Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(5): 2320-2322
Received: 07-07-2017
Accepted: 08-08-2017

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Evaluation of neem products against *Verticillium fungicola* causing dry bubble disease in *Agaricus bisporus* button mushroom

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Abstract
Verticillium fungicola var. *fungicola* is a serious pathogen causing dry bubble disease in button mushroom *Agaricus bisporus*. Present investigations were carried out on both host and pathogen by covering an aspect of *in vitro* management of dry bubble disease causing pathogen. Therefore, the efficacy of neem products *i.e.* neem seed kernel extract, neem oil and neem leaf extracts, at three different concentrations (2.5, 5.0 and 7.5 µl/ml) were determined against *V. fungicola* and *Agaricus bisporus*. The radial growth inhibition recorded was 50.02% at 7.5 µl/ml in case of neem seed kernel extract, followed by 40.99% and 34.94% in neem leaf extract and neem oil, respectively against *V. fungicola*. While, in *A. bisporus* the radial growth inhibition over control was just 8% when neem seed kernel extract was used at concentration 2.5 µl/ml than the others.

Keywords: *Verticillium fungicola*, *Agaricus bisporus*, neem, growth

Introduction
The commercial production of edible mushroom converts different types of agricultural and house-hold wastes into nutrition rich food which helps in addressing the problems of quality food, health and environmental sustainability. In view of increasing demand of high quality food with an increasing world population, mushrooms will be an important source of proteins that can replace meat and vegetables and milk products for a major part (Wani *et al*., 2010) [16]. About 1.5 million species of fungus are known (Hawksworth, 1991) [6] and out of these it has been estimated that 14,000 species produce fruiting bodies that are desirable to be considered as mushrooms (Hawksworth, 2001) [7]. About 7,000 species of edible mushrooms are known out of which 200 are experimentally grown and 10 have been produced at the industrial scale (Chang and Miles, 2004) [4].

In India, mostly four species of edible mushrooms *viz*., *Agaricus bisporus* (white button mushroom), *Volvariella* spp. (paddy straw mushroom), *Pleurotus* spp. (oyster mushroom) and *Calocybe indica* (milky mushroom) are commercially cultivated. Mushroom cultivation is affected by a large number of biotic and abiotic factors. Fungi, bacteria, viruses, nematodes, insects and mites are different biotic factors that damage the mushroom crop directly or indirectly (Sharma *et al*., 2011) [14]. Among the various factors responsible for low production and productivity of mushroom in our country, fungal diseases play a major role. The fungal pathogens, *Verticillium fungicola*, *Mycogone perniciosa*, *Trichoderma* spp. and *Papulaspora byssina* are the predominant mycopathogens. Amongst these, *Verticillium fungicola* var. *fungicola* (Preuss) is the important pathogen of the *Agaricus bisporus* (Lange) Imbach and annual losses to the growers are estimated to be 2–4% of total revenue (Berendsen *et al*., 2010) [1]. The pathogen induces various symptoms like bubbles (undifferentiated spherical masses), bent and/or split stipes (blowout) and spotty caps. Inoculation of *A. bisporus* crop with isolates of *V. fungicola* var. *fungicola* of various degrees of aggressiveness showed that the more aggressive isolates induced higher numbers of bubbles (Largeteau and Savoie, 2008) [9]. The *Verticillium* dry bubble is the most prevalent disease and if left uncontrolled in the mushroom growing environment; the disease can wipe out an entire crop in 2–3 weeks (Sharma *et al*., 2002) [13]. Moreover, the disease may be devastating for years following the initial infection because spores are capable of resting in debris and re-infecting crop year after year (Berendsen *et al*., 2010) [1]. Regarding management of dry bubble disease, an alternative of chemical agents is the use of certain plant derived oils with antifungal properties for disease management. Inhibitory effect of the phyto-chemicals tested both in solid and liquid state against pathogen and different host strains gave different degrees of percentage inhibition.

Oils like neem, citrullina, clove, olive and castor effectively controlled the mycelial growth of *V. fungicola* (Sabharwal and Kapoor, 2014) [12]. Thus the various control measures to be applied *i.e.* cultural practices, sanitation, bio-agents, botanical extracts and chemicals at various stages of crop cycle in order to effectively management of diseases. The chemicals measures significantly inhibit the mycelial growth of cultivated strains and leave harmful residue in fruit bodies. Therefore, it is essential to select safe and ecofriendly control measures of pathogen without affecting the growth of *A. bisporus*.

Materials and Methods

Evaluation of neem extracts and product against *V. fungicola* and *A. bisporus*

The sensitivity of neem extracts and product against the *V. fungicola* and *A. bisporus* were determined by poisoned food technique (Grover and Moore, 1962). Neem products *viz.*, neem seed kernel extract (NSKE), neem oil (NO) and neem leaf extracts (NLE) with different concentrations (µl/ml) *i.e.* 2.5, 5.0 and 7.5 were used against host and pathogen. Stock solutions of these extracts were prepared by dissolving required quantity in sterilized distilled water and neem oil used along with tween-80 to form a stable emulsion. Autoclaved potato dextrose agar (PDA) medium was amended with different stock solutions to obtain the desired concentration of extracts before being poured into Petri plates. The Petri plate with un-amended PDA served as check. Three Petri plates for each concentration of the extracts were inoculated with *V. fungicola* and *A. bisporus* by placing five mm actively growing mycelial disc of 12 days old culture. The radial growth (mm) was recorded after four days interval of incubation at 25±1ºC up to 12 days. The percent growth inhibitions of the pathogen and host at various concentrations of extracts were calculated over the control by using Vincent's formula (1947). [PI =100 (C – T)/C].Where, PI - Per cent inhibition, C - Radial growth (mm) in control, T - Radial growth (mm) in treatment

Results and Discussion

Evaluation of neem extracts and product against *V. fungicola* and *A. bisporus*

The sensitivity of neem extracts and product against the *V. fungicola* and *A. bisporus* were determined by poisoned food technique. Neem products *viz.*, neem seed kernel extract (NSKE), neem oil (NO) and neem leaf extracts (NLE) with different concentrations (µl/ml) *i.e.* 2.5, 5.0 and 7.5 were used respectively. The percent growth inhibitions of the pathogen and host at various concentrations of extracts were calculated over the check. From the Table-1, depicted that percentage inhibition over control towards *V. fungicola* was 50.02% at concentration of 7.5 µl/ml in case of neem seed kernel extract followed by 40.99% and 34.94% in neem leaf extract and neem oil respectively, on the other hand, the percentage inhibition was very low 17.24% in case of neem oil at concentration of 2.5 5 µl/ml after 12 days of incubation (Plate-1). Whereas in *A. bisporus* percentage inhibition over control (Table-2) was 7.77% at concentration 2.5 µl/ml with neem seed kernel extract followed by 8.69% and 10.11% in neem leaf extract and neem oil, respectively. While, percentage inhibition over control of host *A. bisporus* was maximum 24.17% at concentration of 7.5 µl/ml in case of neem oil followed by 15.42% (neem leaf extract) and 14.82% (neem seed kernel extract) after 12 days of incubation.

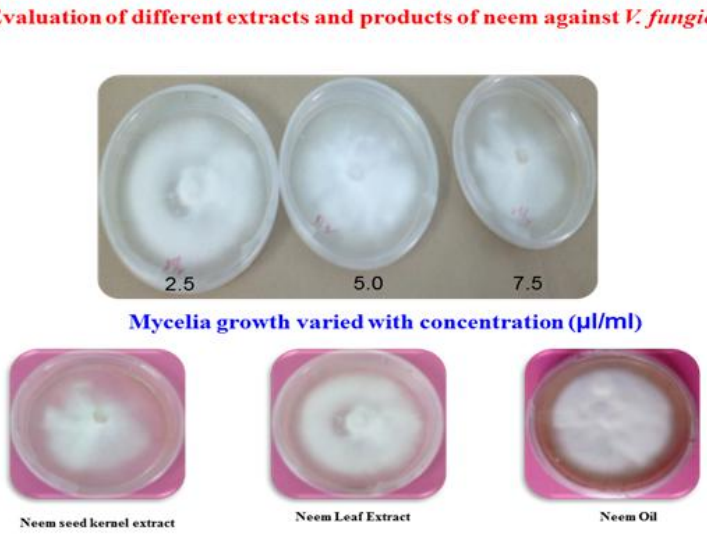

Plate 1

Table 1: Evaluation of neem product and extracts against *Verticillium fungicola*

Conc. (ul/ml)	Observation** on r**adial growth* of *Verticillium fungicola***														
	Neem seed kernel extract					**Neem oil**					**Neem leaf extract**				
	4DAI	**8DAI**	**12DAI**	**Mean**	**PIOC#**	**4DAI**	**8DAI**	**12DAI**	**Mean**	**PIOC#**	**4DAI**	**8DAI**	**12DAI**	**Mean**	**PIOC#**
2.5	12.67	24.33	32.33	23.11	27.02	14.00	27.17	36.67	25.94	17.24	13.33	24.17	34.17	23.89	22.89
5.0	9.50	18.33	27.83	18.56	37.23	12.17	22.00	32.33	22.17	26.98	11.00	21.83	31.50	21.44	28.94
7.5	6.67	11.50	22.17	13.44	50.02	10.33	17.67	28.83	18.94	34.94	9.50	15.83	26.17	17.17	40.99
Control	16.50	30.67	44.33	30.50		16.50	30.67	44.33	30.50		16.50	30.67	44.33	30.50	
Mean	11.33	21.21	31.67			13.25	24.38	35.54			12.58	23.13	34.04		
Factors	A	B^{1}	A× B^{1}			A	B^{II}	A× B^{II}			A	B^{III}	A× B^{III}		
CD at 5%	1.04	1.20	2.08			1.49	1.72	2.98			1.18	1.36	2.36		

*Average of three replications, ** observation (DAI) days after inoculation # PIOC- percent inhibition over control (12DAI) A- Concentration, B^{I} – NSKE observation, B^{II} –Neem oil observation, B^{III} – Neem leaf extract observation, Control- pathogen *Verticillium fungicola*

Table 2: Evaluation of neem product and extracts against *Agaricus bisporus*

Conc. (ul/ml)	Observation** on radial growth* of *Agaricus bisporus*														
	Neem seed kernel extract					Neem oil					Neem leaf extract				
	4DAI	8DAI	12DAI	Mean	PIOC#	4DAI	8DAI	12DAI	Mean	PIOC#	4DAI	8DAI	12DAI	Mean	PIOC#
2.5	12.67	28.33	42.00	27.67	7.77	14.33	25.33	39.00	26.22	10.11	12.67	26.33	41.33	26.78	8.69
5.0	12.33	28.00	40.00	26.78	7.82	14.00	24.00	34.00	24.00	21.69	12.33	25.67	38.33	25.44	11.59
7.5	12.00	26.33	37.00	25.11	14.82	12.33	23.67	33.00	23.00	24.17	11.33	23.33	36.67	23.78	15.42
Control	14.67	29.17	43.50	29.11		14.67	29.17	43.50	29.11		14.67	29.17	43.50	29.11	
Mean	12.92	27.96	40.63			13.83	25.54	37.38			12.75	26.13	39.96		
Factors	A	B^{I}	A× B^{I}			A	B^{II}	A× B^{II}			A	B^{III}	A× B^{III}		
CD at 5%	1.92	2.21	N/A			1.63	1.88	3.26			2.14	2.47	N/A		

*Average of three replications, ** observation (DAI) days after inoculation # PIOC- percent inhibition over control (12DAI) A- Concentration, B^{I} – NSKE observation, B^{II} –Neem oil observation, B^{III} – Neem leaf extract observation, Control- host *Agaricus bisporus*

In an attempt to find ecological safe method for management of disease the sensitivity of neem leaf and seed extracts as well as neem oil was evaluated against *V. fungicola* and *A. bisporus* at three different concentrations (µl/ml) *i.e.* 2.5, 5.0 and 7.5 both towards host and pathogen. The neem seed kernel extract at concentration 7.5 µl/ml inhibited the radial growth of *V. fungicola* to the extent of 50% followed by neem leaf extract (41%) and neem oil (35%), respectively. In *A. bisporus* the radial growth inhibition over control was just 8% when neem seed kernel extract was used at concentration of 2.5 µl/ml than the others. These findings are in complete agreement with those of Sabharwal and Kapoor, (2014) [12] who reported that botanical extracts and oils like neem, citrullina, clove, olive and castor used in different concentration effectively controlled the mycelial growth of mushroom mycopathogen. Similarly, Tanovic *et al*. (2009) also observed inhibition of *V. fungicola* by using few essential oils from aromatic and medicinal plants. Several botanicals and oils have been evaluated against many fungal pathogens throught the world by various researchers (Jahan *et al*., 2013) [8] found that botanicals like garlic and henna also reduced the radial colony diameter of pathogen appreciably at different concentrations. Pattnaik *et al*. (2012) [11] reported that the application of *A. indica* extract resulted in reduction of leaf spot diseases up to extent of 40% in *Lycopersicum esculentum*. Similarly, Minz *et al*. (2012) [10] observed that mycelia growth inhibitiont was upto extent of 58-99% when thirteen plant extracts were used for antifungal assay against wilt disease in ginger. The bio-efficacy of neem extract against pathogens attributed to the fact that neem has active compounds such as azadirachtin, nimbin, nimbidin, nimbinene and azadirone which were antifungal, antibactrial and anti-insecticidal in nature (Bohra *et al*., 2006) [2]. Similar, findings were also obtained against *fusarium* wilt of carnation by application of different botanicals (Chandel and Tomar, 2008) [3].

Summary and Conclusion

In *in vitro* evaluation of neem products *viz.,* neem seed kernel extract, neem oil and neem leaf extracts at different concentrations (2.5, 5.0 and 7.5 µl/ml) against *V. fungicola* revealed maximum radial growth inhibition of 50.02% and minimum 14.82% in *A. bisporus* at concentration of 7.5 µl/ml with neem seed kernel extract. On the other hand, neem oil and neem leaf extract were showed quite inhibitory to mycelial growth of *A. bisporus* and least effective against pathogen.

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