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Morphogenetic characterization of *Fusarium oxysporum* associated with storage rot of ginger in Assam and *in vitro* evaluation of botanicals

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Abstract
Post-harvest rot of ginger is an important disease which resulted in huge losses in quality as well as quantity of ginger ultimately leads monetary loss to farmers as well as traders. In study of storage rot of ginger, survey under three agro-climatic zones (Upper Brahmaputra Valley zone, Hill Zone and Lower Brahmaputra Valley Zone) of Assam revealed that *Fusarium* sp. was majorly associated with storage rot of ginger. Their pathogenicity on ginger was determined and the microbes have been identified based on morphogenetic characterization. Morphological features of the isolate were described and Internal Transcribed Spacer (ITS regions) was used to characterize the isolate at molecular level. Molecular phylogenetic analyses revealed that these isolate was assignable to *Fusarium oxysporum.* Ten botanicals (*Acorus calamus, Allamanda cathertica, Allium cepa, Allium sativum, Curcuma longa, Datura wrightii, Lasia spinosa, Laurus nobilis, Ocimum sanctum* and *piper betle*) were evaluated against the pathogen. Aqueous extracts (20%) of *A. sativum, A. cathertica* and *L. nobilis* significantly inhibited the growth of *F. oxysporum.* These three most effective botanicals were further tested at four different concentrations (5, 10, 15 and 20 per cent) and *A. sativum* (92.22%) at 20 per cent concentration exhibited highest inhibition followed by *A. sativum* (84.44%) at 15 per cent and *A. cathertica* (78.22%) at 20 per cent. This was followed by *A. sativum* (74.44%) at 10 per cent, *A. cathertica* (73.33%) at 15 per cent and *L. nobilis* (72.22%) at 20 per cent.

Keywords: storage rot of ginger, *Fusarium oxysporum,* internal transcribed spacer (ITS), botanicals, inhibition

Introduction
Ginger (*Gingiber officinale* Rosc.) under the family Zingiberacae, a plant indigenous to South-Eastern Asia (Purseglove *et al*., 1981) [27] is known treasured spice esteemed for its pungency and aroma, viewed as a healing gift from God by Indian Ayurvedic systems. Medicinal uses of Ginger has been reported as anti-nausea, anti-clotting agent, antifungal, anti-inflammatory, antiseptic, antibacterial antiviral, antitussive, analgesic, circulatory stimulant, blood flow increasing agent and so on (Gunathilake and Rupasinghe, 2015) [14]. Ginger is the fourth most important spice in India with a production of 1047.19 thousand tons from an area of 160.48 thousand ha (Annon, 2016-17) [1]. Assam produces 156.66 thousand tons of ginger with an average productivity of 8.88 t/ha (Annon, 2016-17) [1]. In North Eastern states, Assam ranks first in acreage as well as in production (Annon, 2016-17) [1]. After harvesting, ginger rhizomes has to be stored for seed and commercial purpose in different types of storage structures at least for a period of 6-7 months (From Jan-Feb to Aug-Sep) for day to day fresh consumption under Assam condition. Ginger has been affected by many pathogens in pre-harvest as well as post-harvest. Post-harvest deterioration is the most important cause of loss of ginger during storage due to rotting, resulting into considerable loss to farmers as well as traders. *Fusarium oxysporum*, *Pythium aphanidermatum* and *Pseudomonas solanacearum* have been reported as the major causal agents for the storage rot of ginger in India (Dake and Edison, 1989; Dohroo, 1989; Sharma *et al.*, 2017) [11, 12, 35]. Fungi taxonomy is traditionally based on comparative morphology and development of sexual reproduction structures which are laborious and time-consuming and also non or low-sporulating fungi are more difficult to identify by means of conventional taxonomy (Wu *et al*. 2003) [46]. The 18S and 28S ribosomal genes and the internal transcribed spacer (ITS) regions have been the site targets in study of fungi. The information contained in these sequences have been extensively used in population diversity and characterization studies (Smit *et al.* 1999; van Elsas *et al.* 2000; Schabereiter-Gurtner *et al*. 2001; Sterflinger and Prillinger 2001) [38, 42, 33, 39], identification and detection (Brookman *et al.* 2000; Redecker 2000; Abd-Elsalam *et al.* 2003; Wu *et al.* 2003; Ablitz *et al.*

2004) [6, 31, 2, 54, 3] and for the establishment of phylogenetic relationships (LoBuglio and Taylor 1995; Peintner *et al*. 2003) [20, 26]. Eco-friendly botanical extracts have shown to be potential alternative to synthetic fungicides (Janisiewiez and Korsten, 2002; Zhang and Zheng, 2005) [17, 48]. Several works have been done on management of post-harvest disease that include microbial antagonists and botanical extracts as potential alternatives to synthetic fungicides (Chauhan and Joshi, 1990; Sarvamangla, 1993; Chaudhary, 2003; Bhardwaj *et al.*, 2013) [7, 32, 9, 5]. On the other hand, Assam as well as other North East states of India is well known for the rich biodiversity specially plants. Hence, use of rich potential of the botanicals available in the state may be a comparative advantage and an alternative for the management of ginger storage rot. They are also best suited for use in organic food production in industrialized countries but can play a much greater role in developing countries like India as a new class of botanical pesticide for controlling diseases (Isman, 2006) [15]. The aim of the present study was to morphogenetic characterization of *Fusarium oxysporum* associated with storage rot of ginger in Assam and *in vitro* evaluation of botanicals.

Materials and Methods

Isolation and morphological characterization of pathogen

Ginger showing typical rotting symptoms were collected from different district *viz.*, Karbi anglong, Joraht, Golaghat and Darrang under three agro-climatic zones (Upper Brahmaputra Valley zone, Hill Zone and Lower Brahmaputra Valley Zone) of Assam. Isolation of pathogen was carried out by using PDA (Potato Dextrose Agar) and pathogenicity test were performed by inoculating healthy ginger rhizome with mycelial disc with the help of 5mm cork borer. Fungi showing positive pathogenicity test was identified based on the spore morphology and colony characters of the fungus by referring to the standard literature available (Barnett and Hunter, 1972) [4].

Molecular characterization

Further molecular approaches like signature sequence (ITS) of fungi has been analyzed for their identification at species level. The genomic DNA from the mycelial pure cultures was extracted using a method previously described by Cheng *et al*. (2003) [8] with minor modifications. The hyphae were crushed using liquid nitrogen and lysed at 65 °C in a lysis buffer containing CTAB. The tissue lysate was then extracted subsequently with phenol/chloroform/isoamyl alcohol (25: 24: 1) and chloroform/isoamyl alcohol (24: 1) and then DNA was precipitated from the aqueous phase using ethanol. The DNA pellet was washed with 70% ethanol and finally re-dissolved in 1X TE buffer. The ITS genomic region of the fungal DNA samples was amplified using PCR with universal ITS pimers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990) [45]. Sequencing of the PCR product was done at BioServe Biotechnologies India Pvt. Ltd., Hyderabad. The ITS gene sequences of the isolates was searched for their homologue sequences in public domain databases. Basic Local Alignment Search Tool (BLAST) was used to determine the phylogenetic relationship from the nucleotide sequence available in the database of National Centre for Biotechnology Information (NCBI). The obtained sequences were aligned using multiple sequence alignment in MEGA 6.0 (Tamura *et al*., 2013) [40]. A phylogenetic tree was constructed using Maximum-Likelihood method included in MEGA 6.0 by taking 500bootstrap.

Evaluation of botanicals *in vitro*

In the evaluation of botanicals, different plant parts like fresh leaves of *Acorus calamus, Allamanda cathertica, Datura wrightii, Lasia spinosa, Laurus nobilis, Ocimum sanctum* and *Piper betle*, bulbs of *Allium cepa* and *Allium sativum* and rhizome of *Curcuma longa* were collected from various localities of Jorhat, Assam for the preparation of aqueous plant extracts. Collected plant parts were washed thoroughly in sterile distilled water and grinded by adding equal amount (100ml) of sterilized distilled water (1:1 W/V). After grinding, the extract was filtered through muslin cloth and finally the extracts were centrifuged at 10,000 rpm for 20 minutes at room temperature and the supernatant was taken as standard plant extract solution (100%). The method reported by Shekhawat and Prasad (1971) [36] was followed with certain modifications. The plant extracts were evaluated at 20 per cent concentration (for preliminary screening) against *Fusarium oxysporum* by 'poisoned food technique' (Nene and Thapliyal, 2000) [25]. Three most promising botanicals were further tested against fungus in four different concentrations *viz.*, 5, 10, 15 and 20 per cent, respectively.

The diameter of the colony is measured when the mycelium fully covered the Petri plates of control plate and the per cent inhibition of the mycelial growth was calculated by the formula of Vincent (1927) [44].

$$I = \frac{(C - T)}{C} \times 100$$

Where, I = Inhibition of mycelial growth (%)
C = Growth in control (mm)
T = Growth in treatment (mm)

Results and Discussion

Morphological and molecular characterization of the pathogen

Colony morphology of isolate on potato dextrose agar (PDA) varies widely. Isolate produced a pale to dark violet or dark magenta pigment in PDA but sometimes produces no pigment at all. Macroconidia were four to five septate, short to medium length (29.42 - 38.6 µm X 4.30 - 5.25 µm), straight to slightly curved, relatively slender and thin-walled with tapered and curved apical cell and pointed basal cell. Micro conidia were oval or elliptical (4.80 - 6.18 µm X 1.65 – 3.30 µm) and usually single septate. Chlamydospores formed in clusters or in short chains. The fungus was tentatively identified as *Fusarium* sp. based on the morphological and colony characteristics. The results of morphological characterization of the present study are in agreement with those reported by several workers (Negi, 1998; Ram and Thakur, 2010; Sharma *et al*., 2017) [24, 30, 35] who also reported the colony color, size of macro-conidia, micro-conidia in the range as obtained in the present investigation. Sharma *et al*. (2017) [35] described macro-conidia (10-27 µm x 2-4 µm) as abundant, falcate to erect with 1- 4 septa. They found microconidia were oval to fusiform and measured 4.2-6.3 µm x 2.4µm and chlamydospores were formed both terminally as well as intercalary.

In molecular characterization sequencing results were successfully obtained from Bio Serve Biotechnologies India Pvt. Ltd., Hyderabad. BLAST results of partial sequencing of Internal Transcribed Spacer (ITS regions) revealed that *Fusarium* sp. having 100 per cent homology with *Fusarium oxysporum* (MK463988.1) isolate. Similarly, White *et al*. (1990) [53] verified the identity of fungi through amplification and sequencing of the ribosomal *Internal transcribed spacer* (ITS) region using the universal primer set ITS1/ITS4. Hence isolated fungus was confirmed as *Fusarium oxysporum* through both morphological and molecular

characterization which was in accordance with report of Lee *et al*. (2000) [19] who suggested that the ITS is more useful in identification of *Fusarium* species. Phylogentic tree analysis indicating the genetic relationship among different *F. oxysporum* isolates (Fig 1). Our isolate (*Fusarium oxysporum* Assam isolate) showed nearest relationship with Manipur isolate (MH911389.1).

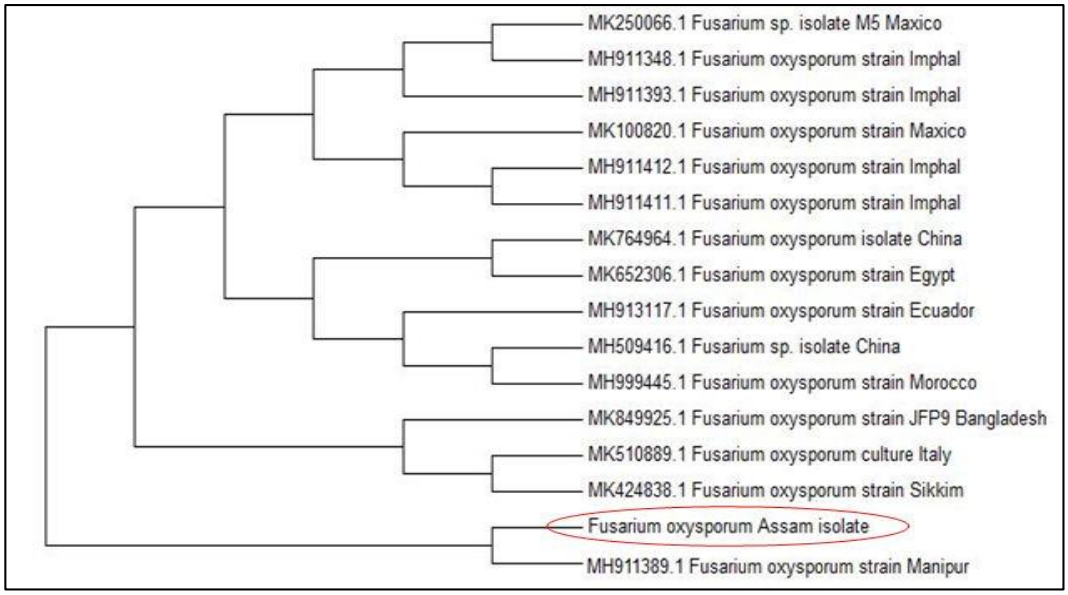


Fig 1: Phylogenetic tree showing the genetic relationship of the *F. oxysporum* isolate to other isolates by using maximum likelihood method with 500 bootstrap replicate

Evaluation of botanicals *in vitro*

The results presented in the Table 1 revealed that some botanicals significantly reduced the mycelial growth of the pathogen over control (90 mm). Among all the botanicals tested, the maximum per cent mycelial inhibition was recorded in *A. sativum* (92.22%) which was found to be significantly superior over rest of the botanicals which are in agreement with those reported by earlier workers (Vijaya *et al.*, 2007; Ram and Thakore, 2009; Jadhav *et al.*, 2013; Chaudhary *et al.*, 2017) [43, 29, 16, 10] who reported that *A. sativum* was highly effective against many fungal pathogens. The effectiveness of *A. sativum* might be due to the presence of some bioactive compounds like *garlicin*, *phytocides*, *allicin*, which act as an inhibitor of respiratory SH- group enzymes, *ajoene* which destroys the integrity of cell wall (Shekhawat and Prasad, 1971; Yoshida *et al*., 1987; Singh *et al*., 1995) [36, 47, 37].

A. cathertica (78.22%) extract was found as the second most effective botanical which was demonstrated by earlier workers (Jannat, 2006; Tania, 2007 and Masuduzzaman *et al.*, 2008) [18, 41, 21] against a number of fungal pathogens including *Phomopsis vexans*, *Phytophthora capsici*, *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii*). Antifungal activity of Allamanda is might be due to presence of a compound known as plumieride which has the inhibitory effect on plant pathogenic fungi (Mone *et al*., 2014) [22]. *L. nobilis* (72.22%) was the third most effective botanicals followed by *D. wrightii* (62.22%) and *L. spinosa* (53.11%). This finding is in agreement with the report of earlier workers like Nayak *et al.* (2006) [23] who reported that leaf extracts of *L. nobilis* promote wound healing activity and antifungal (Qamar and Chaudhary, 1991) [28] and antibacterial (Seyed *et al.*, 1991) [26, 38] activities. The per cent inhibition for the rest of the plant extracts on mycelial growth of the pathogen ranged from 52.22 to 32.22 per cent respectively. The lowest inhibition was recorded in *A. cepa* (3.22%).

Based on the results of this preliminary screening, three most effective botanicals (which exhibited above 70 per cent inhibition over the control) *viz.*, *A. sativum*, *A. cathertica* and *L. nobilis* were selected for further evaluation of their efficacy at different concentrations against the pathogen. Results (Table 2, Fig 2) indicated that irrespective of different concentrations, all the botanicals showed significantly higher inhibitory effect on the mycelial growth of *F. oxysporum* compared to control.

Among the three botanicals tested at four concentrations, the maximum growth inhibition was recorded in treatments involving *A. sativum* (92.22%) at 20 per cent concentration which was significantly superior over rest of the treatments followed by *A. sativum* (84.44%) at 15 per cent and *A. cathertica* (78.22%) at 20 per cent. This was followed by *A. sativum* (74.44%) at 10 per cent, *A. cathertica* (73.33%) at 15 per cent and *L. nobilis* (72.22%) at 20 per cent. Present results are in agreement with the findings of Ram and Thakore (2009) [33] who tested aqueous extracts of 19 botanicals at 5, 10, 15 and 20 per cent concentration against *F. solani* and *P. aphanidermatum* and found *A. sativum* most effective at 20 per cent concentration. Per cent inhibition in *A. cathertica* (73.33%) at 15 per cent was *at par* with *L. nobilis* (72.22%) at 20 per cent and *A. sativum* (74.44%) at 10 per cent. Irrespective of concentrations of plant extracts tested, the treatments involving *A. sativum* recorded maximum mean per cent mycelial inhibition (79.83%) followed by *A. cathertica* (69.61%) and minimum mycelial inhibition was recorded in *L. nobilis* (60.61%).

Table 1: Efficacy of different botanicals (20%) on mycelial growth of *F. oxysporum*

Treatments	Mycelial growth* (mm)	Mycelial growth inhibition over control (%)
T1: *Acorus calamus*	43.00	52.22 (46.24) **
T2: *Allamanda cathertica*	19.60	78.22 (62.21)
T3: *Allium cepa*	61.00	32.22 (34.56)
T4: *Allium sativum*	7.00	92.22 (73.77)
T5: *Curcuma longa*	46.00	46.88 (44.34)
T6: *Datura wrightii*	34.00	62.22 (52.05)
T7: *Lasia spinosa*	42.20	53.11 (46.76)
T8: *Laurus nobilis*	25.00	72.22 (58.17)
T9: *Ocimum sanctum*	53.40	40.66 (39.60)
T10: *Piper betel*	44.40	50.00(45.32)
T11: Carbendazim (0.1%)	0.00	100.00 (89.55)
T12 : Control	90.00	00.00(3.69)
SEd (±)	0.70	
CD (p= 0.05)	1.41	

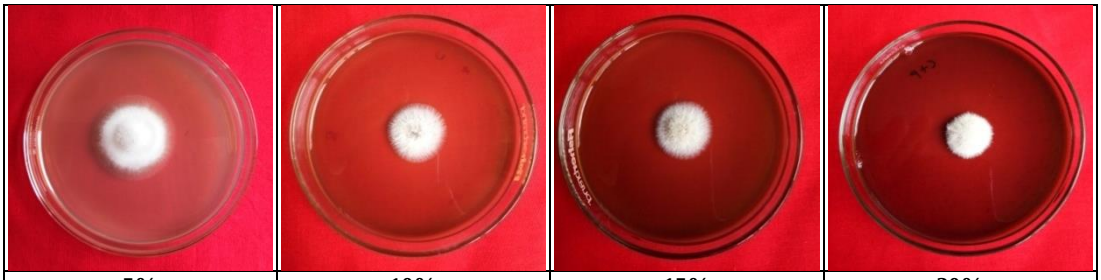
* Mean of five replications

** Data in parentheses represents angular transformation

Table 2: Efficacy of botanicals (5, 10, 15 and 20%) on mycelial growth of *F. oxysporum*

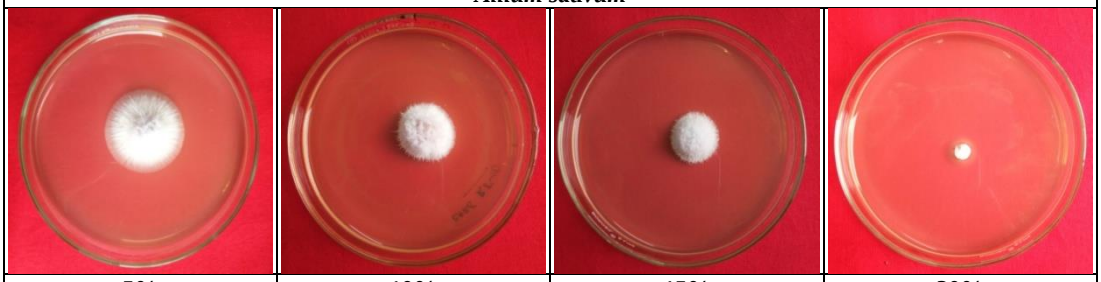
SI. No.	Treatments	Mycelial growth inhibition over control (%)				Mean (mm)
		5	10	15	20	
1	*Allamanda cathertica*	59.78 (50.64)*	67.11 (53.83)	73.33 (58.90)	78.22 (62.24)	69.61 (56.40)
2	*Allium sativum*	65.78 (54.20)	74.44 (59.64)	84.44 (66.96)	92.22 (73.77)	92.22 (73.77)
3	*Laurus nobilis*	48.89 (44.36)	58 (49.60)	63.33 (52.73)	72.22 (58.20)	60.61 (51.22)
	Carbendazim (0.1%)	100.00	100.00	100.00	100.00	
	Control	---	---	--	--	
		Botanicals (B)		Concentrations (C)		Interaction (B X C)
	SEd (±)	0.34		0.89		0.67
	CD (p=0.05)	0.84		0.97		1.67
	CV (%)	--		--		2.30

* Data in parentheses represents angular transformation



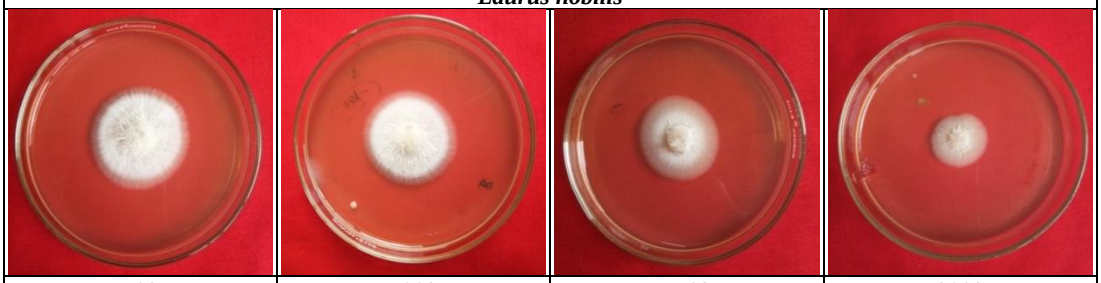
5% **10%** **15%** **20%**

Allium sativum



5% **10%** **15%** **20%**

Laurus nobilis



5% **10%** **15%** **20%**

Fig 2: Efficacy of botanicals (5, 10, 15 and 20%) on mycelial growth of *F. oxysporum* (after 9 days of inoculation)

Conclusion

The present study showed the association of *F. oxysporum* with storage rot of ginger based on morphogenetic characterization. Eco-friendly management approach like use of botanicals more particularly *A. sativum*, *A. cathertica* and *L. nobilis* may be used as an integral part of integrated disease management and it also has prospect as an alternative to reliance only on synthetic fungicide.

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