodule colonization and growth promotion in chickpea inoculated with bacterial endophytes in cups

Endophytic bacterial isolates	15d			30d			50d			
	Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}		Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}		Endophytes log CFU (per plant)		Fresh weight g plant^{-1}	
		Roots	Shoots		Roots	shoots	Roots	Nodules	Root	Shoot
Uninoculated	-	2.11	2.12	-	2.99	2.77	-	-	3.01	3.06
Mesorhizobium (*Meso)*		2.34	2.98		3.00	3.47		-	3.92	3.48
Meso + CNE1	-	2.94	3.47	-	3.11	3.99	-	-	3.98	4.71
Meso + CNE215	-	3.24	3.16	3.08	4.64	4.97	3.29	-	**4.96**	**5.97**
Meso + PRE8	-	2.96	3.82	-	4.24	4.61	-	-	4.57	4.74
Mseo + PNE17	-	2.99	3.03	-	4.11	4.65	-	-	4.29	4.98
Meso + PNE92	-	2.44	3.33	-	4.05	4.30	2.98	-	4.86	5.96
Meso + LRE3	-	2.22	3.04	-	3.90	4.00	-	-	4.17	4.21
Meso + LRE7	-	3.00	3.13	-	4.03	4.13	2.94	-	4.94	5.22
Meso + WRE4	-	3.10	3.30	-	4.20	4.26	-	-	4.23	4.49
Meso + WRE20	-	3.18	3.48	-	3.99	4.05	2.82	-	4.80	5.38
Meso + ORE27	1.18	3.00	3.96	2.90	4.64	4.96	2.56	-	**4.96**	5.08
Meso +ORE35	-	2.79	3.11	2.45	4.72	4.98	2.34	-	4.95	5.56
SE(m)	**0.01**	**0.17**	**0.16**	**0.18**	**0.17**	**0.20**	**0.07**		**0.29**	**0.40**
CD at 5%	**0.03**	**0.49**	**0.32**	**0.36**	**0.35**	**0.40**	**0.25**		**0.7**	**0.90**

Table III: Root and nodule colonization and growth promotion in field pea inoculated with bacterial endophytes in cups

Endophytic bacterial isolates	15d			30d			50d			
	Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}		Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}		Endophytes log CFU (per plant)		Fresh weight g plant^{-1}	
		Roots	Shoots		Roots	shoots	Roots	Nodules	Root	Shoot
Uninoculated	-	0.82	-	-	1.19	1.22	-	-	1.48	1.25
*Rhizo**		0.83	-	-	2.08	2.96	-	-	3.05	3.91
Rhizo + CNE1	-	1.08	1.65	1.65	2.98	4.05	1.09	-	**3.96**	5.26
Rhizo + CNE215	1.17	1.37	3.81	3.81	2.96	3.95	3.43	-	3.89	5.19
Rhizo + PRE8	-	0.96	2.44	2.44	3.22	4.19	2.34	-	3.45	4.99
Rhizo + PNE17	-	1.04	2.86	2.86	2.91	3.95	2.64	-	**3.96**	5.08
Rhizo + PNE92	-	0.86	3.79	3.79	2.93	4.27	3.97	-	3.95	4.97
Rhizo + LRE3	-	1.12	3.33	3.33	2.98	4.28	3.78	-	3.70	**5.28**
Rhizo + LRE7	1.12	1.71	3.86	3.86	3.17	4.28	3.65	-	3.65	5.19
Rhizo + WRE4	1.01	1.18	1.66	1.66	2.80	4.39	1.54	-	3.90	4.98
Rhizo + WRE20	-	1.06	3.43	3.43	2.96	4.29	3.45	-	3.88	4.88
Rhizo + ORE27	1.14	1.29	2.81	2.81	2.96	4.31	2.01	-	3.80	5.27
Rhizo +ORE35	1.09	1.14	2.82	2.82	3.08	4.42	2.89	-	**3.96**	4.92
SE(m)	**0.03**	**0.10**	**0.08**	**0.08**	**0.19**	**0.16**	**0.08**		**0.19**	**0.07**
CD at 5%	**0.09**	**N/A**	**0.25**	**0.25**	**0.57**	**0.47**	**0.25**		**0.56**	**0.20**

Rhizo = Rhizobium leguminisarum biovar trifoli strain PS-43*

Table IV: Root colonization and growth promotion in wheat inoculated with bacterial endophytes in cups

Endophytic bacterial isolates	15d			30d			50d		
	Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}		Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}		Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}	
		Roots	Shoots		Roots	Shoots		Root	Shoot
Uninoculated	-	0.11	0.14	-	0.19	0.18	-	0.82	0.23
CNE1	-	0.38	0.37	-	0.49	0.69	-	0.99	1.69
CNE215	-	0.31	0.42	-	0.40	0.62	-	1.08	1.53
PRE8	-	0.47	0.47	-	0.57	0.63	-	0.92	1.69
PNE17	-	0.38	0.44	-	0. 48	0. 70	-	0.96	1.70
PNE92	-	0.35	0.41	-	0.37	0.48	-	0.95	1.05
LRE3	-	0.39	0.40	-	0.40	0.48	2.99	1.58	1.97
LRE7	-	0.30	0.49	-	0.48	0.69	2.54	1.37	1.76
WRE4	-	0.51	0.51	-	0.51	0.64	-	1.08	1.70
WRE20	-	0.39	0.40	-	0.42	0.68	2.09	0.99	1.53
ORE27	-	0.57	0.57	-	0.62	0.72	3.37	**1.88**	**1.99**
ORE35	-	0.48	0.49	-	0.51	0.69	-	1.09	1.56
SE(m)		**0.17**	**0.22**	-	**0.19**	**0.20**	**0.056**	**0.33**	**0.34**
CD at 5%		**0.44**	**0.60**		**0.50**	**0.52**	**0.162**	**0.90**	**0.95**

Table V: Root colonization and growth promotion in oat inoculated with bacterial endophytes in cups

Endophytic bacterial isolates	15d			30d			50d		
	Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}		Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}		Root endophytes log CFU (per plant roots)	Fresh weight g plant^{-1}	
		Roots	Shoots		Roots	Shoots		Root	Shoot
Un inoculated	-	0.12	0.13	-	0.16	0.20	-	0.25	0.24
CNE1	-	0.19	0.22	-	0.20	0.29	-	0.48	0.59
CNE215	-	0.48	0.57	-	0.57	0.76	2.54	1.24	1.88
PRE8	-	0.46	0.59	-	0.50	0.69	2.25	1.42	1.48
PNE17	-	0.40	0.50	-	0. 52	0. 74	-	1.29	1.10
PNE92	-	0.46	0.54	-	0.52	0.76	-	1.00	1.15
LRE3	-	0.42	0.53	-	0.59	0.60	3.15	1.44	1.81
LRE7	-	0.40	0.58	-	0.59	0.66	2.98	1.49	1.96
WRE4	-	0.49	0.51	-	0.50	0.69	-	1.18	1.22
WRE20	-	0.40	0.59	-	0.59	0.68	2.95	1.73	**2.01**
ORE27	-	0.49	0.50	-	0.50	0.71	**3.98**	1.50	1.89
ORE35	-	0.46	0.52	-	0.56	0.69	-	1.11	1.47
SE(m)		**0.16**	**0.17**	-	**0.19**	**0.19**	**0.26**	**0.25**	**0.29**
CD at 5%		**0.40**	**0.42**		**0.50**	**0.50**	**0.77**	**0.86**	**0.94**

CONCLUSION

It seems that there is no host specificity in the entry of different endophytic isolates in different host and non-host roots and it was independent of the source from which these were isolated. Environmental and ecological conditions are determining the prevalence of different genera and their entry into roots or nodules. Whether the endophytes were entering in plant roots or remaining outside as rhizospheric are benefiting the plants by enhanced root and shoot growth

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