Response of seed endophytic bacteria for the management of chickpea wilt, *Fusarium oxysporum* f. sp. *ciceris*

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Summary

Vascular wilts are devastating plant diseases that can affect both annual crops as well as woody perennials, hence inducing major food losses and damaging valuable natural ecosystems. Because of ecological and economic reasons, the management of vascular wilt diseases by conventional chemical methods is raising concerns. More environmentally friendly alternatives such as the use of microbial antagonist to control phytopathogens are now of growing interest. The fact that bacterial endophytes are able to colonize an ecological niche similar to that of vascular wilt pathogens favours them as potential biocontrol agents against wilt diseases. Several possible disease suppression mechanisms of beneficial bacteria were proposed, among them induction of systemic resistance, growth promotion and competition. In this view, we studied out the seed endophytic bacteria (SEB) for the management of chickpea wilt Caused by *Fusarium oxysporum* f. sp. *ciceris.* Among the treatment of seed entophytic bacterial isolate in SEB-5 was found highest germination percent (92% in JG 11), minimum mortality percent at pre and post–emergence (7.69 and 9.53% in JG 11 and JG 16, respectively). The minimum total mortality percent 17.82 % was found in JG 11 of SEB-5. The other seed entophytic bacterial isolate were found statistically at par with SEB-5. The minimum germination percent (78.5% in JG 62) as well as highest total mortality percent approximate 58% in JG 11 was found in T_6 (treated control). Similarly, all the treatments of seed entophytic bacterial had higher root length and shoot length as compared to T_6. Among the treatments the SEB-5 was found significantly highest by 23 cm in root length and 48 cm in shoot length as well as highest vigour index (6613) over the T_6 in JG 11 variety. Among the varieties grown the JG 11 was perfumed better and found resistant and JG 62 was found susceptible to wilt. The treatment of seed endophytic bacterial isolate SEB-5, SEB-3, SEB-2, SEB-1 and SEB-4 was identified against wilt pathogen in reduce disease incidence, plant mortality and severity as well as promoting plant growth and health.

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) commonly known as "Chana" in Hindi belongs to family Leguminosae and is believed to be originated from southwest Asia.[20] Chickpea seed has 9 per cent protein, 38-59 per cent carbohydrate, 3 per cent fibre, 4.8-5.5 per cent oil, 3 per cent ash, 0.2 per cent calcium and 0.3 per cent phosphorus.[8, 7] There are two distinct types of cultivated chickpea *i.e.* Desi and Kabuli. It is an important pulse crop and grown in temperate as well as subtropical regions of the world. It is one of the most important food legumes grown worldwide. Asia covers largest area (89.7%) of chickpea cultivation followed by Africa (4.3%), Oceania (2.6%), America (2.9%) and Europe (0.4%).[5, 6] India is one of the leading producers and consumer of chickpea in the world accounting for about 70.57 per cent of the world area and 69.21per cent of the world's production.[16] The Major chickpea growing states are Madhya Pradesh, Maharashtra, Uttar Pradesh, Karnataka, Andhra Pradesh, Chhattisgarh, Gujarat, Jharkhand, Bihar and Tamil Nadu.

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Madhya Pradesh has contributed 34 per cent of the total chickpea area and 41 per cent of the total chickpea production in the country.[1]

It is attacked by several air, seed and soil borne fungal diseases.[13] Chickpea wilt was first reported by Butler (1918)[3] in his book entitled 'Fungi and Disease in plants. Fusarium wilt epidemics can devastate crops and cause up to 100 per cent losses in highly infested fields under favourable conditions. The eight pathogenic races of *Fusarium oxysporum* f. sp. *ciceris* (0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been reported worldwide. Prasad and Padwick (1939)[14] made 300 isolates of *Fusarium* and classified them into non-pathogenic types, wilt causing types and seed rotting types. It suffers badly due to drying of plants from the seedling stage up to flowering and podding stage. Several pathogens have been found associated with infected plants, which cause different diseases ultimately resulting in drying of plants. Chickpea plants may show wilt symptoms like wilting, necrosis, damping off, withering, drooping, dull-green discoloration, yellowing, loss of turgidity in leaves, browning of vascular system and eventual collapse of whole plant.

Pathogen penetrates the host plant through root apices or wounds and multiplies to plug the vascular bundles. Initially symptoms appear on upper leaves, flowers and twigs which show discoloration, desiccation, drooping of leaflets, rachis and petioles. After few days' whole plants wither and collapse. Early wilt symptoms observed after 20-30 days of sowing are termed as early wilt and during flowering to podding stage is known as late wilt. The plant growth-promoting endophytic bacteria isolated from chickpea plants stimulated the growth, nutrient acquisition, symbiotic performance and stress tolerance of chickpea plants in saline soil. Bacterial communities present in seeds can support the development of their host.[4] The anti-fungal activity was observed against *Curvularia* spp., *Fusarium oxysporum* and *Phytium ultimum* in several strains of *Pantoea, Microbacterium*, *Pseudomonas, Paenibacillus* and *Curtobacterium*.[22] Seed endophytes bacteria can play important roles during germination, seedling development and plant growth as they possess plant growth promoting and bio-control properties. The study of their application in diverse processes such as bio-fertilization and bio-energy production should be encouraged. This study will help to raise an understanding about the management of chickpea wilt.

Material and Methods

Sample collection

Chickpea plants showing the symptoms of wilt at seedling, flowering and pod formation stage were collected from sick plots, Department of Plant Breeding and Genetics, JNKVV, Jabalpur. *Fusarium oxysporum* f. sp. *ciceris* is characterized by the appearance of preliminary symptoms such as drooping and pale-coloured leaves. Later they collapse to a prostrate position and will be found to have shrunken stems both above and below ground level. When adult plants are affected, they exhibit wilting, the older leaves develop chlorosis while the younger leaves stay a dull green. Samples were brought into the laboratory and symptoms were identified and confirmed.

Isolation and identification of seed endophytic bacteria

Samples were washed with tap water, surface sterilized with 5% sodium hypochlorite followed by sterile-distilled water. 100 µl of the final wash (sterile-distilled water) was spread onto nutrient agar medium for control. For the test plate, 5.0 g surface sterilized seeds were macerated in 10 ml of sterile distilled water and 100 µl of suspension was cultured onto nutrient agar media. Plates were incubated at 28°C for 4 days, under consistent observation. Morphologically distinct colonies were marked and pure cultures of each marked colonies were prepared. Pure cultures were stored as Agar slant at –20°C until used. Each bacterial pure cultures were subjected to morphological characterization. Morphological characteristics observed were pigmentation, shape, elevation, edge, consistency and colony surface of bacterial colonies. Bacterial strains were identified as per described by (BMDB) Bergey's Manual of Determinative Bacteriology.[2] Bacterial cultures were then transferred to flask containing 150 ml of Nutrient Agar Broth (NAB) and incubated at room temperature on an orbital shaker for 24 hours at 200 rpm. Bacterial cells were harvested by centrifuging at 7000 rpm for 10 minutes and re-suspended in 10 ml $MgSO_4$. The separated cells were used for assay of seed endophytes.[15]

Pathogenicity test

The pathogen was mass multiplied on sand maize meal medium (90 g of sand, 10 g of maize meal and 40 ml of water) in 250 ml conical flasks. The flasks were autoclaved for 20 minutes at 15 psi. The medium was inoculated with two discs of 1.0 cm diameter mycelial growth of three days old culture of *Fusarium oxysporum* f.sp. *ciceris* grown on PDA. The flasks were incubated at 28 ± 2°C. After 3 weeks of incubation, inoculum was added to soil in pots @ 100 g kg-1. The seeds of chickpea were sown simultaneously with pathogen inoculation @

15 seeds per pot and an un-inoculated control was maintained. The plants were observed for wilt symptoms. Each treatment was replicated three times.

Test of antagonism in rolled towel test and pot experiment

The pot experiment was conducted at College of Agriculture, Jabalpur. This experiment was planned in CRD with seven treatments and JG11, JG16, JG315 and JG 62 varieties were used, so as to assess the efficacy of five different seed endophytic bacteria against *Fusarium oxysporum* f.sp. *ciceris* in sick pots. A control was sown without any seed treatment. (Table1)

Table 1: List of seed endophytic bacteria used in the study

S. No.	Isolate	Seed endophytic bacteria
1	SEB-1	*Pseudomonas chlororaphis*
2	SEB-2	*Bacillus subtilis.*
3	SEB-3	*Pseudomonas* sp.
4	SEB-4	*Bacillus sp.*
5	SEB-5	*Pseudomonas flurescens*

Results and Discussion

Effect of seed endophytic bacteria on growth of chickpea in case of rolled towel method

All the seed endophytic bacteria had positive effect on germination per cent, root and shoot length and vigour index across the four genotypes. The detailed findings of this experiment are mentioned below:

Table 2A: Evaluation of selected seed endophytic bacteria for chickpea plant growth under *Fusarium oxysporum* f. sp. *ciceris*

S. No.	Treatment	Germination %				Root length (cm)			
		JG16	JG315	JG11	JG62	JG16	JG315	JG11	JG62
1	SEB-1	78.32	82.52	82.66	70.60	8.16	9.62	9.66	6.30
2	SEB-2	83.02	86.52	86.66	74.20	8.46	10.66	8.66	7.33
3	SEB-3	86.60	88.32	87.33	76.20	9.96	11.01	10.66	8.20
4	SEB-4	76.70	81.12	79.33	68.60	7.96	8.12	7.66	6.10
5	SEB-5	88.24	91.72	88.00	78.80	10.96	11.87	12.66	8.26
6	Treated control	70.82	74.12	76.00	59.20	4.56	5.90	4.86	3.49
7	Un-treated control	74.4	78.32	82.00	65.00	6.96	8.00	6.66	5.25
SEm±		0.60	1.16	1.21	1.38	0.83	0.35	0.31	0.21
CD		1.85	3.55	3.71	4.24	2.56	1.09	0.95	0.65

Table 2B: Evaluation of selected endophytic bacteria for chickpea plant growth under *Fusarium oxysporum* f. sp. *ciceris*

S. No.	Treatment	Shoot length (cm)				Vigour index			
		JG16	JG315	JG11	JG62	JG16	JG315	JG11	JG62
1	SEB-1	7.56	10.60	11.00	5.01	1232.28	1668.55	1707.75	798.98
2	SEB-2	8.06	11.46	11.66	6.23	1372.65	1915.03	1760.93	1006.59
3	SEB-3	9.06	11.63	13.00	7.10	1648.34	1999.83	2066.22	1165.86
4	SEB-4	7.46	9.52	10.83	4.78	1183.78	1432.09	1466.81	746.57
5	SEB-5	10.06	12.06	15.00	7.66	1856.04	2196.14	2434.08	1255.59
6	Treated control	5.06	5.96	7.23	3.57	682.27	879.58	918.84	418.78
7	Un-treated control	6.06	9.96	8.79	4.33	969.72	1407.17	1266.90	622.70
SEm±		0.60	0.91	0.86	0.07				
CD.		1.85	2.79	2.65	0.22				

All the five seed endophytic bacteria, which were tested for their effect on plant growth parameters in presence of wilt pathogen and all the endophytic bacteria, had displayed positive effect on germination percent, root and shoot length and vigour index across the four genotypes of chickpea. This study was in confirmation with the work of Rangeshwaran *et al*. (2002)[15] who reported that out of twenty-five endophytic bacteria were identified as *Pseudomonas fluorescens* (PDBCEN 3), *Bacillus subtailis* (PDBCEN3), *Bacillus* sp. (PDBCEN9) *Pseudomonas* sp. (PDBCEN 2, 4, 6, 8) and screened these endophytes against *Fusarium oxysporum* f. sp. *ciceris* and *Sclerotium rolfsii*. They observed that endophytic isolates were able to promote better growth of chickpea and high germination percent and vigour index as compared to treated control on rolled towel test. (Table 2A, 2B).

Determination of the interactive effect of seed endophytic bacteria against *Fusarium oxysporum* f. sp. *ciceris*

The individual effect of different SEB's were evaluated against fungal pathogen treated and untreated controls in pot culture experiment. The results of pot culture experiment are mentioned under following heading.

Table 3A: Effect of SEB and their combination on Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceris*) disease incidence

Treatment	Treatment Combination	Germination (%)				Pre-emergence mortality (%)			
		JG11	JG16	JG315	JG62	JG11	JG16	JG315	JG62
T1	SEB-1	80.11	78.65	79.65	72.15	19.89	17.59	20.35	21.67
T2	SEB-2	83.24	81.41	80.16	76.67	16.76	16.14	19.84	20.00
T3	SEB-3	86.55	85.63	83.33	77.10	13.45	12.22	16.67	18.34
T4	SEB-4	79.65	76.56	76.33	70.12	20.35	19.82	23.67	23.34
T5	SEB-5	92.31	89.33	88.55	78.55	7.69	9.42	11.45	16.67
T6	Treated control	71.55	70.15	75.56	67.11	28.45	25.92	24.44	25.84
T7	Un-treated control	72.65	72.10	77.56	70.50	27.35	22.50	22.44	23.34
SEm±		1.79	2.02	1.90	2.36	1.27	1.50	1.44	0.86
CD		5.48	6.18	5.83	7.25	3.89	4.59	4.47	2.65

Table 3B: Effect of SEB and their combination on Fusarium wilt (*Fusarium oxysporum* f. sp. *ciceris*) disease incidence

Treatment	Treatment Combination	Post-emergence mortality (%)				Total mortality (%)			
		JG11	JG16	JG315	JG62	JG11	JG16	JG315	JG62
T1	SEB-1	17.11	17.00	19.15	18.82	37.00	34.59	39.50	40.49
T2	SEB-2	14.44	14.75	18.41	13.47	31.20	30.89	38.25	33.47
T3	SEB-3	12.64	12.82	17.51	12.49	26.09	25.04	34.18	30.83
T4	SEB-4	19.05	18.68	22.17	21.09	39.40	38.50	45.84	44.43
T5	SEB-5	10.13	9.53	12.52	10.87	17.82	18.95	23.97	27.54
T6	Treated control	30.29	23.95	27.69	26.32	58.74	49.87	52.13	52.16
T7	Un-treated control	27.53	22.46	26.50	21.36	54.88	44.96	48.94	44.70
SEm±		1.21	1.30	1.21	0.98	-	-	-	-
C.D.		3.71	4.06	3.71	3.00	-	-	-	-

All the treatments had higher germination percentage as compared to T_6 (Treated control). The highest germination percent was recorded in T_5 (92.31, 89.33, 88.55 and 78.55%) followed by T_3, T_2, T_1 and T_4 among all isolates compared with treated control T_6 (71.55, 70.15, 75.56 and 67.11%) in JG 11, JG 16, JG 315 and JG 62 respectively. The minimum pre-emergence mortality percent was found in T_5 (7.69, 9.42, 11.45 and 16.67%) followed by T_3, T_2, T_1 and T_4 as compared with T_6 (28.45, 25.92, 24.44 and 25.84%) in JG 11, JG 16, JG 315 and JG 62 respectively. Similarly, the minimum post-emergence mortality percent was found in T_5 (10.13, 9.53, 12.52 and 10.87%) followed by T_3 T_2, T_1 and T_4 as compared with T_6 (30.29, 23.95, 27.69 and 26.32%) in JG 11, JG 16, JG 315 and JG 62 respectively. The minimum total mortality percent was found in T_5 (17.82, 18.95, 23.97 and 27.54%) followed by T_3 T_2, T_1 and T_4 as compared with T_6 (58.74, 49.87, 52.13 and 52.16%) in JG 11, JG 16, JG 315 and JG 62 respectively.

Similar findings were reported by Zhu *et al.* (2017)[21] who observed that germination of *Ammodendron bifolium* seeds after inoculation with three different seed endophytic isolates named as AY3, AY9 and AG18 where AY3 significantly promoted seed germination at 10 days (43%) and 15 days (62%) followed by AY9 and AG18 (32 and 37% at 10 days and 50 and 53% at 15 days,

Table 4A: Effect of SEB and their combinations on Fusarium wilt disease and phenotypic parameter

Treatment number	Treatment Combination	Root length (cm)				Shoot length (cm)				Fresh weight (g/plant)			
		JG11	JG16	JG315	JG62	JG11	JG16	JG315	JG62	JG11	JG16	JG315	JG62
T1	SEB-1	16.12	11.44	12.56	10.50	40.21	34.50	35.76	32.20	12.00	15.45	12.33	8.87
T2	SEB-2	19.50	11.66	13.26	10.66	44.64	36.40	37.15	34.66	13.01	16.00	13.21	9.33
T3	SEB-3	21.25	12.33	15.53	11.10	45.54	38.56	40.90	35.33	14.01	17.19	13.33	10.22
T4	SEB-4	13.33	10.51	11.00	10.50	37.56	33.40	35.80	31.20	11.00	14.88	12.00	8.21
T5	SEB-5	23.12	13.50	16.00	11.50	48.52	40.21	42.25	37.50	15.33	18.20	14.00	10.33
T6	Treated control	9.33	9.33	9.10	8.00	35.22	25.60	30.22	26.55	8.66	12.00	8.67	6.67
T7	Un-treated control	11.01	9.68	10.25	9.33	36.23	30.20	32.25	28.28	9.66	13.23	11.33	8.00
SEm±		1.44	0.69	0.69	N/A	1.32	1.44	1.44	1.27	0.46	0.63	0.34	0.23
CD		4.42	2.12	2.12	0.86	4.06	4.42	4.42	3.89	1.41	1.94	1.06	0.70

Table 4B: Effect of EB and their combinations on Fusarium wilt disease and phenotypic parameters

Treatment number	Treatment Combination	Dry weight (g/plant)				Vigour Index (%)			
		JG14	JG16	JG315	JG62	JG14	JG16	JG315	JG62
T1	SEB-1	2.57	3.45	3.33	2.20	4512.59	3613.19	3848.68	3080.80
T2	SEB-2	2.44	4.12	3.67	2.30	5339.01	3912.57	4040.86	3474.68
T3	SEB-3	2.63	4.18	3.67	2.33	5780.67	4357.72	4702.31	3579.75
T4	SEB-4	2.10	3.12	2.83	2.02	4053.38	3361.75	3572.31	2924.00
T5	SEB-5	3.01	5.10	4.00	2.57	6613.08	4797.92	5158.03	3848.95
T6	Treated control	1.33	2.81	2.00	1.33	3187.55	2450.34	2971.01	2318.65
T7	Un-treated control	1.83	3.11	2.55	1.83	3431.98	2875.35	3296.3	2651.50
SEm±		0.23	0.34	0.23	0.17		-	-	-
C.D.		0.70	1.06	0.70	0.53		-	-	-

respectively). In the control, the germination percentage was found to be 3 per cent at 10 days and 13 per cent at 15 days. Nautiyal (1997)[12] observed that bio-control potential of *Pseudomonas sp.* against *F. oxysporum* f. sp. *ciceris, R. bataticola* and *Pythium sp.* in chickpea crop. Treatment of chickpea seeds with *Rhizobium sp.* and *Pseudomonas sp.* increased the germination by 22 to 34 per cent, survival 24 to 43 per cent, dry weight by 21 to 44 per cent, shoot length by 16 and 29 per cent and root length by 16 to 34 per cent respectively compared to non-bacterial strain. Muthu kumar *et al.* (2011)[11] reported that nine bacterial endophytes, EBC 5, EBC 7 and EBC 6 recorded the minimum mycelial growth with maximum inhibition zone of pathogen over control. In the present study, chilli seeds treated with these endophytes in combination (EBC 5 and EBC 6) recorded the lowest incidence of pre and post-emergence damping-off (9.10 and 12.33%, respectively) at seven and 14 days after sowing when compared to individual treatment. This was followed by seed treatment with EBC 5 and EBC 7 in combination. The combination (EBC 5 and EBC 6) treatment also increased the germination percentage (87.66%), shoot length (13.89 cm) and root length (4.0 cm) of chilli plants.

Effect of endophytic bacteria and their combination on growth parameters

All the treatments had higher root length as compared to T_6 (Treated control). The highest root length was recorded in T_5 (23.12, 13.50, 16.00 and 11.50 cm) followed by T_3, T_2, T_1, and T_4 among all isolates is compared with treated control T_6 (9.33, 9.33, 9.10 and 8.00 cm) in JG 11, JG 16, JG 315 and JG 62 respectively.

All the treatments had higher shoot length as compared to T_6 (Treated control). The highest shoot length was recorded in T_5 (48.52, 40.21, 42.25 and 37.5 cm) followed by T_3, T_2, T_1 and, T_4 among all isolates compared with treated control T_6 (35.22, 25.60, 30.22 and 26.55cm) in JG 11, JG 14, JG 315 and JG 62 respectively. All the treatments had higher vigour index as compared to T_6 (Treated control). The highest vigour index was recorded in T_5 (6613.08, 4797.92, 5203.47 and 3848.95) followed by T_3 (5780.67, 4357.72, 4832.10 and 3579.75), T_2 (5339.01, 3912.57, 4103.87 and 3474.68), T_1 (4512.59, 3613.19, 3800.36, 3080.805), T_4 (4053.38, 3361.75, 3583.00 and 2924.00) among all isolates in compared with treated control T_6 (3187.55, 2450.34, 2758.29 and 2318.65) in JG 11, JG 16, JG 315 and JG 62 respectively. Similar findings were obtained by Saravanan *et al.* (2013)[18] who reported that inoculation with fluorescent *Pseudomonas* induced a significant increase in root and shoot length (123 and 96% respectively) over the uninoculated control. In contrast, pathogen treatment caused a reduction in root and shoot length (4 to 24% and 13 to 23% respectively). Pathogen induced reduction in root and shoot length was nullified by fluorescent Pseudomonas inoculation. That is, the levels of root and shoot length in fluorescent Pseudomonas + pathogen infested plants reached next only to that of fluorescent Pseudomonas alone inoculated plants (100 to 104% increase in root length and 74 to 91% increase in shoot length over the control).

The data is in accord with the findings of Egamberdieva *et al*. (2017)[4] who evaluated four isolates (*Bacillus cereus* NUU1, *A. xylosoxidans* NUU2, *Bacillus thuringiensis* NUU3 and *B. subtilis* NUU4) for their ability to improve plant growth along with chickpea rhizobia symbiotic performance in pots under saline soil conditions found a significant improvement in the shoot height and nodule number compared to the un-inoculated plants. Reetha *et al*. (2014)[17] and Kumari and Khanna (2018)[9] studied antagonists *Pseudomonas* (Ps44, Ps45 and Ps47) and *Bacillus* (Ba1a and Ba19) selected on the basis of antagonistic activity. Evaluated under greenhouse conditions to control Fusarium wilt in chickpea varieties GPF-2 and JG 41 and found increment in seed emergence in GPF2 (86.6%) than negative control (66.6%). Stajkovic *et al.* (2011)[20] reported that co-inoculation with *Rhizobium* and *Pseudomonas* sp. LG or *Bacillus* sp. Bx improved root-shoot fresh and dry weight, nitrogen and phosphorus contents in bean plants, compared to inoculation with *Rhizobium* alone. It was observed that *Pseudomonas* sp. LG promoted bean growth and particularly phosphorus uptake more efficiently than *Bacillus* sp.

Conclusion

The seed endophytic bacteria have great potential in promoting plant health and providing defence to many plant pathogens as well as abiotic stresses. Therefore, the present study was attempted to isolate seed endophytic bacteria and evaluate their efficacy against wilt fungal diseases of chickpea. The treatment of seed endophytic bacterial isolate SEB-5, SEB-3, SEB-2, SEB-1 and SEB-4 was identified against wilt pathogen in reduce disease incidence, plant mortality and severity as well as promoting plant growth and health. The validation through rolled towel test and evaluation under pot experiment in glass house condition revealed significant differences among the treatment across the *Fusarium oxysporum* f. sp. *ciceris* pathogens.

Declaration of interests

The authors have no conflict of interest to declare.

Data sharing

All relevant data are within the manuscript.

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