Research Article

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Isolation, characterization and antagonistic efficacy of fungal endosymbionts from allied genera of cardamom

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Abstract

Small cardamom (*Elettaria cardamomum* Maton) is a major spice crop cultivated for its economic, culinary and medicinal values. Rhizome/clump rot, caused by *Pythium vexans, Fusarium oxysporum* and *Rhizoctonia solani*, is one of the destructive fungal diseases accounting to 30 per cent crop loss. Deployment of beneficial microbes possessing growth promotion activity and antagonistic potential against pathogens could be a viable and sustainable approach to nullify the deleterious effects of synthetic molecules on nature and to control the disease effectively. In this study, an effort was made to isolate the endosymbiotic fungi associated with allied genera of cardamom and evaluating their antagonistic efficacy under *in vitro* conditions against the rhizome rot pathogens. Among the endophytic fungi isolated, maximum inhibition of *P. vexans* was noticed in AsuL4 with 72.4 per cent, followed by HcoL1 with 60.3 per cent, while AmeR2 recorded maximum inhibition 65.3 per cent over control against *R. solani* followed by HcoL1 with 55.1 per cent inhibition. Among the 17 isolates tested against *F. oxysporum*, endophytes isolated from *Amomum subulatum,* AsuLV3 recorded maximum inhibition of 73.8 per cent followed by AsuL4 with 69.9 per cent. The shortlisted efficacious isolates need to be further evaluated under glasshouse and field conditions to confirm their efficacy and could be employed as integral components in cardamom production system to manage rhizome-root rot efficiently, economically and eco-friendly in a sustainable manner.

Keywords: Antagonistic fungi, cardamom, disease management, endophyte, rhizome rot

Introduction

Small cardamon (*Elettaria cardamomum* Maton) also known as queen of spices, is one of the most valuable spice crops grown in Western Ghats owing to its high national and international market demand. In India, cardamom is cultivated in 70000 hectares. Approximately 18 thousand tonnes (Spices Board Estimate, 2014) is produced every year and India is a traditional producer and exporter of small cardamom. Diseases are among the most significant constraints to cardamom production in the subtropics: more than 20 pathogens (fungal, bacterial and viral) are known to attack this crop, but less than a dozen can cause substantial economic loss. Among them, rhizome/ clump rot (caused by *Pythium vexans, Rhizoctonia solani* and *Fusarium oxysporum*) and leaf blight (caused by *Colleotrichum gloeosporoides*) are the major fungal diseases causing about 30 per cent crop loss (Thomas *et al.,* 1988; Vijayan and Thomas, 2002). Indiscriminate use of fungicides invites rejection of export consignment due to Chemical residues that are above the maximum residue level (MRL).

Symptoms of rhizome rot initially starts as water soaked lesions at the collar region, followed by decay of the tillers and ultimately leading to toppling of tillers. Infected tillers can be easily pulled out with a little force. Use of naturally available antagonistic microorganisms against rhizome rot pathogens is a substitution approach to grow cardamom on a sustainable basis. Plants express multiple traits including that are expressed by symbiotic microbes, they provide food or minerals to plants and resistance

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against herbivores and pathogens (Barrett and Heil, 2012). Several characters act directly against these pathogens like competition, repellent, and some indirect effects by producing antimicrobial compounds or function as mechanical barriers (Heil, 2008).

The tactical use of naturally occurring microbes to ward off pathogens and augment production of major crops represents an alternative and feasible option to induce host plant resistance and pesticide free pest and disease control. Glenn *et al.* (1996) found that endophytes are systemic, seedborne and non-pathogenic, belonging to genus *Neotyphodium* and were highly competitive and antagonist.

Marshall *et al.* (1998) characterized ten species of endophytes obtained from *Triticum.* Most of them *were Acremonium* and *Neotyphodium*. *Paenibacillus polymyxa* and *Citrobacter* sp. isolated from wild relatives of maize showed antagonistic activity against *F. oxysporum* (Mousa *et al.,* 2015). The present study was formulated with the objective to characterize endophytes from wild genera of small cardamom free from rhzome rot and test its efficacy.

Materials and methods

Isolation of rhizome rot pathogens

The pathogen was isolated by using tissue dissection methods as described by Prema *et al.* (2011). Infected rhizomes showing typical symptoms were cut into small bits and surface sterilized in 0.1 per cent mercuric chloride solution for about a minute, followed by washing thrice in sterile distilled water. Tissues were transferred to sterile Petri plates, pre plated with potato dextrose agar (PDA) medium. The inoculated Petri plates were incubated in BOD incubator at (28±2ºC) and observations were taken at regular intervals. The pathogen was identified based on their cultural and morphological characters. A bit of fungal culture was microscopically examined at 40X magnification for the presence of conidia (*F. oxysporum*), constriction in daughter mycelia (*R. solani*) and sporangia (*P. vexans*).

Isolation of fungal endosymbionts

Endophytic fungi present in wild genera of cardamom were isolated from leaves, stem, root and rhizomes of three wild genera *viz.*, *Aframomum melegueta* (Ame)*, Amomum subulatum* (Asu) and *Hedychium coronarium* (Hco). For study of endophytic fungi growing symptomless in healthy plant tissues, cultivation technique was followed as explained by Unterseher and Schnittler (2009). Plant organs were dissected into small fragments, surface sterilized and plated onto PDA medium. Cultural characters like colony growth, colour, zonation, substrate colour, margin of colony and topography were recorded.

In vitro inhibition of pathogens

Fungal endosymbionts were evaluated for their ability to inhibit mycelial growth of *F. oxysporum, P. vexans and R. solani* by following the dual culture technique (Dennis and Webster, 1971). The antagonist culture was inoculated at one side of Petri plate at about a cm from one edge of the plate on PDA medium and mycelial disc (8 mm diameter) of seven days old culture of pathogens was placed on the opposite side perpendicular to the antagonist plug. The plates were incubated at room temperature (28±2^{o}C) for four days in case of *P. vexans* and *R. solani* and for seven days in case of *F. oxysporum*, and the radial mycelial growth (mm) of the pathogen was recorded. Per cent inhibition of the mycelial growth was then calculated by using formula as follows.

$$\text{Per cent inhibition over control} = \frac{C - T}{C} \times 100$$

where, C is the mycelial growth of pathogen in control and T is mycelial growth of pathogen in dual plate

Data analysis

The *in vitro* bioassay experiments were laid out in completely randomized design (CRD), the per cent data was transformed using arc sine transformation and statistical analysis was carried out using the software package AGRES version 7.01 (1994 Pascal Intl Software Solutions).

Results and discussion

Rhizome rot in small cardamom is one of the major diseases known to farmers growing the crop in Kerala and Karnataka and is also referred as clump rot disease, caused by pathogen trio *P. vexans, R. solani* and *F. oxysporum.* The disease is a serious menace to cultivation of small cardamom in tropics. At present, the disease is controlled by drenching huge quantity of pesticides before and after the onset of the monsoon. Alternatively, the use of fungal endophytes can be a remedy for the disease and also biologically safe.

Endophytes are well thought-out as plant mutualists, as they obtain nourishment and security from the host plant, while the host gets benefit from improved cut-throat abilities and augmented confrontation to various biotic and abiotic stresses (Saikkonen *et al.,* 1998). An attempt was made to isolate the endosymbiotic fungi associated with wild genera of cardamom, namely, *Aframomum melegueta* (Ame)*, Amomum subulatum* (Asu) and *Hedychium coronarium* (Hco). Isolation of endophytic fungi was carried out from leaves, petiole, stem, root and rhizomes of these species and characterized. Totally 17 isolates of endosymbiotic fungi were isolated from the three allied genera of cardamom and grown in PDA medium. Greyish white coloured colonies were observed in the isolates AsuLV1, AsuLV2, AsuL2 and AsuL4. Grey coloured colonies were observed in AsuLV3, AmePe2, white coloured colonies were observed in AsuL2, HcoL2, AmeL, AseLV, AmePe1 and Ame R1, while brown an brownish orange coloured colonies were observed in AmeR2 and AmePs, respectively.

Variations were observed in substrate colour, three isolates (AsuLV1, HcoL1, AmeL) were cream coloured, brown to browish yellow coloured substrates were observed in isolates, AsuLV3, AsuL5 and AmeR2. Margins of isolates also showed variations, four isolates were irregular and other were regular and mixed reaction of wavy and smoothness were also seen. Considering the topography, 11 isolates were raised and fluffy, five isolates were flat, one isolate (AmePS) was raised and cottony and one (AseLV) was powdery.

Clear zonation was noticed only in AmeL isolate, with regard to pigmentation, seven isolates showed characteristic pigmentation (Table 1, Fig. 1). Maximum colony diameter (90 cm) was observed in eleven isolates on seventh day after sub-culturing. Colony appearance, colony growth rate and colony morphology are important traits for identification and characterization of fungi (Prema *et al.,* 2011). There was wide variation in the colony characters *viz.,* colour, topography, pigmentation, zonation, sporulation and mycelial growth of different isolates in PDA media. In the present study, isolates were characterized by blackish white, grey and white coloured colonies. Margins were smooth as well as irregular, all the isolates differed with respect to sporulation, pigmentation, margin and topography. Lu *et al.* (2012) characterized the endophytic fungi isolated from Chinese medicinal plant *Actinidia* and identified based on moprhological and cultural characters.

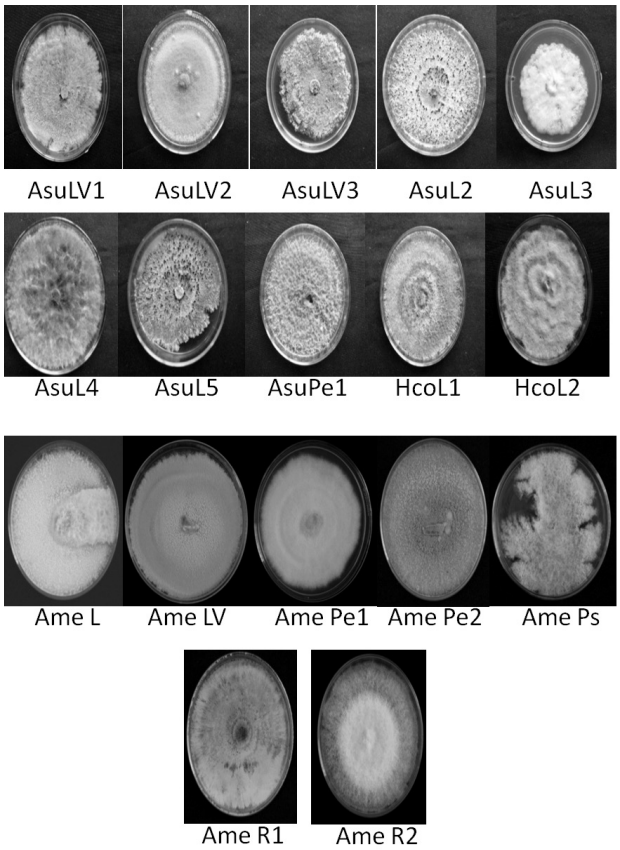

Fig. 1. Cultural characters of endophytic isolates from wild relatives of small cardamom

Table 1. Cultural characters of endophytic isolates from *Amomum subulatum* (Asu), *Hedychium coronarium* (Hco) and *Aframomum melegueta* (Ame) on PDA medium

S. No.	Isolate	Colony colour	Substrate colour	Margin	Topography	Zonation	Pigmentation	Colony diameter (mm)
1	AsuLV1	Greyish white	Cream	Wavy	Raised and fluffy	No zonation	—	85
2	AsuLV2	Greyish white	White	Smooth	Raised and fluffy	No zonation	—	90
3	AsuLV3	Grey	Brown	Wavy and irregular	Raised and fluffy	No zonation	Brown	78
4	AsuL2	Greyish white	Light brown	Smooth	Flat	No zonation	Black	90
5	AsuL3	White	White	Wavy and regular	Raised and fluffy	No zonation	—	70
6	AsuL4	Greyish black	Black	Smooth and regular	Raised and fluffy	No zonation	—	90
7	AsuL5	Whitish brown	Brown	Wavy and irregular	Flat	No zonation	Black	83
8	AsuPe1	Whitish grey	Yellow	Wavy and regular	Raised and fluffy	No zonation	Yellow	90
9	HcoL1	Blackish grey	Greyish yellow	Wavy and regular	Raised and fluffy	No zonation	Whitish yellow	90
10	HcoL2	White	Cream	Wavy and irregular	Raised and fluffy	No zonation	—	90
11	AmeL	White	Cream	wavy	Raised and fluffy	Clear zonation	—	90
12	AseLV	White	Creamy white	Smooth	Powdery	No zonation	—	90
13	AmePe1	White	White	Smooth	Flat	No zonation	—	90
14	AmePe2	Grey	Blackish grey	Smooth	Flat	No zonation	Black	90
15	AmePs	Brownish orange	Orange	Wavy and irregular	Raised and cottony	No zonation	—	75
16	AmeR1	White	White	Smooth and regular	Raised and fluffy	No zonation	—	90
17	Ame R2	Brown	Brownsih yellow	Wavy and irregular	Flat	No zonation	Dark brown	83

The results of *in vitro* antagonistic activity of endosymbionts against mycelial growth of *P. vexans* revealed that AsuL4 isolate significantly reduced the mycelial growth of *P. vexans* with mean mycelial growth of 21.3 mm accounting for 72.4 per cent inhibition over control. It was followed by HcoL1 with a mean mycelial growth of 30.7 mm and 60.3 per cent inhibition over control. Least inhibition of 28.0 per cent was recorded in AsuPe1 with the mycelial growth of 55.7 mm (Table 2; Fig. 2).

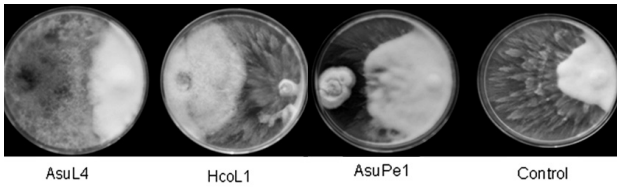

Fig. 2. *In vitro* antagonistic activity of endophytes against *Pythium Vexans*

Similarly, *in vitro* efficacy of endosymbionts against the mycelial growth of *R. solani* revealed that the isolate AmeR2 significantly recorded higher inhibition of 65.3 per cent, followed by HcoL1 recording 55.1 per cent inhibition over control. The lowest inhibition was recorded by AsuL3 with a mycelial growth and per cent inhibition of 67.0 mm and 10.7, respectively (Table 3; Fig. 3).

The result of *in vitro* efficacy of endosymbionts against mycelial growth of *F. oxysporum* showed that among the 17 isolates tested for mycelial

Table 2. ***In vitro*** **antagonistic activity of endosymbionts against** ***P. vexans***

Sl. No.	Isolate	Growth of pathogen (mm)	Percentage inhibition over control	Growth of endophyte (mm)	Interaction type
1	AsuLV1	34.7	55.2^{b}	30.0	Overlapping
2	AsuLV2	43.7	43.5^{c}	18.0	Inhibition
3	AsuLV3	45.0	41.8^{c}	15.7	Inhibition
4	AsuL2	33.3	56.9^{b}	25.7	Inhibition
5	AsuL3	35.0	54.7^{b}	30.3	Overlapping
6	AsuL4	21.3	72.4^{a}	30.3	Inhibition
7	AsuL5	32.7	57.7^{b}	34.7	Inhibition
8	AsuPe1	55.7	28.0^{d}	11.0	Inhibition
9	HcoL1	30.7	60.3^{b}	39.0	Inhibition
10	HcoL2	35.0	54.7^{b}	34.7	Overlapping
11	Ame L	36.0	53.4^{b}	34.3	Inhibition
12	Ame LV	31.0	59.9^{b}	13.7	Inhibition
13	Ame Pe1	32.7	57.7^{b}	32.3	Overlapping
14	Ame Pe2	32.0	58.7^{b}	29.0	Overlapping
15	Ame Ps	36.0	53.4^{b}	33.7	Overlapping
16	Ame R1	32.7	57.5^{b}	30.3	Inhibition
17	Ame R2	37.0	52.8^{b}	21.0	Overlapping
18	Control	77.3	0.0		

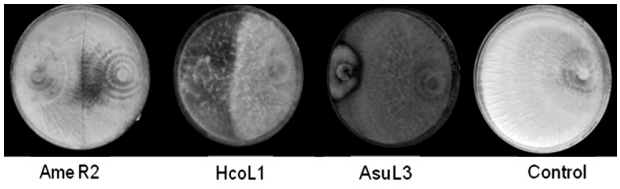

Fig. 3. ***In vitro*** **antagonistic activity of endophytes against** ***R. solani***

inhibition, the isolate AsuLV3 recorded significantly higher inhibition of 73.8 per cent over control, followed by AsuL4 with 69.9 per cent inhibition over control. The isolate AmeP2 recorded the lowest per cent inhibition (40.6) (Table 4; Fig. 4). Very few studies in endophytes from the wild relatives of cultivated crops have been isolated, characterized

Table 3. ***In vitro*** **antagonistic activity of endosymbionts against** ***R. solani***

S. No.	Isolate	Growth of pathogen (mm)	Percentage inhibition over control	Growth of endophyte (mm)	Interaction type
1	AsuLV1	52.3	30.2^{i}	13.3	Overlapping
2	AsuLV2	48.3	35.6 ^{fghi}	14.3	Overlapping
3	AsuLV3	49.0	34.7 ^{ghi}	11.3	Overlapping
4	AsuL2	45.0	40.0 ^{rfg}	16.7	Overlapping
5	AsuL3	67.0	10.7 ^{k}	3.0	Overlapping
6	AsuL4	38.7	48.4 ^{cd}	22.0	Inhibition zone
7	AsuL5	43.0	42.7 ^{de}	19.3	Overlapping
8	AsuPe1	44.3	40.9 ^{ef}	15.7	Overlapping
9	HcoL1	33.7	55.1 ^{b}	18.7	Inhibition zone
10	HcoL2	46.0	38.7 ^{efgh}	15.7	Overlapping
11	Ame L	45.0	40.0^{hi}	18.7	Overlapping
12	Ame LV	50.3	32.9^{efgh}	18.0	Overlapping
13	Ame Pe1	46.3	38.1^{bc}	17.3	Overlapping
14	Ame Pe2	36.3	51.5^{j}	20.7	Overlapping
15	Ame Ps	61.7	17.8^{j}	9.3	Inhibition
16	Ame R1	45.0	40.0^{efg}	12.3	Inhibition
17	Ame R2	26.0	65.3^{a}	28.7	Inhibition
18	Control	75.0	0.0		

Table 4. ***In vitro*** **antagonistic activity of endosymbionts against** ***F. oxysporum***

S. No.	Isolate	Growth of pathogen (mm)	Percentage inhibition over control	Growth of endophyte (mm)	Interaction type
1	AsuLV1	42.0	45.0^{hi}	24.7	Overlapping
2	AsuLV2	36.7	52.0^{efgh}	30.3	Overlapping
3	AsuLV3	20.0	73.8 ^{a}	45.3	Overlapping
4	AsuL2	28.0	63.3 ^{bcd}	35.3	Overlapping
5	AsuL3	30.3	60.3 ^{cde}	37.0	Overlapping
6	AsuL4	23.0	69.9 ^{ab}	34.3	Inhibition zone
7	AsuL5	37.3	51.1 ^{fgh}	38.0	Overlapping
8	AsuPe1	25.0	67.2 ^{abc}	22.7	Overlapping
9	HcoL1	42.0	45.0 ^{hi}	34.0	Inhibition zone
10	HcoL2	32.7	57.2 ^{def}	34.3	Overlapping
11	Ame L	36.0	52.8^{efgh}	30.0	Inhibition
12	Ame LV	32.0	58.1^{def}	31.0	Overlapping
13	Ame Pe1	35.0	54.1^{efg}	23.3	Inhibition
14	Ame Pe2	45.3	40.6^{i}	21.0	Overlapping
15	Ame Ps	41.3	45.9^{egi}	26.0	Inhibition
16	Ame R1	34.0	55.5^{def}	25.0	Inhibition
17	Ame R2	23.3	69.4^{ab}	69.0	Overlapping
18	Control	76.3	0.0		

and evaluated against plant pathogens, their efficacy against pathogens operates through various mechanisms. Yue *et al.* (2000) identified several derivatives of indole, terpene, and amide from *Epichloe festucae*, however, the inhibition of pathogens *in vitro* and disease resistance exhibited *in vivo* could not be linked clearly. Turf grasses, which had *E. festucae* as an endophtye, showed considerable confrontation over turf grasses which did not harbour *E. festuca,* to leaf spot pathogens *Sclerotina homeocarpa* (Clarke *et al.,* 2006) and *Laetisaria fusiformis* (Bonos *et al.,* 2005). Still, the mystery is unsloved that the mechanism of enhanced disease confrontation with the presence of certain endophytes, is associated with antimicrobial compounds produced by the endophyte or by physical exclusion mechanism or by competition or combination of all these mechanisms, in order to ward off the pathogen.

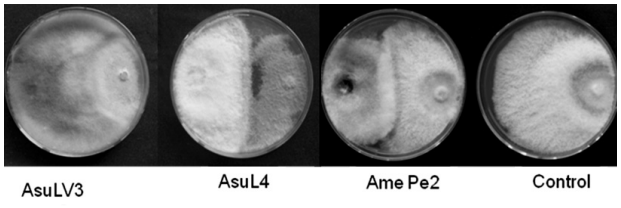

Fig. 4. ***In vitro*** **antagonistic activity of endophytes against** ***F. oxysporum***

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