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Inhibitory effect of botanicals on growth and sporulation of *Fusarium oxysporum* inciting wilt of Chilli (*Capsicum annuum* L.)

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Abstract
The antifungal activity of twelve botanicals including commercial formulations of neem and garlic at 1, 2, 5 and 10% concentrations was tested against *Fusarium oxysporum* (*i.e.*, Isolate Fo8) under *in vitro* conditions. The botanicals revealed marked reduction in mycelial growth and sporulation of the *F. oxysporum* isolate. Growth inhibition of *F. oxysporum* increased linearly with an increase in concentration of the botanicals. Among the botanicals, neem oil formulation (Nemazal) and garlic oil exhibited significant effect on the test fungus. The neem oil (Nemazal) and garlic oil at 10 per cent concentration completely inhibited the mycelial growth that was followed by mustard oil (69.26%), *Datura* (46.67%), *Withania somnifera* (34.44%), whereas, the effectiveness of rest of the leaf extracts *viz*., *Chrysanthemum* (30.37%), *Duranta erecta* (28.15%), *Bougainvillea* (26.30%), *Clerodendron enerme* (24.44%), *Parthenium* (20.37%), *Cannabis sativa* (18.52%) and *Eucalyptus* (16.30%) thereby indicating less effectiveness. Garlic oil was highly effective (100%) even at 5 per cent concentration, whereas, neem oil was comparatively less effective (59.63%). Similarly, complete inhibition in sporulation was observed by the use of neem oil and garlic oil at 10 per cent that were statistically at par to mustard oil (93.75%), *Datura* (90.94%), *Withania somnifera* (87.50%), whereas, the effectiveness of rest of the botanicals *viz*., *Chrysanthemum*, *Duranta erecta*, *Bougainvillea*, *Clerodendron enerme*, *Parthenium*, *Cannabis sativa* and *Eucalyptus* varied between 12.82 to 70.00 per cent. The rate of sporulation of *F. oxysporum* decreased linearly with an increase in concentration and the type of botanicals.

Keywords: *capsicum annuum, fusarium oxysporum*, botanicals, growth inhibition, sporulation

Introduction
India also known as "the land of spices" is the largest producer, consumer and exporter of variety of spices in the world. Chilli (*Capsicum* spp.) is one of the most important Indian spices, which is grown worldwide and consumed as fresh or after process. India is the leading producer and consumer of chilli with production capacity of 1.49 million tonnes from 0.77 milllion hectare and the productivity is 1.92 MT/ha (Anonymous, 2014) [8]. A number of biotic and abiotic factors are a constraint in production of chilli. Among the biotic factor, Fusarium wilt has emerged as a serious problem in past decade with the disease incidence of 2-85 per cent in different regions of India (Anonymous, 2005) [7]. The yield loss due to the disease is known to vary from 10-80 per cent throughout the world (Loganathan *et al*., 2013) [33] based on the varieties selected and the prevailing climatic conditions. *Fusarium oxysporum*, *F. solani*, *F. moniliforme* and *F. pallidoroseum* have been reported as the as wilt causing agents from chilli growing areas but *F. oxysporum* and *F. solani* are the most prevalent species of *Fusarium* found associated with wilt of chilli in India (Naik, 2006) [39]. The yield losses due to the disease is known to vary from 10-80 per cent worldwide (Loganathan *et al*., 2013) [33] depending upon the variety being grown and prevailing climatic conditions. The pathogen is typically soil-borne (Booth, 1971) [15] with dry weather condition and excessive soil moisture is conducive to the disease development. The characteristic symptoms of the disease are brown vascular discoloration followed by upward and inward rolling of the upper leaves and subsequently wilting of the plant (MacHardy and Beckman, 1981; Rivelli, 1989) [34, 48]. The wilting symptoms appear as a result of severe water stress, mainly due to the vessel occlusion. The mycelial texture vary from fluffy to fibrous; buff, umber, loteous, pale loteous, ochreous and dark brown based on mycelia colour; and long, medium and short macro-conidial length. Daami-Remadi *et al.* (2006) [19] observed that the temperature range from 25 to 30°C was optimum for maximum mycelial growth and sporulation of *F. oxysporum* f.sp. *tuberose*. Among the different available options for the management, chemicals are neither economically

viable, nor safe for the environment. Plants extracts and essential oils show antifungal activity against a large number of fungal diseases (Bowers and Locke, 2000; Chandel and Deepika, 2010; Ahila Devi *et al*., 2013; Neela *et al*., 2014; Javaid and Rauf, 2015) [16, 3, 18, 28, 40]. The plant extracts provide an effective measure for Fusarium wilt disease management and it represents an alternative to reliance on fungicides. The fungitoxic properties of different plant extracts against *F. solani* have been investigated by Shivpuri *et al*. (1997) [52]. Different botanicals *viz*., neem, garlic, datura leaf extract and different plant oils are effectively used in disease management strategies due to their eco-friendly nature as well as insensitivity to the non-target organisms (Shivpuri *et al.*, 1997; Ragab *et al.*, 2012; Enespa and Dwivedi, 2014) [52, 21, 44]. Plant extracts and essential oils have also shown antisporulant activity against a large number of fungal diseases including Fusarium wilt (Katan *et al*., 1997; Rachappa *et al*., 2007; Sharma and Pandey, 2010; Jaruhar and Prasad, 2011; Dey *et al*., 2013; Amadi *et al*., 2014; Bhushan, 2014; Hossain *et al*., 2015) [29, 43, 27, 5, 50, 26, 20]. Evaluation of different botanicals for inhibition of growth and sporulation of the test fungus as an alternate to Fusarium wilt disease management of chilli that may form an integral part of integrated management is therefore undertaken.

Materials and Methods

Plant based pesticides *i.e*., botanicals which are relatively economical, safe and non-hazardous have been used against the wilt causing pathogenic fungi *Fusarium oxysporum*. A total of twelve plant extracts/ botanicals (Table 1) were selected to know their efficacy against mycelial growth and sporulation of *F. oxysporum* isolate Fo8 (virulent one). These extracts/botanicals were tested by using poisoned food technique (Nene and Thapliyal, 1973) [41] at 1, 2, 5 and 10% concentrations, respectively.

Preparation of plant extracts: Fresh plant material were collected and washed first in tap water and then in distilled water. Hundred grams of fresh sample was chopped and then crushed in a surface sterilized mixer & grinder by adding 100 ml sterile water (1:1 w/v). The extract was filtered through Whatman filter paper No.1, thereafter centrifuged at 8000-10000 rpm and filtered through bacteria-proof MF-Millipore membrane filters (thickness, 0.22 µm) under aseptic conditions. The filtrate was used as stock solution. One, two, five and ten ml of stock solution was mixed with 99, 98, 95 and 90 ml of sterilized molten Czapek's Dox agar (CZA) medium respectively, so as to get 1, 2, 5 and 10 per cent concentrations, respectively. The medium was thoroughly shaken for uniform mixing of the extracts. Twenty ml of medium was poured into sterile Petri plates (90mm). The mycelial discs of five mm size from actively growing (4 days old) culture of the *F. oxysporum* isolate (as virulent one) were cut by using sterile cork-borer and one such disc was placed in inverted position at the centre of each Czapek's Dox agar (CZA) plate and incubated at 27±1°C for seven days in BOD incubator with alternate light and dark for 12 hrs.

The per cent inhibition of the radial growth of the pathogen over control was calculated by using the formula given by Vincent (1947), as depicted below.

$$I = \frac{C - T}{C} X\ 100$$

Where,

I = Per cent inhibition; C = Growth in control; T = Growth in treatment

The conidial density and sporulation pattern of the isolate Fo8 with different concentrations of botanicals was studied on the incubated CZA plates. Ten ml of sterile distilled water was added to culture plate and using a sterile glass slide, the culture surface was gently scrapped to make a conidial suspension. The number of conidia were counted using Neubauer haemocytometer. Conidia produced per unit surface area and spore density (= Number of conidia/ml suspension) were estimated using the formula given below.

$$\text{Conidia produced per unit surface area (mm}^2\text{)} = \frac{\text{Number of conidia ml}^{-1}\text{ suspension x Total surface area from which conidial suspension was derived}}{\text{Volume of water to make suspension}}$$

Spore density (spores/ml) = (n) x 25 x 10^4

Where, n = the average cell count per small square in a central large square

Three replications were maintained for each concentration of the treatments in a completely randomized design. The data was analyzed by OPSTAT package of programs (Sheoran, 2006) [51] after arcsine transformation.

Results

The antifungal activity of twelve botanicals including commercial formulations of neem and garlic at 1, 2, 5 and 10% concentrations was evaluated against *F. oxysporum* (*i.e*., Isolate Fo8) under *in vitro* conditions by poisoned food technique. After seven days of incubation the fungal isolate exhibited growth inhibition and reduction in sporulation in a dose dependent manner (Table 1, Figure 1). The statistical analysis showed that botanicals at different concentrations significantly affected ($P \geq 0.05$) radial growth and sporulation of the fungus. The growth inhibition of *F. oxysporum* increased linearly with an increase in concentration of the botanicals. Perusal of the data revealed that the botanicals *viz*., neem oil formulation (Nemazal) and garlic oil had significant effect on the *F. oxysporum* isolate. The neem oil (Nemazal) and garlic oil at 10 per cent concentration completely inhibited the mycelial growth that was followed by mustard oil (69.26%), *Datura* (46.67%), *Withania somnifera* (34.44%), whereas, the effectiveness of rest of the leaf extracts *viz*., *Chrysanthemum* (30.37%), *Duranta erecta* (28.15%), *Bougainvillea* (26.30%), *Clerodendron enerme* (24.44%), *Parthenium* (20.37%), *Cannabis sativa* (18.52%) and *Eucalyptus* (16.30%) thereby indicating less effectiveness. The treatments with 1 and 2 per cent aqueous emulsions of the botanical extracts did not cause any significant inhibition in the radial growth of *F. oxysporum* as compared to control, whereas, 5 and 10 per cent aqueous emulsions resulted in significant inhibition of radial growth (Tables 1). The botanicals evaluated at 10 per cent concentration were significantly superior to 5 per cent concentration, however, garlic oil was highly effective (100%) even at 5 per cent concentration, whereas, neem oil was comparatively less effective (59.63%). The inhibition levels both with garlic oil and neem oil (100% each) were statistically at par and were superior to rest of the treatments in inhibition of mycelial growth, whereas, *Eucalyptus* leaf extract was least effective (16.30%) against the test pathogen (Table 1, Figure 1).

The sporulation of the test fungus (*i.e*., Isolate Fo8) varied greatly with different botanicals used at different concentrations. The sporulation at 10 per cent concentration decreased linearly with an increase in concentrations and the type of botanicals. Complete inhibition in sporulation was found by the use of neem oil and garlic oil that were

statistically at par to mustard oil (93.75%), *Datura* (90.94%), *Withania somnifera* (87.50%), whereas, the effectiveness of rest of the botanicals *viz*., *Chrysanthemum* (70.00%), *Duranta erecta* (58.76%), *Bougainvillea* (54.07%), *Clerodendron enerme* (42.82%), *Parthenium* (29.70%), *Cannabis sativa* (20.63%) and *Eucalyptus* (12.82%) varied between 12.82 to 70.00 per cent (Table 1, Figure 2). However, the minimum reduction in sporulation was recorded in *Eucalyptus* leaf exract (12.82%), which was significantly lower than rest of the treatments.

Table 1: *In vitro* evaluation of botanicals against mycelial growth and sporulation of *F. oxysporum*

Botanicals	Percent inhibition of mycelial growth at different concentrations					Mean sporulation† [spores/ml (x 10^4)]	Reduction in Sporulation (%)
	1%	2%	5%	10%	Mean		
Neem oil (*Azadirachta indica*)	8.15(16.57)*	19.63(26.29)	59.63(50.53)	100.00(89.39)	46.85(45.70)^{b}	0.0^{a}	100.00
Garlic oil (*Allium sativum*)	6.30(14.44)	11.80(20.00)	100.00(89.39)	100.00(89.39)	54.53(53.31)^{a}	0.0^{a}	100.00
Mustard oil (*Brassica juncea*)	11.85(20.04)	15.19(22.89)	31.48(34.11)	69.26(56.31)	31.95(33.34)^{c}	16.7^{b}	93.75
Parthenium (*Parthenium hysterophorus*)	13.70(21.69)	14.44(22.32)	17.41(24.64)	20.37(26.81)	16.48(23.87)^{f}	187.5^{i}	29.70
Eucalyptus (*Eucalyptus grandis*)	8.15(16.57)	11.85(20.10)	14.07(22.01)	16.30(23.78)	12.59(20.62)^{g}	232.5^{k}	12.82
Bhang (*Cannabis sativa*)	13.33(21.39)	12.96(21.05)	14.44(22.32)	18.52(25.46)	14.81(22.56)^{f}	211.7^{j}	20.63
Clerodendron (*Clerodendron enerme*)	14.07(22.00)	16.30(23.78)	18.15(25.19)	24.44(29.62)	18.24(25.15)^{e}	152.5^{h}	42.82
Duranta (*Duranta erecta*)	16.67(24.08)	17.04(24.36)	21.48(27.59)	28.15(32.02)	20.84(27.01)^{e}	110.0^{f}	58.76
Bougainvillea (*B. spectabilis*)	14.81(22.59)*	16.67(24.08)	19.63(26.28)	26.30(30.83)	19.35(25.95)^{e}	122.5^{g}	54.07
Ashwagandha (*Withania somnifera*)	17.41(24.65)	18.89(25.75)	25.56(30.35)	34.44(35.92)	24.08(29.17)^{d}	33.3^{d}	87.50
Datura (*Datura stramonium*)	18.52(25.46)	21.48(27.59)	30.74(33.66)	46.67(43.07)	29.35(32.45)^{c}	24.2^{c}	90.94
Chrysanthemum (*C. morifolium*)	17.04(24.35)	17.78(24.92)	23.70(29.11)	30.37(33.42)	22.22(27.95)^{d}	80.0^{e}	70.00
Control	---	---	---	---	---	266.7^{l}	---
Mean	13.33(21.15)	16.17(23.59)	31.36(34.60)	42.90(43.00)			
	Botanicals (B)	Concentration (C)			Interaction (B x C)		
SEm ±	0.34	0.20			0.68	1.18	---
CD (p = 0.05)	(0.96)	(0.55)			(1.92)	3.45	---
CV (%)	---	---			3.89	1.85	---

*Figures in parentheses indicate arcsine transformed values; Values indicated by similar letters are statistically not different; †Sporulation at 10% concentration; All values represent means of three replicates.
Note: 0.01 value is added to zero per cent of values, while 0.01 value is reduced from hundred per cent of values for each observation for the statistical analysis.

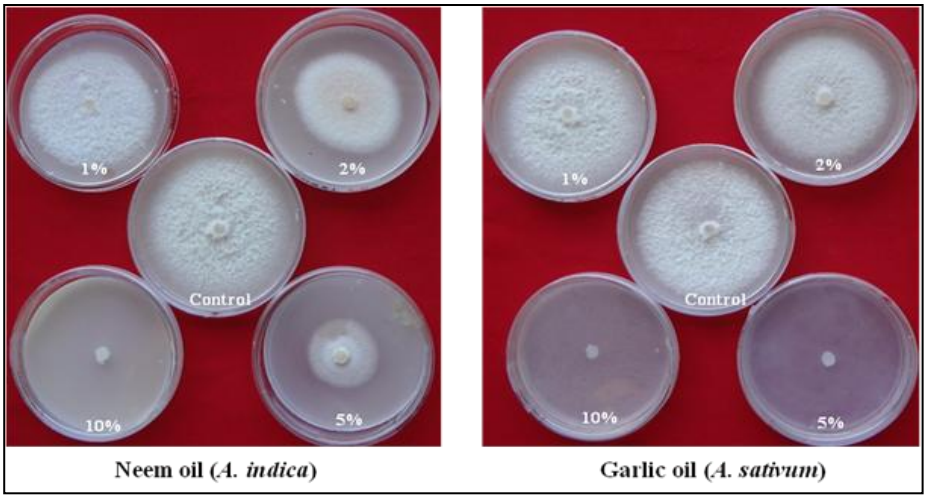

Fig 1: Mycelial growth inhibition (%) of *F. oxysporum* (Isolate Fo8) with different botanicals at different concentrations.

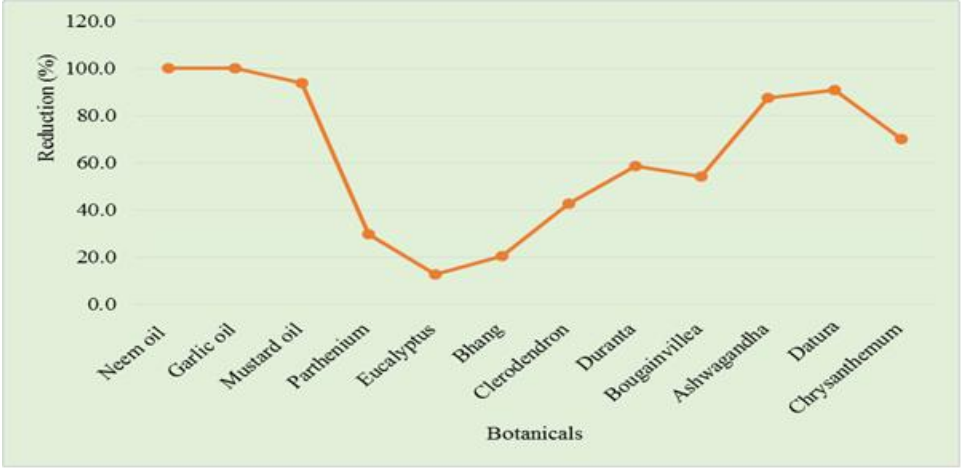

Fig 2: Reduction (%) in sporulation of *F. oxysporum* with different botanicals at 10 per cent concentration

Discussions

In the present investigation, twelve botanicals/plant extracts with four concentrations *viz.*, 1, 2, 5 and 10 per cent were evaluated under *in vitro* condition against *F. oxysporum* to know their fungitoxic nature. The aqueous emulsions of the extracts at 5 and 10 per cent resulted in significant inhibition in radial growth. Although, garlic oil was also effective (100%) at 5 per cent concentration, while neem oil was comparatively less effective (59.63%). Among the botanicals, garlic oil or neem oil (10%) significantly reduced the mycelial growth and sporulation up to 100 per cent, whereas, the least growth (16.30%) and reduction in sporulation (12.82%) was observed with *Eucalyptus* extract. These results are in agreement with Sahayaraj *et al.* (2006) [49] who reported the anti-fungal activity of *Allium sativum*, which varied from 80-95 per cent. Similar results were reported by Mishra & Dixit (1976) [37], Arya *et al.* (1995) [10] and Bowers & Locke (2000) [16] who reported that the clove extract of *Allium sativum* was 85-100 per cent effective against eighteen different fungi including *Fusarium* spp. The present findings are also in agreement with those of several other workers (Shivpuri *et al.*, 1997; Bansal and Gupta, 2000; Abdulrahman and Alkahail, 2005; Kishore, 2007; Taskeen-Un-Nisa *et al*., 2011; Shukla and Dwivedi, 2012; Gopi and Thangavelu, 2014; Mamun *et al*., 2016) [52, 11, 22, 35, 56, 31]. The inhibitory effect of the plant extracts might be attributed to the presence of antifungal/antimicrobial compounds. The differences might be due to the difference in nature, quality and quantity of the inhibitory substances present in the botanicals (Bashar and Chakma, 2014) [13]. Many investigations have shown that garlic (*Allium sativum* L.) contains a sulphur-containing antibiotic, toxic to plant pathogen and its effect on many diseases has already been reported by several other workers (Ark and Thompson, 1959; Anonymous, 1987; Wilson *et al*., 1997; Perello *et al*., 2013) [8, 9, 42, 58]. The inhibitory action of garlic extract on fungal growth has been attributed to the presence of allicin as the major antifungal component (Cavallito and Balley, 1944; Muhsin *et al*., 2000) [17, 38]. Allicin (diallyl thiosulfinate), the main thiosulphinate from garlic, is a volatile phyto-anticipin that has been shown to be responsible for the anti-microbial effects of garlic. Moreover, it was shown that the cyto-morphological modifications or changes, particularly the accumulation of lipid bodies and thickening of cell wall have been induced by garlic extracts (Hippe, 1991; Alberto *et al*., 1997; Khan and Zhihui, 2010) [24, 4, 30]. The garlic extracts induce the disruption in fungal cell metabolism, increased permeability of fungal plasma membrane and destruction of the conidial wall structure (Baron and Tansey, 1977; Tariq and MaGee, 1990; Horev-Azaria *et al*., 2009) [12, 25, 55].

The leaf extracts of neem (*Azadirachta indica*) have been reported as highly toxic to *Fusarium oxysporum* showing complete inhibition of mycelial growth and spore germination (Shivpuri *et al*., 1997; Rai *et al*., 2002; Hassanein *et al*., 2008; Yelmame *et al*., 2010; Enespa and Dwivedi, 2014; Abd-El-Ghany *et al*., 2015; Ramaiah and Raj Kumar, 2015) [52, 45, 46, 59, 23, 21, 1]. The fungicidal spectrum/bioactivity of *Azadirachta indica* has been attributed to various compounds such as nimbin, nimbidin and salannin and the most important antifungal compound was azadirachtin, which belongs to C25 terpenoides (Subramaniam, 1993; Lale and Abdulrahman, 1999; Ramaprasad Shresti, 2005) [54, 32, 2]. Spore yield among fungicides treatments depend upon its inhibitory action on colony growth. The conidial count was less in *Azadirachta indica* and *Allium sativum* oils treated plate, as its inhibitory action was strong and it did not allow fungus to grow and sporulate. The complete inhibition in sporulation was found with neem oil and garlic oil, however, the minimum percent inhibition in sporulation was recorded in *Eucalyptus* extract (12.82%). The activity of plants extracts and essential oils as anti-sporulant agent have been revealed against a large no of fungal diseases as reported by several other workers (Katan *et al*., 1997; Sharma and Pandey, 2010; Taskeen-Un-Nisa *et al*., 2011; Mamzaa *et al*., 2012; Dey *et al*., 2013; Amadi *et al*., 2014; Hossain *et al*., 2015) [29, 50, 5, 56, 20].

Conclusions

Plant based pesticides *i.e*., botanicals being relatively economical, safe and non-hazardous show antifungal activity against a large no of fungal diseases (Bowers and Locke, 2000; Chandel and Deepika, 2010; Javaid and Rauf, 2015) [16, 18, 28]. These effective botanicals/ plant extracts may provide an effective measure for management of Fusarium wilt of chilli that may form an integral part of integrated management and it also has prospect as an alternative to reliance only on fungicides.

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