*Phytopathol. Mediterr.* (2010) 49, 179–186

# *In vitro* efficacy of bacterial endophytes against the chilli damping-off pathogen *Pythium aphanidermatum*

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**Summary.** Damping-off of chilli caused by *Pythium aphanidermatum* is a major nursery disease in vegetables. The effect of ten endophytic isolates of *Pseudomonas fluorescens* was tested against *P. aphanidermatum* under *in vitro* conditions. All the isolates tested were short rods and produced bright fluorescence when exposed to UV light. Among the isolates, EBS 20 produced the largest inhibition zone and the least mycelial growth of *P. aphanidermatum*. With regard to the secondary metabolites, *P. fluorescens* EBS 20 produced most salicylic acid, siderophore and hydrogen cyanide. The culture filtrate of this isolate at a 15% concentration totally inhibited mycelial growth of *P. aphanidermatum*. In addition, chilli seeds treated with culture filtrate of this isolate also led to the greatest plant growth in terms of root length, shoot length and vigour index.

**Key words**: biological control, hot pepper, plant growth promotion, *Pseudomonas fluorescens*.

**Abbreviations**: EBS, endophytic bacteria stem; EBR, endophytic bacteria root.

## Introduction

Chilli (*Capsicum annuum* L.) is a universal spice in India. It belongs to the genus *Capsicum*, family Solanaceae, and is popularly known as "red pepper". The red colour of chilli is caused by a pigment capsanthin, and its pungency is attributed to an alkaloid capsaicin. Capsaicin is used in the pharmaceutical and cosmetic industries to cure colds and throat and chest congestion. Chilli also contains vitamins A, C and E. The chilli crop is affected by a number of fungal, bacterial and viral diseases. Among the fungal diseases, damping-off caused by *Pythium aphanidermatum* (Edson) Fitzp. is very common and produces serious loss in yield. It causes 60 per cent mortality of seedlings both in the nursery and in the main field (Manoranjitham *et al*., 2000). Though fungicides and insecticides control damping-off, they are not a long-term solution because of concerns over exposure, health and environmental hazards, and residue persistence. Moreover, the frequent use of pesticides may cause tolerance in the target organism. As a result, in recent years the focus has shifted to the control of insect pests using bio-control agents, which are a safe and promising alternative to synthetic pesticides.

There is some evidence that endophytes contribute to the control of plant diseases (Kloepper *et al*., 1992). Endophytic bacteria are prokaryotes that colonize the internal tissues of healthy plants but do not cause any disease symptoms (Wilson, 1995). Bacterial endophytes may promote plant growth and improve the host's capacity to with-

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stand pathogen attack by competing with the pathogens, and by competition, antibiosis and by causing systemic resistance. In India, some limited work has been done isolating the endophytic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis* from the stems and roots of chilli seedlings (Muthukumar, 2008); and isolating *Bacillus* sp., *P. fluorescens* and *Pantoea* sp. from chickpea (Rangeshwaran *et al*., 2008). The internal tissues of plants provide a uniform and safe environment when compared to the rhizosphere and the phylloplane, where an invasing bacterial population must compete for nutrients and is subjected to temperature changes and UV rays. These advantages of endophytic bacteria suggest that they can be exploited for the biological control of plant diseases (Sturz and Christie, 1995; Nejed and Johnson, 2000). We undertook a study to determine the biological control potential of endophytic bacteria isolated from the stem and root portions of chilli seedlings.

## Materials and methods

### Seed material, endophytic bacterial isolates and pathogen

The experiments were conducted in the Department of Plant Pathology, Annamalai University, Annamalainagar, India. Chilli plants cultivar Coimbatore1 (Co 1), were obtained from the Department of Olericulture, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, India. Bacterial endophytes were isolated from stem and root portions of these plants. Isolation was done following the method described by Rajendran *et al*. (2006). A total of 45 stem isolates and 35 root isolates were obtained from the chilli plants and designated as EBS (Endophytic Bacteria Stem) (1–45) and EBR (Endophytic Bacteria Root) (1–35). The pathogenic fungal strain used in all the experiments was isolated from the soil of a nursery in Annamalainagar, purified in plain agar by the single hyphal tip method (Rangaswami, 2005), and identified as *P. aphanidermatum* at the National Centre of Fungal Taxonomy (NCFT), Indian Agricultural Research Institute (IARI), New Delhi, India. Based on the dual culture technique, those endophytic bacterial isolates that were effective were used for further study.

### Identification of effective endophytic bacteria

In vitro *bioassay*

The antagonistic activity of the endophytic bacteria against *P. aphanidermatum* was tested by the dual culture technique (Dennis and Webster, 1971) on PDA. Effective endophytic bacterial isolates were selected on the basis of how well they inhibited the growth of the pathogen. The percent inhibition of mycelial growth was calculated according to Vincent (1929).

Inhibition % (I) = C-T / C × 100

Where: C, radial growth in control; T, radial growth in treatment; I, percent inhibition.

*Biochemical tests*

Effective endophytic bacterial isolates were identified with various biochemical tests (gram staining, KOH test, motility, gelatin hydrolysis, nitrate reduction, fluorescent pigment and starch hydrolysis) given by Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

*Morphological studies of effective endophytic isolates of* P. fluorescens

The morphological characters (shape and colony colour) of the effective endophytic isolates were performed with the method described by Buchanan and Gibbson, 1974.

### Production of secondary metabolites

Salicylic acid, siderophore and HCN produced by the effective endophytic *P. fluorescens* isolates were determined by the method of Meyer *et al*. (1992); Reeves *et al*. (1983); Siddiqui Imran *et al*. (2006). Each test was carried out in three replications.

### Effect of culture filtrates on the mycelial growth of *P. aphanidermatum*

*Pseudomonas fluorescens* isolates were grown on King's B broth under constant shaking at 100 *g* for 48 h at room temperature (28±2°C). Bacterial cells were harvested by centrifugation at 12,000 *g* for 15 min, and resuspended in PB (0.01 M, pH 7.0). The concentration was adjusted to approximately $10^8$ cfu $mL^{-1}$ ($OD_{595}$=0.3) with a spectrophotometer and used as inoculum for seed bacterization (Thompson, 1996) while culture filtrates were used to evaluate the effects on mycelial growth.

Culture filtrates of the effective bacterial isolates were separately incorporated into sterilized

PDA at 5, 10 and 15% concentration by adding the calculated quantity of the culture filtrate to the medium using a sterile pipette. Fifteen mL of bacterized medium was transferred separately to sterile Petri dishes and allowed to solidify. Each Petri dish was inoculated at the centre with a three-day-old (six mm) disc of *P. aphanidermatum* grown on PDA. Each treatment was replicated three times. Sterile water served as a control. The diameter of the mycelial growth (in mm) of *P. aphanidermatum* was measured when the mycelium fully covered the control plates (Nene and Thapliyal, 2000).

**Plant growth promotion**

Seed bacterization was done by the method of Rajendran *et al*. (2006).

The plant growth promoting activity of the effective bacterial isolates was assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). The vigour index was calculated following Abdul Baki and Anderson (1973), and the germination percentage was also calculated.

**Statistical analysis**

The effect of treatments on pathogen growth, production of secondary metabolites and the vigour index was analysed by analysis of variance (ANOVA), and treatment means were compared by Duncan's multiple range test (DMRT). The data on germination were arcsine transformed before statistical analysis (Gomez and Gomez, 1984).The IRRISTAT package version 92-1, developed by the International Rice Research Institute Biometrics Unit, Philippines, was used for analysis.

## Results

**Identification of effective endophytic bacteria**

*Biochemical tests*

The gram reaction and biochemical tests performed to identify the effective endophytic isolates showed that all isolates gave similar results with regard to gram staining, KOH test, nitrate reduction and starch hydrolysis (which showed negative results), and motility, gelatin hydrolysis and fluorescent pigment (which produced positive results), thus confirming the identification as *P. fluorescens*.

In vitro *inhibition of P. aphanidermatum by the bacterial isolates*

Table 1 lists the varying degrees of antagonism to *P. aphanidermatum* by the effective *P. fluorescens* isolates. Among the isolates tested, *P. fluorescens* EBS 20 produced the widest inhibition zone, 13.66 mm, with a minimum of 21 mm of mycelial growth of *P. aphanidermatum*, reducing mycelial growth by 76.7% compared with the control. This was followed in order by *P. fluorescens* isolate EBS 4. The other isolates (EBS 1, 14, 27, 29, 36 and EBR 10, 19, 22) were less effective in inhibiting mycelial growth of *P. aphanidermatum in vitro*.

*Morphological characteristics of effective isolates*

The ten effective bacterial isolates tested showed variations in colony type, colony colour and growth type but were similar in cell shape and reaction to UV light fluorescence. All the isolates were short rod in shape and produced bright fluorescence when exposed to UV light. Colony type varied from round to irregular. Colony colour varied from yellowish to greenish yellow, yellowish green and dull yellow. The growth type varied from fast to slow (Table 2).

**Secondary metabolites produced by effective isolates**

Table 2 shows that all the *P. fluorescens* isolates produced salicylic acid, siderophore and hydrogen cyanide. *P. fluorescens* EBS 20 produced the greatest amount of salicylic acid, siderophore and hydrogen cyanide (13.1 $\mu$g $mL^{-1}$, 11 $\mu$mol $mL^{-1}$ and 0.08 respectively) followed by *P. fluorescens* EBR 4 (9.3 $\mu$g $mL^{-1}$, 10.2 $\mu$mol $mL^{-1}$ and 0.07 respectively). The lowest amount of salicylic acid, siderophore and hydrogen cyanide was produced by EBR 19 (0.88 $\mu$g $mL^{-1}$, 1.3 $\mu$mol $mL^{-1}$ and 0.01 respectively).

**Effect of culture filtrates of isolates on the mycelial growth of *P. aphanidermatum***

Different concentrations of culture filtrate of *P. fluorescens* isolates varied in their effect on the mycelial growth of *P. aphanidermatum in vitro*. Culture filtrate of *P. fluorescens* isolate EBS 20 and EBR 4 totally inhibited mycelial growth of *P. aphanidermatum* at a concentration of 15% *in vitro*, followed by *P. fluorescens* isolate EBS 1, which gave a 90.4% inhibition of mycelial growth at that concentration. However, a 5% concentration of culture filtrate was not effective against *P.*

Table 1. *In vitro* inhibition of mycelial growth of *Pythium aphanidermatum* by effective endophytes.

| Isolate | Mycelial growth[a] (mm) | Inhibition zone[a] (mm) | Percent inhibition over control |
|---|---|---|---|
| EBS - 1 | 25.00 c | 11.33 bc | 72.22 |
| EBS - 14 | 29.33 fe | 9.30 fg | 67.41 |
| EBS - 20 | 21.00 a | 13.66 a | 76.66 |
| EBS - 27 | 31.33 gf | 9.20 fgh | 65.18 |
| EBS - 29 | 27.66 ed | 9.73 e | 69.26 |
| EBS - 36 | 32.00 hg | 9.08 ih | 64.44 |
| EBR - 4 | 23.33 b | 12.00 b | 74.07 |
| EBR - 10 | 28.00 ed | 9.33 fe | 68.88 |
| EBR - 19 | 33.66 hd | 9.00 igh | 62.20 |
| EBR - 22 | 26.33 cb | 10.43 cd | 70.74 |
| Control | 90.00 i | 90.00 j | - |

[a] Values are means of three replications. Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P$=0.05.

Table 2. Cultural characteristics and secondary metabolites produced by effective endophytes.

| Isolate | Colony type | Colony colour | Salicylic acid production[a] ($\mu$g mL$^{-1}$) | Siderophore production[a] ($\mu$mol benzoic acid mL$^{-1}$) | HCN production[a] (absorbance at 625 nm) |
|---|---|---|---|---|---|
| EBS - 1 | Round | Yellowish | 5.56 c | 7.86 bc | 0.05 bc |
| EBS - 14 | Round | Greenish yellow | 1.73 f | 3.16 def | 0.04 cd |
| EBS - 20 | Round | Yellowish green | 13.10 a | 11.00 a | 0.08 a |
| EBS - 27 | Round | Yellowish | 1.24 g | 2.43 ef | 0.02 de |
| EBS - 29 | Round | Dull yellowish | 4.33 e | 5.26 cde | 0.03 cde |
| EBS - 36 | Round | Yellowish | 1.16 h | 2.30 ef | 0.02 de |
| EBR - 4 | Round | Yellowish green | 9.30 b | 10.20 b | 0.07 ab |
| EBR - 10 | Irregular | Dull yellowish | 1.24 g | 2.43 ef | 0.02 de |
| EBR - 19 | Irregular | Yellowish | 0.88 i | 1.30 f | 0.01 e |
| EBR - 22 | Round | Greenish yellow | 5.10 d | 6.03 cd | 0.05 bc |

[a] See Table 1.

*aphanidermatum*, irrespective of the isolate from which the culture filtrate was derived (Table 3). The culture filtrate of all the isolates inhibited *P. aphanidermatum*. Generally, an increase in the concentration of the culture filtrate reduced the mycelial growth of the pathogen.

**Effect of effective bacterial endophytes on plant growth**

The culture filtrate of the *P. fluorescens* isolates did not inhibit the germination of chilli seeds. However, soaking the seeds in the culture filtrate significantly increased shoot and root length compared with the untreated control. Root length, shoot length and the vigour index were greatest when the chilli seeds were treated with the 48-h-old culture filtrate of the *P. fluorescens* isolate EBS 20 (4.26 cm, 8.20 cm and 1136.27 respectively). The other isolates also significantly increased chilli shoot and root length, with the exception of the control (Table 4).

## Discussion and conclusions

Damping-off of chilli causes severe yield loss. The control of damping-off with cultural practices and toxic chemicals has both advantages and disadvantages. Controlling damping-off by biological methods has become increasingly important recently. Among biological control methods, endophytic bacteria can serve as an alternative to chemical pesticides.

The endophytic *P. fluorescens* isolate EBS 20 produced the greatest inhibition of *P. aphanidermatum in vitro*. Similarly, Muthukumar (2008) reported that *P. fluorescens* stem isolate 5 significantly reduced colony growth of *P. aphanidermatum*. Muthukumar and Bhaskaran (2007) stated that among 12 isolates of *P. fluorescens* they tested, isolates 3 and 4 were highly effective in inhibiting mycelial growth of *Pythium* sp. Nakkeeran *et al*. (2006) reported that *P. chlororaphis*

Table 3. Effect of culture filtrate of effective endophytes on the growth of *Pythium aphanidermatum*.

| Isolate | pH of the culture filtrate | Concentration of culture filtrate | | | | | |
|---|---|---|---|---|---|---|---|
| | | 5% | | 10% | | 15% | |
| | | Mycelial[a] growth (mm) | Inhibition % over control | Mycelial growth (mm) [a] | Inhibition % over control | Mycelial growth (mm) [a] | Inhibition % over control |
| EBS - 1 | 6.6 | 30.00 c | 66.66 | 20.66 cb | 77.04 | 8.66 d | 90.37 |
| EBS - 14 | 6.5 | 38.66 e | 57.04 | 29.00 fe | 67.77 | 19.33 f | 78.52 |
| EBS - 20 | 6.8 | 20.00 a | 77.77 | 11.33 a | 87.41 | 0.00 c | 100.00 |
| EBS - 27 | 6.5 | 39.00 fe | 56.66 | 30.00 f | 66.66 | 22.33 g | 75.18 |
| EBS - 29 | 6.4 | 36.66 ed | 59.26 | 25.66 ed | 71.48 | 24.33 g | 72.96 |
| EBS - 36 | 6.4 | 40.66 gf | 55.55 | 31.33 gf | 65.18 | 18.66 f | 79.26 |
| EBR - 4 | 6.7 | 25.33 b | 71.85 | 17.66 b | 80.37 | 0.00 c | 100.00 |
| EBR - 10 | 6.3 | 37.66 fe | 58.15 | 28.00 fe | 68.88 | 19.00 f | 78.88 |
| EBR - 19 | 6.4 | 43.00 g | 52.22 | 36.33 g | 59.63 | 25.00 g | 72.22 |
| EBR - 22 | 6.5 | 33.33 dc | 62.96 | 24.00 dc | 73.33 | 11.66 e | 87.04 |
| Control | 7.0 | 90.00 g | - | 90.00 h | - | 90.00 h | - |

[a] See Table 1.

Table 4. Effect of effective endophyte isolates on chilli seedling growth.

| Isolate | Germination[a, c] (%) | Shoot length[a] (cm) | Root length[a] (cm) | Vigour index | Increase (%) of vigour index over control |
|---|---|---|---|---|---|
| EBS - 1 | 88.33 (70.02) a | 3.96 b | 7.66 c | 1026.39 | 58.81 |
| EBS - 14 | 87.66 (69.43) b | 3.67 d | 6.70 fe | 909.03 | 40.65 |
| EBS - 20 | 89.33 (70.93) a | 4.26 a | 8.20 a | 1136.27 | 75.81 |
| EBS - 27 | 87.33 (69.14) b | 3.60 ed | 6.50 gfe | 900.96 | 39.40 |
| EBS - 29 | 88.00 (69.73) b | 3.70 d | 7.43 dc | 979.44 | 51.54 |
| EBS - 36 | 87.66 (69.43) b | 3.53 fh | 6.33 hgf | 880.00 | 36.16 |
| EBR - 4 | 88.00 (69.73) b | 4.00 b | 7.86 b | 1043.68 | 61.48 |
| EBR - 10 | 87.33 (69.14) b | 3.66 ed | 7.00 ed | 930.93 | 44.04 |
| EBR - 19 | 87.00 (68.86) c | 3.43 hfg | 6.23 igh | 862.40 | 33.43 |
| EBR - 22 | 88.66 (70.32) a | 3.80 c | 7.50 dc | 1001.85 | 55.01 |
| Metalaxyl (0.1%) | 89.00 (70.63) a | 4.33 a | 8.23 a | 1135.64 | 75.71 |
| Control | 82.33 (65.14) | 2.23 i | 5.56 | 646.29 | - |

[a] See Table 1.
[b] Values are means of three replications.
[c] Values in parentheses are arcsine transformed.

strain PA23 and *B. subtilis* strain BSCBE4 most strongly inhibited mycelial growth of *P. aphanidermatum* causing chilli damping-off. The mycoparasitic potential of *Pseudomonas* spp. has been well documented by earlier workers (Anitha and Tripathi, 2001; Bhowmik *et al*., 2002). *P. fluorescens* isolate EBS 20 produced higher levels of extracellular metabolites like siderophore, salicylic acid and HCN when compared with other isolates. Similarly, antifungal compounds such as pseudobactin, HCN, salicylic acid and 2-hydroxy phenazine produced by fluorescent pseudomonads suppressed plant pathogenic fungi (Ongena *et al*., 1999; Velazhahan *et al*., 1999; Dave and Dube, 2000; Gupta *et al*., 2001; Pandey *et al*., 2006; Hofte and Bakker, 2007; Reddy *et al*., 2008).

Chilli seeds soaked with the culture filtrate of *P. fluorescens* EBS 20 produced the greatest shoot length and the highest vigour index. This may be due to the growth promoting substances, gibberellin, cytokinin and indole-acetic acid that produces *P. fluorescens* (Jagtap, 2002). Similarly, Valarmathi (2007) reported that tomato seeds treated with *P. fluorescens* increased seed germination, seedling growth and vigour (Rangeshwaran and Prasad, 2000).

Preliminary mass spectrum analysis through a NIST search suggested that the *P. fluorescens* isolates used in this paper produce some compounds close related to phenazine (data not published). Phenazine derivatives were reported to be strong inhibitors of plant pathogens, especially the oomycete group of fungi including *Pythium* species (Turner and Messenger, 1986). More recently, Kavitha *et al*. (2005) reported that phenazine derivatives produced by *P. fluorescens* were effective against *P. aphanidermatum*, disorganizing hyphal morphology by causing vacuolation, cell degeneration and lysis. Similarly, it is hypothesized that metabolites produced by *P. fluorescens* reduced *P. aphanidermatum* growth in the current *in-vitro* study, which suggests the need of further investigation.

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*Accepted for publication: March 14, 2010*