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Isolation and Identification of fungi from post-harvest stored onion varieties and their control measures with organic formulations

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ABSTRACT: ***The research work entitled "Studies on preventive measures to reduce the postharvest losses in onion (Allium cepa L.)" was carried out at NIFTEM, Sonepat, Haryana, India during Rabi seasons of 2014-15 and 2015-16. The research work was carried out with objectives of studying the effect of application of organic formulations (applied as pre-harvest foliar spray viz., Neem based formulation (Besara) at the rate of 20ml/L; Panchgavya at the rate of 100ml/L; Trichoderma viride (Bio Shield) at the rate of 50g/L) on growth period of selected onion varieties (Pusa Madhavi, Pusa Riddhi, Pusa Red, NHRDF Red and Agri found Light Red) grown during Rabi season in Haryana region. The formulations were applied as four pre-harvest foliar sprays starting from 30 days after transplanting and repeated at an interval of 15 days. The main aim of this experiment was to isolate and identify the pathogenic fungi causing spoilage of stored onion bulbs under cold and ambient conditions. Additionally, the efficacy of organic formulations against the isolated pathogenic fungi was studied under both the onion storage conditions. Fungal isolates responsible for disease and rotting as identified under cold storage condition were Penicillum purpurogenum, Penicillum griseofalvum, Penicillum citrinum and as Aspergillus niger under ambient storge contion. Under in-vitro condition, Neem based formulation (Besaara) was most effective for inhibition of fungal growth. The efficacy of organic formulations in restricting the fungal growth was higher with higher concentration (i.e. at 20%).***

Keywords: Onion, Fungi, Storage Condition, Organic Formulation, Inhibition

1. INTRODUCTION

Onion suffers from many diseases from pre harvest to post harvest period. After harvesting, inappropriate storage conditions leads to higher losses where in poor aeration, relative humidity plays important role. Although onion possess a good nutritive value, but a high-quality check over its nutritive consistency relies mostly on the adequate storage conditions. Once these crops are harvested they are threatened by the post-harvest diseases mostly caused by bacterial and fungal pathogens that may serve as a vehicle for disease propagation which in turn reduces their shelf life (Kumar and Neeraj, 2015).

A survey conducted at the international level revealed that about 35-40 % onion is lost due to damage caused by different diseases (pre and postharvest). The extensive losses in onion bulbs during marketing due to post harvest diseases have also been reported by Fatima et al. (2015). Among various entities responsible for post-harvest decay, many microorganisms have also been found responsible for rotting of onion bulbs and among various microorganisms, fungi are the main causal agent responsible for pre and post-harvest losses in the onion (Currah and Proctor, 1990). It is generally assumed that the disease occurs severely during storage because of fungi, which contaminates onion bulbs during harvest by moving through wounds caused by topping, finally damage the whole stored onion.

Therefore, if onion bulbs are infected in the field and carried into the warehouse, severe damage of onion bulbs may occur. The majority of associated fungi (isolates) with rotting of onion bulbs have been found pathogenic (Jacobs et al., 2008). The control of such fungal pathogen involves application of heavy doses of antifungal inorganic chemicals which poses severe health risks. As antifungal compounds from plant origin are less toxic and more environmentally friendly, these days' preferences to such organic compounds are given as compared to inorganic chemicals. Extracts from many plants have been derived and screened for their antifungal activities and valuable results have been achieved (Bansal and Gupta, 2000). An attempt was made to identify the major microbial pathogen associated with storage rot during storage period.

2. MATERIAL AND METHODS

The experiment was laid out in completely randomized design (CRD) with three replications. The main aim of this experiment was to isolate the pathogens from rotten onion and identify the pathogens responsible for rotting of bulb. There were three treatments of organic formulations for inhibition of pathogens. Each treatment was applied at two concentrations to check the efficacy of application.

Isolation and identification of fungi using microscopic and biochemical evaluation methods:

The experiments were conducted to isolate and identify the post-harvest micro-organisms responsible for spoilage of onion bulbs during storage. The samples were collected from rotten onion bulbs of five varieties namely Pusa Madhavi, Pusa Riddhi, Pusa Red, NHRDF Red, and AGLR. The samples collected were inoculated on PDA media and were allowed to proliferate followed by sub culturing to obtain pure culture. The purified fungal isolates were then identified using microscopic and biochemical evaluation. After the identification pathogenic fungi were exposed to various organic formulations (*viz.,* Neem based formulation, Panchgavya and *Trichoderma viride*) under *in-vitro* conditions to evaluate their anti-fungal activity.

The collected samples were inoculated on PDA media and were allowed to proliferate followed by sub culturing to obtain pure culture. The purified fungal isolates were then identified on the basis of morphological and cultural characteristics using microscopic and biochemical evaluation. Fungal identification and isolation was confirmed from Indian type culture collection (ITCC), Indian Agricultural Research Institute, New Delhi, India.

3. RESULTS AND DISCUSSION

Biochemical and morphological characterization of the fungal isolate which was collected from rotten onion bulbs under two different storage conditions (cold and ambient respectively) and grown on PDA media were identified as fungal pathogens. The details are presented in Table No- [A]

Table No: [A] Fungi isolated and identified from rotten onion bulbs

	Organism code	**Storage condition**	**Type of Isolates**
1.	F1	Cold	*Penicillum purpurogenum*
2.	F2	Cold	*Penicillum griseofalvum*
3.	F3	Cold	*Penicillum citrinum*
4.	F4	Ambient	*Aspergillus niger*

Fungal isolates were characterized based on their morphological appearance viz., fruiting body and mycelial characteristics.

The other reports in review of literature also indicate that the dominant fungi associated with post harvest spoilage of onion bulbs belonged to the genera *Penicillum* and *Aspergillus*.

In our study *Aspergillus niger* was found to be responsible for spoilage of onion bulbs under ambient storage conditions. The other reports in literature also indicate that the *Aspergillus* spp. are the most widely associated fungal pathogens associated with post harvest spoilage of onion stored under ambient storage conditions (Varga et al., 2011). It is also to mention that the significant percentage of the losses of vegetables during post harvest period is attributed to spoilage caused by fungi. Tournas (2005) and Prajapati and Patil (2015) have reported that *Aspergillus* spp. was the most prominent fungal pathogen involved in post-harvest spoilage of onion in Karnataka. *Aspergillus awamori* (Oh et al., 2016), *Penicillium georgiense* (Oh et al, 2015), *Penicillium brasilianum* (Sang et al, 2014) and *Aspergillus ochraceus* (Perrone et al., 2007) have also been isolated and reported to be associated with post harvest spoilage of onion and garlic under different storage conditions (cold and ambient).

In our study three species of *Penicillum viz., Penicillum purpurogenum, Penicillum griseofalvum* and *Penicillum citrinum* were identified from onion stored under cold storage. There are very limited instances of previous reports on prevalence of *Penicillum purpurogenum* and *Penicillum griseofalvum* infecting onion stored in low temperature conditions. Welke et al. (2011) also reported *Penicillum griseofalvum* to be infecting apples stored under low temperature conditions.

The genus *Penicillum* generally causes a soft watery rot on the surface of stored onion bulbs, which bear broom like conidiophores with long chains of conidia of blue green color (Onions and Brady, 1987; Duduk et al. 2017). *Penicillum digitatum* has been reported to be the most frequently isolated fungus from onion bulbs stored under cold storage conditions (Raju and Naik, 2006). *Penicillum* causes post harvest losses in fruits and vegetables during storage. The genus *Penicillium* is an important and extensively described genus with both beneficial and spoilage of vegetables (Moss, 2008).

Pathogenicity test

A pathogenicity test was carried out using the technique as described by Jabeen et al. (2012). In this technique, healthy onion bulbs were washed in sterile distilled water and surface was sterilized with 0.1 % mercury chloride solution. A sharp razor blade was used to cut onion bulb and then culture of the isolates (*Penicillum purpurogenum, Penicillum griseofalvum,*

Penicillum citrinum and *Aspergillus niger*) were inoculated into the open cut of onion bulb and replaced with the core. These were incubated for 24-72 hours in respective storage conditions i.e. cold storage for *Penicillum griseofalvum* and *Penicillum citrinum* and in ambient storage condition for *Aspergillus niger*. On establishment of disease symptoms, inoculums from the rotten onion bulb were taken and were subjected to curing. It was noticed that, the isolates of *Penicillum purpurogenum* could not be re-grown on artificially inoculated onion bulbs and were neither culturable on artificial laboratory media. Literature reports that *Penicillum purpurogenum* depending upon the strain, at times is difficult to culture. Further, in one of the recent report it is mentioned that *Trichoderma* spp. has inhibitory effect on its growth (Gwa and Abdulkadir, 2017). In our study we have applied *Trichoderma viride* in pre-harvest stage.

The outcome showed that the symptoms of rotting obtained as a result of artificial inoculation of fungal pathogens were identical to those as were observed in naturally rotten onion bulbs during respective storage conditions and by respective fungal pathogen (i.e. *Penicillum griseofalvum* and *Penicillum citrinum* in cold storage and *Aspergillus niger* under ambient storage condition). The morphological characteristics of conidia and mycelia of the fungi that were re-isolated from inoculated onion bulbs were also confirmed to be same fungus as inoculated to established infection thereby confirming the Koch's postulates (2000).The finding also revealed that all the isolated and identified fungi collected from rotten onion were pathogenic in nature.

Application of organic formulations (used during pre-harvest stage as foliar spray) as control measures on growth inhibition of identified pathogens (in- vitro conditions)

Two fungi namely *Penicillum griseofalvum* and *Penicillum citrinum* were isolated from the onions stored in cold storage (Temp- 5^0C± 2^0C, RH-80-85%) and one fungal species *Aspergillus niger* was isolated from the onions stored in ambient storage (Temp-30^0C/24^0C ±2^0C, RH-75-80%). Isolated fungi were cultured on PDA for further experiments. The isolates of *Penicillum purpurogenum* could not be re-grown on artificially inoculated onion bulbs and also not on artificial laboratory media.

The current objective was set to assess the impact of selected organic formulations on inhibiting the growth of fungi isolated and identified as pathogens (*viz., Penicillum griseofalvum* and *Penicillum citrinum* and *Aspergillus niger*) associated with post harvest spoilage during the storage of onion in cold and ambient conditions. These formulations were applied on onion crop as pre harvest sprays during growth period. The three different organic formulations used were: Neem based formulation (Besara), Panchgavya, and *Trichoderma viride* and were applied at 10% and 20% concentration. Untreated fungi served as experimental control *i.e.* without application of any organic formulation. In order to compare the extent of efficacy of organic formulation on growth inhibition of isolated fungal pathogens under *in-vitro* conditions, a synthetic fungicide (carbendazim) was used as positive control at 10% and 20% concentration. The experimental control was characterized by no application of either any organic formulation or synthetic fungicide. After the inoculation the samples were placed under respective storage conditions i.e. *Penicillium* spp. in cold storage and *Aspergillus* sp. in ambient conditions. The effects of different formulations were assessed on the radial growth (mm) of respective fungi as per the procedure given by Ansari (1995).

The impact of different concentrations (%) of Neem based formulation and carbendazim on inhibition in growth (%) of fungal pathogens:

The colony diameter in all the tested fungi was found highest in the case of experimental control which was subjected to no treatment of organic formulation (NBF) or synthetic fungicide (carbendazim). The data pertaining to this have been presented in Table No: 1

Penicillum griseofalvum exposed to 20% Neem based formulation (NBF) exhibited a reduced fungal mycelial growth with a diameter of less than 45cm. Therefore, NBF treatment inhibited the mycelial growth of *Penicillum griseofalvum* (47.05%).
Penicillum griseofalvum exposed to 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 30cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum griseofalvum* (66.65%).
Penicillum citrinum exposed to 20% NBF exhibited a lowest fungal mycelial growth with a diameter of less than 8cm. Therefore, NBF treatment inhibited the mycelial growth of *Penicillum citrinum* (85.18%).
Penicillum citrinum exposed to 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 45cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum citrinum (*35.71%).
Aspergillus niger exposed to 20% NBF exhibited a reduced fungal mycelial growth with a diameter of less than 50cm. Therefore, NBF treatment inhibited the mycelial growth of *Penicillum citrinum* (44.45%).
Aspergillus niger exposed to 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 47cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum citrinum (*47.78%).
The overall results indicate that the Neem based formulation (NBF) was effective in controlling *Aspergillus niger* as compared to carbendazim by showing 80.55% mycelia growth inhibition (mean basis) as compared to 46.11% (mean basis) by carbendazim. The similar trend was also observed in *Penicillum citrinum* when treated with NBF as compared to carbendazim.
Neem based formulation holds at least 35 biologically active ingredients of which triterpenoides, e.g. azadirachtin, nimbidine and nimbin, are the highest vigorous fungicidal and insecticidal ingredients (Mondal et al., 2009).

Similar results were also confirmed by Krishanti and Prianto (2017). They studied five types of neem seed oil formulation which showed antifungal activity against

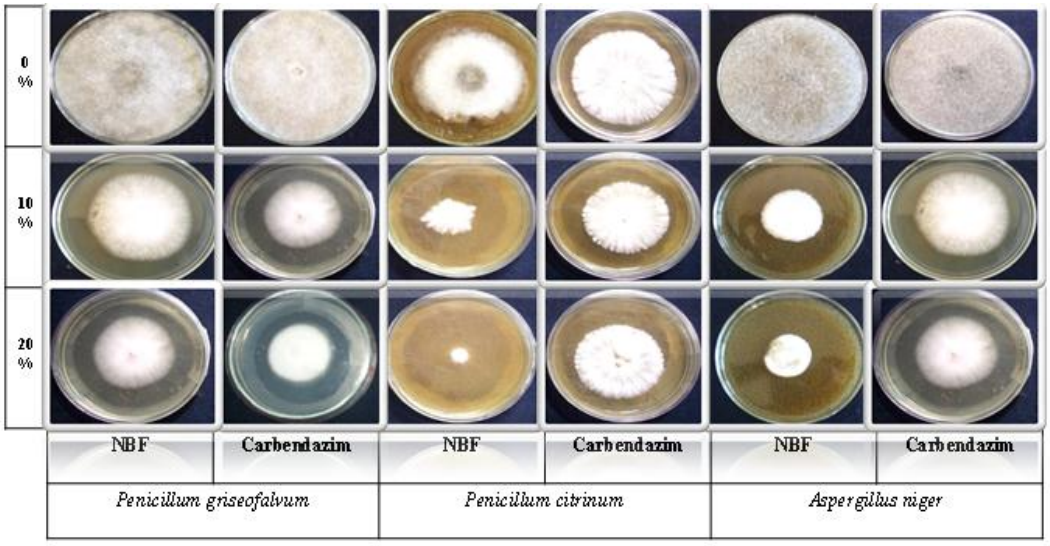


Table No: 1 Impact of different concentrations (%) of neem based formulations and carbendazim on inhibition in growth (%) of fungal pathogens

Fungi	Treatment	Colony diameter (mm)	Inhibition in growth (%)
Penici	Control	85	00.00

	NBF@10%	50	41.17
	NBF@20%	45	47.05
	Mean (NBF)	**47.5**	**44.11**
	Control	90	00.00
	Carb@10%	40	55.56
	Carb@20%	30	66.65
	Mean (Carb)	**35**	**61.10**
Penicillum citrinum	Control	54	00.00
	NBF@10%	20	62.96
	NBF@20%	8	**85.18***
	Mean (NBF)	**14**	**74.07**
	Control	70	00.00
	Carb@10%	45	35.71
	Carb@20%	46	34.28
	Mean (Carb)	**45.50**	**34.95**
Aspergillus niger	Control	90	00.00
	NBF@10%	20	77.78
	NBF@20%	15	83.33
	Mean (NBF)	**17.50**	**80.55**
	Control	90	00.00
	Carb@10%	50	44.45
	Carb@20%	47	47.78
	Mean (Carb)	**48.5**	**46.11**
NBF= Neem based formulation (Besaara); Carb= Carbendazim			

post-harvest fungi and they could prevent the growth of mycelial pathogenic fungi, *Fusarium oxysporum*, effectively. The results indicated that neem seed formulations have potential to be developed as bio-fungicide and it requires a further analysis about active compounds that shows important role in fungal growth inhibition. Antifungal activity of *Azadirachta indica* (Neem) leaf extract against three fungal species - *Aspergillus flavus*, *Cladosporium* and *Alternaria solani* was also confirmed by Shrivastava and Swarnkar (2014).

Neem formulations for managing the black mould disease of onion were also reported by Gupta et al. (2012). Neem extract has been used to prevent numerous fungal plant pathogens, such as *Aspergillus flavus*, *Alternaria alternata*, *Fusarium oxysporum*, *Fusarium solani*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani* (Obongoya et al., 2010).

The confirmation of ours results was also supported by work done by Mahmoud et al. (2011) while assessing the effect of aqueous, neem leaves extracts on growth of fungal pathogens (*Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Aspergillus terreus and Microsporum gypseum Candida albicans*) *in -vitro condition.*

3.2 The impact of different concentrations (%) of Panchgavya and carbendazim on inhibition in growth (%) of fungal pathogens:

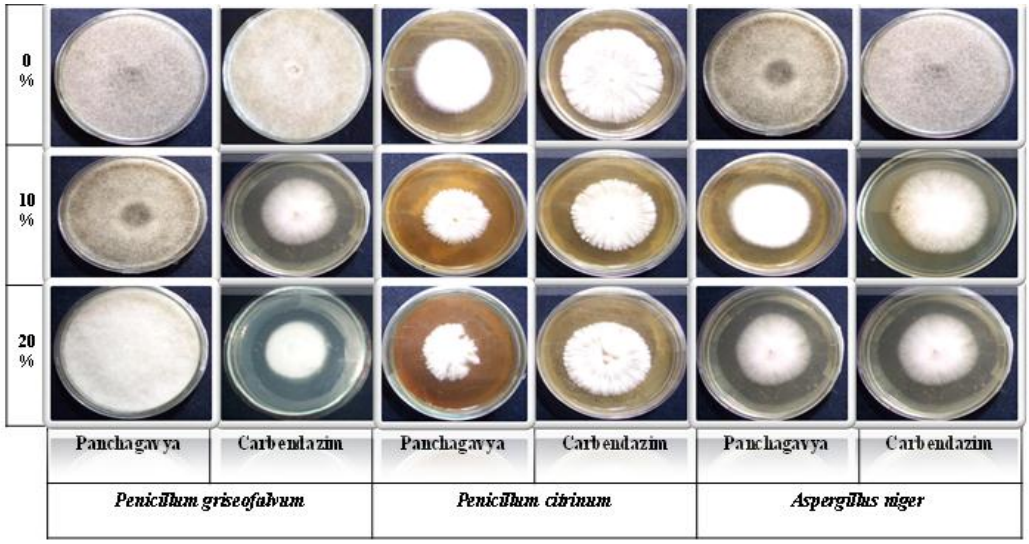
The colony diameter in all the tested fungi was found highest in the case of experimental control which was subjected to no treatment of organic formulation (panchgavya) or synthetic fungicide (carbendazim). Table No: 2

Penicillum griseofalvum exposed to 20% panchgavya exhibited a reduced fungal mycelial growth with a diameter of more than 88cm. Therefore, panchgavya treatment inhibited the mycelial growth of *Penicillum griseofalvum* only by 2.23%.

Penicillum griseofalvum exposed to 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 30cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum griseofalvum* by 66.65%.

Table No: 2 Impact of different concentrations (%) of panchgavya and carbendazim on inhibition in growth (%) of fungal pathogens

Fungi	Treatment	Colony diameter (mm)	Inhibition in growth (%)
Penicillum griseofalvum	Control	90	00.00
	Panch@10%	85	05.56
	Panch@20%	88	02.23
	Mean (Panch)	**86.50**	**3.89**
	Control	90	00.00
	Carb@10%	40	55.56
	Carb@20%	30	66.65
	Mean (Carb)	**35**	**61.10**
Penicillum citrinum	Control	50	00.00
	Panch@10%	48	04.00
	Panch@20%	35	30.05
	Mean (Panch)	**41.50**	**17.02**
	Control	90	00.00
	Carb@10%	40	55.56
	Carb@20%	30	66.65
	Mean (Carb)	**35**	**61.10**
Aspergillus niger	Control	80	00.00
	Panch@10%	45	43.75
	Panch@20%	44	45.36
	Mean (Panch)	**44.50**	**44.55**
	Control	90	00.00
	Carb@10%	40	55.56
	Carb@20%	30	66.65
	Mean (Carb)	**35**	**61.10**
Panch= Panchgavya; Carb= Carbendazim			



Penicillum citrinum exposed to 20% panchgavya exhibited a lowest fungal mycelial growth with a diameter of less than 35cm. Therefore panchgavya treatment inhibited the mycelial growth of *Penicillum citrinum* by 30.05%.
Penicillum citrinum exposed 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 30cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum citrinum* by 66.65%.
Aspergillus niger exposed to 20% panchgavya exhibited a reduced fungal mycelial growth with a diameter of less than 44cm. Therefore, panchgavya treatment inhibited the mycelial growth of *Penicillum citrinum* by 44.45%.
Aspergillus niger exposed 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 30cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum citrinum* by 66.65%.
The overall results indicate that the carbendazim was effective on controlling *Aspergillus niger* as compared to panchgavya by showing 61.10% reduction in mycelia growth (mean basis) as compared 44.55% (mean basis) by carbendazim. The similar trend was observed in all the fungi when treated with carbendazim as compared to panchgavya.
Antifungal properties of Panchagavya against fungal pathogens *i.e. Sclerotium rolfsii, Rhizoctonia solani*, *Fusarium oxysporium* was also confirmed by Jandaik and Sharma (2016). The laboratory experiment results showed that Panchagavya revealed antifungal activity against most of the post-harvest fungal Pathogens.

The validations of our finding were also confirmed by Ashlesha and Paul (2014). They used Panchgavya against important fungal pathogens of bell pepper namely *Sclerotium rolfsii, Fusarium solani, Phytophthora nicotianae, Fusarium oxysporum* f.sp. *capsici, Sclerotinia sclerotiorum* and *Rhizoctonia solani* under *in- vitro* and *in vivo* conditions.

The antifungal potential of Panchgavya against fungus pathogens was also confirmed by Sugha (2009) in *R. solani, S. rolfstt, S. sclerotiorum, F. solam* and *Phytophthora colocasiae in -vitro* studies.

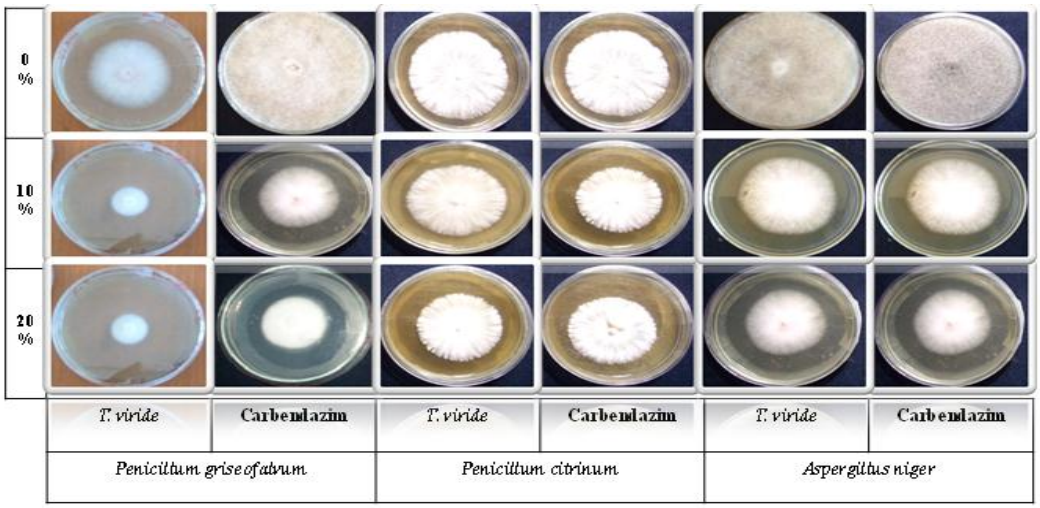
Current research also found effective response at high concentration of panchgavya formulations. It may be due to presence of antifungal properties of panchgavya formulations. The higher concentrations of Panchagavya are promising source for simple and naturally derived less expensive media with antifungal effect and growth promotion.

3.3 The impact of different concentrations (%) of *Trichoderma viride* and carbendazim on inhibition in growth (%) of fungal pathogens

The colony diameter in all the tested fungi was found highest in the case of experimental control which was subjected to no treatment of organic formulation (*Trichoderma viride*) or synthetic fungicide (carbendazim). Table No: 3
Penicillum griseofalvum exposed to 20% *Trichoderma viride* exhibited a reduced fungal mycelial growth with a diameter of less than 14cm. Therefore, *Trichoderma viride* treatment inhibited the mycelial growth of *Penicillum griseofalvum* by 71.10%.
Penicillum griseofalvum exposed to 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 30cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum griseofalvum* by 66.65%.
Penicillum citrinum exposed 20% *Trichoderma viride* exhibited a lowest fungal mycelial growth with a diameter of less than 50cm. Therefore *Trichoderma viride* treatment inhibited the mycelial growth of *Penicillum citrinum* by 28.55%.

Table No: 3 Impact of different concentrations (%) of *Trichoderma viride* and carbendazim on inhibition in growth (%) of fungal pathogens

Fungi	Treatment	Colony diameter (mm)	Inhibition in growth (%)
Penicillum griseofalvum	Control	50	00.00
	T.viride@10%	15	70.05
	T.viride@20%	14	71.10
	Mean (*T.viride*)	**14.50**	**70.57**
	Control	90	00.00
	Carb@10%	40	55.56
	Carb@20%	30	66.65
	Mean (Carb)	**35**	**61.10**
Penicillum citrinum	Control	70	00.00
	T.viride@10%	60	14.25
	T.viride@20%	50	28.55
	Mean (*T.viride*)	**55**	**21.40**
	Control	90	00.00
	Carb@10%	40	55.56
	Carb@20%	30	66.65
	Mean (Carb)	**35**	**61.10**
Aspergillus niger	Control	90	00.00
	T.viride@10%	56	44.45
	T.viride@20%	35	61.15
	Mean (*T.viride*)	**45.5**	**52.80**
	Control	90	00.00
	Carb@10%	40	55.56
	Carb@20%	30	66.65
	Mean (Carb)	**35**	**61.10**
T.viride = Trichoderma viride; Carb= Carbendazim			



Penicillum citrinum exposed 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 30cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum citrinum* by 66.65%.

Aspergillus niger exposed to 20% *Trichoderma viride* exhibited a reduced fungal mycelial growth with a diameter of less than 35cm. Therefore, *Trichoderma viride* treatment inhibited the mycelial growth of *Penicillum citrinum* by 61.15%.
Aspergillus niger exposed to 20% carbendazim exhibited a reduced fungal mycelial growth with a diameter of less than 30cm. Therefore, carbendazim treatment inhibited the mycelial growth of *Penicillum citrinum* by 66.65%.
The overall results indicate that the *Trichoderma viride* was effective on controlling *Penicillum griseofalvum* as compared to carbendazim by showing 70.57% (mean basis) reduction in mycelial growth as compared to 61.10% (mean basis) by carbendazim. The similar trend was also observed in *Penicillum citrinum* when treated with *Trichoderma viride* as compared to carbendazim.
Trichoderma viride showed significant difference and had promising potential for biological control of all the fungi (*Penicillum purpurogenum, Penicillum griseofalvum, Penicillum citrinum, Aspergillus niger*). But, it had less inhibitory effect as compare to other treatments.

Antagonistic properties of *Trichoderma spp* is due to the production of effective antifungal compounds make them useful as bio-control agents. *Trichoderma viride* (UC 4785) contains Dermadine, Heptelidic acid and *Trichodermin* compounds. Godtfredsen and Wangedal (1965) and Joseph & Sankarganesh (2011) also tested the antagonistic effect of *Trichoderma spp* and found the similar inhibitory results on fungal pathogens.

The overall results indicate that among various organic formulations tested in the current study, *in-vitro* application of Neem based formulation (Besaara) was most effective in controlling the fungal growth. The efficacy in restricting the fungal growth was higher with higher concentration (i.e. at 20%)

4. CONCLUSION

Fungal isolates responsible for disease and rotting as identified under cold storage condition were *Penicillum purpurogenum, Penicillum griseofalvum, Penicillum citrinum* and as *Aspergillus niger* under ambient storge contion. Under *in-vitro* condition, Neem based formulation (Besaara) was most effective in controlling the fungal growth. The efficacy of organic formulations in restricting the fungal growth was higher with higher concentration (i.e. at 20%).

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