

Original Research Article

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## Chickpea Survivability after Endophytic Seed Bacterization and Soil Application of Fe<sup>3+</sup> Edta in Green House Condition

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### A B S T R A C T

A greenhouse experiment was conducted to study the suppression of Fusarium wilt in suppressive and conducive soils. The soils were rendered suppressive by addition of endophytic bacterial antagonists through seed inoculation. Conducive soil was made by soil-inoculation *Fusarium oxysporum f. sp. ciceris* (FOC). An average of 64% to 96.4% of plant germinated in all the ten treatments where seed bacterization with endophytic bacteria was done. In case of control (only FOC) only 26% of plants germinated and in normal control (without any treatment) 60% of the plant survived. *Pseudomonas fluorescens* (EN-1) treated pots amended with 100 µM Fe<sup>3+</sup> Edta showed maximum (96.4%) germination in conducive soils. The next highest germination (88.0%) was observed in *Bacillus substalis* (EN-3) treated with 50µM Fe<sup>3+</sup> Edta amended conducive soils. The number of plant surviving after 40 days was recorded in all the treatments. None of the plant survived, in pathogen treated FOC but in normal soil 35.2% of plant survived.

#### Keywords

*F. oxysporum* f.sp.  
*Iycopersici*,  
*Pseudomonas*  
*fluorescens*

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### Introduction

Wilt caused by *Fusarium spp* is a very serious problem affecting all five continents supporting vegetation *Fusarium oxysporum* incite vascular wilt in crops that reduces the yield by as much as 80 to 90 percent among other species the vascular wilt caused by *Fusarium oxysporum*. Schlecht f sp ciceri (Padwick) of chickpea. *Fusarium udum* of

pigeonpea and *F. oxysporum* f.sp. *Iycopersici* in tomato are economically important in India. Recent studies indicate that endophytic bacteria colonizing the internal tissues of plants show promise in conferring systemic resistance to plants against plant diseases. The endophytic bacteria are present in various plant parts such as seed, leaves stem and fruits. The discovery of internal bacterial population in plant parts has been used to

support the modification of the rhizosphere concept to encompass soil microorganism that is able to penetrate plant roots. Some workers, went further to classify endophytic bacteria as a part of the rhizosphere community. Endophytic bacteria have a natural and intimate association with plants. The internal tissues of plants provide a uniform and safe environment when compared to the rhizosphere and phylloplane where the introduced brachial population must compete for nutrients with other microbes and endure fluctuation of temperature and exposure to UV rays. These advantages envisage the use of endophytic bacteria for more successful bio-corral of plant diseases. (Elad and Backer, 1985, Mahaffee and Kleopper, 1994; Sturtz and Christie 1995; Sushmitha and Gaikwar, 1995; Nautiyal *et al.*, 2001)

Selection of a successful bio-agent has been largely empirical since only 1 to 4% or less of the bacteria tested as potential bio-control agent and contribution of endophytic bacteria is an attractive possibility especially the control of vascular wilt diseases. The use of fungicides has proven to be unsatisfactory particularly against *Rhizoctonia solani*, *Sclerotium rolfsii*, *Pythium* spp., *Verticillium dahliae*, *Fusarium oxysporum* f.sp. *ciceri*, *Fusarium oxysporum* f.sp. *Lycopersici*, *Fusarium udum*. Few recent studies have actually shown that endophytic bacteria are able to significantly control vascular wilt and other diseases (Nejed, 1995, 2000; Nautiyal, 1997; Paultiz and Looper, 1991; Pleben *et al.*, 1995; Kieopper, 1997).

Selection of an effective antagonist is tedious since bacterial antagonists produce antibiotics *in vitro* but may be unfit and ineffective under the natural environment moreover some exhibit deleterious effect on plant health. We undertook a study to know the biocontrol potential of endophytic bacteria isolated from root and stem portions of different plant

samples to test the endophytic isolates for ability to inhibit wilt pathogens under *in vitro* and *in vivo* conditions.

## Materials and Methods

Endophytic bacteria were isolated from healthy roots and stem of commonly grown crops viz. Chickpea, Sunflower, Mustard, Niger and Bell pepper, where root rot, wilt and other soil-borne disease were prevalent. Diseased samples exhibiting collar rot/root rot/wilt symptoms were collected from campus.

## Isolation of endophytic bacteria

Whole plants of above mentioned various crop varieties were manually uprooted and transported immediately to laboratory. (For long distance transport the plants were kept at 10 °C). Root and stem sections (2-3 cm long) were made using a sterile scalpel. For younger plants (14 day) root samples were taken just below the soil line and 5-10 cm below the soil line were taken for older plants (21 day).

Stem sections were taken 1-2 cm above the soil line in younger plants and 10cm above the soil line in older plants. Stem samples were first weighed and surface sterilized with 20% hydrogen peroxide for 10 minutes and rinsed four times with 0.02 µM potassium phosphate buffer (pH 7.0) Root samples were surface disinfected with 1.05% sodium hypochlorite and washed in four changes of 0.02 µM phosphate buffer solution. Measured quantity of 0.1-mi aliquot from the final buffer wash was removed and transferred in 9.9 ml Tryptic-Soya broth to serve as sterility check. Samples were discarded, if growth was detected in the sterility check within 48 hr. Selected samples were triturated in 9.9 ml of buffer in a sterile pestle and mortar. The triturate was serially diluted in potassium phosphate buffer solution.

The dilutions whole poured on plates containing sterilized Tryptic Soy. Agar (TSA) medium. Representatives of Colony morphology were transferred to fresh TSA plates to blushed pure cultures. Shake culture of purified strains were prepared at room temperature or 18-24hr in Tryptic Soya Broth (TSB) and centrifuged at 5000 rpm for 7 minutes. Pellets were suspended in 2.0 ml TSB and re-suspended in (1:1 ratio) 20% glycerol and maintained at -80 °C in cryo-vials for later identification.

### **Seed-bacterizatton with endophytes**

For seed bacterization, the mix method of Baker and Cook (1986) was used. The surface sterilized seeds of chickpea were examined for the colony forming unit (cfu) on TSA plates, after 48 hr of incubation at 25 °C. The coated seed of chickpea carried ( $1 \times 10^7$ ) cfu per seed. The treated seeds were than sown in plastic containing soil and left for germination.

### **Assay for disease suppressiveness of soils**

The suppressive/conducive nature was confirmed by using the greenhouse assay. Test pathogen *Fusarium oxysporum f.sp ciceris* was grown for two weeks on sterile chickpea seeds which were than shaken in required quantity of sterile water in rotary shaker for the separation of mycelia mat and conidia. Measured amount of inoculum load of aqueous conidial suspension was added per pot (13 cm in dia) containing 1000g of soil. Ten chickpea seeds of Annigiri variety were planted with three replicates, pots containing uninoculated soil served as control. Pots were maintained in greenhouse and watered on alternate days with 50ml of sterile water.

### **Effect of iron<sup>iii</sup> on fusarium wilt**

To assay the disease conduciveness / suppressiveness of soil seed bacterization with

antagonistic bacterial endophyte and the effect of iron in soil, a greenhouse experiment was conducted One hundred milliliter of 50 µM and 100 µM of Ethyldiaminetetraacetatoferate (Fe III Edta) was added on alternate days to the pots containing approximately 1 Kg disease conducive / suppressive soil inoculated with 25 ml of *Fusarium oxysporum f. sp. ciceris* (FOC) inoculum ( $1 \times 10^5$  spores per ml.).

Ten selected endophytic bacteria were tested. Seed bacterization with endophytic bacteria was done as described by above.

### **Treatment details**

$T_1 = Pseudomonas fluorescens$  (EN-1) + FOC + different doses of Edta Fe(3+)

$T_2 = Pseudomonas spp.$  (EN-3) + FOC + different doses of Edta Fe(3+)

$T_3 = Bacillus subtilis$  (EM-3) + FOC + different doses Edta Fe(3+)

$T_4 = Pesudomonas sp.$  (EN-4) + FOC + different doses of Edta Fe(3+)

$T_5 = Endophyte$  (EN-5) + FOC + different doses of Edta Fe(3<sup>+</sup>)

$T_6 = Pseudomonas fluorescens$  + FOC + different doses of Edta Fe(3<sup>+</sup>)

$T_7 = Endophyte$  (EN-7) t FOC + different doses of Edta Fe(3+)

$T_8 = Pseudomonas sp.$  (EN-8) + FOC + different doses of Edta Fe(3+)

$T_9 = Bacills subtilis$  (EN-9) + FOC + different doses of Edta Fe3+

$T_{10} = Pseudomonas fluorescens$  (EN-10) + FOC + different doses of Edta Fe(3+)

Control: a. FOC alone, b.Edta F3+, c.CMC alone. Without FOC (normal)

Fourteen days after, soil inoculation with *Fusarium oxysporum f.sp. ciceris*, and seed inoculation with antagonistic bacteria wilt symptoms were recorded. For the measurement of wilt disease-index, the modified version (Chen *et al.*, 1995) was used. The bacterized plants were visually compared with non-bacterized plants infected with *Fusarium oxysporum f.sp. ciceris*

#### Disease severity index was rating by using 0-4 rating scale

0 = No disease (healthy seedling)

1 = 1-25% leaves symptom (wilting & chlorosis of plant with necrotic tip)

2 = 26-50% leaves symptom (wilting & chlorosis of plant with necrotic area above root tip)

3 = 51-75% partially necrotic stem and root less development of plants

4 = >75% severe necrosis of root and stem total wilting.

#### Relative disease reduction

For different bioassays the relative disease suppression (RDS) by bacterial antagonist was calculated by using equation  $R=CC-W/Cx100$ , Were R=represents the relative disease suppression. C=represents the average of percent diseased plants in control. W=represents the percent diseased plants in the bacterial treatment (Raijmaker *et al.*, 1995).

#### Mean Disease suppression

Disease suppression is the ability of endophytic bacteria to suppress the effect of a

disease on fresh weight, which is a relative index of disease suppression when as in case. This was calculating by using equation  $S=T-D/H-D$ , Where S=disease index, T=mean fresh weight (g) of treated plant, D=mean fresh weight (g) of healthy plant, D=mean fresh weigh (g) of control diseased plant (Chen *et al.*, 1995).

#### Results and Discussion

A greenhouse experiments' was conducted to study the suppression of FOC in suppressive and conducive soils. The soils were rendered suppressive (wilt resistant) by addition of antagonists by seed inoculation. Conducive (wilt sick) soil was made by soil inoculation with FOC. Both the types were amended with ferric iron to study the diseases progress since bacteria play a role in chelating ferric iron. The results are presented in Table 1 and 2. The number of surviving seedling at 40 days was recorded in suppressive and non-suppressive (conducive) soils challenge with FOC.

An average of 64.9 to 96.4 percent of the plants germinated in all the ten treatments where seed bacterization with endophytic bacteria was done. In case of pathogen control (only FOC), only 26 percent of the plants germinated and in normal control (without any treatment) 60 percent of the plants survived. *Pseudomonas fluorescens* (EN-1) treated pots amended with 100  $\mu M$   $Fe^{3+}$  Edta showed maximum (94.4 percent) germination in conducive soils (Table 1). The next highest germination (88.0 percent) was observed in *Bacillus substillis* (EN-3) treated and 50  $\mu M$   $Fe^{3+}$  Edta amended conducive soils. The numbers of plants surviving after 40 days were recorded in all the treatments. Results revealed that maximum number of surviving plants (72.9 percent) was recorded with *Pseudomonas fluorescens* (EN-1) treated pots amended with 100  $\mu M$   $Fe^{3+}$  Edta in FOC inoculated soils (conducive). In soils amended

with 50  $\mu\text{M}$   $\text{Fe}^{3+}$  Edta and inoculated with *Bacillus subtilis* (EN-3) 58.4 percent of the plants survived. In pots treated with endophyte (EN-5) amended with 50  $\mu\text{M}$   $\text{Fe}^{3+}$  Edta only 30.9 percent of the plants survived. None of the plants survived in pathogen control but normal soil 35.2 percent of the plants survived. With the above results per cent wilt suppression was calculated as per the method adopted by Chen *et al.*, (1095). Data presented in Table 1 reveal that all the endophytic bacteria could suppress wilt. Maximum wilt suppression (92.6 percent) was exhibited by the *P. fluorescens* endophyte (EN-6) when amended with 50  $\mu\text{M}$   $\text{Fe}^{3+}$  Edta. The next

highest was observed with *Pseudomonas sp* (EN-2) treated soil amended with 100  $\mu\text{M}$   $\text{Fe}^{3+}$  Edta. Wilt suppression was slow (13.7 percent) in *P. fluorescens* (EN-10) treated and 100  $\mu\text{M}$   $\text{Fe}^{3+}$  Edta amended soils. Even with 50 M  $\text{Fe}^{3+}$  Edta amended soils wilt suppression was low (21.8%) with *P. fluorescens* (EN-10) treated pots.

In normal pots (without any treatments) wilt suppression was calculated to be 96.7 percent but this data is not conclusive since no pathogen was added to the soil. Wilt suppression was 0 percent in pathogen control since none of the plants survived.

**Table.1** Survival of chickpea seedlings after seed bacterization with isolate bacteria endophytes before planting and soil application of  $\text{Fe}^{3+}$  Edta in conducive soils in green house

Treatment	Percent Germination		Percent increase		Percent wilt suppression	
	A	B	A	B	A	B
<i>Pseudomonas fluorescens</i> (EN-1)	77.5 *(62.43)	96.4 (77.58)	50.6 (46.80)	72.9 (56.43)	25.4 (28.90)	<b>21.3</b> <b>(25.92)</b>
<i>Pseudomonas sp.</i> (EN-2)	79.9 (63.14)	89.4 (70.87)	41.1 (39.89)	60.1 (52.08)	85.8 (67.53)	<b>81.8</b> <b>(63.83)</b>
<i>Bacillus subtilis</i> (EN-3)	88.0 (71.45)	95.5 (81.75)	58.4 (49.32)	70.2 (55.69)	68.7 (55.70)	<b>62.6</b> <b>(52.43)j</b>
<i>Pseudomonas sp.</i> (EN-4)	84.4 (67.50)	89.0 (72.00)	49.5 (44.78)	60.0 (50.46)	27.0 (27.68)	<b>21.2</b> <b>(27.35)</b>
<i>Endophyte</i> (EN-5)	69.0 (62.46)	80.0 (64.00)	39.9 (34.10)	60.7 (50.58)	57.8 (44.12)	<b>48.0</b> <b>(43.53)</b>
<i>P. fluorescens</i> (EN-6)	73.3 (59.39)	76.6 (63.80)	48.2 (43.06)	60.5 (50.60)	92.6 (73.96)	<b>59.7</b> <b>(50.70)</b>
<i>Endophyte</i> (EN-7)	73.0 (59.38)	79.0 (63.14)	51.3 (45.80)	52.6 (46.88)	68.8 (56.11)	<b>54.8</b> <b>(47.55)</b>
<i>P. fluorescens</i> (EN-8)	70.8 (55.66)	70.4 (55.60)	57.7 (48.62)	60.7 (51.12)	61.5 (51.71)	<b>37.9</b> <b>(37.85)</b>
<i>B. subtilis</i> (EN-9)	64.9 (54.16)	78.3 (62.43)	40.5 (39.89)	52.8 (46.80)	32.3 (34.73)	<b>25.9</b> <b>(30.26)</b>
<i>P. fluorescens</i> (EN-10)	71.6 (56.04)	78.3 (63.60)	45.9 (45.12)	56.2 (48.38)	21.8 (27.86)	<b>pr3"7</b> <b>(2T93~</b>
<i>Fe 3+Edta</i>	70.0 (59.36)	73.0 (59.02)	92.5 (73.29)	77.7 (62.23)	25.2 (29.35)	<b>20.2</b> <b>(27.14)</b>
<i>CMC +FOC</i>	46.6 (43.53)		25.4 (30.16)		13.7 (21.90)	-
<i>CMC alone</i>	56.6 (50.08)		22.6 (27.95)		34.5 (35.25)	-
<i>FOC alone</i>	26.0 (33.76)		0.00		0.00	-
<i>Normal (control)</i>	60.0 (58.12)		35.2 (36.25)		96.7 (80.76)	-
<i>CD at 5%</i>	<b>4.802</b>		<b>3.866</b>	<b>2.670</b>	<b>1.742</b>	<b>1.688</b>

FOC – *Fusarium oxyrium oxysporum f.sp. ciceri* test pathogen

Edta – Ethylendiaminetetraacetoferrate A – 50  $\mu\text{M}$

Edta conc. B-100 M Edta conc.

CMC – Carboxy Methyl Cellulose

\*Figures in parenthesis are angular transformed values.

**Table.2** Combined influence of seed-bacterization with isolated bacterial endophytes and soil application of Fe<sup>3+</sup> Edta and FOC on growth parameter of chickpea in green house

Treatment	Plant height (cm)		Fresh weight (g)		Dry weight (g)		Vigor Index	
	A	B	A	B	A			
<i>Pseudomonas fluorescens</i> (EN-1)	36.4	28.7	18.4	18.0	16.4	15.2	2356.0	<b>2766.61</b>
<i>Pseudomonas j sp.</i> (EN-2)	49.9	44.6	21.5	19.8	16.5	15.6	3987.0	<b>3986.2</b>
<i>Bacillus subtilis</i> (EN-3)	33.8	33.4	19.9	18.8	14.7	13.5	2974.0	<b>3189.7</b>
<i>Pseudomonas sp.</i> (EN-4)	54.3	43.8	17.6	16.1	16.0	14.5	4604.6	<b>3933.2</b>
<i>Endophyte</i> (EN-5)	42.9	50.2	19.0	17.3	14.5	13.6	2960.1	<b>4056.0</b>
<i>P. fluorescens</i> (EN-6)	43.7	39.6	23.0	19.3	15.0	13.5	3203.2	<b>3033.3</b>
<i>Endophyte</i> (EN-7)	28.4	26.5	20.0	17.8	11.4	9.7	2073.2	<b>2093.5</b>
<i>P. fluorescens</i> (EN-8)	23.4	22.7	20.4	18.5	13.2	10.0	1656.7	<b>1598.0</b>
<i>B. subtilis</i> (EN-9)	23.8	20.9	19.0	17.4	8.7	7.7	1544.6	<b>1636.4</b>
<i>P. fluorescens</i> (EN-10)	22.9	22.3	18.1	16.4	10.0	19.0	1639.6	<b>1746.0</b>
<i>Fe 3+Edta</i>	28.1	26.6	13.4	13.9	11.0	10.0	1967.0	<b>1941.8</b>
<i>FOC alone (control)</i>	0.0		0.0		0.0		0.0	
<i>Normal (control)</i>	37.6		24.5		5.0		2274.0	
<i>CMC+FOC</i>	16.9		2.2		6.7		787.5	
<i>CMC alone</i>	29.36		10.2		6.4		1031.0	
<b>CD at 5%</b>	<b>4.807</b>	<b>4.660</b>	<b>3.601</b>	<b>3.084</b>	<b>2.358</b>	<b>2.060</b>	<b>13.548</b>	<b>15.642</b>

FOC – *Fusarium oxysporum f.sp. ciceris* -test pathogen

Edta – Ethylenediaminetetraacetoferrate A – 50 µM Edta conc.

B-100 µM Edta conc. CMC- Carboxy Methyl Cellulose

Growth parameters of chickpea plants from the above described experiment were recorded and the results are presented in Table 2. Data revealed that maximum plant height (54.3cm) was noticed with *Pseudomonas sp.* (EN-4) treatment amended with 50 µM Edta in soil. In normal (control) treatment 24.5 cm plant height was recorded but lowest plant height of 20.9 cm was recorded with *B subtilis* (EN-9) treatment amended with 100 µM of Fe<sup>3+</sup>. Maximum fresh weight (23.0 g) was recorded in *P. fluorescens* (EN-5) treated pots amended with 50 µM of Fe<sup>3+</sup>. Edta but in case of normal, it was 24.5 g. Lowest fresh weight was recorded in *Pseudomonas sp.* (EN-4) treatment amended with 100 µM of Fe<sup>3+</sup>. Dry weight was maximum (16.5g) with *Pseudomonas sp.*

(EN-2) in 50 M Edta amended soil but in case of normal it was 15.0 g Plants treated with *B subtilis* (EN-9) and amended with 100 µM of Fe<sup>3+</sup> exhibited again lowest dry matter content.

In an experiment conducted, the low plant growth parameters observed in some of the endophyte treated plants might be due to increased concentration of iron in soil. Increased iron concentration also affects production of siderophores by antagonistic bacteria. Similar observations were reported by Kloepper *et al.*, (1980). Baker and Cook (1988) and Howell and Stipanovic (1980). Vigour index was also calculated treatments with *Pseudomonas sp.* (EN-4) at 50 µM gave maximum vigour indices (4604.6) whereas,

lowest (1544.6) was with *B. subtilis* (EN-9) at 50 µM Edta in conducive soils. The addition of seed bacterization with endophytic bacteria and soil application of Fe 3+ Edta at 50 µM and 100 µM did cause increase growth except in FOC inoculated conducive soils.

The above results indicate the possibility of converting a conducive soil to a suppressive soil by inoculating with endophytic bacteria and addition of Edta Fe<sup>3+</sup> inoculation of healthy chickpea seeds with a known suspension of endophytic bacterium before sowing resulted increased seed germination (30% to 100%), when compared uninoculated seeds in presence of FOC. When the two doses Edta Fe<sup>3+</sup> (50 µM and 100 µM) were used as soil treatment at alternate days in FOC inoculated conducive soils the soils remained conducive. Hence, addition of endophytic bacteria through seed bacterization to a FOC inoculated conducive soils converted them to become suppressive in nature. The differential effect on plant survival and wilt suppression indicate that elimination of suppressiveness in all the treatments by the adition of Fe<sup>3+</sup> Edta and seed inoculation with endophytic bacteria suggest that, siderophores which are only produced by microorganisms in response to iron limiting conditions make it unavailable to the pathogen in the soil(Kleopper *et al.*, 1980).

Endophytic bacteria residing in roots and stems of plants play role in disease suppression. Some of them are also plant growth promoters. The ability to convert conducive soils into suppressive soils implies that selected endophytic bacteria, fluorescent Pseudomonads or other siderophore producing microorganisms may be successfully used for the biological control of Fusarium wilts and wilt like disorders. The capacity of these endophytic bacteria to protest plants from disease by merely treating

seeds is indicative of their root/plant colonizing ability and seed treatment with endophytes prior to sowing is recommended for biocontrol of wilt diseases Moreover treatment of seeds with endophytes prior to sowing should be advocated to farmers and this will go a long way in strengthening biocontrol approaches to combat plant diseases.

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