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Harnessing the diversity of bacterial endophytes isolated from wild and cultivated taro plants against *Phytophthora colocasiae*

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Abstract

Taro (*Colocasia esculenta* (L) Schott), a tuber crop which belongs to Araceae family is an important staple or subsistence crop for millions of people in developing countries. The crop capitulates to several fungal, bacterial, and viral diseases as well as some diseases of uncertain etiology. Taro leaf blight, a threatening disease of taro caused by *Phytophthora colocasiae* is associated with 90% and 50% loss in leaf and corm yield of taro, respectively. The preventive measures used by crop rotations and the use of improved disease resistant varieties have failed to completely eradicate the disease. Chemical fungicides are not only costly but have harmful effects on humans and the environment. Endophytes with antifungal activity can be exploited as excellent biocontrol agents against phytopathogenic fungi. Hence, this study was centred on evaluating the antagonistic activity of endophytic bacteria and fungi associated with wild and cultivar taro plants itself against *Phytophthora colocasiae*. The study involves isolation of endophytic bacteria and evaluation of antagonism against the pathogen using *in vitro* dual culture method. A total of 97 bacterial endophytes were isolated from taro plants and they were evaluated for their antagonistic activities against *Phytophthora colocasiae*. The *in vitro* study indicated that among the bacterial isolates, KV9 showed the highest antagonistic activity of 84.07±1.04%. This research study demonstrates that these endophytes can be exploited to create a promising biocontrol agent against *P. colocasiae* in the taro field.

Keywords: Taro, *Phytophthora colocasiae*, endophytes, biocontrol, antagonism, dual culture method

Introduction

Taro (*Colocasia esculenta* (L.) Schott), is one of the important tropical tuber crops which belongs to the family Araceae. It is widely cultivated for its edible underground corms, which is the main source of carbohydrates, starch, ash, phytochemicals, vitamins etc and leaves as a staple food for millions of people in developing countries like Asia, Africa, and Central America (Nath et al., 2012, Rashmi et al., 2018). Two commonly cultivated varieties of taro are *Colocasia esculenta var. esculenta* and *Colocasia esculenta var. antiquorum* (Ahmed et al., 2020). Taro ranks second among the staple root crops in terms of consumption after sweet potato with about 12 million tonnes produced globally from about 2 million hectares with an average yield of 7 tonnes per hectare (FAOSTAT 2021; http://faostat.fao.org). Although taro is planted for the corm, the leaves, stem, and flowers are all edible and have an exceptional nutritional value.

The crop capitulates to several fungal, bacterial, and viral diseases as well as some diseases of uncertain etiology.

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Major among them, *Phytophthora colocasiae* Rac., an oomycete, is primarily a foliar pathogen which affects taro and causes one of the most ruinous diseases of taro called taro leaf blight (TLB). The initial symptoms of TLB are shown on leaves as small purple or brown water-soaked speckles which enlarge to form yellow marginated dark brown lesions which cause defoliation and ultimately lead to death of crop. The disease is associated with 95% and 50% loss in leaf and corm yield of taro, respectively (Singh et al., 2012). Despite the normal 40 days life span of healthy taro leaves, infected leaves devastate within 20 days. The severity of leaf blight is shown maximum at areas having high relative humidity and frequent rainfall compared to warmer areas. Under cloudy weather conditions with patchy rains and temperature around 28°C, disease spreads at tremendous speed and the entire field gives a blighted appearance (Jackson et al.,1980; Misra et al., 2008; Singh et al., 2012). As taro is a vegetatively propagated crop, TLB is frequently spread through the usage of planting materials inflamed with *P. colocasiae* sporangia and zoospores. The pathogen could carry over to the next season through mycelium in dead and dying plant tissues and infected corms, and through encysted zoospores or as chlamydosporesin soil (Okereke, 2020; Nelson et al., 2011).

Diverse approaches have been employed for the sustainable management of TLB, using resistant cultivars developed in India, *viz*., Muktakeshi, Bhu Kripa, Thamarakannan (ICAR-CTCRI, 2020), Poonam pat, Sakina V and by using chemical fungicides etc. (Misra et al., 2008). Metalaxyl and Mancozeb based phenylamide fungicides are the most effective and commonly used ones by the farmers against TLB in India (Nath et al., 2013). Although this strategy has produced encouraging results, phytotoxicity and chemical residues are significant issues that put the environment and human health at risk. Also, fungicides are too costly to be affordable by the marginal farmers and the development of resistant strains against the fungicide is another threat. Thus, the need for an alternative for managing taro leaf blight becomes inevitable. Recently the trend of using chemical free products has been increased. Among those, bio-control agents are getting special attention by scientists as they are eco-friendly, economical, sustainable, and potent alternative to control many virulent plant pathogens (Hong et al., 2021). One of the most successful biological control methods in agriculture is the use of fungal and bacterial endophytes (Moise et al., 2018).

Endophytes are microorganisms, most commonly bacteria and fungi, which colonize the internal tissue of living plants without causing any harm, at least while they are in the endophytic stage of their life cycle (Kushwaha et al., 2020). Numerous studies have revealed an enormous diversity of endophytic bacteria in plant systems and have pinpointed their potential contribution to disease resistance and plant growth promotion (Martinez et al., 2017). Bacterial endophytes have been noticed to impede the onset of disease by enabling the de novo synthesis for novel phytochemicals and secondary metabolites which may have antimicrobial, antifungal, anticarcinogenic, immune-suppressant or antioxidant activity. Harnessing endophyte-plant interactions could enhance plant health and be an essential aspect of low-input sustainable agriculture applications (Ryan et al., 2008).

The goal of this study is to identify and describe bacterial endophytes that were isolated from taro plants and to investigate the potential of these endophytes to act as a biocontrol agent against *Phytophthora colocasiae*, which is responsible for causing taro leaf blight disease.

Materials and Methods

Isolation of fungal pathogen, *Phytophthora colocasiae*

Leaf blight infected leaf samples of different taro plants were collected from various regions of Kerala, India for the isolation of fungal pathogen *Phytophthora colocasiae.* Infected leaf samples were cut into small bits such that the bits contain both infected region and healthy region and washed with sterile distilled water. Surface sterilization was carried out with back-to-back washing using 3% sodium hypochlorite solution for 2 min, 70% ethanol for 1 min., followed by three consecutive washes using sterile distilled water (Anjum and Chandra, 2015). Sample bits were allowed to dry using Whatman filter paper and transferred aseptically to potato dextrose agar medium with 100 μl ampicillin. The Petri plates were incubated at 28±2°C in the BOD for 4-6 days. Mycelia from the growing verge were subcultured on carrot agar medium plates. The pure cultures were primarily confirmed by microscopically examining the mycelium and sporangia. The cultures were maintained on carrot agar slants at 4°C for further study. Pathogenicity assay was done by detached leaf assay to choose the most virulent pathogen strains from the isolated ones as per Nath et al., (2016).

Confirmation of pathogen using species specific primers

The total genomic DNA was extracted from the fungal pathogen by Cetyl Trimethyl Ammonium Bromide (CTAB) method (Karthikeyan et al., 2010). The genomic DNA was amplified using universal fungal specific ITS1 and ITS4 primer pairs and *Phytophthora colocasiae* specific primer pairs such as PCSP-RF and PCSP-RR. PCR assays were carried out in an automated temperature cycling device (Agilent Tech). The amplified PCR products were size fractionated on a 1.5% agarose gel stained with ethidium bromide and the image was analysed by Gel Doc System (Alpha Innotech Corporation, San Leandro, CA, USA).

Collection of taro plant samples for bacterial endophyte isolation

For the isolation of bacterial endophytes, healthy wild and cultivable taro plant samples were collected from fourteen different regions of Kerala. The uprooted plant samples were carried over to laboratory in sterile polythene sampling bags and used for further experiments.

Isolation of bacterial endophytes

Healthy tissues of leaves, petiole, corm, and roots of taro samples were used for isolation of bacterial endophytes. They were washed separately under running tap water to remove adhering soil particles and dirt. Each of the plant parts were then excised inside the laminar flow, into 1-2 cm bits using a sterile scalpel. These samples were surface sterilized using 3% sodium hypochlorite solution for 2 min, 70% ethanol for 1 min, followed by washing three times with sterile distilled water and the water in surface sterilized samples was removed with filer paper (Araujo et al., 2002, Anjum and Chandra, 2015). Each bit was placed on nutrient agar medium plates with three replications. By imprinting cultured aliquots of water from the most recent rinse onto nutrient media, the surface sterilisation technique was validated. They served as the control plates. The inoculated plates were incubated at 28±2°C in a growth incubator for 24 to 72 h and observed. The individual bacterial colonies with visible differences in their morphology were sub-cultured in nutrient agar medium to maintain the pure cultures. Finally, all the purified endophytes were stored at 4°C until they were employed. All chosen isolates were subcultured in nutrient agar slants.

In vitro* screening of bacterial endophytes for antagonistic activity against *P. colocasiae

Isolated bacterial endophytes were assessed for their antifungal activity against *P. colocasiae* by modified dual culture technique on potato carrot agar plates (Shastri et al., 2020). After standardisation, potato carrot agar plates were made by potato dextrose agar and carrot agar in the ratio 1:3. A small circular mycelial plug of 4mm of *P. colocasiae* taken from an actively growing 7-day old culture on carrot agar plate was placed at the centre of the sterile 90 mm plate containing potato carrot agar. Simultaneously, the endophytic bacterial strains were rectilinearly streaked 30 mm away from fungal plug on opposite sides. The fungal culture grown on the potato carrot agar plate without any bacterial isolate served as control. The above setup was done in three replications. The plates were incubated for 6-8 days at 28±2°C in BOD incubator. The radial growth of *P. colocasiae* mycelium was measured, and the percentage inhibition of mycelium radial growth of *P. colocasiae* over control was calculated with the following formula.

$$\text{Percentage of mycelial growth inhibition} = \frac{(C-T)}{C} \times 100$$

Where C was the radial growth (mm) of the control mycelium colony and T was the radial growth (mm) of the mycelium growing in presence of antagonist endophytic bacterial isolate.

Statistical analysis

The data were analysed wherever necessary using the free online software, (https://sreejyothi.shinyapps.io/agrianalyticsr) developed by ICAR-CTCRI, Thiruvananthapuram, Kerala, India and the treatments were compared.

Results and Discussion

A total of fifteen isolates of *P. colocasiae* were obtained from diseased taro leaf samples collected from different regions of Kerala, ICAR-CTCRI (Table 1). The isolates were tentatively recognised as *P. colocasiae* based on morphological traits such as colony morphology and sporangial characteristics (Fig. 1). According to Misra et al., (2008), the mycelium of *P. colocasiae* are hyaline, coenocytic, aseptate and the sporangia are elongated, slender and narrow ended.

Table 1. Different isolates of Phytophthora colocasiae

Sl. No.	Isolate code	Location	District/Sampling site
1	PCKA	Kerala	Thiruvananthapuram/ Vamanapuram
2	PCTH	Kerala	Kollam/Thalavur
3	PCSA	Kerala	Kollam/Sadanandapuram
4	PCCH	Kerala	Thiruvananthapuram/ Cheruvakkal
5	PCPA	Kerala	Thiruvananthapuram/Palode
6	PCCTA	Kerala	Block II/ICAR-CTCRI Field
7	PCCTB	Kerala	Block I/ICAR-CTCRI Field
8	PCHK	Kerala	Kollam/Kottarakkara
9	PCNA	Kerala	Malappuram/ Nilambur
10	PCPB	Kerala	Idukki/Kumily
11	PCKP	Kerala	Thrissur/Punna
12	PCSD	Kerala	Wayanad/Sultanbathery
13	PCNY	Kerala	Thiruvananthapuram/ Neyyatinkara
14	PCRM	Kerala	Kollam/Kulathupuzha
15	PCTL	Kerala	Kollam/Thenmala

The results of the pathogenicity assay are summarized in Table 2. All the isolates were virulent and reproduced serious infection on detached taro leaf discs except those isolated earlier. There were no lesions in the control leaf discs (Fig. 2). The isolates-initiated lesion development after 1-4 days of inoculation and the inoculated sites showed water-soaked lesions at the beginning which turned brown upon progression of the disease. There was a significant difference in the lesion diameter among the isolates (Fig. 3). The isolate PCHK which exhibited the highest virulence was used further for *in vitro* screening.

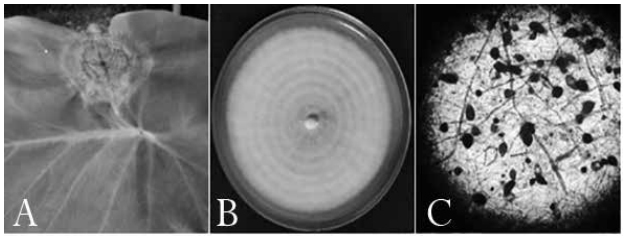

Fig.1. (a) Taro leaf blight symptom, (b) Eight days old culture of *P. colocasiae* on carrot agar medium, (c) Sporangia of *P. colocasiae* observed under 40×

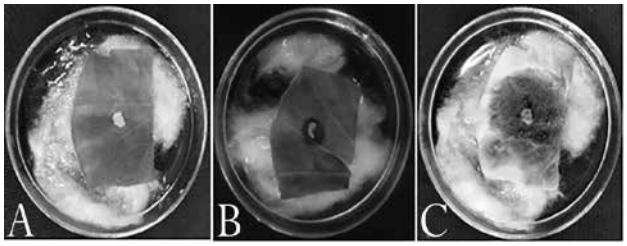

Fig. 2. Pathogenicity assay on detached taro leaves (a)1st day of *P. colocasiae* mycelial plug inoculation. (b) Lesion appeared on 2 days after inoculation (c) Fully infected leaf on 5 days after inoculation

Table 2. Pathogenicity assay by different *P. colocasiae* isolates

Isolate code	Days taken for lesion developed	Lesion diameter (cm)
PCKA	1	4.53±0.06
PCTH	4	2.02±0.13
PCSA	2	4.00±0.20
PCCH	2	3.80±0.30
PCPA	3	3.33±0.29
PCCTA	1	4.82±0.08
PCCTB	4	2.47±0.06
PCHK	1	5.33±0.20
PCNA	2	3.63±0.15
PCPB	2	3.58±0.14
PCKP	3	3.33±0.14
PCSD	3	3.53±0.15
PCNY	2	3.72±0.20
PCRM	3	3.63±0.12
PCTL	4	3.13±0.23

CTAB method was used for the isolation of DNA from *P. colocasiae* isolates. The extracted genomic DNA was run on 1% agarose gel and visualized in Alpha Imager to observe the bands. The concentration of DNA obtained was 380 ng μL^{-1} and absorbance ratio were 1.6. The *P. colocasiae* specific primer pairs PCSP-RR and PCSP-RF successfully amplified all the *P. colocasiae* isolates and yielded an amplicon of size 206 bp approximately when resolved on 1.5% agarose gel.

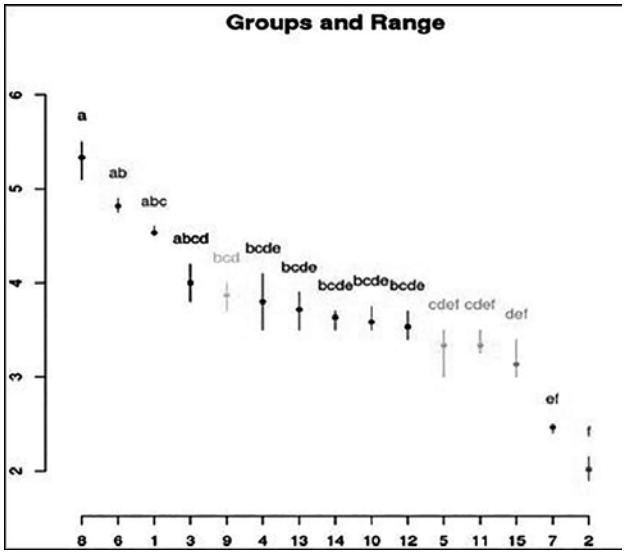

Fig. 3. Multiple comparison plot of virulence of *P. colocasiae* isolates

Endophytic microorganisms have attracted interest in agriculture in recent years due to their prospective impact on crop growth promotion, biocontrol, and disease resistance against diverse phytopathogens (Dwibedi et al., 2022). To obtain a broad diversity of endophytes, wild and cultivated kinds of taro plant samples have been analysed in this work to isolate diverse endophytes and investigate various functional features. Twelve taro plant samples were collected from different parts of Kerala, among them each seven were wild and cultivable taro plants. Root, corm, petiole, and leaves of the taro plants were used for the isolation of bacterial endophytes. The presence and diversity of bacterial endophytes identified from wild and cultivated taro types under natural circumstances are significant because these endophytes can benefit plant health. A total of 97 distinct bacterial endophytes were successfully isolated from roots, corms, petioles, and leaves from wild and cultivated varieties of taro plant samples, of which 33 from root, 27 from corm, 17 from petiole and 20 from leaves (Table 3).

One of the key and essential phases in the isolation approach is effective sterilisation and media selection (Shastri et al., 2020). As there was no growth on the control plates, the isolates can be confirmed as endophytic bacterium of taro.

Endophytic bacterial diversity varies significantly within different plant tissues such as root, corm, petiole, and leaf and between wild and cultivated varieties of plants. Endophytic diversity is greater in root tissues when compared to corm, petiole, and leaf tissue in both types (Fig. 4). The abundance of bacteria in root tissues may be owing to their ability to produce root exudates that promote the proliferation and colonization of bacteria (Karnwal and Dohroo, 2018).

Table 3. Distribution of bacterial endophytes in various plant tissues

Sl. No.	Place of sample collection	Wild/Cultivar	Number of bacterial endophytes			
			Root	Corm	Petiole	Leaf
1	Azhoor/ Pathanamthitta	Cultivated	2	1	2	0
2	Kariavattom/Thiruvananthapuram	Wild	4	2	5	2
3	Kumily/Idukki	Cultivated	2	1	1	2
4	Mavelikkara/Alappuzha	Wild	3	3	2	1
5	Ottapalam/Palakkad	Cultivated	1	2	1	0
6	Pala/Kottayam	Cultivated	2	2	0	0
7	Palode/Thiruvananthapuram	Cultivated	3	2	1	3
8	Periya/Kasaragod	Wild	3	2	1	3
9	Punna/Thrissur	Wild	4	3	0	2
10	Sultanbathery/Wayanad	Wild	3	4	1	3
11	Thenmala/Kollam	Wild	3	2	1	1
12	Ulloor/Thiruvananthapuram	Cultivated	3	3	2	3

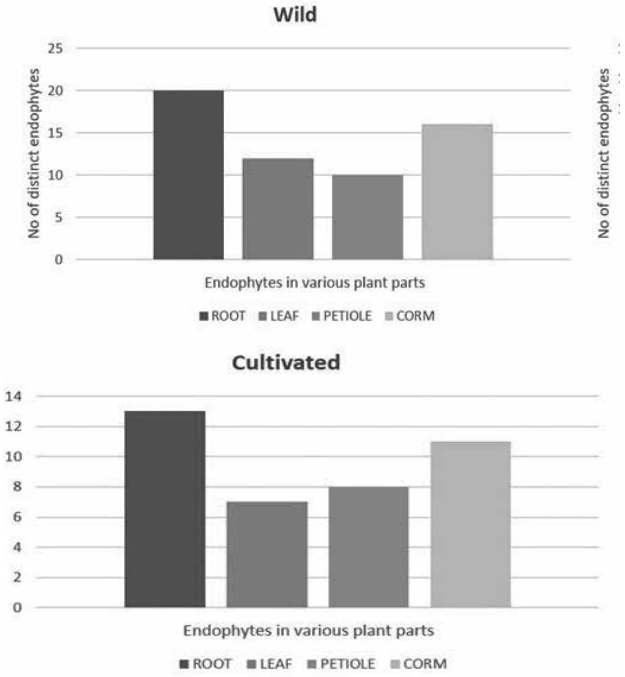

Fig. 4. Number of distinct endophytic bacteria isolated from various plant tissues of wild and cultivated taro samples

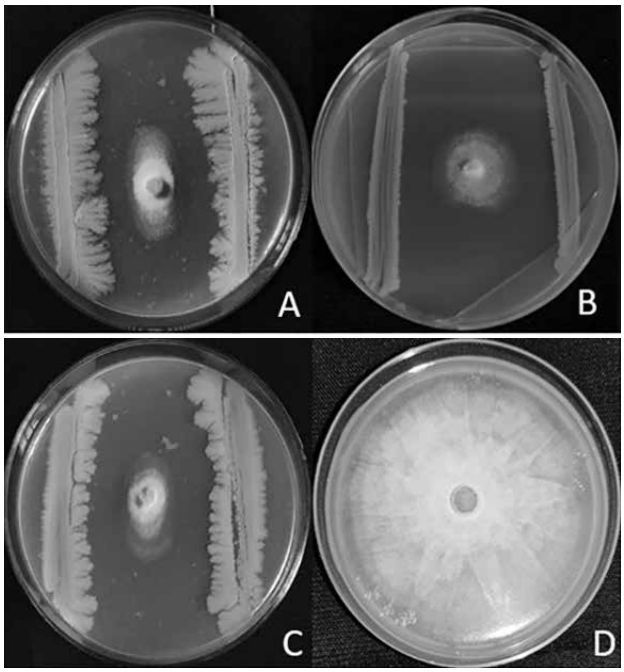

Fig. 5. Antagonistic actvity of bacterial endophytes (A) PA3, (B) UL4, (C) KV9, (D) Control plate

In vitro screening of antagonistic activity of ninety-seven bacterial isolates by dual culture assay against *P. colocasiae* showed that seventy-three endophytic bacteria exhibited antagonistic activities against *P. colocasiae* in varying degrees (Table 4 and Fig. 5). Among those, 55% (40 bacterial endophytes) were from wild taro plant samples and rest (33 bacterial endophytes) from cultivated taro plant samples. The percentage of mycelial growth inhibition was ranged from 7.08% (KV3) to 84.07% (KV9). Twenty-five isolates showed more than 50% mycelial growth inhibition (Fig. 6). Among the isolates,

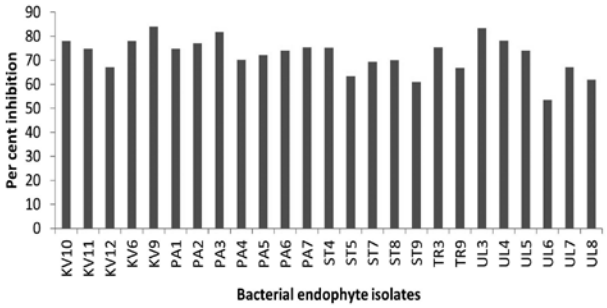

Fig. 6. Percentage inhibition of bacterial endophytes (>50% inhibition) against *P. colocasiae*

Table 4. Mycelial inhibition of *P. colocasiae* by isolated bacterial endophytes

Sl. No.	Isolate code	Tissue used for isolation	Percentage inhibition	Sl. No.	Isolate code	Tissue used for isolation	Percentage inhibition
1	PT1	Root	48.97±0.340	49	PA8	Root	41.3±1.76
2	PT2	Petiole	22.5±0.51	50	PA9	Root	0.0±0.00
3	PT3	Root	0.0±0.00	51	KG1	Root	0.0±0.00
4	PT4	Corm	45.2±1.28	52	KG2	Corm	11.3±1.02
5	PT5	Petiole	47.1±0.32	53	KG3	Corm	47.9±0.29
6	KV1	Petiole	13.95±0.290	54	KG4	Root	34.99±0.510
7	KV2	Root	0.0±0.00	55	KG5	Root	33.5±0.77
8	KV3	Corm	7.08±1.55	56	KG6	Leaf	0.0±0.00
9	KV4	Petiole	26.0±1.65	57	KG7	Petiole	17.5±1.35
10	KV5	Root	0.0±0.00	58	KG8	Leaf	10.3±0.64
11	KV6	Corm	77.95±0.690	59	KG9	Leaf	13.6±0.49
12	KV7	Leaf	44.2±0.23	60	TR1	Root	0.0±0.00
13	KV8	Leaf	28.9±1.36	61	TR2	Corm	39. 9±0.15
14	KV9	Petiole	84.1±1.04	62	TR3	Root	75.4±0.52
15	KV10	Petiole	77.95±0.260	63	TR4	Leaf	31.3±2.70
16	KV11	Petiole	74.8±0.26	64	TR5	Root	41.8±0.82
17	KV12	Root	67.0±0.25	65	TR6	Root	0.0±0.00
18	KV13	Root	0.0±0.00	66	TR7	Corm	39.4±0.88
19	IK1	Leaf	12.9±2.06	67	TR8	Corm	0.0±0.00
20	IK2	Root	44.3±0.38	68	TR9	Leaf	66.5±1.14
21	IK3	Leaf	38.1±0.51	69	ST1	Root	32.5±1.34
22	IK4	Corm	24.3±0.64	70	ST2	Corm	0.0±0.00
23	IK5	Petiole	17.3±1.28	71	ST3	Root	28.3±2.12
24	IK6	Root	28.7±1.34	72	ST4	Corm	75.2±0.69
25	MV1	Corm	30.0±1.02	73	ST5	Corm	63.3±0.78
26	MV2	Root	43.6±0.15	74	ST6	Root	0.0±0.00
27	MV3	Petiole	18.5±1.79	75	ST7	Leaf	69.4±0.90
28	MV4	Corm	41.7±1.17	76	ST8	Leaf	69.99±0.450
29	MV5	Corm	0.0±0.00	77	ST9	Leaf	60.9±0.69
30	MV6	Root	0.0±0.00	78	ST10	Petiole	20.95±0.10
31	MV7	Petiole	22.5±1.02	79	ST11	Corm	0.0±0.00
32	MV8	Leaf	20.7±1.97	80	UL1	Root	36.5±0.78
33	MV9	Root	0.0±0.00	81	UL2	Corm	0.0±0.00
34	OT1	Corm	45.8±1.55	82	UL3	Root	83.3±0.90
35	OT2	Root	0.0±0.00	83	UL4	Corm	78.1±0.94
36	OT3	Corm	0.0±0.00	84	UL5	Corm	73.9±1.20
37	OT4	Petiole	25.5±0.41	85	UL6	Petiole	53.5±1.45
38	KM1	Root	19.2±0.58	86	UL7	Leaf	67.0±0.24
39	KM2	Root	26.9±0.51	87	UL8	Leaf	61.9±0.52
40	KM3	Corm	18.5±2.40	88	UL9	Root	0.0±0.00
41	KM4	Corm	0.0±0.00	89	UL10	Petiole	43.3±1.55
42	PA1	Root	74.8±0.26	90	UL11	Leaf	14.8±0.29
43	PA2	Leaf	77.0±0.69	91	TK1	Root	43.3±0.58
44	PA3	Leaf	81.7±0.45	92	TK2	Root	35.1±0.96
45	PA4	Leaf	70.2±0.25	93	TK3	Corm	0.0±0.00
46	PA5	Corm	72.2±0.45	94	TK4	Root	0.0±0.00
47	PA6	Corm	73.9±0.45	95	TK5	Petiole	32.3±1.47
48	PA7	Petiole	75.4±0.52	96	TK6	Corm	0.0±0.00
				97	TK7	Leaf	27.9±0.29

KV9 isolated from a wild taro plant sample showed the highest inhibitory effect of 84.07±1.04 %. A wide range of antagonistic biologically active compounds have been reported to be produced by the endophytic bacteria which may inhibit the growth of fungal pathogens (Jha et al., 2013). In the dual culture, the most effective endophytes which inhibit pathogen growth displayed a significant zone of inhibition. Endophytes isolated from leaves showed the highest number of antagonistic isolates among wild taro plant samples, followed by petiole, corm, and root. In the case of cultivated taro plants, endophytes isolated from roots had the highest number of antagonistic isolates, followed by corm, leaf, and petiole (Fig.7). Even though the number of bacterial endophytes isolated from wild and cultivated taro samples differed significantly, there was no significant variation in antagonistic activity between the isolates from wild and cultivated taro plant samples. The biocontrol activities of rhizobacterial strains from the genera *Bacillus* and *Pseudomonas* against a wide range of plant diseases have been widely researched (Chen et al., 2020; Borriss, 2011; Beneduzi et al., 2012, Kumar et al., 2012).

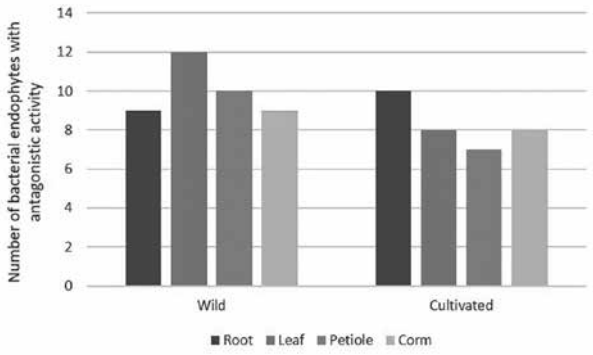

Fig. 7. Distribution of antagonistic bacteria from differenty taro plant tissues

Conclusion

As concerns about the use of agrochemicals in agriculture grow, the use of bacterial endophytes as biocontrol agents shows tremendous promise for quick adoption to control plant diseases, including *P. colocasiae*, the causal agent of taro leaf blight. Many bacterial endophytes have been identified and tested for antagonistic activity against various plant diseases. However, there was limited information on biocontrol of *P. colocasiae* with biocontrol agents. The current study was successful in identifying 25 most promising endophytic bacteria from taro plants that might be used in integrated disease management. As a result, more research is needed to evaluate the bio control efficacy and plant growth stimulating effects of these promising bacteria before incorporating them into the management of taro leaf blight.

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