International Journal of Agriculture, Environment & Biotechnology
Citation: IJAEB: 7(2): 305-312 June 2014
DOI: 10.5958/2230-732X.2014.00248.4

Plant Pathology

Exploring Entagonistic Effect of Endophytic Microorganisms Against *Xanthomonas axonopodis* pv. *dieffenbachiae* (McCulloch & Pirone) Vauterin Causing Bacterial Blight of *Anthurium*

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Paper No. 212 Received: February 17, 2014 Accepted: April 29, 2014 Published: May 30 2014

Abstract

One of the major constraint in anthurium cut flower production is bacterial blight incited by *Xanthomonas axonopodis* pv. *Dieffenbachiae* (Xad). It causes considerable economic loss. Considering the serious nature of the disease the present investigation was undertaken to harness the potential of antagonistic endophytes against bacterial blight pathogen. Endophytic microorganisms were isolated from healthy anthurium plants collected from different locations. A quantitative estimation of endophytic micro organisms revealed the abundance of bacteria than fungi. Moreover, more number of endophytes were isolated from roots than from petiole and leaves. Based on cultural characters of endophytic microorganisms, 14 fungal and 37 bacterial endophytes were selected. Antagonistic action of these endophytes against pathogen was studied in comparison with that of reference culture of *Pseudomonas fluorescens* (KAU), *P. fluorescens* (TNAU), *Trichoderma viride* (KAU) and *T. harzianum* (IISR). The study revealed that, only eight bacterial and two fungal cultures showed antagonistic action against the pathogen. Since fungal endophytes showed less inhibition, they were not selected for further studies. The selected endophytes were subjected to various tests for understanding parameters that may act to produce antagonism as well as enhanced growth of the plants. All the antagonists inhibited growth of the pathogen, promoted plant growth, produced IAA and ammonia. Only two isolates *viz.*, EB-14 and EB-31 are found cyanogenic. Only four isolates *viz.*, EB-15, EB-31, EB-26 and EB-12 were found capable of solubilizing phosphate. All isolates except EB-12 were found capable of producing siderophores.

Highlights

- EB-14 and EB-31 were found cyanogenic. EB-15, EB-31, EB-26 and EB-12 were capable of solubilizing phosphate. All isolates except EB-12 were producing siderophores.

Keywords: Antagonism, Anthurium, Cyanogenic, Endophytes, Siderophores

Anthurium is an important cut flower crop of Kerala. It has got good market within and outside the State as well as in international market. Bacterial blight caused by *Xanthomonas axonopodis* pv *dieffenbachiae* is one of the major constraints in anthurium cultivation. This disease was reported from Kauai Islands, Hawaii in early 1970s. In India,

the occurrence of the same was reported in imported anthurium plants by Satyanarayana *et al.*, (1998). Although application of antibiotics may lesser the incidence and severity of the bacterial diseases, it cannot be continued as long term solution because of the chance of development of resistant strains of the pathogen (Anjana, 2005). Nowadays more emphasis is being given on biological management of plant diseases. Biocontrol agents isolated from rhizosphere and phylloplane have been extensively studied for the management of plant disease. But their success greatly depends on the host, environment and many other conditions. These problems can be overcome to a great extent by use of antagonistic endophytes, since the internal habitat ensure supply of nutrients and protects them from competition with other microbes (Manjula *et al*., 2002). In tea, the rhizosperic endophytes already screened for their multiple plant growth promoting activities including indole acetic acid production (IAA) production, phosphate solubilization, ammonia production and siderophore production (Ratul nath *et al.,* 2013). A perusal of the literature reveals that no attempts have been made so far from Kerala for the management of bacterial blight of anthurium using endophytic microorganisms. Thus the present investigation is focused to harness the potential effect of endophytic microbes on the management of the dreaded bacterial blight of anthurium caused by *Xanthomonas axonopodis* pv. *dieffenbachiae.*

Materials and Methods

Isolation of the pathogen: The pathogen causing bacterial blight of anthurium was isolated from naturally infected plants collected from two locations *viz.,* Nelliyampathi area of Palakkad and Vellanikkara area of Thrissur districts of Kerala. The infected leaf samples were washed thoroughly and subjected to ooze test. The diseased leaves with profuse ooze were selected, the infected areas were cut into small bits, surface sterilized with 1 per cent sodium hypochlorite solution for one minute. These bits were then washed in three changes of sterile water and teased apart to get a bacterial suspension. The suspension was streaked on Potato Sucrose Peptone Agar (PSPA) medium to get well isolated colonies of the bacterium. The plates were incubated for 48 h at room temperature. Characteristic single colonies were selected on the basis of its colour, fluidity and slime and purified by repeated streaking on PSPA medium.

Isolation and enumeration of endophytic microbes: Endophytic microorganisms were isolated from stem, leaves and roots of healthy anthurium plants. For this, plants were collected from anthurium growing areas of Thrissur, Kannur, Kasargod and Thiruvananthapuram districts of Kerala. Stem ,leaf and root samples were washed in tap water, weighed separately (1 g each) and then surface sterilized with one per cent Sodium hypochlorite solution for three minutes, followed by four changes in 0.02 M sterile potassium phosphate buffer (pH 7). Following surface disinfection, the samples were triturated with mortar and pestle in 9 ml of buffer. From this serial dilutions upto 10^{-2} of the triturate were made in potassium phosphates buffer. From each dilution one ml was transferred to Petri plate. Three replications were maintained for each dilution and plates were incubated at room temperature. To decrease the possibility of recovery of surface contaminants, sterility checks were also maintained by transferring 0.1 ml of final buffer wash to Petri dishes containing solidified NA and PDA. Bacterial colonies developing after 24 h and that of fungal colonies after 48 h were selected and transferred to respective media. Thus 37 bacterial isolate and 14 fungal isolates were maintained for further studies.

***In vitro* evaluation of endophytes for their antagonistic effect against the pathogen:** The antagonistic effect of isolated bacterial and fungal endophytes against *Xanthomonas axonopodis* pv. *dieffenbachiae* was studied under *in vitro* condition by adopting dual culture method (Dennis and Webster, 1971). Standard cultures of *Pseudomonas fluorescens* (KAU), *Trichoderma viride* (KAU), *T. harzianum* (IISR) were also used for comparison.

Preliminary screening of bacterial endophytes: For preliminary screening, Nutrient Agar (NA) plate seeded with 48 h old culture of pathogen in Petri plate was spot inoculated with endophytic bacterial isolate. In each plate four different bacterial isolates were inoculated at equidistant points two cm away from the periphery of the plate. The plates were incubated at room temperature and observed for inhibition of the pathogen after 48 h. The plates with pathogen alone served as control. Eight isolates which showed zone of inhibition were selected for further studies. The bacterial isolates which showed antagonism in preliminary screening were further tested individually. These selected antagonists were again spot inoculated at the center of NA plate seeded with pathogen. The plates were incubated at room temperature and observed for inhibition of the pathogen after 48 h. The plates with pathogen alone served as control. Inhibition zone was measured and per cent inhibition calculated.

Preliminary screening of fungal endophytes: A total of 14 fungal endophytes were tested for their antagonistic property against the pathogen. Agar blocks of 10mm diameter containing seven day old growth of fungal endophyte were inoculated at equidistant points, two cm away from periphery of the NA plate seeded with 48h old culture of pathogen. The plates were incubated at room temperature and observed for inhibition of pathogen after 48h. The plates with pathogen alone served as control. Only two isolates which showed zone of inhibition of pathogen were selected for further studies. The fungal isolates which showed antagonism in preliminary screening were tested individually by spot inoculating at centre of NA plate seeded with pathogen and incubated at room temperature. The plates with pathogen alone served as control. Inhibition zone was measured and per cent inhibition was calculated.

Selection of antagonistic endophytes: Since fungal endophytes did not show much inhibition, only eight bacterial endophytes were selected for further studies in comparison with standard *P. fluorescens* isolate from KAU.

Mechanism of antagonism

Selected antagonist where further subjected to various tests for understanding the parameters that may contribute to disease control.

Vigour Index: The selected endophytes along with reference culture of *P. fluorescens* (KAU) were bio assayed for their ability to promote seedling growth using the method suggested by Shende *et al.,* (1977) and Elliot and Lynch (1984) with few modifications. Sorghum seeds were surface sterilized with 0.1 per cent mercuric chloride for three minutes followed by successive washing with sterile water. Water was decanted and the seeds were soaked for 30 min in 48 h old cultures grown in nutrient broth, after which medium was decanted. Sterile filter paper discs soaked in sterile water was placed in the Petri dish. Soaked seeds were placed equidistantly on the filter paper and incubated for 72 h.Three replications were maintained and the seeds treated with sterile nutrient broth alone served as control. Germination percentage, length of epicotyl and hypcotyl were measured after 72 h.The Vigour Index (VI) was calculated using the formula:

VI = (Mean root length + Mean shoot length) x Germination percentage.

The endophytes were scored based on vigour index (VI) was as follows

VI of >1<2 = 1; VI of >2<3 = 2; VI of >3<4 = 3; VI of >4 = 4

Production of HCN: Production of HCN by isolates was tested by following method of Wei *et al.,* (1991). Log phase of bacterial culture was inoculated to 25ml of Kings'B broth supplemented with 4.4 g^{-1} of glycine taken in a sterile Petri plate. Sterile filter paper strips soaked in picric acid solution was placed in the lid of each plate. Petri plates were sealed with paraffin and incubated for 72 h. Change in colour of filter paper from yellow to brown indicates production of HCN.

Production of ammonia: The selected bacterial endophytes were grown in 25ml of peptone water and incubated at 30^0C for 4 days. Three replications were maintained for each isolate. After incubation 1 ml of Nessler's reagent was added to the broth. Presence of faint yellow or deep yellow or brown colour of the broth culture from yellow or brown indicates production of ammonia.

P solubilization: The P solublization capacity of bacterial endophytes was tested *in vitro* using Pikovskaya's agar medium. Ten ml of log phase of isolates were spot inoculated at centre of plate containing medium and incubated at 28^0 C for five days. Plates were observed for clearing zone around colony and its diameter measured. Three replications were maintained for each isolate.

Qualitative estimation of IAA: IAA production was estimated using modified protocol suggested by Bric *et al.,* (1991). A loop full of bacterial culture was inoculated in 25ml of Luria Bertini medium and incubated for 24 h, at 28^0C in rotary shaker. After 24h the broth cultures were centrifuged at 10,000 rpm for 15 minutes. Two ml of supernatant was taken and 2 drops of Orthophosphoric and 2 ml of Salkowsky's reagent (1ml of 0.5M $Fecl_3$ in 50ml of 35 percent $HClO_4$) was added and incubated at 28^0 C for 30 minutes and absorbance was measured at 530 nm. A standard curve was prepared with different concentrations of IAA and was used to quantify the IAA production.

Detection of siderophores: Siderphore production was detected by UV fluorescence method. Log phase of the endophyte cultures including reference culture were streaked on King's B (KB) plate and incubated at 28^0C for 48h. The plates were observed on UV trans-illuminator to view the fluorescence (Kloepper *et al.,* 1980)**.** Iron dependent production of siderophores was also estimated. The King's B medium was amended with two

concentrations of $FeCl_3.6H_2O$ @ 1 and 10 mg l^{-1}. The sterilized medium was poured into each Petri plate. The test cultures were streaked on the surface of the medium. Three replications were maintained. The inoculated plates were incubated at room temperature for 48h. Observations on production of greenish yellow fluorescent pigment were recorded.

Results and Discussion

Isolation and enumeration of endophytes: Biocontrol agents isolated from rhizosphere and phylloplane in many cases showed inconsistent performance due to their dependence on environmental conditions. This problem can be overcome by endophytes by way of internal habitat, assured nutrient supply and protected environmental conditions. Hence, attempts were made to select endophytes from healthy anthurium plants collected from various locations. It is established that the total number of endophytes that invade plant is controlled by plant type and environmental conditions. Isolation of endophytic microbes yielded more number of bacteria than fungi. Further there was variation in the population of endophytes in samples collected from different locations. This result is in line with earlier reports by Kloepper *et al.*, (1980) and Fisher *et al.*, (1992). According to Hallmann *et al.*, (1997), plant and soil factors especially pH, affect endophytic microbes indirectly by altering saprophytic community in rhizosphere thus preselecting potential endophytes. Out of the 51 representative endophytes selected for further studies 37 were bacterial and rest were fungal endophytes. It was also noticed that, root portion yielded more endophytes compared to leaf and petiole, which is in agreement with the reports of many other workers (Tripathi *et al.*, 2006; Rajendran *et al*., 2006; Saisree, 2007 and Aravind *et al.*, 2009) (Table 1).

***In vitro* evaluation of endophytes for their antagonistic effect against the pathogen:** Selection and identification of growth promoting and disease suppressive biologically efficient microbes through *in vitro* and *in vivo* assay is crucial and is the first step towards the development of an effective biocontrol agent before launching its field evaluation (Weller *et al*., 1988). Thus, it is pertinent that the efficacy of such microbes should be established under lab conditions and the most effective ones are selected for further pot/ field evaluations. Hence, the *in vitro* antagonistic effect of the isolated endophytes against *Xanthomonas axonopodis* pv. *dieffenbachiae* were tested in comparison with the standard cultures of fungal (*T. harzianu*m IISR and *T. viride*) and bacterial (*P. fluorescens* from TNAU and KAU) bioagents. Of the 14 endophytic fungi selected only two (EF-2, EF-5) showed slight zone of inhibition. Thus they were not selected for further studies. Among the 37 endophytic bacteria screened, most of the isolates were found to be ineffective and only eight were beneficial and exhibited antagonistic activity against the pathogen. The highest inhibition of the growth of pathogen was observed with the endophytic bacterial isolates, EB -15 and EB-31. The inhibitory action may be due to production of secondary metabolites and siderophores by the bacterial endophytes. Such inhibitory effect of endophytic microbes against bacterial and fungal pathogens was reported by Rahman and Khan (2002), Bacon and Hinton (2002), Liu *et al.*, (2003) Ji *et al*.(2008)and Muthukumar (2008) Table 2).

Table 1: Quantitative estimation of endophytic microorganisms of anthurium in different parts of Kerala

Sl. No.	Location	Part used	Fungi*($x10^2$ cfu/g)	Bacteria*($x10^2$ cfu/g)
1	Thiruvananthapuram	Root	1.67	7.33
		Petiole	1.33	3.67
		Leaf	1.67	4.33
2.	Thrissur	Root	2.67	5.67
		Petiole	1.67	2.67
		Leaf	2.33	3.33
3.	Kannur	Root	1.67	3.67
		Petiole	1.33	2.33
		Leaf	1.33	3.33
4.	Kasargod	Root	1.67	3.67
		Petiole	0.67	1.33
		Leaf	1.33	1.67

* Average of three replications

Table 2: *In vitro* inhibitory effect of bacterial endophytes against the pathogen, *Xanthomonas axonopodis* pv. *dieffenbachiae*

Sl. No.	Endophytes	*Diameter of inhibition zone (mm)	Per cent inhibition over control	***Score
1.	EB-8	9.3	10.23[g]	1
2.	EB-12	17.3	19.06[d]	2
3.	EB- 13	15.0	16.50[e]	2
4.	EB-14	9.0	9.9[g]	1
5.	EB-15	24.3	26.76[a]	3
6.	EB-26	18.3	20.16[c]	2
7.	EB-31	22.0	24.20[b]	3
8.	EB-32	12.3	13.56[f]	2
9.	**_Pf_ KAU	18.3	20.16[c]	2

* Mean of three replications
** _Pf_1: _Pseudomonas fluorescens_ (KAU)
*** Score: inhibition zone >1<10mm = 1, >10<20mm = 2, >20<30mm = 3, >30 = 4

Mechanism of antagonism

Endophytic bacterial isolates which showed antagonistic effect were further subjected to various tests for understanding the parameters that contribute to antibacterial action. Consequently, they were tested for their growth promoting action, production of hydrogen cyanide, ammonia, growth regulators and siderophores and also their effect on P solubililzation in comparison with reference strain of *Pseudomonas fluorescens* (KAU).

Vigour Index: The eight endophytes along with reference culture of *P. fluorescens* (KAU) were bio assayed for their ability to promote or inhibit growth of sorghum seeds. In general, seeds bacterized with endophytes exhibited better germination per cent thereby indicating that the endophytes have the potential to enhance plant growth. Such growth promoting effects of endophytes in various crops have been reported by many workers. (Sturz *et al*., 1998; Sharma and Nowak,1998;Varma *et al*., 1999; Nejad and Johnson, 2000;Surette *et al*.,2003; Kulinseky *et al*., 2004 and Vijayaraghavan, 2007) (Table 3).

Production of HCN: With regard to the production of HCN, it was seen that two of them *viz.*, EB-14 and EB-31 were cyanogenic. Rest of the isolates were non cyanogenic when tested with supplemented King's B medium. Bano and Mussarrat (2003) noticed low HCN Production under iron limiting conditions. Production of HCN by endophytes in various crops is well documented (Nejad and Johnson, 2000 and Paul, 2004).

Production of ammonia: The ability of endophytes for the producting of ammonia, a volatile compound having

Table 3: Vigour Index of sorghum seeds due to treatment with selected endophytes

Sl. No.	Isolate	*Per cent Germination	*Shoot length (cm)	*Root length (cm)	Vigour Index (VI)	Score
1.	EB-8	93.33	4.81	2.58	6.89	4
2.	EB-12	86.67	3.17	2.98	5.33	4
3.	EB-13	86.67	2.87	2.18	4.37	4
4.	EB-14	90.00	3.50	2.22	5.14	4
5.	EB-15	96.67	4.97	2.68	7.39	4
6.	EB-26	86.67	4.13	2.79	5.99	4
7.	EB-31	93.33	5.12	2.12	6.75	4
8.	EB-32	86.67	2.28	1.91	3.63	3
9.	*P.f* (KAU)	90.00	3.91	3.21	6.40	4
10.	Control	83.33	2.32	1.41	3.10	3

* Mean of three replications, *P.f- Pseudomonas fluorescens* (KAU)
Score: VI of >1<2 = 1; VI of >2<3 = 2; VI of >3<4 = 3; VI of >4 = 4

direct effect on biocontrol activity, is well documented (Paul, 2004). The present study revealed that all the selected endophytes exhibited varying levels of ammonia production. The isolates (EB-15 and EB-31) which gave the highest inhibitory effect against the pathogen showed the highest level of ammonia production also. The production of ammonia by bacterial biocontrol agents in suppressing plant pathogens, triggering growth promotion and induction of systemic resistance in host plants have been documented (Ryu *et al.*, 2003) (Table 4).

Table 4: Production of ammonia by selected endophytes of anthurium

Sl. No.	Isolate	Score
1.	EB-8	2
2.	EB-12	3
3.	EB-13	3
4.	EB-14	3
5.	EB-15	4
6.	EB-26	3
7.	EB-31	4
8.	EB-32	2
9.	*P. fluorescens* (KAU)	3
10.	Control	1

Score chart: Nil:1, Low: 2, Medium: 3, High: 4

Phosphorous (P) solubilization: One of the important criteria to assess the efficiency of plant growth promoting bacteria is their ability to transform unavailable 'P' to available form. So in the present study, an attempt was made to assess P solubilizing capacity of selected endophytes along with reference culture of *P. fluorescens* (KAU). Among the eight isolates, only 4 *viz.*, EB-15, EB-31 EB-12 and EB-8 showed P solubilisation ability on Pikokvsya's medium. The maximum P solubilization was with EB-15. Such ability of endophytes in solubilizing P has been well documented (Kulinsky *et al.*, 2004). Vijayaraghavan (2007) noticed P solubilization capacity of PGPR isolates of ginger. Further, conferring resistance to plants to stress conditions by mobilizing P for plant growth was reported (Table 5).

Table 5: Phosphorous solubilization by selected endophytes of anthurium

SI.No.	Isolate	*P solubilization zone (mm)	Score
1.	EB 8	0	0
2.	EB 12	7.5	3
3.	EB 13	0	0
4.	EB 14	0	0
5.	EB 15	10.0	4
6.	EB 26	6.0	2
7.	EB 31	8.3	3
8.	EB 32	0	0
9.	*P.fluorescens* (KAU)	7.3	3
10.	Control	0	0

*Mean of three replications

Score: >1<3 mm = 1; >3<6 mm = 2; >6<9 mm = 3 and >9 mm = 4

Assay of growth promoting hormone: It is well established that the application of bio agents has a positive effect in promoting growth of plants. This effect is attributed to the production of phytohormones like auxin, gibberilin and cytokinins by bioagents. Among this IAA plays an important role in growth promotion. Production of IAA by different endophytic microbes from various crops has been reported. (Holland, 1997; Tripathi *et al.*, 2006; Saisree 2007;Vijayaraghavan, 2007 and Ratul nath *et al.*, 2013). Hence, in this study the capacity of eight endophytes in IAA production was assessed. All the isolates produced IAA ranging from 7-200 µg ml^{-1}. The highest IAA production was noticed in case of EB-15. This was reflected in the vigour index of seedlings treated with this isolate (Table 6)

Table 6: Production of Indole Acetic Acid by selected endophytes of anthurium

SI. No.	Isolates	IAA (µg/ml)	Score
1.	EB-8	30.00	1
2.	EB-12	55.67	2
3.	EB-13	7.02	1
4.	EB-14	15.00	1
5.	EB-15	200.00	4
6.	EB-26	190.33	4
7.	EB-31	180.33	4
8.	EB-32	75.00	2
9.	Control	-	0

*Mean of three replications

Score: >0<50 µg/ml =1; >50<100 µg/ml=2; >100<150 µg/ml =3; >150 µg/ml =4

Detection of siderophores: Bacteria under iron limiting conditions produce siderophores to escape from deleterious effects due to unavailability of iron. They have evolved mechanisms which under iron limiting condition selectively chelate iron for their own purpose and make it unavailable to others. Hence, the production of siderophore is correlated with the antagonistic potential. So the potential antagonistic endophytes selected were tested for their capacity to produce siderophores. All the isolates except EB-12 produced siderophores as evidenced by their fluorescence. This implies that EB-12 is less competitive than other endophytes and may explain the weak antibiosis of this isolate compared to the other siderophore producing endophytes. Similar findings of siderophore production were documented by many workers (Kloepper *et al*., 1980; Banu and Mussarrat, 2003; Paul, 2004; Tripathi *et al*., 2006; Vijayaraghavan, 2007 and Ratul nath *et al*., 2013). The study also revealed that the capacity of the isolates to produce siderophores reduced as the concentration of $FeCl_3$ is increased.

Conclusion

Thus the present study throws light on the potentiality of using endophytic bacteria for the management of bacterial blight of anthurium. Among the endophytes used EB-15, identified as *Bacillus* sp. performed very well both under *in vitro* and *in vivo* conditions. The significant reduction of bacterial blight incidence by endophytes especially by EB-15 may be due to the production of inhibitory substances like ammonia, siderophores, antibiotics etc. However, investigations on the potential of the isolate in different agro climatic situation need to be ascertained

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