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In-vitro evaluation of Phyto-extracts and bio-agent against *Aspergillus niger*

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Abstract
Aqueous phyto extracts (leaves and clove) of 7 botanicals were evaluated *in vitro* (each @ 10%) against *Aspergillus niger* and the results obtained on its mycelial growth and inhibition were significantly effective with mycelial growth inhibition were recorded with Garlic and Nilgiri / Eucalyptus (100%). This was followed by the Phyto-extracts *viz.*, Tulsi (92.74%), Neem (76.10%) and Onion bulb (52.05%).While least mycelial inhibition was recorded with *viz.*, Ginger (91.70%) and Turmeric (50.72%) were found comparatively less effective. Seven bio-control agents *Trichoderma hamatum, T. lignorum, T. koningi, T. harzianum, T. viride, Pseudomonas fluorescence and Gliocladium virens* were evaluated *in vitro* for their efficacy against *Aspergillus niger* by applying dual culture technique (Arora and Upadhay, 1978) and using PDA as basal medium. The results obtained on colony diameter and percent inhibition of *Aspergillus niger* with seven fungal antagonists. Amongst the eight fungal antagonists tested, *Trichoderma harzianum* was found the most effective and recorded significantly least linear mycelial growth (0.57mm) with highest percent mycelial inhibition (99.93%) of the test pathogen. The second best antagonist found was *Trichoderma viride*, which recorded mycelial growth 1.17mm and percent mycelial inhibition 98.75%.

Keywords: *Aspergillus niger*, Phyto-extracts, bioagents, mycelial growth and inhibition

Introduction
Onion (*Allium cepa* L.) is one of the most important commercial vegetable crop grown in India. It belongs to the family Liliaceae. As a food stuff they are usually cooked or used as a vegetable, but can also be eaten raw or used to make pickles or chutneys (Anonymous, 2013a). In world India rank second after China with total production of 19.401 million tonnes (2014). China rank first with 22.3 million tonnes production of onion. According to UN data, the total harvested area is 1,025,000 Ha. In India onion occupies an area of 1.2704 million hectare with production of 21.56 million tonnes and productivity is 17.0 (MT/ha) (Anonymous Database 2017). The major onion producing states of India are Maharashtra, Karnataka, Gujrat, Bihar and Madhya Pradesh. Among these Maharashtra is a largest onion producing state with contribution of 28% followed by Karnataka (17%) and Madhya Pradesh (15%). In Maharashtra area under onion is 15.25 million hectare, production 270.38 metric tonnes and productivity of 14.36 metric tonnes/ha (Anonymous Database 2017). The major onion producing districts of Maharashtra are Nashik, Pune, Satara, Jalgaon, Dhule, Ahmednagar, Nagpur, Solapur, Aurangabad and Jalna.

Several field and storage diseases affecting productivity of onion have been reported, among which black mould rot of onion is an important post-harvest disease. Onion black mould disease is the most destructive disease of storages and in the field (Wani *et al*., 2011). Rajam (1992) reported that among the post-harvest diseases of onion, black mould rot caused by *Aspergillus niger* was the predominant one. Qadri *et al*. (1982) [9] revealed that the spoilage caused by *A. niger* was as high as 80 percent. *Aspergillus niger*, a soil saprophyte being ubiquitous in occurrence attacks onion by producing various enzymes and toxins and establishes itself in bulb and other tissues.

Materials and Methods
1. *In vitro* evaluation of plant extract
Preparation of Phytoextracts
Fresh healthy plant parts (leaves /cloves /bulbs /rhizomes) collected from fields were washed with distilled water and air-dried and 100 g crushed in 100 ml of distilled water (Garlic and

Onion crushed in 100 ml of distilled water by w/v method). The extract was filtered through double layered, muslin cloth and further filtrated through Whatsman No.1 filter paper using funnel and volumetric flasks (100 ml cap.). The extract obtained formed 100 percent concentration of 10 percent. An appropriate quantity of each plant extract (10%) was separately mixed thoroughly with autoclaved and cooled (40 ºC) PDA medium in conical flasks (250 ml cap.) to obtained desired concentrations (10 percent).

The PDA medium amended separately with plant extracts was then poured (20 ml / plate) into sterile glass petri plates (90 mm dia.) and allowed to solidify at room temperature. For each test botanical extract and their respective concentrations, three plates / treatment / replication were maintained. Each plant extract and its respective concentration were replicate thrice. Upon solidification of PDA, all the treatment and control plates were aseptically inoculated by placing in the centre a 5 mm mycelial disc obtained from a week old actively growing pure culture of *A. niger*. Plates containing plain PDA without any botanical extract and inoculated with mycelial disc of the test fungus served as untreated control. All these plates were then incubated at 27 ± 1^0C temperature for a week or till the untreated control plates were fully covered with mycelial growth of the test fungus.

Details of experiment
Design: CRD
Replication: Three
Treatments: Eight

Table 1: Details of botanicals

Sr. No.	Botanical name	Common Name	Family	Plant Part Used	Conc. (%) (w/v)
1.	*Zingiber officinale*	Ginger	Zingiberaceae	Rhizome	10%
2.	*Curcuma longa*	Turmeric	Zingiberaceae	Rhizome	10%
3.	*Azadirachta indica*	Neem	Meliaceae	Leaves	10%
4.	*Allium sativum L.*	Garlic	Liliaceae	Clove	10%
5.	*Eucalyptus globules*	Nilgiri	Myrtaceae	Oil	10%
6.	*Allium cepa*	Onion	Liliaceae	Bulb	10%
7.	*Ocimum sanctum*	Tulsi	Lamiaceae	Leaves	10%
8.	Control	-	-	-	-

All these plants extract were evaluated @10% observation on radial mycelial growth of the test pathogen was recorded at 24 hrs. interval and continued till growth of test pathogen in untreated control plate is fully covered. Percent inhibition of test pathogen was also calculated by applying the formula given by (Vincent 1927) [16].

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

Where
C= growth of the test fungus in untreated control plates (mm).
T= growth of the test pathogen in treated plates (mm).

2. *In-vitro* evaluation of bio-agents against *A. niger*
Seven bio-control agents *viz., Trichoderma hamatum, T. lignorum, T. koningii, T. harzianum, T. viride, Pseudomonas fluorescence, Gliocladium virens* known to antagonists were evaluated *in-vitro* against *Aspergillus niger* by applying dual culture technique (Arora and Upadhay, 1978) [1] on PDA medium in Petri plates. Seven days old cultures of test bio-agents and test fungus (*A. niger*) grown on (PDA) were used for the study. Discs (5 mm dia.) of PDA along with culture growth of the test fungus and bio-agents were cut out with sterilized cork borer. Then two culture discs, one each of the test fungus and bio-agent were placed at equidistance and exactly opposite with each other on solidified PDA medium in Petri plates aseptically and plates were incubated at 27 ± 1^0 C. PDA plates inoculated only with culture disc of the test fungus was maintained as untreated control.

Details of Experiment
Design: CRD
Replications: Three
Treatments: Eight

Fungal antagonists
Trichoderma hamatum, T. lignorum, T. koningii, T. harzianum, T. viride, Pseudomonas fluorescence, Gliocladium virens and Control (Monoculture of test fungus without bioagent) used for evaluation of their effects against *A. niger*.
Observations were made on radial growth of test pathogen and antagonism when fungus in control plate reached to rim of plate. The percent growth inhibition of test pathogen was calculated by following formula.
The Percent Inhibition of test pathogen will be calculated by applying formula given by Arora and Upadhay (1978) [1].

$$\text{Percent growth inhibition} = \frac{C - T}{C} \times 100$$

Where
C= Growth of the test fungus in (mm) untreated control plates.
T= Growth of the test fungus in (mm) treated plates.

Results and Discussion
3. *In vitro* evaluation of plant extract/botanicals
Aqueous extracts (leaf and clove) of 7 botanicals were evaluated *in vitro* (each @ 10%) against *Aspergillus niger* and the results obtained on its mycelial growth and inhibition are presented in Table 2, Fig. 1. Results revealed that all the 10 botanicals extracts evaluated were fungistatic / antifungal to *Aspergillus niger*, which significantly reduced mycelial growth and increased its inhibition over untreated control. The mycelial growth was found to be decreased and its inhibition was increased with increase in concentrations of the botanicals tested.

Radial mycelial growth
At 10 percent concentration of different botanicals, radial mycelia *Aspergillus niger* was recorded from 00 mm (Garlic and Nilgiri) and to 44.35 mm (Turmeric), as against 90 mm in

untreated control. However, significantly least mycelial growth was recorded by Garlic and Nilgiri (00). This was followed by the botanicals *viz*., Tulsi (06.53 mm), Ginger (07.47 mm), Neem (21.51 mm) Onion bulb (43.15 mm) comparatively highest mycelial growth was recorded with the phyto-extracts *viz.*,Onion bulb (43.15 mm) and Turmeric (44.35 mm).

Mycelial inhibition

Results obtained on mycelial growth inhibition of *Aspergillus niger* with the aqueous extracts of the test botanicals tested are presented in the Table 1 and depicted in the Fig. 1 and. Results (Table 2) indicated that all the botanicals tested (@ 10% each) significantly inhibited mycelial growth of the test pathogen, over untreated control and it was increased with increase in concentrations of the botanicals tested.

At 10 percent, mycelial growth inhibition of *Aspergillus niger* was recorded from 50.72 (Turmeric) to 00.00 (Garlic and Turmeric) percent. However, significantly maximum mycelial growth inhibition was recorded with Garlic and Nilgiri (100%). This was followed by the botanicals *viz.*, Tulsi (92.74%), Neem (76.10%) and Onion bulb (52.05%).While least mycelial inhibition was recorded with *viz.*, Ginger (91.70%) and Turmeric (50.72%) were found comparatively less effective.

The results of present investigation resembling the findings of earlier workers *viz.*, Wani and Kurucheve (2004) [8] reported garlic bulbs extract inhibited 100% mycelium growth of *A. niger* and *A. flavus in vitro.* Mohamed *et al.* (2012) and Sheth *et al.* (2010) observed that garlic cloves, onion bulb and neem leaf extracts have the ability to cause reduction in the mycelial growth of *A. niger*.

Table 2: *In vitro* efficacy of plant extracts against *Aspergillus niger*

Treatment	Colony Dia.* (mm) at 10% Conc.	% inhibition at 10% Conc.
Ginger (*Zingiber officinale*)	07.47	91.70 (73.22)
Turmeric (*Curcuma longa*)	44.35	50.72 (45.38)
Neem (*Azadirachta indica*)	21.51	76.10 (60.70)
Garlic (*Allium sativum*)	00.00	100.00 (90)
Nilgiri *(Eucalyptus globules)*	00.00	100.00 (90.00)
Onion (*Allium cepa*)	43.15	52.05 (46.15)
Tulsi *(Ocimum sanctum)*	06.53	92.74 (74.33)
Control	90.00	00.00 (0.00)
SE±	0.207	0.231
C.D. @ 0.01	0.627	0.698

*: Average of three replications.

Figures in parenthesis are arcsine transformation values

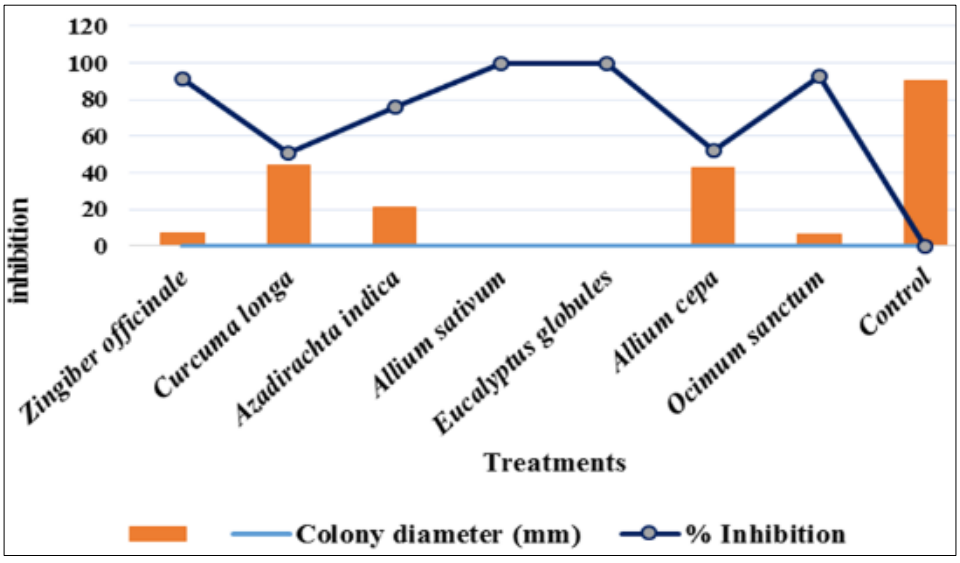

Fig 1: *In-vitro* efficacy of different Phyto-extracts against *Aspergillus niger*.

4. *In-vitro* Evaluation of bio-control agents against *Aspergillus niger*

Seven bio-control agents *Trichoderma hamatum, T. lignorum, T. koningi, T. harzianum, T. viride, Pseudomonas fluorescence and Gliocladium virens* were evaluated *in vitro* for their efficacy against *Aspergillus niger* by applying dual culture technique (Arora and Upadhay, 1978) [1] and using PDA as basal medium.

The results obtained on colony diameter and percent inhibition of *Aspergillus niger* with eight fungal antagonists are presented in Table 3 and Fig.2 revealed that all the bio-agents evaluated exhibited fungistatic / antifungal activity against *Aspergillus niger* and significantly inhibited its growth over untreated control.

Amongst the eight fungal antagonists tested, *Trichoderma harzianum* was found most effective and recorded significantly least linear mycelial growth (0.057 mm) with highest percent mycelial inhibition (99.93%) of the test pathogen. The second best antagonist found was *Trichoderma viride*, which recorded mycelial growth of (1.117 mm) and inhibition of (98.75%), which was statistically at par with treatment *Trichoderma harzianum*. The next best treatment was *Trichoderma koningii*, which recorded mycelial growth of (10.40 mm) with percent inhibition of (88.43%). This was

followed by *Trichoderma hamatum* having colony diameter (20.03 mm) and percent inhibition of (77.74%), *Trichoderma lignorum* having colony diameter (24.72 mm) and percent inhibition (72.52%).The fungal antagonist, *Pseudomonas fluorescence* (T_6) was found less effective, which recorded (44.46) mm linear mycelial growth and (50.59%) mycelial inhibition. The antagonist, *Gliocladium virens* was also found least fungistatic and recorded (29.91mm) colony diameter and (66.76%) percent growth mycelial inhibition respectively.

Table 3: *In vitro* efficacy of bio-agents against *Aspergillus niger*

Treatments	Colony Dia.* of bioagent (mm)	Colony Dia.* (mm) of the test pathogen	% Inhibition of the test pathogen
Trichoderma hamatum	69.97	20.03	77.74 (61.81)
Trichoderma lignorum	65.28	24.72	72.53 (58.36)
Trichoderma koningii	79.60	10.40	88.44 (70.09)
Trichoderma harzianum	89.94	0.57	99.93 (88.48)
Trichoderma viride	88.88	1.17	98.75 (83.56)
Pseudomonas fluorescence	45.54	44.46	50.60 (45.32)
Gliocladium virens	60.09	29.91	66.76 (54.77)
Control	00.00	90.00	00.00 (00.00)
SE ±	0.257	0.226	0.250
CD @ 0.01	0.778	0.683	0.757

*: Average of three replications.

Figures in parenthesis are arcsine transformation values

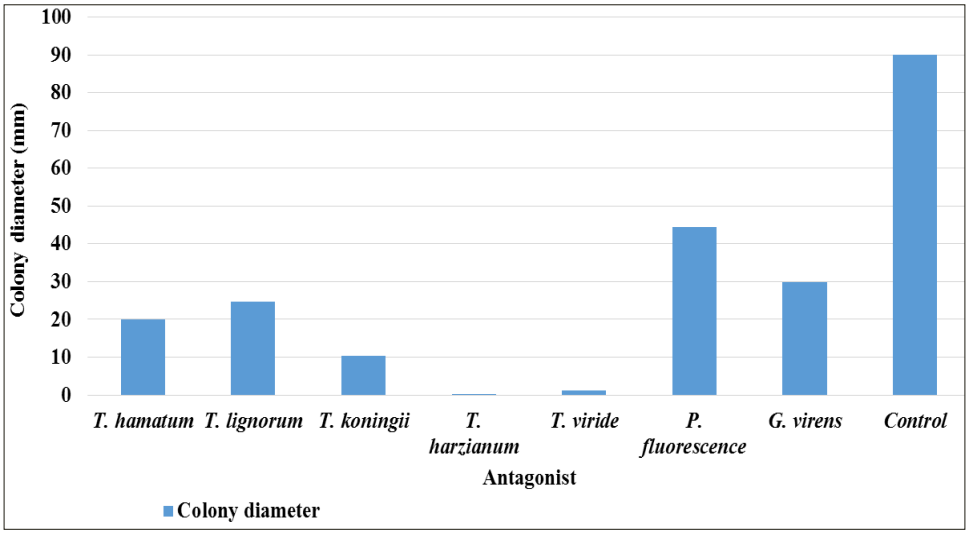

Fig 2: *In-vitro* efficacy of different bio-agents on growth and inhibition of *Aspergillus niger.*

The results of present investigation resembling the findings of earlier workers *viz.*, Rao and Sitaramaiah (2000) and Prabhu and Urs (1998) also documented that *Trichoderma* isolates significantly inhibited the growth of *A. niger*. Kishore *et al.*, (2001), whoreported that the *T. viride* and *T. harzianum* were found to be effective in reducing the radial growth of *A. niger in vitro*. Shalini *et al.*, (2006) [14]. *Trichoderma harzianum* is an efficient bio-controlling agent commercially produced to thwart the development of several soil born pathogenic fungi.

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