# Endophytic bacteria from tomato and their efficacy against *Fusarium oxysporum f.sp. lycopersici*, the wilt pathogen

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## ABSTRACT

Endophytic bacteria have been found in virtually every plant studied, in which they are reported to help in plant growth promotion and fight against plant pathogens in addition to contribution to yield of crop plants. The colonies of endophytes in the internal tissues also produce a variety of natural products which could be exploited for potential pharmaceutical, agricultural and industrial use. Bacterial endophytes were isolated from surface sterilized root, stem, leaves and fruits of healthy tomato plants. Several isolates belonging to four bacterial genera *viz., Bacillus, Pseudomonas, Klebsiella* and *Citrobacter* were obtained and identified using standard biochemical methods. Of the bacterial endophytes isolated in the study, only 50% of the isolates showed antagonistic activity against *Fusarium oxysporum f. sp. lycopersici*, the pathogen causing wilt disease in tomato. Bacterial metabolites like siderophore, hydrogen cyanide, indole acetic acid and salicylic acid in the culture media were studied. The result showed that comparatively the maximum quantity of siderophore (53.6%), hydrogen cyanide (45%), and salicylic acid (48.7%) was produced by *Pseudomonas* and indole acetic acid (48.2%) by *Bacillus*. Among the isolated endophytes, *Pseudomonas* was found to exhibit superior antagonistic activity against the test pathogen.

**Keywords**: Endophytes, *Fusarium*, HCN, IAA, salicylic acid, siderophore.

## INTRODUCTION

A large number of microorganisms are in continuous association with plants and animals under natural habitat. The plant interior is now recognized as a prolific environment for the discovery of endophytes with new biological activities especially biocontrol capabilities (Favaro *et al.,* 2012). While bacteria living in the gut of animals play significant role in stimulating immune response (Hopper *et al.,* 2001), plant associated endophytic bacteria could trigger defense mechanism against various stress conditions. Some bacteria associate with the roots of the plants and establish a mutualistic relationship benefiting the plants in nutrition and deriving their nutritive support from root exudates without any harm to the host (Hallmann *et al.,* 1997; Azevedo *et al.,* 2000). Bacon and White (2000) defined endophytes as "microbes that colonize living, internal tissues of the plants without causing any immediate, overt negative effects". However, the population density of the endophytes are comparatively lower than the rhizophere colonizing bacteria and plant pathogens (Hallmann *et al.,* 1999; Rosenblueth and Martinez-Romero, 2004). Endophytic bacteria promote plant growth by producing growth regulating compounds; act as biocontrol agents against a variety of other harmful microbes in addition to the production of medically and industrially important metabolites in the internal tissues. The commonly occurring endophytes include *Pseudomonas* and *Bacillus* and these genera have been well studied and established as candidates for the production of a variety of products such as antibiotics, antitumor compounds, organic substances, antiviral principles and compounds of insecticidal property. Effect of endophytic bacteria influence the nitrogen fixation that benefits the plants (Hurek *et al.,* 2002; Iniguez *et al.,* 2004) or the production of phytohormones and also participate in the biocontrol of pathogenic microbes by inducing systemic host resistance, producing siderophores to chelate iron and creates a deficiency situation to pathogens (Sessitsch *et al.,* 2004). Endophytes from tomato plants were demonstrated to have antagonistic activity against

pathogenic fungi (Sessitch *et al.*, 2004). The *in vitro* production of antibiotics and siderophores has also been reported (Sessitsch *et al.*, 2002). The bacterial antagonists could also detoxify the toxins produced by pathogenic microbes and thus aid in the plant growth and production.

The endophytes, which are systemically present inside the plant, pose a competition and antagonism against systemic pathogens like *Fusarium.* The study included endophytes from various tissues including fruits, since control of pathogenic establishment inside fruit tissues has greater impact on the quality of the tomato fruit and product derived out of it. Hence, the present study is focused with a long term objective of possible elimination of pathogen from internal tissues of tomato rendering the crop healthy and productive.

In this paper we reported the isolation of endophytic bacteria from tomato plants and screening for their antagonistic activity against wilt pathogen *in vitro.* Production of siderophore, hydrogen cyanide, indole acetic acid and salicylic acid in the *in vitro* cultured endophytes were quantified and reported with the objective of determinining their growth promotion and antagonistic characteristics.

## MATERIALS AND METHODS

### Sample collection

Four week old healthy tomato plants were collected from two different agricultural fields in Katpadi and Bagayam of Vellore District, South India. Random sampling was done carefully, by uprooting the plants from field. Wilt affect plants were collected separately. The plants were bagged in sterile polythene bags and transported to laboratory and processed within 4 hrs of collection.

### Isolation of bacterial endophytes from tomato plants

Root, stem, leaf and fruit portions were split into longitudinal section and excised to 1cm diameter pieces and washed in running tap water to remove soil particles. The tissues were sterilized by sequential immersion in 70% (v/v) ethanol for 5 min, and sodium hypochlorite solution (1%, w/v, available chlorine) for 20 min, and placed in 0.05% w/v triton X-100 for 10 min, and rinsed four times in 0.02 M sterile potassium phosphate buffer (PPB), pH 7.0.

The samples were then washed in sterile distilled water for three times to remove surface sterilization agents. The samples were soaked in 10% (w/v) $NaHCO_3$ solution for 10 min to retard the growth of endophytic fungi. Each sample (0.5 g) was homogenized in sterile pestle and mortar using 9.5 mL of the buffer. Serial dilutions of the homogenate up to ($10^{-10}$) were made in PPB. Dilutions of all samples were plated separately (0.1 mL) on three different media viz., tryptic soy agar (Hi Media, India), nutrient agar and King's B medium with three replications. The plates were incubated at 28ºC for 48-72 hrs. Single colonies were further sub-cultured in respective media. Isolated endophytic bacteria were identified up to genus level based on standard biochemical tests (Gram's staining, catalase, oxidase, indole, methyl red, Voges Proskauer and citrate utilization tests).

### Validation of surface sterilization protocol

Surface sterilized samples were finally rinsed with PPB. From this final buffer wash, 0.1mL aliquot was taken and transferred to 9.9 mL tryptic soy broth (TSB) which served as sterility check. Samples were discarded if growth was detected in the sterility check samples (agitating samples in TSB, Hi media, India) at 28ºC within 48 hrs to check the growth of endophytes and to eliminate the epiphytes.

### Isolation of Fusarium from tomato plant

*Fusarium* was isolated from stem and root tissues of infected tomato plants. Sections of lateral stem were surface sterilized with 10% sodium hypochlorite for 5 min and washed three times in sterile distilled water and blotted on sterile filter paper to remove excess water. Tissue pieces were inoculated on PDA plates and incubated at 28ºC. Single spore culture technique was followed to obtain pure culture. The culture was identified as *Fusarium oxysporum f. sp. lycopersici* at Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India, by comparing the morphological, cultural, spore and mycelia characteristics with standard culture.

***In vitro* antagonistic activity of endophytes against *Fusarium***

*Fusarium* and isolated bacterial endophytes was studied by the paired Petri dish technique (Laha *et al.,* 1996). Two day old fresh culture of isolated bacterial endophytes strains were uniformly streaked on PDA medium in a Petri dish. In another set PDA plate inoculated with pathogen (upper) was paired with bacterial endophytes (lower) and sealed with parafilm and incubated at 28ºC. PDA plates inoculated with the fungus alone and paired with PDA plate without biocontrol agents served as control. The diameter of the fungal growth was measured 5 days after incubation and expressed as percent growth inhibition over control. The experiment was also done in King's B media. Although King's B supported growth of bacteria, it failed to support growth of *Fusarium* even in the control plates. Hence results of the experiment on PDA media were documented. The metabolic determinants of antagonism and possible growth promotion by endophytic isolates obtained in the study were assessed by their antagonistic activity and by *in vitro* production of siderophores, hydrogen cyanide, salicylic acid and indole-1,3-acetic acid.

**Siderophore production**

Strains were grown in selective liquid media specific to each genus of endophyte under study for 3 days and centrifuged at 200 rpm for 10 min. The pH of supernatant was adjusted to 2.0 with 0.1N HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and solvent fraction was collected. This process was repeated three times to bring the entire quantity of siderophores from the supernatant. The solvent fractions were pooled, air-dried and dissolved in 5 mL of ethanol (50%). A 5 mL of solvent fraction was mixed with 5 mL of Hathway's reagent (1.0 mL of 0.1 M $FeCl_2$ in 0.1 N HCl to 100 mL of distilled water + 1.0 mL of potassium ferricynaide). The absorbance for dihydroxy phenol was read at 700 nm (Reeves *et al.,* 1983). A standard curve was prepared using dihydroxy benzoic acid. The quantity of siderophore produced was expressed as µg mL $^{-1}$ of culture filtrate. Production of siderophores by bacterial endophytic strains was performed by plate assay. The tertiary complex chrome azural S (CAS) / Fe 3+ / hexadecyl trimetyl ammonium bromide served as an indicator. Forty eight hour old culture of the strains was streaked on to the succinate medium amended with indicator dye. The resultant blue liquid was observed for the formation of bright zone with yellowish fluorescent coloured medium indicating siderophore production. The result was scored either positive or negative to this test (Schwyn and Neilands, 1987).

**Hydrogen cyanide production**

Production of HCN was determined by using modified procedure of Millar and Higgins (1970). Strains were grown on trypticase soy agar (TSA Hi media, India). Filter paper soaked in picric acid solution (2.5g of picric acid; 12.5g of $Na_2CO_3$, 1000 mL of distilled water) was placed in the lid of each Petri dish. Dishes were sealed with parafilm and incubated at 28ºC for 48 hrs. A change in colour of the filter paper from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong in producing HCN by each strain respectively.

Strains were grown on trypticase soy broth (TSB, Hi media, India). Filter paper was cut into uniform strips of 10cm long and 0.5cm wide saturated with alkaline picrate solution and placed inside the test tubes in a hanging position. After incubation at 28 ºC for 48 hrs, the sodium picrate in the filter paper was reduced to a reddish compound in proportion to the quantity of hydrocyanic acid evolved. The colour was eluted by placing the filter paper in a clean glass test tube containing 10 mL of distilled water and absorbance was measured at 625nm (Sadasivam and Manickam, 1992).

**Salicylic acid production**

Salicylic acid (SA) production of the isolated endophytic bacterial strains was determined as per the method described by Meyer and Abdallah (1978). The strains were grown in the standard succinate medium at 28ºC for 48 hrs. Cells were collected by centrifugation at 8000 rpm for 5 min and were resuspended in 1 mL of 0.1 M phosphate buffer. A 4 mL of cell free culture filtrate was acidified with 1 N HCl to pH 2.0 and SA was extracted in $CHCl_3$. 4 mL of water and 5 µl of 2 M $FeCl_2$ were added to the pooled $CHCl_3$ phases. The absorbance of the purple iron-SA complex, which was developed in the aqueous phase, was read at

527 nm. A standard curve was prepared with SA dissolved in succinate medium and quality of SA produced was calculated (Meyer *et al.*, 1992).

**Table 1.** Inhibition of *Fusarium* by bacterial endophytes

| Bacterial endophytes | Percent growth inhibition of *Fusarium* (%) |
|---|---|
| *Pseudomonas* | 70 |
| *Bacillus* | 50 |
| *Klebsiella* | 10 |
| *Citrobacter* | 5 |
| Control | - |

**Indole acetic acid production**

The isolated bacterial strains were inoculated with TSB with tryptophan as a precursor (100 µg/mL) on shaker for 30 mins. Supernatants of the culture were collected after centrifugation at 2000 rpm for 10 min and 1 mL of cell free culture filtrate was mixed with 2 mL of Salkowsky reagent (1 mL of 0.5 M $FeCl_3$ on 50 mL of 35% perchloric acid) and incubated at 28ºC for 30 min. Quantification was done colormetrically in 530nm comparing with IAA standard curve (Gorden and Paleg, 1957).

## RESULTS

**Bacterial isolates obtained from tomato**

Fourty bacterial isolates were obtained from root, stem, leaves and fruit of tomato plant. We were able to group the isolates into four genera according to the standard biochemical tests as *Bacillus* (TEB6) from root, *Klebsiella* (TEK1) from leaves, *Pseudomonas* (TEP3) from stem, and the growth of endophytes without contaminating epiphytes was confirmed.

**Antagonistic activity on *Fusarium***

In the dual culture tests, bacterial endophytic strain of *Pseudomonas* (TEP3) significantly inhibited the pathogenic *Fusarium*. The growth inhibition was to the tune of 70% (Table 1). Based on the antagonistic activity of bacterial endophytes determined in terms of percent inhibition of pathogenic *Fusarium*, their biotechnological potential was further assessed based on *in vitro* production of siderophores, hydrogen cyanide, salicylic acid and the plant growth hormone indole-1, 3-acetic acid.

**Production of siderophore, HCN, salicylic acid and IAA**

*In vitro* production of siderophore was measured qualitatively and quantitatively. Qualitative production shows the blue colour liquid formation with bright zone in the dark coloured medium. Day after inoculation of bacterial endophytes and the effect of siderophore production absorbance was read at 700nm in a spectrophotometer (Table 2) and the percent increase over control was statistically analyzed. *Pseudomonas* had produced more amount of siderophore from the isolated endophytes. HCN showed a change of colour of filter paper from yellow to brown. HCN production at different time points of growth was read at 625 nm in a spectrophotometer (Table 2) and the percent increase over control was calculated statistically. Salicylic acid production was studied by reading at 527 nm and IAA was read at 530 nm.

**Table 2.** Siderophore production by tomato endophytes

| Bacterial endophytes | Siderophore production (Percent increase over control) | Hydrogen cyanide production (Percent increase over control) | Salicylic acid production (Percent increase over control) | Indole acetic acid |
|---|---|---|---|---|
| ***Pseudomonas*** | 0.512 (53.6) | 0.150 (45.00) | 0.162 (48.7) | 0.161 (48.2) |
| ***Klebsiella*** | 0.459 (47.6) | 0.144 (43.12) | 0.135 (40.4) | 0.149 (44.8) |
| ***Citrobacter*** | 0.391 (36.2) | 0.123 (37.00) | 0.101 (30.2) | 0.131 (39.0) |
| ***Bacillus*** | 0.251 (25.3) | 0.107 (32.22) | 0.043 (13.0) | 0.110 (32.9) |
| **Control** | 0.160 | 0.101 | 0.024 | 0.101 |

Values with same alphabets in superscript are statistically insignificant

## DISCUSSION

Plants are in continuous association with microbes which interact with them in positive, negative or neutral ways. Endophytes are microbial entities that colonize living plant tissues and most of them live in a relationship with their host plant as symbionts and mutualistic association. Many endophytes are capable of producing compounds that serve as defense chemicals against pathogenic microbes infecting the plants. By cultivating the endophytes outside the plant under laboratory conditions, these bioactive compounds can be harvested in large quantities for commercial use. Cheplick and Feath (2009) have reviewed the role of endophytes of grass species which have been shown to affect host plant growth and reproduction, photosynthetic physiology, abiotic stress tolerance, and competitive ability. Endophytes not only promote plant growth, but also contribute to yield of the crop, suppress pathogens, help in bioremediation of contaminated soils and solubilization of phosphates and nitrogenous nutrients. The review of Rosenblueth and Martinez-Romero (2006) summarized the endophytes and their host plants, they also reported that some of the endophytes are seed borne while others are those that colonize the plant roots directly from soil (Rosenblueth and Martinez-Romero, 2006).

Not much work has been done on tomato endophytes other than the report of *Salmonella enterica* (Islam *et al.,* 2004), *Streptomyces* (Cao *et al.,* 2004) root colonizing *Rhodococcus* species, *Pseudomonas aeroginosa*, *Serratia marcesens*, *Burkholderia cepacia* and *Psukamurella inchonensis*. However, exogenous applications of endophytes like *Pseudomonas fluorescens* have been reported to induce systemic resistance to *Fusarium oxysporum* in tomato (Duijff *et al.,* 1997).

Rini and Sulochana (2007) have reported a similar dual culture technique and assessed the antagonistic activity of *Trichoderma* and *Pseudomonas* against *Rhizoctonia solani* and *F. oxysporum* in tomato. They observed that the native isolates obtained from tomato were more efficient. In the present study also, the *Pseudomonas* obtained from tomato stem showed greater growth inhibition of *Fusarium* and the efficiency could be attributed to its nativity.

Natural suppressiveness of *Fusarium* by non pathogenic fungi of some species and fluorescent *Pseudomonas* has been reported. Biological control of *Fusarium* by exogenous bacteria *viz.*, *Pseudomonas* species *Bacillus* species (Grosch *et al.,* 2001; Baysal *et al.,* 2008) are extensively investigated. Since the possible role of growth inhibition by the bacterial antagonists, either as an endophyte or exogenous colonizer was attributed to the production of diffusible and volatile metabolites (Dujiff *et al.,* 1997; M'piga *et al.,* 2002; Rini and Sulochana, 2007; Baysal *et al.,* 2008), we evaluated the metabolites produced by the isolated endophytes from tomato.

*Pseudomonas* ranked as the major producer of HCN and salicylic acid, however endophytic isolate of *Bacillus* was found to be superior in IAA production. Cao *et al.* (2004) observed host specificity of plant growth promoting as well as biocontrol endophytes. The Streptomyces isolates were found to produce metabolites and the growth promotion and enhanced resistance to disease was observed in tomato but a similar effect was not found in cucumber plants. The growth inhibition of *F. oxysporium* by *Trichoderma* and *P. fluorescens* was due to volatile and non- volatile antibiotic compounds that varied, among the isolates (Rini and Sulochana, 2007). The growth promotion by the endophytes can be the effect of changes in nitrogen fixation abilities (Sevilla *et al.,* 2001; Hurek *et al.,* 2002; Iniguez *et al.,* 2004) or the protection of plant pathogens in the rhizosphere (as a consequence of antimicrobial metabolites, siderophores, competition for nutrients and induced systemic resistance and plant immunity) and making unavailable nutrients into available forms (Sturz *et al.,* 2000; Sessitsch *et al.,* 2002).

Our studies clearly demonstrated the relative efficacy of endophytes in the production of metabolites like HCN, siderophore, salicylic acid and IAA which are involved in plant growth promotion and induced systemic resistance. It would be interesting to determine if volatile compounds could be produced inside plants. Some

research has been done to find endophytes that could contribute to the yield increase after artificial inoculation (Rosenblueth and Martinez-Romero, 2006). To study the effects of endophytes, inoculation experiments have been performed, but it has been a problem to eliminate resident or indigenous endophytes from plants in order to have bacteria-free plants. Functional redundancy of resident endophytes and added inocula may limit the effects observed from inoculation. Endophyte inoculation under field conditions may also suffer from the complexity of plant-microbe interactions, rhizosphere competence with native microbes (Sturz *et al.*, 2000), and fluctuations in the number of bacterial colonies as influenced by environmental factors also could limit the applicability.

Endophytic colonization differs from species to species and plant parts (Rosenblueth and Martinez-Romero, 2006). Early endophytic colonization differed from one cultivar to another, but later endophytes were recovered in approximately similar numbers from the different cultivars. Strain variations of efficient colonization have been reported for *Rhizobium* strains (Rosenblueth and Martinez-Romero, 2004). In general, endophytic isolates are having abilities to colonize as well as recolonize internal tissues of plants when compared to microbial isolates at the root surface (van Peer *et al.*, 1990; Rosenblueth and Martinez-Romero, 2004).

Although the endophytic bacteria are found in almost all parts of the plants including roots, stems, leaves, seeds, fruits, tubers, ovules and also inside legume nodules (Hallmann *et al.*, 1997; Sturz *et al.*, 1997; Benhizia *et al.*, 2004), the below-ground parts of plants have been reported to have the higher numbers of endophytes as against above-ground tissues (Rosenblueth and Martinez-Romero, 2004). In contrast to the report by Hurek *et al.* (1997), that bacterial endophytes do not inhabit living vegetative cells, we observed the most efficient endophytes in the stem tissues. Tomato endophytes isolated and characterized in the study look promising to play significant role in increasing yields, remove contaminants, inhibit pathogenic microbes of tomato, and produce novel substances for commercial exploitation. The challenge in frontage is to manipulate and manage the microbial communities so as to offer a favorable environment for successful establishment of endophytes under *in planta* conditions. This would be possible when a better fundamental knowledge on *Pseudomonas* biology and their ecology in tomato with better understanding of molecular level interactions is attained. Systemically induced resistance and microbial competitive exclusion as a means of biocontrol has been reported recently (Martinuz *et al.*, 2012). Hence, the contributions of our research may have economic and environmental impacts when further in depth investigation into the molecular plant-microbe interactions of their system is undertaken.

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